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The expression of miRNA in HPV-associated and HPV-independent head and neck tumors

Exprese miRNA u nádorů hlavy a krku asociovaných a neasociovaných s HPV

Doctoral thesis

Supervisor: RNDr. Ruth Tachezy, Ph.D.

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Prohlášení:

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Praha, 19.11.2018 Mgr. Zuzana Vojtěchová

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LIST OF ABBREVIATIONS

A_E	early polyadenylation
AFP	α-fetoprotein
Ago	argonaute protein
AKT	protein kinase B
A_L	late polyadenylation
AML1/ETO	acute myelogenous leukemia 1/for eight-Twenty One
APOBEC	apoliprotein B mRNA editing enzyme, catalytic polypeptide-like
APOT	Amplification of Papillomavirus Oncogene Transcripts
ARE	AU-rich element
B-CLL	B cell chronic lymphocytic leukemia
bp	base pair
BPV	bovine papillomavirus
CAF1	chromatin assembly factor-1
CBP	CREB-binding protein
CCR4-NOT	carbon catabolite repressor 4-negative on TATA
CDKI	cyclin-dependent kinase inhibitor
CDKs	cyclin-dependent kinase
CFSs	common fragile sites
CIN	cervical intraepithelial neoplasia
COX-2	cyclooxygenase-2
CR	conserved region
CRPV	cottontail rabbit papillomavirus
DBS	double-stranded break
DCP2	decapping enzyme 2
DDX6	DEAD-box RNA helicase
DGCR8	DiGeorge syndrome critical region 8 protein
DICER1	double-stranded RNA-specific endoribonuclease 1

DNA	deoxyribonucleic acid
DSS	disease specific survival
E region	early region
E1BS	E1 binding site
E2BS	E2 binding site
E6AP	E6-associated protein
EGFR	epidermal growth factor
eIF4G	eukaryotic translation-initiation factor 4G
EMT	epithelial-to-mesenchymal transition
ER	endoplasmatic reticulum
EV	epidermodysplasia verruciformis
FF	fresh frozen
FFPE	formalin-fixed paraffin-embedded
FGFR1	fibroblast growth factor 1
FHWT	Favorable Histology Wilms Tumor
FXR1	fragile X mental retardation-related protein 1
GA	Golgi aparatus
GTP	guanosintriphosphate
HCV	hepatitis C virus
HDACs	histone deacetylases
HER2	human epidermal growth factor receptor 2
HGL	high-grade lesion
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	high risk
HSPGs	heparan sulphate proteoglycans
hTERT	human telomerase reverse transcriptase

IFN	interferon
IRES	internal ribosome entry site
IRF	interferon regulatory factor
L region	late region
LCR	long control region
LGL	low-grade lesion
LNA	locked nucleic acid
LR	low risk
MDM	mouse double minute 2
MHCI	major histocompatibility complex I
miRISC	microRNA-induced silencing complex
miRNA	microRNA
mlncRNA	mRNA-like noncoding RNA
NCT	number of clinical trial
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NIH	National Institute of Health
ORF	open reading frame
ORI	origin of replication
pA	polyadenylation
PABP	polyA-binding protein
PACT	kinase-R-activating protein
PAN	polyA nuclease
PARN	poly(A)-specific ribonuclease
PI3K	phosphatidylinositol-3-kinase
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PSA	prostate-specific antigen

PV	papillomavirus
RACE	Rapid Amplification of cDNA Ends
RLB	reverse line blot
RNAP	RNA polymerase
RNS	reactive nitrogen species
ROS	reactive oxygen species
RRP	recurrent respiratory papillomatosis
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SA	splice acceptor
SCC	squamous cell carcinoma
SD	splice donor
SNP	single nucleotide polymorphism
SSB	single-stranded break
TCGA	The Cancer Genome Atlas
TLDA	TaqMan Low Density Array
TNF	tumor necrosis factor
TRBP	transactivating response RNA-binding protein
TSGs	tumor suppressor genes
TUTase	terminal uridylyl transferase
URR	upstream regulatory region
UTR	untranslated region
V-ATPase	vacuolar ATPase
VEGF	vascular endothelial growth factor
VLPs	virus-like particles
Xpo5	exportin 5
XRN1	5′-3′ exoribonuclease

ABSTRACT

Head and neck cancers represent a group of tumors with two different etiologies. The first type is associated with the viral HPV infection, the second one is virus-independent and it is associated with smoking and alcohol consumption as two main risk factors. Numerous studies show that HPV-positive tumors are more frequent in younger patients, as well as that the prognosis and overall survival of these patients is remarkably better. Therefore, the modification of the treatment is considered. For this, however, specific, sensitive and clinically relevant biomarkers for accurate identification of tumor etiology is needed. Suitable candidates for such biomarkers are miRNAs, small non-coding regulatory molecules stable in archived samples, that have been shown as differentially expressed in human cancers and the expression pattern seems specific for tumors of different origin.

The submitted thesis focuses on miRNA profiling in HPV-positive and HPV-negative tonsillar tumors and cervical carcinomas with the aim to find out the differences between regulation of important carcinogenetic pathways of tumors of viral and non-viral etiology. Our data have shown very large heterogeneity of the miRNA expression profiles of these tumors. Despite the well characterized and uniform samples collection, we have found very small overlap of the HPV-specific miRNAs in comparison to both our model system and to other studies. Therefore, we focused on the reasons for such heterogeneity, this study has shown the importance of the homogeneity of analyzed samples and standardization of the type of clinical material and normalization approach for data analyses.

Overall, the submitted thesis presents our results from miRNA research of HPV-related cancers. During the research, several important observations were made which allow for the improvement of the next experiments. Finally, the HPV-core miRNAs were identified and will be evaluated in a following project in a set of HPV-related and non-related samples from malignant tissues of other anatomical locations. These selected miRNAs will be then analyzed functionally.

ABSTRAKT

Nádory hlavy a krku představují skupinu nádorů dvojí etiologie. První skupinou jsou nádory asociované s infekcí HPV, druhou skupinou jsou nádory nevirové etiologie, které jsou spojeny s dvěma hlavními rizikovými faktory, a tím je kouření a konzumace alkoholu. Z publikovaných studií je zřejmé, že HPV-pozitivní nádory se vyskytují častěji u mladších pacientů a zároveň tito pacienti vykazují lepší prognózu a celkové přežívání. Z tohoto důvodu je zvažována modifikace léčby na základě etiologie nádorového onemocnění. Nicméně je důležité zajistit specifický, citlivý a klinicky významný biomarker pro přesné určení etiologie nádoru. Vhodným kandidátem pro takové biomarkery jsou miRNAs, jakožto malé nekódující regulační molekuly, které jsou stabilní i v archivních vzorcích, a jejichž rozdílná exprese byla detekována v řadě lidských nádorů a je specifická pro nádory různého původu.

Předkládaná práce je zaměřena na miRNA profilování v HPV-pozitivních a HPV-negativních nádorech krčních mandlí a nádorech děložního hrdla s cílem najít rozdíly v regulaci důležitých karcerogenních drah nádorů virové a nevirové etiologie. Naše výsledky ukazují velkou heterogenitu v expresních profilech miRNAs u těchto nádorů. I přes využití velmi dobře charakterizovaného a jednotného souboru vzorků jsme detekovali malý překryv HPV-specifických miRNAs ve srovnání s naším modelovým systémem, a stejně tak ve srovnání s jinými studiemi. Z toho důvodu jsme hledali příčiny takové heterogenity a naše výsledky ukazují na důležitost stejnorodosti analyzovaných vzorků a standardizaci typu klinického materiálu a normalizačních přístupů pro vyhodnocování získaných dat.

Souhrnně lze říci, že předkládaná práce představuje naše výsledky studia miRNA v HPV-asociovaných nádorech. Během výzkumu jsme narazili na několik důležitých poznatků, které nám pomůžou vylepšit další experimenty. Identifikovali jsme tzv. „HPV-core“ miRNA specifické pro HPV-asociované nádory a získaná data budou využita a zhodnocena v rámci následujícího projektu zabývajícího se miRNA analýzou HPV-asociovaných a neasociovaných nádorů dalších anatomických lokalizací. Dále pak budou identifikované miRNAs analyzovány funkčně.

CONTENTS

1. PREFACE

Head and neck cancer (HNC) is the ninth most common cancer type worldwide with more than 500 thousand of new cases annually (Ferlay *et al.*, 2015; Gupta *et al.*, 2016). In the Czech Republic, HNC is the twelfth most common tumor with almost 2000 new cases annually, and the incidence of the disease is still increasing (www.svod.cz, www.uzis.cz). HNC is often diagnosed in the late stage of the disease and thus the survival of patients is unsatisfying. The main risk factors for the development of this disease are smoking and alcohol consumption. In recent years, the role of HPV was noticed, especially with oropharyngeal tumors. The proportion of HPV-caused tumors varies worldwide, however, in the Czech Republic, around 68 % of the oropharyngeal tumors are high-risk HPV-positive (Tachezy *et al.*, 2009). Although the disease is diagnosed mostly in the late stage, patients with HPV-associated tumors have better prognosis and overall survival (Dayyani *et al.*, 2010), and the treatment could be optimized for each group according to the etiology. Therefore, it is important to find some specific and sensitive biomarker for the distinction of the carcinogenetic mechanisms of tumors of different etiologies. Such biomarker should help to improve diagnostics prognostic as well as therapeutic approaches.

The thesis focuses on the study of head and neck tumors of both etiologies and on the determination of such biomarker. The suitable candidates for it could be miRNAs, short non-coding RNAs with posttranscriptional regulatory function. Their expression profiles differ in cancer tissues and in non-malignant ones, and the expression is also tissue specific. The expression of miRNA has been studied in various tumors, also in HNC, however, the distinction of etiology of these tumors has not been included yet. Thus, our research focused on the analysis of the group of well characterized HNC samples of single anatomical location in comparison to cervical cancer samples, where tumor relates to HPV infection in almost 100 %. Our results revealed some new findings in the field of miRNA research, which will, hopefully, contribute to determination of the right HNC biomarkers and help to improve the therapy and prognosis of the patients.

2. INTRODUCTION

2.1 Basic facts about human papillomaviruses

Human papillomaviruses (HPVs) belong to the family *Papillomaviridae* and together with the family *Polyomaviridae* and *Adenoviridae* are referred as small DNA tumor viruses. HPVs have small non-enveloped, icosahedral capsid with 55 nm in diameter (Figure 2-1) which contains double-stranded DNA with the length up to 8000 bp. These viruses infect epithelial cells of the skin or mucosa and may cause benign proliferations, such as papillomas or warts. Further, some types of HPVs have also oncogenic potential and participate in the oncogenic progression of premalignant lesions.

Since the clinical manifestation of genital warts has been known since the times of Hippocrates, until the 50s of the 20th century they had not been considered as sexually transmitted disease. The first papillomaviruses were identified by Shore and Hurst in 1933 in the cottontail rabbit (cottontail rabbit papillomavirus, CRPVs) (Shope and Hurst, 1933). Soon afterwards, Rous and Beard described the oncogenic potential of this papillomavirus (PV) type (Rous and Beard, 1935). The viral particles in genital warts were first demonstrated by electron microscopy in 1969 (Waterson and Almeida, 1969). The milestone in the HPVs research came at the beginning of the 80s of the 20th century with the isolation of HPV16 and HPV18 from the cervical carcinoma cells in the laboratory of Harald zur Hausen (Durst *et al.*, 1983; Boshart *et al.*, 1984). Since then many research groups became interested in the investigation of HPVs, their molecular biology, the relation to the carcinogenesis and the research aimed at development of the vaccine. In 2008, Harald zur Hausen received the Nobel Prize in Physiology or Medicine for his discovery of HPV causing cervical cancer.

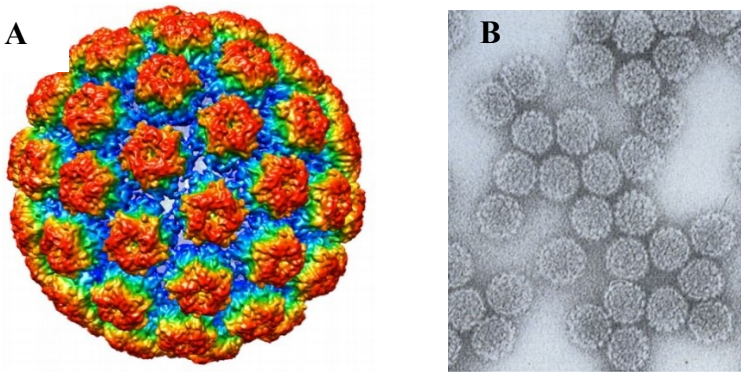

Figure 2-1: (A) Model of HPV16 capsid (Cardone *et al.*, 2014); (B) Electron micrograph of papillomavirus particles (Doorbar *et al.*, 2015).

2.1.1 Viral classification

With the development of the molecular biology methods in the 1980s, the new period of HPV research started. To date, up to 200 HPV types have been identified along with over 100 of animal PV types including mammals, birds, snakes or turtles (http://pave.niaid.nih.gov). For PVs classification, the gene for the major capsid protein L1 is used standardly since the L1 open reading frame (ORF) is the most conserved region in HPV genome (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007). To be identified as a new papillomavirus type, the DNA sequence of the L1 ORF must differ in more than 10 % from the closest known PV type (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007). HPVs are organized into 5 genera (Figure 2-2). The largest PV groups are: Alpha-, Beta-, Gamma-papillomaviruses and also Nu- and Mu-papillomaviruses.

Alpha-papillomaviruses are classified as cutaneous and/or mucosal types, according to the tissue tropism. Based on the oncogenic potential, HPV types are classified as high-risk (HR), which can cause pre- and malignant lesions, and low-risk (LR), causing benign lesions. The most frequent mucosal LR-types are HPV6 and 11 which cause genital warts (condyloma accumitatum) or recurrent respiratory papillomatosis (RRP) (Ball *et al.*, 2011; Omland *et al.*, 2014). The most common HR-HPVs are HPV16, 18, 31, 33, 45, 51 or 58. Many studies confirmed the role of HR-HPV types in the development of cervical cancer and cancer of other anogenital regions (Rubin *et al.*, 2001; Daling *et al.*, 2002; Munoz *et al.*, 2003), as well as their etiological role in the development of head and neck cancer (Snijders *et al.*, 1996; Gillison *et al.*, 2000).

Beta-papillomaviruses mostly include cutaneous types of HPVs and were found to be associated with the cutaneous squamous cell carcinomas (SCC) of epidermodysplasia verruciformis (EV) patients (Pfister, 2003). EV is a hereditary skin disease with characteristic skin lesions which can progress to SCC and is associated mostly with HPV5 and 8, rarely with HPV14, 17, 20 or 47. However, beta-papillomaviruses are also widely spread on the skin of healthy population with the prevalence up to 91 % and the most prevalent type HPV23 (de Koning *et al.*, 2009). Gamma-papillomaviruses comprehend mostly cutaneous types and are represented by e. g. HPV4, 48, 50 or HPV60.

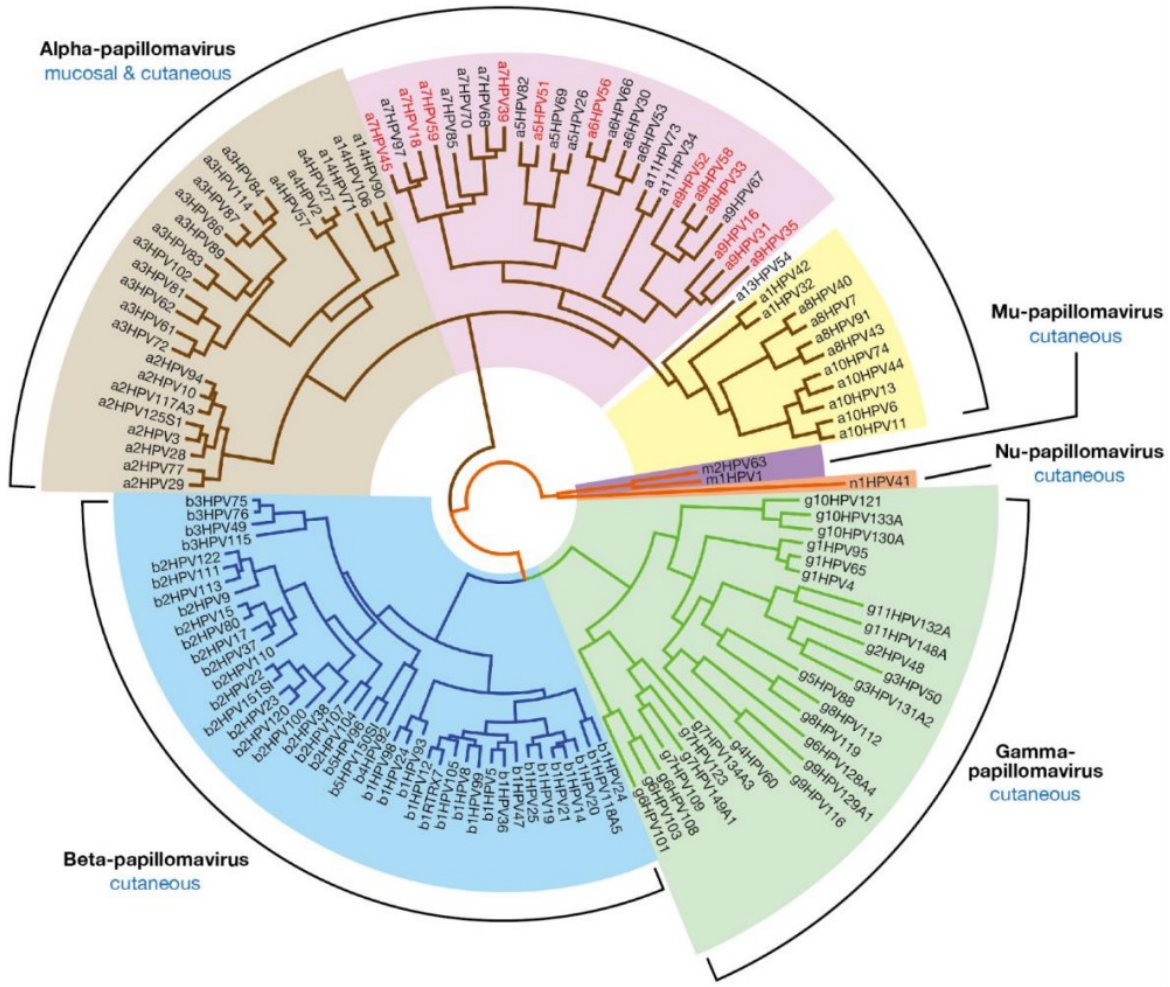

Figure 2-2: Phylogenetic relationship between human papillomaviruses. Alpha-papillomaviruses are classified as low-risk cutaneous (brown), low-risk mucosal (yellow) and high-risk mucosal (pink). The highlighted types (text in red) are confirmed as "human carcinogens". Adapted from Egawa *et al.*, 2015.

2.1.2 Genome of human papillomaviruses

HPVs´ viral capsid contain approximately 8 kb long circular double-stranded DNA genome, which is divided into three main regions and contains eight or nine ORFs (Figure 2-3); the early region (E) that encodes the early proteins E1-E7, the late region (L) that encodes two capsid proteins L1 and L2, and the upstream regulatory region (URR), also known as long control region (LCR), which contains regulatory sequences, such as promotor and enhancer elements as well as the viral origin of replication (ORI) (Seedorf *et al.*, 1985). The regions are separated by two polyadenylation (pA) sites; early pA (A_E) and late pA (A_L). In the early stages of the viral life cycle, the viral early transcripts are driven by early promotor (e.g. p97 in HPV16, p99 in HPV31) (Smotkin and Wettstein, 1986; Ozbun and Meyers, 1998) and polyadenylated at the pA_E. As the cell

differentiates, the late promotor (e.g. p670 in HPV16, p742 in HPV31) (Grassmann *et al.*, 1996; Ozbun and Meyers, 1998) is activated and the shortened variants of the early proteins E1, E2 and E4 are transcribed. During the terminal differentiation of the host cells, the activity of pA_E is downregulated, the transcription of the true late region is activated and the mRNAs for L1 and L2 proteins polyadenylated at the pA_L are transcribed (reviewed in Johansson and Schwartz, 2013). The genome of all HPVs is transcribed from a single DNA strand and the polycistronic mRNAs undergo alternative splicing utilizing various splice sites.

The number of ORFs differ among HPVs, however, the core genes encoding the early proteins E1 and E2 involved in replication, and genes for late proteins L1 and L2 involved in packaging, are well-conserved across the HPV types. The early region further encodes other four early genes (E4, E6, E7, E5). In some HPV types, the fusion protein E8^E2C has been proven (Stubenrauch *et al.*, 2000; Lace *et al.*, 2008; X. Wang *et al.*, 2011; Sankovski *et al.*, 2014).

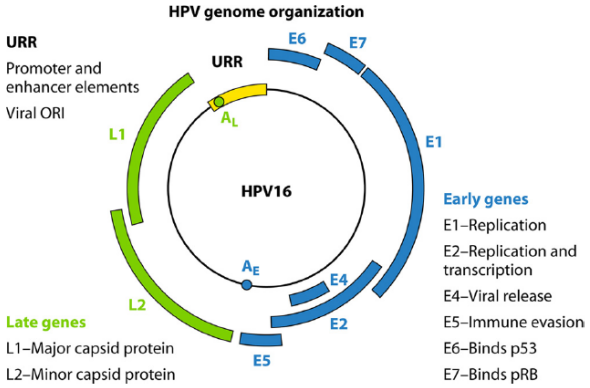

Figure 2-3: Illustration of the HPV16 genome organization (Stanley, 2012).

The E1 protein has helicase and ATPase activity, it is necessary for viral replication when it binds to the specific viral DNA sequence (E1 binding site, E1BS) and together with the protein E2 it initiates the DNA unwinding (Wilson *et al.*, 2002; reviewed in Bergvall *et al.*, 2013). The E2 protein has triple role. First, its domain in carboxyl terminus binds to four conserved E2 binding sites (E2BSs) in the LCR sharing a consensus DNA sequence. The binding of different levels of this protein to E2BSs regulates transcription of the early viral genes, including E2 itself (Romanczuk *et al.*, 1990). The E2 protein is also a negative regulator of the expression of the main HPV oncoproteins E6 and E7. Furthermore, E2 protein binds to the specific motifs in the viral genome and attaches it to the mitotic chromosome. This mechanism ensures the right

segregation of the viral genome between daughter cells during cell division (Bastien and McBride, 2000; McBride *et al.*, 2006). The middle region of the protein supports the stability of E2 proteins itself and is required for the efficient nuclear localization (Zou *et al.*, 2000). Finally, the N-terminus is essential for the interaction with the protein E1 and as such it plays role in regulation of the viral replication. Recently, Cruz-Gregorio *et al*. have revealed that proteins E1 and E2 modulate cellular redox state and thus influence DNA damage, which could be an important event in establishment of persistent infection and potential cellular transformation (Cruz-Gregorio *et al.*, 2018).

The gene for E4 protein overlaps in the HPV genome with the *E2* gene, but it is expressed in a different ORF. The protein E4 is expressed as a fusion protein E1^E4 (Doorbar *et al.*, 1990; X. Wang *et al.*, 2011) and the most abundant expression occurs during the later stages of the viral life cycle (Doorbar *et al.*, 1997; Peh *et al.*, 2002; Middleton *et al.*, 2003), therefore the expression of the E4 protein is consider as a biomarker of vegetative viral genome amplification (Peh *et al.*, 2002). Similarly, Wilson *et al*. wanted to determine the role of E1^E4 protein of HPV18. They revealed that this E1^E4 protein plays role in regulating the HPV late functions, however, it also participates in the early phase of the replication cycle (Wilson *et al.*, 2007). Contrary to this study, Fang *et al*. have reported that the expression of full-length E1^E4 protein is not necessary for efficient genome amplification of HPV11 (Fang *et al.*, 2006). That suggests that there may exist type-specific differences between the functions of E1^E4 proteins. The E1^E4 protein also plays role in virus escape form the epithelial cells, since it associates with keratin filaments and participates on reorganization of the keratin cytoskeleton (Wang *et al.*, 2004; McIntosh *et al.*, 2010). Thereby, it disrupts structural integrity of infected cells and facilitates the release of newly synthetized virus particles.

Several types of HPV (e.g. HPV16, HPV18 or HPV31) express also a spliced mRNA that links the *E8* gene with the *E2* gene and encodes the fusion protein E8^E2C (Stubenrauch *et al*., 2000; Lace *et al*., 2008; Wang *et al*., 2011; Sankovski *et al*., 2014). This protein functions as a repressor of viral replication and transcription, and it interferes with the E2-dependent transcriptional activation by the full-length E2 proteins (Stubenrauch *et al.*, 2001; Lace *et al.*, 2008; Straub *et al.*, 2014).

The HPV early proteins E5, E6 and E7 are the main viral oncoproteins, the mechanisms of their function are described in detail in 2.1.4.

HPVs encode two structural proteins - the major late protein L1 and the minor late protein L2. The protein L1 forms 72 pentameric capsomers and together with up to 72 single L2 proteins forms an infectious virion (Buck *et al.*, 2008). The L1 protein facilitates the attachment with the host cell, whilst the L2 protein is essential for the viral genome encapsidation or it participates in multiple steps during the viral entry to the host cell (reviewed in Buck *et al.*, 2013; Wang and Roden, 2013). Moreover, the major capsid protein L1 is able to spontaneously self-assemble into "virus-like particles" (VLPs) exhibiting morphological and immunological features of native virions (Zhou *et al.*, 1991; Ghim *et al.*, 1992). Since the VLPs are only the empty capsids without the infectious genome, they are used for development of VLP-based vaccines (Kirnbauer, 1996; Vonka and Hamsikova, 2007). Until 2015, two types of VLP-based prophylactic vaccines were available in the Czech Republic - bivalent vaccine Cervarix™, designed to prevent against the two most common HR-HPV types 16 and 18, and quadrivalent vaccine Gardasil™ (also known as Silgard®), targeting additionally against HPV6 and HPV11 causing genital warts and low-grade lesions (reviewed in Cutts *et al.*, 2007). After years of development, the current quadrivalent vaccine was enlarged to the nonavalent vaccine targeting five additional HR-HPV types (HPV31, -33, -45, -52, -58) and as Gardasil 9® was licensed in 2014 (Pils and Joura, 2015). This nonavalent vaccine has been available in the Czech Republic since 2015. The vaccination in the Czech Republic is endowed for the young of both genders at the age of 13-14 years.

2.1.3 Life cycle of HPVs

Human papillomaviruses are intraepithelial pathogens. Their life cycle is strictly dependent on differentiation process of infected keratinocytes and on the replication apparatus of the host cell (Figure 2-4). HPVs infect the basal layers of epithelium through the small wounds or abrasions. The mitotic cell division, which occurs during the wound healing, is necessary for the entry of the virus into the host cell and into the cell nucleus (Pyeon *et al.*, 2009). The virus initially bounds to the heparan sulphate proteoglycans (HSPGs) situated on the basement membrane of epidermis (Combita *et al.*, 2001; Giroglou *et al.*, 2001). The interaction between the HPV capsid and the heparan sulphate on the membrane is mediated by the HPV protein L1 and is necessary for the efficient infection of the cells (Knappe *et al.*, 2007; Surviladze *et al.*, 2015). This attachment induces the conformational changes in the virus capsid leading to the exposure of the

amino terminal portion of capsid protein L2, which is then cleaved by convertase furin and other, non-HSPGs receptors, are uncovered (Richards *et al.*, 2006; Day and Schiller, 2009). The exact way, how the virus enters the host cell and reaches the nucleus, is unknown, however Spoden *et al*. suggest that the HR-HPV types share similar novel requirements for the endocytic mechanism of the entry (Spoden *et al.*, 2013) independent on the well-studied clathrin-, caveolin- or dynamin-mediated pathways.

In the basal cells of the epithelium, the virus maintains its genome in low copy number as an extrachromosomal genome called episome. The viral DNA replication is regulated by the proteins E1 and E2 and the genome is amplified to 50-100 copies per cell (Parish *et al.*, 2006; McBride, 2008), while the expression of the potent viral oncogenes E6 and E7 is under very tight control. When the host cells begin to differentiate the viral gene expression and DNA replication is upregulated, the expression of E6 and E7 proteins allows the infected cell to re-enter the S-phase of the cell cycle and the genome copy number increases to thousands. During the terminal differentiation of the epithelium, the viral late promotor is activated and the genes encoding the capsid proteins are expressed. Afterwards, the viral genomes are encapsidated and exit the cell along with the shredding keratinocytes as an infectious particle. The life cycle of HPVs is at least three-weeks long and the release of the viral particles does not cause the cell death.

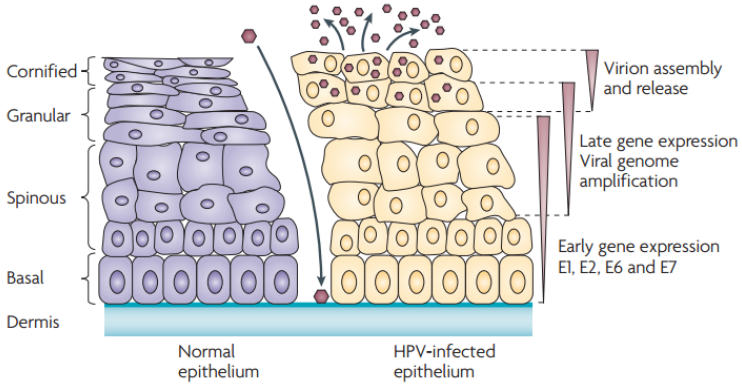

Figure 2-4: Life cycle of human papillomaviruses (Moody and Laimins, 2010).

2.1.4 Human papillomaviral oncoproteins

2.1.4.1 Protein E6

Protein E6 is - along with the other protein, E7, one of the most important HPV oncoprotein and it participates in cellular transformation. It targets critical regulators of cellular processes, which leads to unlimited proliferation, evasion of apoptosis, induction

of invasive and metastatic properties or sustained angiogenesis (McLaughlin-Drubin and Munger, 2009b). The most well-characterized function of this protein is the ability to interfere with the activity of the protein p53. Protein p53 is a tumor suppressor regulating cellular pathways which mediate cell cycle arrest or induce cellular apoptosis in response to DNA damage or other cellular stress. HPV oncoprotein E6 forms a complex with p53 and cellular E3 ubiquitin ligase called E6-associated protein (E6AP). This results in ubiquitination and degradation of the p53 via proteasome (Scheffner *et al.*, 1990; Mortensen *et al.*, 2015). The E6AP interacts with the LXLL motif situated in the amino terminal region of the E6 oncoprotein.

Protein E6 plays role also in p53-independent pathways resulting in cellular transformation. One of them is the ability to activate telomerase (hTERT), which maintains the telomeric DNA at the end of linear chromosomes and, therefore, telomeres are not shortened upon each cell division, which leads to immortalization of the host cells. Veldman *et al*. published that HR-HPV E6 protein activates the hTERT promoter via interactions with c-Myc and thus increases the enzyme activity (Veldman *et al.*, 2001; Veldman *et al.*, 2003). In addition, there is also some evidence that protein E6 associates with the repressor of the hTERT promoter NFX1 leading to the increased hTERT transcription (Gewin *et al.*, 2004; Katzenellenbogen *et al.*, 2007).

The carboxyl terminal region of the E6 protein contains PDZ-binding motifs which interact with the specific PDZ domains of cellular proteins. These proteins often play role in signaling pathways and regulation of cellular polarity. The interaction of E6 and proteins harboring PDZ domains leads to their degradation and thus cellular transformation (Nguyen *et al.*, 2003; Watson *et al.*, 2003). Webb Strickland *et al*. have also reported that E6 interaction with PDZ domains of several proteins modulates the activation and nuclear localization of transcriptional regulator YAP1 and thus contributes to oncogenesis (Webb Strickland *et al.*, 2018). E6 proteins of LR-HPVs lack the ability to associate with the PDZ proteins (Kiyono *et al.*, 1997).

HR-HPV protein E6 can also interact with other cellular proteins such as interferon regulatory factor 3 (IRF-3), which normally activates the expression of interferons (IFNs) - cytostatic cytokines playing role in immune response to viral infections. The association of E6 protein and IRF-3 leads to the inhibition of IRF-3 transcriptional activity (Ronco *et al.*, 1998). Further, E6 protein interacts with the transcriptional coactivators CREB-binding protein and p300 (CBP/p300), tumor

suppressors promoting apoptosis and inhibition of the cell cycle progression and cellular differentiation (Goodman and Smolik, 2000). The binding of the E6 protein to CBP/p300 displaces the protein p53 from its binding site on CBP/p300 and thus it is another viral mechanism how to abrogate the p53 function (Zimmermann *et al.*, 1999).

2.1.4.2 Protein E7

Other important protein of human papillomaviruses is oncoprotein E7 which along with the oncoprotein E6 participates in the cellular transformation. It is a small polypeptide containing two conserved regions CR1 and CR2 at the amino terminal domain. These regions are necessary for the transforming activities of the protein and have the sequence similarities with adenovirus protein E1A as well as polyomavirus SV40 large T antigen. The main target of E7 is retinoblastoma protein Rb and other "pocket proteins" associated with pRb, namely p107 and p130. Protein Rb is a tumor suppressor protein regulating cell cycle progression since it controls the G1/S transition. It forms the pRb-E2F repressor complex which arrests the cell cycle progression into the S-phase. When the cell is ready to divide, Rb protein is phosphorylated by cyclin-dependent kinases (CDKs), the transcription factor E2F is released from the repressor complex and the cell cycle can progress to the S-phase. During M/G1 transition, pRb is dephosphorylated and it returns into the "active" hypophosphorylated state with linked E2F (reviewed by Munger and Howley, 2002).

The primary mechanism of E7-mediated cell cycle deregulation is the association of E7 protein with the hypophosphorylated pRb and its targeting for the proteasomal degradation through the interaction with cullin 2 ubiquitin ligase complex (Huh *et al.*, 2007). The pRb is bound to the conserved LXCXE motif in CR2 region of E7. Simultaneously, the transcription factor E2F is released from the complex with pRb and the E2F-mediated transcription is activated facilitating the cell cycle progression leading to cellular transformation.

In addition to pRb, the oncoprotein E7 interacts also with other cellular proteins involved in the cell cycle regulation. Its inactivation leads to the cellular transformation, including cyclin A and E or cyclin-dependent kinase inhibitor $p21^{CIP1}$ and $p27^{KIP1}$ both of which are activated by anti-proliferative signals and contribute to cell-cycle arrest (McLaughlin-Drubin and Munger, 2009a). Moreover, it has been also shown that E7 can attract the histone deacetylase (HDACs) proteins to the chromatin and thus promote

E2F-mediated transcription (Longworth *et al.*, 2005). The protein E7 binds to the HDACs through the zinc-finger-like regions at the carboxyl terminus. Further, the binding of the E7 protein to HDACs is important for maintenance of the extrachromosomal form of HPV in undifferentiated keratinocytes (Longworth and Laimins, 2004). Analogous to protein E6, the oncoprotein E7 interacts with the interferon regulatory factor 1 (IRF-1) through its pRb-binding domain (Um *et al.*, 2002). IRF-1 is a transcription factor influencing expression of interferon β (IFN-β) and thus the viral ability to escape the host cell immunity.

2.1.4.3 Protein E5

Papillomaviral protein E5 is the least understood of the three papillomaviral oncoproteins. It is rich in hydrophobic amino acid clusters and localized to membranes of Golgi aparatus (GA) and endoplasmatic reticulum (ER) (Oetke *et al.*, 2000; Disbrow *et al.*, 2003). The characteristics of bovine papillomavirus 1 (BPV1) E5 and HPV16 E5 have been studied the most. Although E5 proteins of these two types show minimal sequence similarity with each other and HPV16 E5 is almost twice as large, they both transform cells and overlap in their function and targets.

E5 protein is not encoded in all HPV types, however, it is likely that it plays a significant role in viral replicative life cycle and that its presence in viral genome correlates with the risk of cancer. This suggests that it contributes to the function of oncoproteins E6 and E7 and hence also to the human carcinogenesis (Bravo and Alonso, 2004; Schiffman *et al.*, 2005). It has been shown that HPV16 and HPV18 E5 increases the level of the receptor of epidermal growth factor (EGFR) and in this way sensitizes the cells to EGF and stimulates the transformation and mitogenic activity of the cells (Tomakidi *et al.*, 2000; Kivi *et al.*, 2008; Wasson *et al.*, 2017). HPV16 E5 also influences the EGFR downstream signaling pathways and, through that, also the expression of a number of oncogenes such as c-jun, c-fos, cyclooxygenase-2 (COX-2) or vascular endothelial growth factor (VEGF) contributing to the independent growth (Chen *et al.*, 1996; Kim *et al.*, 2006; Kim *et al.*, 2009). Further, HPV16 E5 downregulates the activity of cell cycle inhibitors p21 and p27 (Tsao *et al.*, 1996; Pedroza-Saavedra *et al.*, 2010).

HPV16 E5 influences the host cells also in EGFR-independent way when it promotes the cell-cell fusion and contributes to transformation by increasing the cellular DNA content. Hu *et al*. demonstrated that HPV16 E5 is necessary for the formation of

bi-nucleated cells which play an initiating role in the early stages of HPV-induced cellular transformation (Hu *et al.*, 2009). Furthermore, HPV16 E5 interacts with the 16K subunit of vacuolar ATPase (V-ATPase) (Conrad *et al.*, 1993) which leads to the decreasing acidification of endosomes followed by increased recycling of EGFR to the cell surface and constitutive signaling (Straight *et al.*, 1995). HPV16 E5 also plays role in evading of immune response since it interacts with the heavy chain of human major histocompatibility complex I (MHCI) antigen and thus restricts its transport to the cell surface (Ashrafi *et al.*, 2005; Ashrafi *et al.*, 2006).

2.1.5 Epidemiology

2.1.5.1 Epidemiology of HPV infection

HPV infection is the most common viral sexually transmitted infection worldwide and it is estimated that approximately 440 million individuals are infected with HPV (Malik *et al.*, 2014). HPV is most often transmitted horizontally by sexual contact, but the penetrative sex is not required. Further, the vertical transmission from infected mothers is also possible, but it is not very common (Burchell *et al.*, 2006). Most of the infections are inapparent and are spontaneously cleared with the immune system of the host without clinical manifestation, nevertheless the clearance rate of HR-HPVs is lower than that of the low risk types (Molano *et al.*, 2003). Only about 25 % of all individuals infected with HPV develop precancerous neoplasia and fewer than 1 % progress to the invasive cancer (Malik *et al.*, 2014). Based on the meta-analysis published by Bruni *et al*., the HPV prevalence worldwide among women with normal cytological findings was ~11 % before the introduction of vaccination (Bruni *et al.*, 2010), but it varied between different continents. The HPV prevalence was the lowest among females who reported never having sex and significantly increased with increasing age (Hariri *et al.*, 2011). The most prevalent types in the Eastern Europe were HR-HPV types 16, 31, 18, 39, 33, and 66 and from the low risk types it was HPV6 (Bruni *et al.*, 2010). In the Czech Republic the HPV prevalence was higher than in other countries of Eastern Europe (Tachezy *et al.*, 2013) and the types detected in the Czech population (HPV16, 56, 39, 45, 18, and 51) were different than those reported by Bruni *et al*. (Bruni *et al.*, 2010). In 2016, Markowitz *et al*. published a study reporting a decrease of prevalence of quadrivalent HPV vaccine types after introduction of vaccination in the population of United States (Markowitz *et al.*, 2016). There was a 64% decrease in the prevalence

among females aged 14 to 19 years and 34% decrease among those aged 20 to 24 years. Similar results were reviewed in meta-analysis by Drolet *et al*. (Drolet *et al.*, 2015) which reflects promising effect of vaccination programs.

Beside HPV DNA, the useful epidemiologic marker of HPV infection is the detection of antibodies to antigens derived from HPV-specific proteins. The presence of antibodies specific to L1 VLPs reflects the past and cumulative exposures to the virus (Herrero *et al.*, 2003; Zhao *et al.*, 2005; Tachezy *et al.*, 2009). Our laboratory published a large population-based seroepidemiological study which revealed that the seroprevalence in the Czech Republic is age-dependent and for the most common HR-HPV types reaches the maximum in 30-39-year-old women and 50-59-year-old men (Hamsikova *et al.*, 2013). This study also provided evidence-based data supporting the implementation of HPV vaccination in the Czech Republic before the age of 13. Antibodies specific to HPV E6 and E7 are rarely present in healthy individuals but their prevalence is high in patients with invasive malignant diseases associated with HPV infection (Zumbach *et al.*, 2000).

2.1.5.2 Epidemiology of HPV-related carcinomas

More than 14 million of cancer cases are diagnosed each year worldwide (Ferlay *et al.*, 2015) and about 5 % is associated with the HPV infection (Plummer *et al.*, 2016). Cervical cancer is the fourth most common cancer in women worldwide and the seventh overall with over 500 thousand new cases in 2012 (Plummer *et al.*, 2016), and is almost in 100 % etiologically linked to the HPV infection. For the development of the premalignant lesions and/or invasive carcinomas the persistent infection with HR-HPVs is necessary (Schiffman and Kjaer, 2003; Radley *et al.*, 2016). An important factor for the progression of precancerous lesions is HPV type. The most frequent type in invasive carcinomas is HPV16 which is responsible for 40 % of high-grade lesions and more than a half of the cervical cancer cases (Crow, 2012; Schiffman and Wentzensen, 2013). The second most common carcinogenic type is HPV18 (Figure 2-5).

A recent study of Schütze *et al*. demonstrated that HPV types with lower prevalence in cervical cancer and precursor lesions show reduced *in vitro* immortalization capacity and require more genetic host cell aberrations than the more prevalent types (Schutze *et al.*, 2016).

Further, to the persistent HPV infection and the progression of the disease contribute behavioral factors such as smoking, multiparity or sexual behavior (International Collaboration of Epidemiological Studies of Cervical Cancer *et al.*, 2006; International Collaboration of Epidemiological Studies of Cervical Cancer, 2006; International Collaboration of Epidemiological Studies of Cervical Cancer, 2009). In the Czech Republic, the incidence of the cervical carcinoma stagnates in time with more than 800 new cases in 2014 (http://svod.cz). The most prevalent type in Czech women is HPV 16 which was detected in 43 % of cervical precancerous lesions and in 73 % of cervical carcinomas (Tachezy *et al.*, 2011).

Although the majority of HPV-related cancers are cervical, the viral infection can also lead to the carcinomas in other parts of the body (Figure 2-5). These cancers include those of vagina, vulva, anus, and penis, with more than 110 thousand of new cases in 2012 (Plummer *et al.*, 2016). There is also the evidence that HPV infection is related to the head and neck tumors, mainly oropharyngeal cancer. The part about head and neck tumors is discussed in chapter 2.2.

Beside the mucosal types from the genus alpha-PVs, the cutaneous HPV types might play role in the pathogenesis of cutaneous cancer. In 2009, two beta-PVs, HPV5 and HPV8, were classified as possibly carcinogenic in patients with epidermodysplasia verruciformis with a high susceptibility to beta-PVs and skin cancer (Bouvard *et al.*, 2009).

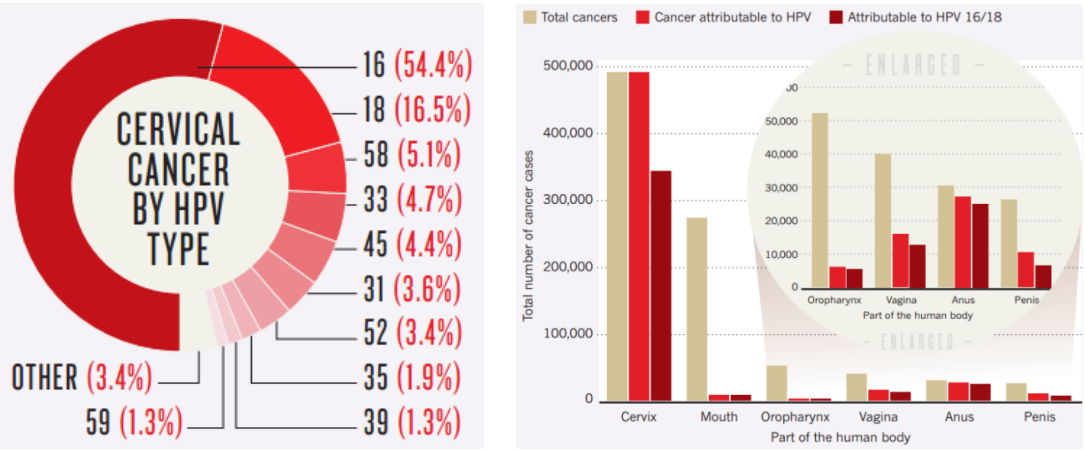

Figure 2-5: HPV and cancer. Left - percentage distribution of HPV types is cervical cancer. Right – comparison of total number of cancer cases at different anatomical location and those related to HPV infection (Crow, 2012).

2.1.6 Molecular mechanisms of HPV-associated carcinogenesis

As mentioned in 2.1.5.2, persistent viral infection with HR-HPV type, especially HPV16/18, is a precondition for the development of premalignant lesions and their progression to malignant carcinomas (Radley *et al.*, 2016). Cervical cancer is preceded by premalignant changes of different histological grading known as cervical intraepithelial neoplasia (CIN). A low-grade lesion CIN (LGL, CIN1) typically disappears within a few months and is caused by transient HPV infection. The cellular proliferative and antiapoptotic effects of cervical inflammation in combination with the low-level expression of HPV oncogenes contribute to the progression of this mild dysplasia to the high-grade lesions characterized as moderate dysplasia CIN2 and/or severe dysplasia CIN3 (Williams *et al.*, 2011). Although most of women are at some time in their lives infected with HPV, the progression into the invasive carcinoma occurs only in the minority of them. It suggests that some other factors are contributing to the cancer development on molecular level such as genomic instability, HPV DNA integration into the host genome, or epigenetic alterations. The mechanisms of HPV-related carcinogenesis was studied mostly in cervical cancer, however, the key events are similar also for the carcinomas of head and neck region.

2.1.6.1 Genomic instability

Primary immune response to the viral infection or infection by other pathogens is an inflammation (Williams *et al.*, 2011). Chronic inflammation is referred to as a cancer promotor (Coussens and Werb, 2002) because it induces cell proliferation and increases formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) which leads to the DNA damage such as DNA single-stranded breaks (SSB) and double-stranded breaks (DSBs). It also increases accumulation of mutations and thus genomic instability. However, infection with HPV does not cause a typical immune response since the virus infects the basal epithelial cells, and the HPV life cycle is nonlytic thus the inflammation leading to oxidative stress does not occur. Moreover, the specific immune response is also influenced in HPV-associated preneoplastic lesions where the right function of antigen presenting cells is decreased (Herfs *et al.*, 2009). The splice variant of the viral oncoprotein E6, E6*, can increase ROS levels in keratinocytes which leads to the increased DNA damage (Williams *et al.*, 2014). Alcohol consumption and tobacco smoking are other factors which contribute to the induction of oxidative stress in

infected cells resulting in DNA damage (Tsuchiya *et al.*, 1993). Mehta and Laimins (Mehta and Laimins, 2018) have revealed that HPVs preferentially recruit DNA repair factors to viral genomes, utilize them for the induction of viral genome amplification upon epithelial differentiation (Gillespie *et al.*, 2012; Anacker *et al.*, 2014; Spriggs and Laimins, 2017) and rapid reparation of DNA breaks in viral genome. Further, HPV maintains this deregulated state with the expression of the oncoprotein E6 which mediates the degradation of p53, a cellular guardian of the cell cycle, and thus disrupts the repair mechanisms of the cellular DNA. Proteins E6 and E7 also induce the accumulation of abnormal numbers of centrosomes leading to the development of aneuploidy and genomic instability (Duensing *et al.*, 2000; Duensing and Munger, 2002).

2.1.6.2 HPV DNA integration

Regardless the anatomical site, the mechanism of HPV-induced carcinogenesis relies on the function of the main HPV oncoproteins E6 and E7. As discussed in 2.1.4, HPV oncoproteins associate with the tumor suppressors p53 and pRb leading to their degradation and thus deregulation of the tumor suppressive function. The integration of the viral DNA into the host genome is considered as a key event in the HPV-related carcinogenesis. The risk of virus integration depends on the level of DNA damage because this event requires DSBs in both the host and virus DNA since HPV do not encode any integrase (Pett and Coleman, 2007). The frequency of integration is also different for each HR-HPV type. The HPV16 is found integrated in 55 % of cervical tumors, by contrast, HPV18 is found integrated in 92 % and HPV31 in 14 % of those tumors (Vinokurova *et al.*, 2008). The study of Vinokurova *et al*. also suggests that the integration is a consequence of the deregulated expression of oncoproteins E6 and E7 (Vinokurova *et al.*, 2008). Moreover, the recent study of Schütze *et al*. has revealed the differences in *in vitro* E6/E7 immortalization capacity of different HR-HPV types where in cells infected with HPV types with reduced immortalization potential (HPV45, -51, -59, -66, -70) higher number of genetic host cell aberrations was found necessary for inducing of immortalization (Schutze *et al.*, 2016). This may explain the fact that there are less cervical carcinomas induced by these HPV types in population.

The frequency of HPV16 integration increases with the severity of the cervical neoplasia, however, only extrachromosomal forms of the virus were also detected in the invasive lesions (Klaes *et al.*, 1999; Arias-Pulido *et al.*, 2006; Vinokurova *et al.*, 2008).

It seems that the integration is important in the HPV-associated oncogenesis, albeit not necessary, and other mechanisms could lead to the cell transformation. In high-grade cervical lesions, the region of early genes E2 and E8^E2 are often disrupted or lost during the HPV integration, leading to the increased upregulation of the expression of the E6 and E7 which favors oncogenesis (Jeon *et al.*, 1995; Klaes *et al.*, 1999; Tsakogiannis *et al.*, 2015). Further, the integration into the E1 or L2/L1 gene regions was also observed in high-grade malignancies, as well as in low-grade lesions (Li H. *et al.*, 2013; Tsakogiannis *et al.*, 2015). Nevertheless, the viral oncoproteins E6 and E7 are always retained and are actively transcribed (Akagi *et al.*, 2014).

In the cases of carcinoma with extrachromosomal form of the virus, the elevation of the copy number of viral episome represents one of the options how the expression of viral oncogenes might be increased (Gray *et al.*, 2010). However, other studies have not observed the increased number of viral oncogenes in cancers with episomal form of HPV. Similarly, our results indicate this tendency, but the results were not statistically significant (Pokryvkova *et al*., manuscript in preparation). Other possibilities how the expression of the viral oncoproteins E6 and E7 can be increased is the epigenetic regulation of the binding sites in URR for the negative regulator E2 (discussed in 2.1.6.3) which was suggested in cervical tumors by Cheung *et al*. (Cheung *et al.*, 2013), or the mutations in the LCR that would preclude the right expression and binding of E2 protein (Chaiwongkot *et al.*, 2013; Cheung *et al.*, 2013; Kahla *et al.*, 2014). Both of these alternative mechanisms were studied also in our laboratory; however, our study has not revealed mutations in the E2 binding sites (Pokryvkova *et al.*, manuscript in preparation), and, in contrary to our expectations, the increased methylation level of E2BSs was observed in tumors with integrated HPV genome (Pokryvkova *et al*., manuscript in preparation).

Two types of integration have been observed in HPV-associated neoplasms (Jeon *et al.*, 1995). In type I, there is a single copy of viral genome integrated into a host chromosome, whilst in type II a tandem of several viral genomes is integrated with the last copy being disrupted. In type II, mostly the 3′ end of the viral genome is transcriptionally active. The other copies are intact, however transcriptionally inactive (Van Tine *et al.*, 2004). It has been suggested by Kalantari *et al*. (Kalantari *et al.*, 2008) that the other copies are silenced by epigenetic modifications. In agreement with Kalantari *et al*. and Chaiwongkot *et al*. (Chaiwongkot *et al.*, 2013), we observed

hypermethylation of LCR in type II integrated HPVs (Pokryvkova *et al*., manuscript in preparation). Moreover, Dooley *et al*. (Dooley *et al.*, 2016; Warburton *et al.*, 2018) have recently revealed that tandemly integrated repeats of HPV genome can develop into a Brd-4 dependent super-enhancer-like element. Brd4 is a chromatin binding factor which regulates cellular transcription by binding to the super-enhancer regions - clusters of traditional enhancers often associated with expression of oncogenes. The authors detected highly enriched marker Brd4 at tandemly integrated copies of HPV16 in cells of cervical neoplasia cell line W12 and have shown that it drives the strong E6/E7 oncogenes transcription promoting the development of neoplasia (Dooley *et al.*, 2016; Warburton *et al.*, 2018).

HPV integration sites are thought to be a randomly distributed throughout almost all chromosomes without specific hotspots (Wentzensen *et al.*, 2004). However, it has been observed that integration may occur also near or within common fragile sites (CFSs) (Thorland *et al.*, 2003; Kraus *et al.*, 2008), the genomic regions prone to chromosome breaks. Wentzensen *et al*. reported HPV integration in fragile sites in 38 % of the analyzed integration sites of primary tumor samples of anogenital tract, head and neck region, and cell lines (Wentzensen *et al.*, 2004). Moreover, the integration of virus into the host genome often leads to the disruption or deregulation of the important target genes including tumor suppressor genes, to chromosomal rearrangements or to upregulation of genes involved in cancer progression (Parfenov *et al.*, 2014; Akagi *et al.*, 2014). The integration near known growth-control genes, including frequently targeted *MYC* locus, *TP63* or telomerase reverse transcriptase *TERT* (Wentzensen *et al.*, 2002; Ferber *et al.*, 2003; Wentzensen *et al.*, 2004; Peter *et al.*, 2006) located in or near translocation breakpoints, or in/near the transcriptionally active regions (Lace *et al.*, 2011; Schmitz *et al.*, 2012; Khoury *et al.*, 2013; Bodelon *et al.*, 2016) were detected. These data suggest that the elevated expression of the viral oncoproteins is not the only mechanism leading to the oncogenesis, but the prompt changes in the host genome due to the integration are an alternative way.

2.1.6.3 Epigenetic alterations

Among other factors that can contribute to the disease progression belong epigenetic alterations. During all stages of carcinogenesis, many types of epigenetic alterations, including DNA methylation or histone modification, may occur in HPV as

well as in host genome. These changes, which do not lead to mutations of DNA can cause the disease as well. DNA methylation is a covalent chemical modification of cytosine by methyltransferase enzyme leading to the formation of 5-methylcytosine and to the inactivation of the target gene. Aberrant methylation has been described in a number of tumor suppressor genes (TSGs), cell cycle regulators or DNA repair genes in CIN lesions and cervical cancer (reviewed in Saavedra *et al.*, 2012). Global DNA hypomethylation in cervical neoplastic tissues and its increase over the progression of neoplasm leading to an activation of certain proto-oncogenes has been also observed (Kim *et al.*, 1994). Furthermore, the altered methylation influencing the malignant transformation has been also observed in HPV genome. Kalantari *et al*. were the first to claim that the methylation of the 3´ region of the L1 gene is associated with neoplastic progression (Kalantari *et al.*, 2004). They detected this region heavily methylated in cervical cancer, however, low level of methylation was observed in low-grade lesions. Later, also the methylation of L2 and E5 genes has been observed in neoplastic lesions (Brandsma *et al.*, 2009).

As mentioned above, epigenetic alterations may be an alternative mechanism through which the expression of the main viral oncoproteins E6 and E7 can be influenced. These oncoproteins are negatively regulated by the viral protein E2 which binds to the four conserved E2BSs in the LCR rich in the CpG motifs and thus contribute to the deregulation of the E6 and E7 expression during disease progression. Methylation of these sites leads to the inability to bind the E2 protein and suppression of its regulatory function (Kim *et al.*, 2003). Chaiwongkot *et al*. have revealed in their study on cervical lesions that the methylation of E2BSs appears to change during neoplastic progression and depends on the integration status of the viral genome (Chaiwongkot *et al.*, 2013). Similarly, Reuschenbach *et al*. (Reuschenbach *et al.*, 2015) have pointed to the association of E2BSs methylation with E2 integrity and viral genome status in HPV-associated oropharyngeal squamous cell carcinomas, and Sen *et al*. have shown differential molecular signatures among cervical carcinoma cases with episomal and integrated HPV16 (Sen *et al.*, 2017). On the other hand, in the study by Bryant *et al*. focusing on samples of vulvar carcinomas, no significant differences in the methylation levels based on the viral status were detected (Bryant *et al.*, 2014). The methylation status of E2BSs as an alternative mechanism leading to the increase of E6/E7 expression and thus cellular transformation in tumors with extrachromosomal form of the virus was also studied in our laboratory. Unlike others, increased level of methylation in E2BSs has been

observed in tonsillar tumors with integrated form of the virus (Pokryvkova *et al*., manuscript in preparation).

2.2 Head and neck cancer

Head and neck cancer is the ninth most common malignancy in the world with more than 500 thousand new cases annually worldwide (Ferlay *et al.*, 2015; Gupta *et al.*, 2016). The incidence and mortality of HNC depends on gender, cancer sub-site and country. Regardless of whether the country is developed or developing, there is an increase in both parameters, except for mortality rate in the USA, which has been decreasing in last four decades (Gupta *et al.*, 2016). More than 90 % of these tumors are characterized as SCC arising from the epithelial cells of the mucosal lining in the head and neck regions (e.g. oral cavity, pharynx, and adjacent regions) (Figure 2-6).

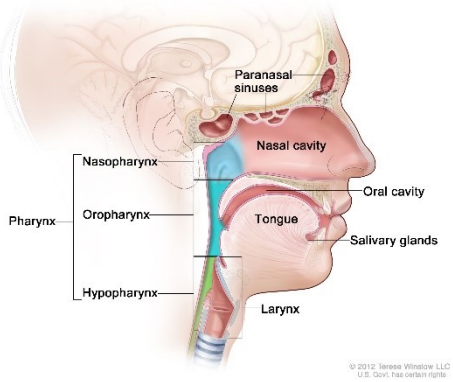

Figure 2-6: Illustration of possible head and neck cancer regions (http://cancer.gov).

The differences in the worldwide incidence and mortality is given by the variations in the exposure to the main risk factors. Most of the cases are associated with smoking and alcohol consumption, both of which increase the mutation rates in important cellular pathways (Hashibe *et al.*, 2009; Zygogianni *et al.*, 2011; Gaykalova *et al.*, 2014). Approximately 33 % of HNC cases are associated with smoking alone (Hashibe *et al.*, 2009). Berthiller *et al*. have demonstrated that already smoking of >0-3 cigarettes per day is associated with a 50% increased risk of HNC. However, the duration in smoking plays also important role since this increased risk has not been found among smokers with smoking duration shorter than 20 years (Berthiller *et al.*, 2016). By contrast to smoking,

nearly 4 % of HNC is attributed to alcohol consumption alone and nearly 35 % of HNC is related to both smoking and alcohol (Hashibe *et al.*, 2009).

Furthermore, HR-HPV infection is also recognized as another primary cause of HNC. The first evidence indicating the relationship between oral carcinogenesis and HPV infection was reported by Syrjanen *et al*. (Syrjanen *et al.*, 1983). The strongest association with HPV has been described for oropharyngeal carcinomas, specifically those involving tonsils and base of tongue (Nordfors *et al.*, 2014). Contrary to cases related to the tobacco use, the incidence rates of oropharyngeal carcinomas associated with HPV is increasing (Chaturvedi *et al.*, 2008; Chaturvedi *et al.*, 2013), which fact is influenced by changes in sexual behavior; earlier age of sexual debut, higher number of sexual partners or insufficient use of barrier protection (Smith *et al.*, 2004; Gillison *et al.*, 2008; Rettig *et al.*, 2015). However, the proportion of HPV-associated oropharyngeal cancers varies around the world. In the USA about 40-80 % of oropharyngeal cases are associated with HPV, whereas in Europe the percentages ranges from 90 % in Sweden to less than 20 % in countries with the highest rates of tobacco use, namely the Netherlands (23-29 %), France (17 %) or Germany (17-63 %) (Nasman *et al.*, 2009; Marur *et al.*, 2010). In the Czech Republic, HR-HPV DNA has been detected in 18 % of oral and in 68 % of oropharyngeal carcinomas, and the seropositivity of antibodies to HPV E6/E7 oncoproteins was 56 % in patients with oropharyngeal tumors, which fact further supports the etiological association of HR-HPV with tumors of this location (Tachezy *et al.*, 2009). The most prevalent HR-HPV type in HNC is HPV16 detected with up to 95 % of oropharyngeal cancers followed by HPV33 and HPV18 (Dayyani *et al.*, 2010; St Guily *et al.*, 2011; Koslabova *et al.*, 2013).

Interesting, and clinically important, fact is that patients with HPV-associated carcinomas are commonly younger and have significantly better disease-specific and overall survival compared to patients with HPV-independent tumors (Hafkamp *et al.*, 2008; Dayyani *et al.*, 2010; Rotnaglova *et al.*, 2011). Moreover, Jung *et al*. have also revealed in their study that the overall survival of the patients depends on the fact whether the virus is transcriptionally active or not (Jung *et al.*, 2010). They claim that HPV-negative and HPV DNA-positive/RNA-negative patients show similar survival rate whilst tumors with transcriptionally active virus are associated with better prognosis. Zhang *et al*. performed an integrative analysis of genomics and transcriptomics data from Cancer Genome Atlas and found that the replication of HPV genome and its invasion into

the host genome may enhance DNA repair mechanisms since the genes involved in DNA mismatch repair pathways were found upregulated in HPV-positive tumors, and as such they limit the accumulation of lethal somatic mutations (Zhang *et al.*, 2016). The differences in survival should be given also by the diverse immunological responses of patients with HPV-positive and HPV-negative tumors. Nevertheless, the underlying mechanisms for this prognostic advantage of HPV-related tumors are unclear and further studies are needed to clarify this observation.

The early-stage tumors are mostly treated with one treatment modality such as surgery or radiotherapy. The treatment of advanced tumors is usually multimodal requiring the combination of surgery with the adjuvant postoperative radiotherapy and/or chemotherapy. Regarding the prognostic advantage of patients with HPV-positive HNC and their better outcome regardless of the treatment strategy (Licitra *et al.*, 2006), it is important to think about the improvement of life of the survivors and eventually de-intensify the treatment regimens based on the HPV status or developing of some new HPV-specific treatment strategies (Klozar and Tachezy, 2014). Several studies are currently testing de-escalation of the treatment in order to reduce the side effects (Kimple and Harari, 2014; Mirghani *et al.*, 2015), and the first results can be expected in the upcoming years. Besides, unlike cervical cancer which develops from clinically well-defined and visible precancerous lesions, oropharyngeal tumors are not usually detected in the early stages, most likely due to the locations of the lesions in tonsillar crypts and their worse accessibility for diagnosis, and the majority of patients present more advanced disease where the radical and mutilating treatment is required. Therefore, better understanding of specific molecular mechanisms in HPV-associated tumorigenesis and identification of useful and clinically relevant biomarkers of HPV-related tumors will provide more opportunities for improvement of diagnostics, target selected pathways during the treatment and specify more precisely the prognosis of patients.

2.2.1 Differences in carcinogenesis of HPV-positive and HPV-negative HNC

As mentioned in chapter 2.1.4, HPV encodes two main oncoproteins E6 and E7 which are the key players in HPV-associated oncogenesis where the main regulators of cell cycle are inactivated through their function. The main mechanisms of HPV oncoproteins functions are illustrated in the Figure 2-7. On the contrary, in HPV-negative

tumors, the malignant progression is associated mostly with the mutations of the cellular cycle key genes. In HPV-positive tumors, one of the most important cellular tumor suppressor p53 is inactivated and degraded by the activity of E6 and thus these tumors basically harbor wild-type p53, in contrast to HPV-negative tumors which are characterized by genetic alterations of the p53 pathways and show p53 mutations occurring in 46-73 % of cases (Riaz *et al.*, 2014). In a great part of HPV-negative tumors, the gene *CDKN2A* encoding the protein p16, is inactivated through homozygous deletions, loss of function mutations or epigenetic alterations (Hayes *et al.*, 2015). This protein is a cyclin-dependent kinase inhibitor (CDKI) that inhibits pRb phosphorylation through the disrupting of cyclin D1/CDK4/6 complex, and thus blocks the progression of cell cycle. Its inactivation in HPV-negative tumors is associated with the worse prognosis of patients (Namazie *et al.*, 2002). Since the expression of p16 occurs because of the inactivation of the pRb, the HPV-positive tumors are characterized by the high expression of p16 as a consequence of the pRb inactivation by oncoprotein E7 when the transcriptional factor E2F is released and positively regulates the expression of *CDKN2A*. For this reason, the p16 has been denoted as a surrogate marker of active HPV infection in cervical tumors (Sano *et al.*, 1998; Cuschieri and Wentzensen, 2008). However, it is not sufficient for the determination of active viral infection in HPV-positive HNC and it is recommended to use the combination of immunohistochemical detection of the p16 and detection of HPV DNA (Rotnaglova *et al.*, 2011; Hoffmann *et al.*, 2012) with the consideration of the tumor location (Lukešová, 2014).

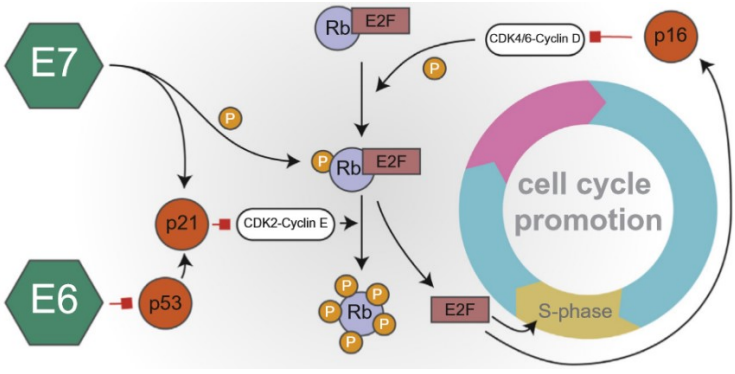

Figure 2-7: Main mechanisms of HPV-induced oncogenesis (adapted from Polanska *et al.*, 2014).

Stransky *et al*. performed a whole-exome sequencing analysis of head and neck tumors of both etiologies and revealed that the mutation rate of HPV-positive tumors was

approximately half of that found in HPV-negative tumors (Stransky *et al.*, 2011). Another large-scale sequencing analysis performed by Agrawal *et al*. revealed fourfold difference in the mutation rate (Agrawal *et al.*, 2011). The discrepancy in the results may arise from the bioinformatics technologies or methodological nuances, however, the results of both studies are consistent with the notion of biological differences between these two types of HNC etiologies.

The main molecular differences between HPV-positive and HPV-negative head and neck tumors and their frequencies are summarized in Table 2-1. In HPV-positive tumors, a broad deletion in chromosome 11q has been detected including region of tumor suppressor gene *ATM* participating in DNA repair and control of the progression of cell cycle (Hayes *et al.*, 2015). The inactivation of the gene *TRAF3* has been revealed in approximately 20 % of HPV-positive tumors which is implicated in the immune response to the viral infection (Oganesyan *et al.*, 2006). Moreover, this gene is also a component of the NF-κB signaling pathway implicated in cell survival (Hacker *et al.*, 2011). HPV-associated tumors display the evidence of induction of apoliprotein B mRNA editing enzyme catalytic popypeptide-like (APOBEC) family of proteins (Henderson *et al.*, 2014). These enzymes catalyze the deamination of cytosine and mediates mutations in genes, including proto-oncogene *PIK3CA*, which plays role in PI3K/AKT/mTOR pathway regulating cell cycle or proliferation.

Table 2-1: Prevalence of genetic alterations in notable genes in HPV-associated and HPV-negative tumors (adapted from Cleary *et al.*, 2016).

	HPV+	HPV-
ATM	very common	common
CCND1	rare	very common
CDKN2A	rare	most
EGFR	rare	common
FGFR1	rare	common
NOTCH1	common	very common
p53	rare	most
PIK3CA	very common	common
Rb	rare	rare
TRAF3	very common	rare

Rare defined as < 5 %, common 10-20 %, very common 20-50 %, most > 50 %.

Very common genetic alteration in HPV-negative tumors is an increase of the copy number of gene for cyclin D1 (*CCND1*) and deletion of cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*) involved in the cell cycle regulation leading to its progression (Lechner *et al.*, 2013; Seiwert *et al.*, 2015). Amplifications of EGFR and receptors for fibroblast growth factor (*FGFR1*) are also predominated in HPV-negative tumors (Sok *et al.*, 2006; Cancer Genome Atlas Network, 2015). Their tyrosine kinase activity initializes the signaling pathways leading to cell proliferation and tumor progression. Another alteration in head and neck tumors is loss-of-function mutations in *NOTCH1* gene playing role as a tumor suppressor. This leads to amplification of transcription factor TP63 regulating cell growth, proliferation or cell adhesion (Candi *et al.*, 2007; Agrawal *et al.*, 2011).

2.3 MicroRNA

MicroRNAs (miRNAs) are a class of short, single-stranded and noncoding RNAs with the length ~21 nucleotides that play a very important role in the post-transcriptional

regulation of gene expression (Bartel, 2004). MiRNAs are involved in many cellular processes, such as development, cell growth, differentiation processes, survival, or regulation of apoptosis in a variety of eukaryotic organisms, and the deregulated expression of miRNAs has been observed in many types of human cancer (Calin *et al.*, 2002; Ambros, 2004; Bartel, 2004; Lu *et al.*, 2005; He *et al.*, 2005). It is suggested that the expression of at least 60 % of human protein-coding genes is regulated by miRNAs since they comprise conserved sites for miRNA binding (Friedman *et al.*, 2009).

The investigation of miRNAs dates back into 1990s, when Victor Ambros with his colleagues Rosalind Lee and Rhonda Feinbaum discovered that the gene *lin-4* does not encode a protein but produces a pair of small RNAs (Lee *et al.*, 1993). Afterwards, they showed that these RNAs had antisense complementarity to the multiple sites in the 3′untranslated region of *lin-14* gene, which fact leads to the repression of the *lin-14* expression involved in the development of the *Caenorhabditis elegans* (Lee *et al.*, 1993; Wightman *et al.*, 1993). Several years later, the discovery of *let-7* small RNA controlling the expression of other genes involved in C. *elegans* development supported these observations (Reinhart *et al.*, 2000). Shortly afterwards in 2001, the term miRNA was used at first in several related papers published in Science (Lee and Ambros, 2001; Lau *et al.*, 2001; Lagos-Quintana *et al.*, 2001). Since that time, up to 48 885 mature miRNA products were identified across 271 species, from that 2 693 mature miRNAs and more than 1 984 precursor hairpins have been identified in humans (miRBase 22 launched in March 2018, http://mirbase.org).

2.3.1 Biogenesis of miRNAs

Genes encoding miRNAs are located throughout the whole genome. Most of the miRNA genes are noncoding and miRNA is the only product. However, miRNAs genes located within the intron or in the untranslated region (UTR) of the gene coding protein, and/or within the exon of mRNA-like noncoding RNAs (mlncRNAs) have been also found (Rodriguez *et al.*, 2004). MiRNAs are not usually located within the coding exons since the excision of the miRNA would lead to the loss of the coding protein transcript. MiRNAs are grouped into clusters that include physically adjacent miRNA genes transcribed from the same primary miRNA (explained below), and to families, which include miRNAs based on the sequence similarities.

Most of the miRNA families undergo the canonical biogenesis pathway converting primary miRNA transcript to the mature miRNA (Figure 2-8). The miRNA genes are transcribed mostly by RNA polymerase II (RNAP II) or RNA polymerase III (RNAP III) in the nucleus of the cell as a primary miRNA transcript (pri-miRNA) (Lee *et al.*, 2004; Borchert *et al.*, 2006). The pri-miRNAs have a stem-loop structure with hundreds of nucleotides in length and may contain monocistronic transcript producing single miRNA or polycistronic cluster coding two or more miRNAs. The pri-miRNAs are typically spliced and have 7′methylguanosine cap on the 5′end and polyA structure on the 3′end (Calin *et al.*, 2004). Further, the long pri-miRNA is processed by a microprocessor complex including RNAse III family enzyme Drosha and its cofactor DiGeorge syndrome critical region 8 protein (DGCR8), which generates 60 - 70 nucleotides long stem-loop precursor miRNAs (pre-miRNAs). Drosha contains two tandem RNAse III domains - RIIIa and RIIIb, and double-stranded RNA-binding domain crucial for the processing (Han *et al.*, 2004). The pre-miRNA is then exported out of the nucleus to cytoplasm by Exportin 5 (Xpo5), a RanGTP-dependent protein, which mediates the export of pre-miRNAs with simultaneous hydrolysis of guanosintriphosphate (GTP) bounded on the cofactor Ran (Yi *et al.*, 2003; Bohnsack *et al.*, 2004).

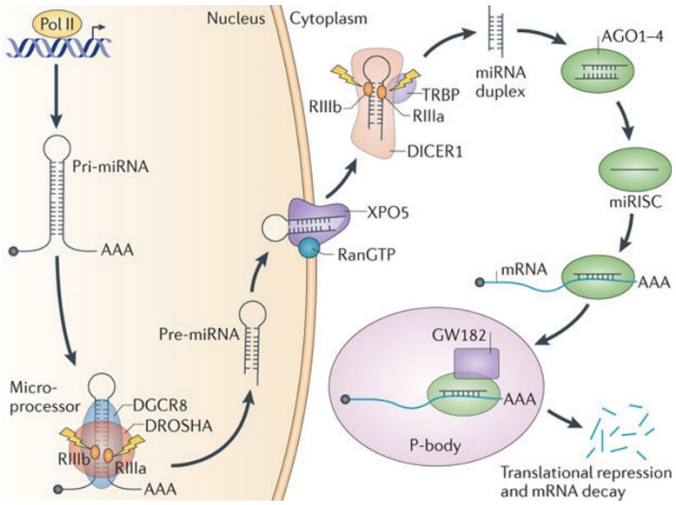

Figure 2-8: Canonical miRNA biogenesis pathway (S. Lin and Gregory, 2015).

The second part of the miRNA biogenesis occurs in the cytoplasm. The pre-miRNA is further cleaved by the complex composed of RNase III endonuclease (DICER1) which leads to transactivation of the RNA-binding protein (TRBP) and kinase-R-activating protein (PACT) response generating a mature 20-25 nucleotide long

miRNA duplex with 2-nucleotide 3´overhangs consisting of a guide strand referred as miRNA, and passenger strand referred as miRNA* (Chendrimada *et al.*, 2005; Lee *et al.*, 2006; Feng *et al.*, 2012). The guide miRNA strand is then bounded to the Argonaute proteins 1-4 (Ago 1-4) to form the final RNA-induced silencing complex (miRISC) which guide the mature miRNA to their target genes for post-transcriptional regulation. The miRNA* strand is typically degraded, however, for some miRNAs, both strands generated from the miRNA-miRNA* duplex can be loaded to the miRISC. The strand generated from the 5´end of the precursor is then noted as 5p, and the strand from 3´end as 3p. Consequently, one miRNA gene produces two different mature miRNAs with different targets, and, therefore, different biological functions. The more frequent strand loaded into the miRISC depends also on the cell type.

The Ago 2, mostly found in humans, is supported by the GW182 protein family (Gregory *et al.*, 2005; Rehwinkel *et al.*, 2005), and has a RNA cleavage activity, which facilitates the seeking of the target mRNA. For the target association, 2-7 nucleotides of miRNA known as “seed” region is important (Grimson *et al.*, 2007). Depending on the degree of complementarity, the binding of the target gene, which mostly occurs in the 3´untranslated region, induces the translation inhibition or degradation of the target mRNA occurring in the specific cytoplasmic foci called P-bodies containing untranslated mRNA (Liu *et al.*, 2005).

Beside the most common canonical pathway of miRNA biogenesis, some alternative pathways have been described. The first one is the case of miRNA genes called miRtrons that are encoded within the intronic region of the genes (Ruby *et al.*, 2007; Okamura *et al.*, 2007; Berezikov *et al.*, 2007). In this pathway, the step including Drosha processing of pri-miRNA into pre-miRNA is bypassed. Instead, the miRtron is spliced by the spliceosome and debranching enzymes to generate pre-miRNA. This miRtron-derived pre-miRNA is further processed in the same way as in the canonical pathway. Other alternative pathway is Dicer-independent where the pre-miRNA is at first classically generated by microprocessor complex and exported to the cytoplasm, but later the precursor is directly bound by Ago 2 cleaving the star strand and the mature miRNA trimmed by 3´-5´exonuclease poly(A)-specific ribonuclease (PARN) is loaded on the miRISC (Cifuentes *et al.*, 2010; Cheloufi *et al.*, 2010; Yoda *et al.*, 2013). The combination of both-mentioned alternative pathways is used by miRNAs called simtrons (splicing-independent miRtron-like miRNAs) whose biogenesis is independent on

DGCR8, DICER, Xpo5, or Ago proteins. The synthesis of simtrons is reduced only in the absence of Drosha (Havens *et al.*, 2012).

2.3.2 Mechanisms of miRNA function

The mechanism of miRNA function and binding to the target sequences differ in animals and plants. With animals, miRNA binds to the mRNA target site with partial base pairing which mostly leads to the translational repression with a little or no influence on the abundance of mRNA. With plants, miRNAs perfectly base pairs with their targets which induces the cleavage and degradation of mRNA. Nevertheless, despite these differences in target recognition, there is the evidence that miRNA can induce the mRNA degradation in animals through the interaction with additional proteins, and that the translational inhibition occurs also in plants, but the underlying mechanisms are not yet known (Huntzinger and Izaurralde, 2011). Furthermore, some evidence about posttranscriptional activation of translation caused by miRNA function has been published in recent years (Ørom *et al.*, 2008).

In the case of plants, the fully or almost complementary target sites of miRNAs are localized in coding exons as well as in 3′ UTR (Jones-Rhoades and Bartel, 2004). The cleavage is mediated by PIWI domains of Ago proteins in the miRISC which have RNase activity and cleave the target mRNA in the position facing nucleotides 10 and 11 of the miRNA (Ameres and Zamore, 2013). As for animals, the majority of miRNAs forms partial duplexes with the 3′ UTR of their target sequences (Jones-Rhoades and Bartel, 2004). However, the interaction with the 5′ UTR has been also identified (Ørom *et al.*, 2008). The complementarity is localized in 2-7 nucleotides long region on the 5′ end of the miRNA, referred as “seed” region. Such partial complementarity prevents the cleavage activity of the miRISC and additional effector proteins for the translational repression or mRNA decay are then required (Bartel, 2009).

2.3.2.1 Translational inhibition

The exact mechanisms of how miRNAs block protein production are still intensively studied, but there is an evidence for the inhibition of translational initiation or elongation as well as for direct proteolysis of the synthetized peptide. Several factors are required for the initiation and the correct continuation of translation (reviewed in

Jackson *et al.*, 2010). Messenger RNAs competent for the translation have the 5′ cap structure and 3′ polyA tail. The cytoplasmic polyA-binding protein (PABP) associated with the 3′ polyA interacts with the eukaryotic translation-initiation factor 4G (eIF4G) which through 5′ cap-binding protein eIF4E stabilizes the mRNA, protects it from the degradation and recruits the pre-initiation complex. Protein GW182, which is the component of the miRISC interacts with the PABP and deadenylase complexes CCR4-NOT promoting shortening of the polyA tail and dissociation of PABP from the target mRNA (Zekri *et al.*, 2009; Zekri *et al.*, 2013). This results in the break of the loop structure necessary for the initiation of translation and miRNA-mediated repression of the translation. The other mechanism how protein GW182 mediates the translational repression is its recruitment of downstream translational repressors such as DEAD-box RNA helicase (DDX6) or elongation factor 4E-binding protein 4E-T (Su *et al.*, 2011; Kamenska *et al.*, 2014). Mechanisms independent of the GW182 have been also demonstrated. It has been shown that miRNAs mediate the dissociation of ATP-dependent RNA helicase eIF4A from the cap-binding complex eIF4F which leads to the inhibition of ribosome binding and/or ribosomal scanning (Fukao *et al.*, 2014).

The assumption that the translation might be repressed at the post-initiation stage was supported by the evidence that miRNAs and their targets were found associated with translating polysomes (Maroney *et al.*, 2006; Nottrott *et al.*, 2006). Nottrott *et al*. suggested that specific proteases or factors recruiting proteases might be associated with miRISC leading to the inhibition of nascent polypeptide chain elongation (Nottrott *et al.*, 2006) whereas Petersen *et al*. proposed that miRNAs induce ribosome drop off (Petersen *et al.*, 2006). The support of the idea that miRNAs regulate translation after its initiation has been provided through the observation that repression of translation occurs also during translation initiated independently on the cap structure through an internal ribosome entry site (IRES) allowing cap-independent association of ribosome with mRNA (Petersen *et al.*, 2006).

In plants, miRNAs were initially thought to mediate the gene silencing only through the endonucleolytic activity of Ago proteins since they bind to the fully complementary target sequences and they lack GW182 homolog proteins. Although several reports suggesting translational repression in addition to cleavage have been published (Brodersen *et al.*, 2008; Yang *et al.*, 2012; Li S. *et al.*, 2013), detailed molecular roles of participating proteins in plant miRNA pathways still need to be elucidated.

2.3.2.2 mRNA degradation

The mRNA degradation is the main mechanism of miRNA-mediated posttranscriptional regulation of gene expression in plants. In animal cells, where the complementarity between miRNA and target mRNA is only partial, the mechanism exists but it is less frequently used. In such regulation, miRNAs direct their targets to the cellular 5′-to-3′mRNA decay pathway initiated by the removal of the polyA tail by CAF1-CCR4-NOT and PAN2-PAN3 deadenylase complexes recruited to the target mRNA by GW182 protein through its two conserved motifs (Fabian *et al.*, 2011). Target mRNA is then processed by the decapping enzyme DCP2 and its cofactors recruited by miRISC (Nishihara *et al.*, 2013), and the decapped mRNA is degraded by the major cytoplasmic 5′-to-3′ exonuclease XRN1. This degradation is done in cytoplasmic processing granules known as P-bodies which function as the sites for mRNA decay because they are enriched for the decapping enzymes and other proteins involved in 5′-3′degradation (Sheth and Parker, 2003; Cougot *et al.*, 2004). The P-bodies also do not contain the ribosomal component and may serve as a mRNA storage without translation (Aizer *et al.*, 2014).

Contrary to animal miRNAs, plants miRNAs do not undergo deadenylation but they direct the endonucleolytic target mRNA cleavage through action of Ago proteins (Ameres and Zamore, 2013). Afterwards, the 3′ end of 5′-cleaved fragments are uridylated by terminal uridylyl transferases (TUTases) and are accumulated in P-bodies (Xu *et al.*, 2016). After the decapping, the 5′-cleaved fragments might be degraded in 5′-3′ decay through the exonucleases XRN or in 3′-5′ decay through exonucleolytic complex called exosome (reviewed in Souret *et al.*, 2004; Eulalio *et al.*, 2007). The 3′-cleaved fragments resulting from the endonucleolytic cleavage contain a 5′-monophosphate (instead of 5′cap) and are degraded also by the 5′-3′exonuclease XRN4.

2.3.2.3 Translational activation

Although the function of most miRNAs has negative effect on the mRNA translation, the positive regulation of translation has been also observed. Vasudevan *et al.* revealed in their study that miR-369-3 directs the Ago protein and fragile X mental

retardation-related protein 1 (FXR1) to the AU-rich elements (AREs) of the tumor necrosis factor α (TNFα) and thus activate the translation (Vasudevan *et al.*, 2007). Moreover, Ørom *et al*. reported an association of miR-10a with the 5´untranslated region of mRNA encoding ribosomal proteins which enhanced their expression (Ørom *et al.*, 2008). However, despite such evidence, the miRNA-associated activation of mRNA translation is poorly understood.

2.3.3 miRNA and cancer

Cancer is a condition of cells with abnormal growth and deregulated expression of genes, including miRNAs. MiRNAs influence many steps in tumor development, such as tumor growth, invasion, angiogenesis or formation of metastasis.

The evidence of the role of miRNAs in human tumors was at first reported in 2002 by Calin *et al*. (Calin *et al.*, 2002), who studied a region on chromosome 13q14, frequently found deleted in B cell chronic lymphocytic leukemias (B-CLL). They reported that this region contains two polycistronic miRNA genes, miR-15 and miR-16, and showed that the deletion of this region correlates with the downregulation of these miRNAs expression. Following this study, they mapped 186 miRNAs and showed that miRNA genes are frequently located in cancer-associated genomic regions or in fragile sites (Calin *et al.*, 2004), and they first provided a list of miRNA genes that may play role in cancer.

The fact that miRNAs posttranscriptional regulate gene expression implies their dual role in tumors. First, miRNA genes which are often amplified in tumors negatively, regulate the expression of crucial tumor suppressors genes and thus mediate tumor progression. These miRNAs are commonly denoted as oncomiRs. On the other hand, miRNAs normally maintain the right function of cell cycle progression and negatively regulate oncogene expression. These miRNA genes are frequently located in fragile sites and are often deleted or mutated in tumor cells.

Contrary to mRNA profiles, it has been shown that tumors as well as normal tissues of different origin have specific miRNA expression signatures called miRNome (Lu *et al.*, 2005; Volinia *et al.*, 2006). This fact of uniqueness provides to miRNAs a great potential to be useful biomarkers for diagnosis of cancer. Furthermore, specific miRNA profiles allow to distinguish between different tumor subtypes, for example, in human

breast cancer (Blenkiron *et al.*, 2007), in non-small-cell lung carcinoma (Lebanony *et al.*, 2009), or in ovarian cancer (Iorio *et al.*, 2007).

2.3.3.1 Mechanisms of miRNA deregulation in cancer

During the recent years, the underlying mechanisms of miRNA deregulation in cancer have become clearer, including structural genetic alterations, transcriptional control changes, epigenetic regulation and/or defects in miRNA biogenesis machinery.

The aberrant miRNA expression in tumors is often caused by chromosomal abnormalities of miRNA genes such as amplifications, deletions or translocations. As mentioned above. Calin *et al*. showed that more than half of miRNA genes are located in genomic regions that are altered in cancer (breakpoint regions and fragile sites, amplified regions, common sites for translocation, deletion or viral integration) (Calin *et al.*, 2004; reviewed in Calin and Croce 2006). High proportion of DNA copy number alterations was determined also by Zhang *et al*. in ovarian cancer, breast cancer and melanoma (Zhang *et al.*, 2006). Although the point mutations or single nucleotide polymorphisms (SNP) in miRNA coding genes have been detected at very low level (Saunders *et al.*, 2007), several studies have indicated that these events may cause the deregulation of miRNA expression, for example in prostate (Xu *et al.*, 2010) or breast cancer (Li *et al.*, 2009).

Other mechanism of miRNA expression deregulation is mediated by transcription factors activity, such as c-Myc or p53. O´Donnell *et al*. revealed that c-Myc activates the expression of the oncogenic cluster *miR-17-92* and thus regulates the cell proliferation and apoptosis (O'Donnell *et al.*, 2005). Later, the same group revealed the widespread downregulation of tumor suppressive miRNA expression, such as miR-15a, miR-26, miR-29, miR-30 or let-7 family, induced by oncogenic c-Myc (Chang T.C. *et al.*, 2008). Other example of c-Myc function is the regulation of tumor suppressive miR-122 whose disruption results in development of hepatocellular carcinoma (Wang B. *et al.*, 2014). Contrary to c-Myc, transcription factor p53 has tumor suppressive role. The most significant miRNA family regulated by this protein is mir-34, which promotes cell cycle arrest, cell senescence and apoptosis (He *et al.*, 2007; Hermeking, 2010). Other studies described additional miRNAs regulated by p53, such as miR-107, miR-200, miR-192 or miR-605 regulating angiogenesis, epithelial-to-mesenchymal transition (EMT) and/or expression of ligase MDM2 (Yamakuchi *et al.*, 2010; Pichiorri *et al.*, 2010; Chang *et al.*,

2011; Xiao *et al.*, 2011). Since p53 is often lost or mutated in cancer, miR-34 and other miRNAs regulated by this protein are frequently downregulated in tumor cells.

Just as protein coding genes, miRNA coding genes are regulated also by epigenetic modifications, such as DNA methylation or histone acetylation. Hypermethylation of CpG islands in promotors is one of the most common cause of the loss of tumor suppressive miRNA expression leading to the development of cancer (reviewed in Lopez-Serra and Esteller, 2012). For an example, Fazi *et al*. revealed that fusion protein AML1/ETO triggers heterochromatic silencing of miR-223 expression by CpG methylation (Fazi *et al.*, 2007). Saito *et al*. showed increased expression of several miRNAs after treatment of bladder cancer cells with chromatin modifying inhibitors (Saito *et al.*, 2006). Further, Scott and his colleagues revealed rapid alteration of miRNA levels followed histone deacetylase inhibition in breast carcinoma cells (Scott *et al.*, 2006). Overall, using epigenetic drugs offers the possibility to restore the expression of tumor suppressive miRNAs and, through this process, to treat the tumor.

As mentioned above, the biogenesis pathway of miRNAs is a multistep process including many regulating proteins and enzymes. Therefore, mutations or aberrant expression of these components could lead to changes in miRNA expression and thus contribute to tumor development. The key RNase III endonucleases Drosha and DICER have been found deregulated in tumors. Walz *et al*. reported mutations of Drosha and its cofactor DGCR8 in 15 % of Favorable Histology Wilms Tumors (FHWT) leading to significant decrease in expression of mature let-7a and mir-200 family (Walz *et al.*, 2015). The endonuclease DICER was found upregulated in 81 % of prostate adenocarcinoma leading to global increase of miRNA expression (Chiosea *et al.*, 2006), in precursor lesions of lung adenocarcinoma (Chiosea *et al.*, 2007) and was significantly associated with aggressive form of breast cancer (Caffrey *et al.*, 2013). On the other hand, Karube *et al*. revealed reduced expression of DICER in lung tumors associated with poor prognosis (Karube *et al.*, 2005). Other essential components of miRNA biogenesis machinery are Ago proteins. Zhang *et al*. published a study revealing upregulated Ago2 expression in gastric cancer (Zhang *et al.*, 2013), contrary to Völler *et al*. who identified downregulation of Ago2 expression in melanoma (Voller *et al.*, 2013). Further, export of miRNAs from nucleus to cytoplasm is provided by XPO5 which was found mutated in a subset of human tumors with microsatellite instability (Melo *et al.*, 2010). This genetic defect traps pre-miRNAs in the nucleus and avoids the miRNA inhibiting function.

2.3.3.2 MiRNAs as biomarkers in clinical management of cancer

There is number of characteristics explaining the meaning of the word biomarker as a shortcut for biological marker. The Biomarkers Definition Working Group convened by National Institute of Health (NIH) defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). In terms of cancer research there is an effort to identify useful and reliable biomarkers to provide diagnostic, prognostic and therapeutic information. Several biomarkers are nowadays widely used in clinical practice, such as prostate-specific antigen (PSA) for prostate cancer, human epidermal growth factor receptor 2 (HER2) for breast cancer or α-fetoprotein (AFP) for liver cancer. Nevertheless, there is still a need for biomarkers that could detect early stages of cancer and, based on their presence/absence the individual treatment, could be applied and/or the prognosis of patient´s could be predicted.

As mentioned above, miRNA expression profiles are specific for tumors of different origin, therefore, they have a potential to be useful and sensitive biomarkers. Moreover, due to their small size, miRNAs are more stable than other molecules like long mRNAs (Jung *et al.*, 2010) and are less prone to degradation caused by processing and storage conditions.

First, miRNAs as biomarkers are usable in connection with the precise diagnosis of the primary tumor site since it improves the patients´ outcome and reduces the treatment costs. For example, Feraccin *et al*. performed the study using miRNA profiling as a tool for identification of cancer with unknown primary tissue-of-origin from metastases (Ferracin *et al.*, 2011). Overall, they reached 100% accuracy for primary cancers and 78% for metastasis. Selection of better treatment strategy can be also based on the precise determination of tumor subtype specified by miRNA profiles. Several studies on breast cancer (Blenkiron *et al.*, 2007), ovarian cancer (Iorio *et al.*, 2007), renal cancer (Youssef *et al.*, 2011) or lung cancer (Gilad *et al.*, 2012) have been published distinguishing tumor subtypes by miRNAs expression pattern. Furthermore, the patients´ survival and prognosis depends on the stage of tumor at the time of diagnosis which is possible to determinate by profiling of miRNA expression. Du Rieu *et al*. performed a study indicating that overexpression of miR-21 and miR-205 precedes the phenotypic changes in the pancreatic ducts, which suggests they could function as biomarkers for the

early diagnosis of pancreatic ductal adenocarcinoma (du Rieu *et al.*, 2010). In addition, miRNAs can serve as biomarkers for prediction of patients´ prognosis. For example, Hui *et al*. processed data from The Cancer Genome Atlas database (TCGA) and identified miRNAs potentially associated with survival of patients with head and neck cancer (Hui *et al.*, 2016). Finally, miRNA signatures serve also as predictors of the response to the tumor treatment allowing for timely modifications of the treatment (Giovannetti *et al.*, 2010; Xu *et al.*, 2014).

Besides their presence in solid tumors, miRNAs have been also found and isolated from body fluids such as blood, plasma, serum, saliva, urine, and other, or circulating exosomes (Mitchell *et al.*, 2008; Taylor and Gercel-Taylor, 2008; Michael *et al.*, 2010; Hanke *et al.*, 2010; Wang J. *et al.*, 2014) and they are called circulating miRNAs. In this extracellular environment, miRNAs are associated with RNA-binding proteins such as argonaute proteins, ribosomal proteins, or lipoprotein particles, which protect them from nucleases (Turchinovich *et al.*, 2011; Arroyo *et al.*, 2011; Vickers *et al.*, 2011; Ashby *et al.*, 2014). Moreover, miRNAs can be transported in the endosome-derived extracellular vesicles called exosomes (Valadi *et al.*, 2007). Since it has been observed that the alteration of miRNA expression in tumors is reflected also in miRNA profiles from exosomes, exosomal miRNAs, as well as other circulating miRNAs, seems to be suitable candidates for non-invasive cancer biomarkers (Taylor and Gercel-Taylor, 2008; Schwarzenbach, 2015). Nevertheless, the methods for isolation of circulating miRNAs are still challenging since there is a low level of miRNA fraction in body fluids and it is difficult to separate the exosomes with miRNAs from the rest of other extracellular vesicles. Further, in study of Chevillet *et al*., the most abundant exosomal miRNAs were present on average in less than one copy per exosome (Chevillet *et al.*, 2014). Thus, the improvement and validation of extraction methods of circulating miRNAs is current hot topic of biomarkers´ research.

2.3.3.3 MiRNA profiling studies

MiRNA profiling is based on the measurement of the relative abundance of miRNA expression specific for different species, biological origin or the current state of cells. Nowadays, the main methods for miRNA profiling include reverse transcription quantitative real-time PCR (RT-qPCR) based approaches, microarrays, next-generation sequencing (NGS) based platforms, and/or others (reviewed in Pritchard *et al.*, 2012;

Chugh and Dittmer, 2012; Tian *et al.*, 2015). Each of these methods has its advantages, disadvantages, and limitations, and is suitable for different applications (Mestdagh *et al.*, 2014). One of the important factors influencing the outcome of the miRNA profiling studies is the type of analyzed material; cell lines, fresh frozen (FF) tissues, and formalin-fixed paraffin-embedded (FFPE) tissues, blood or other body fluids. Besides the material preparation, very important for miRNA expression studies is the data analysis, for which there exist a whole range of software programs and different statistical approaches.

The global miRNA profiling of cancers has been extensively studied in clinical samples as well as in cancer cell lines. The aim is to uncover the changes occurring during tumor development and to increase the knowledge about gene regulation of particular disease. However, the published profiling studies are often inconsistent due to the different methodological and data analysis approaches, character of samples or etiology of the studied tumors, and thus, it is difficult to make some precise, unique and usable conclusion about the miRNA gene regulation of the disease. It is, therefore, necessary to standardize all steps in the miRNA profiling processes.

In my thesis, I focus on cervical cancer and head and neck cancer as two types of tumors, both etiologically linked to HPV infection - cervical cancer in almost 100 % of cases, in the case of HNC, in our previous study, HPV infection was associated with more than 60 % of oropharyngeal carcinomas (Tachezy *et al.*, 2009). Thus, these tumors serve as unique model for the study of cancer of the same anatomical location caused by different molecular mechanisms. Several studies focused on miRNA profiling of these tumors have been published recently (Hui *et al.*, 2010; Pereira *et al.*, 2010; Lajer *et al.*, 2012; Wang X. *et al.*, 2014), however, these lack the information about the etiology of the studied tumors. The published miRNA profiling studies of cervical cancer and HNC are discussed in more details in chapter 6.

2.3.3.4 HPV-encoded miRNAs

During tumor development and progression, the host´s miRNA expression is altered. Furthermore, several viruses contributing to the oncogenesis encode their own miRNAs whose expression might influence the cellular mechanisms as well as viral life cycle. Unlike HPV, several groups of human oncoviruses encode their viral specific miRNAs (Epstein-Barr Virus, human herpesvirus 8, Merkel Cell Polyomavirus, or

hepatitis C virus). Two research groups have suggested the existence of HPV-encoded miRNAs (Qian *et al.*, 2013; Weng *et al.*, 2017). First, Qian *et al*. have validated four miRNAs and predicted their target genes suggesting their role in cell cycle regulation, cell adhesion and migration, or development of cancer (Qian *et al.*, 2013). Several years later they detected their expression in cervical samples at very low level (Virtanen *et al.*, 2016). Recently, Weng *et al*. have developed genome-wide sequence analyses and predicted other HPV-encoded miRNAs by bioinformatics approaches (Weng *et al.*, 2017). However, no studies of *in vivo* function of these miRNAs have been published yet. This research area is still unexplored and additional investigation is needed.

2.3.4 MiRNAs for clinical use

As mentioned above, because of their characteristics miRNAs may serve as suitable biomarkers for many biological conditions and have a potential to be used not only for diagnostics but also as a tool for therapy. There are three therapeutic approaches usable for the main required outcomes - the sequestration of upregulated miRNAs by artificial miRNA-binding sites, the inhibition of oncogenic miRNAs by small molecules, or by chemically modified oligonucleotides (Figure 2-9). The first approach utilizes specific miRNA constructs called miRNA sponges (Ebert *et al.*, 2007; Ebert and Sharp, 2010) (Figure 2-9a) containing multiple artificial complementary sequences or binding sites which after the introduction into cells prevent the interaction of nature endogenous miRNAs. The typical miRNA sponge contains 4-10 miRNA binding sites. The second method is the use of synthetic oligonucleotides complementary to endogenous miRNAs, the so-called as antisense oligonucleotides, antagomiRs or anti-miRs which bind miRNAs and block their regulatory function (Figure 2-9b) (Krutzfeldt *et al.*, 2005; Krutzfeldt *et al.*, 2007). Further, the synthetic oligos function also as miRNA mimic when they substitute the mature downregulated miRNA and bind its targets. Since these oligonucleotides are very unstable, they need to be chemically modified, for example by adding of 2′-O-methyl or -methoxyethyl groups on ribose or phosphorothioate modifications which increase their resistance to cellular nucleases. The third approach is the application of small molecules that inhibit or influence various steps of miRNA biogenesis or expression and the mature miRNA is thus functionally incompetent (Velagapudi *et al.*, 2014) (Figure 2-9c). Important question of this research field is the delivery of therapeutics into the cell which can be based on viral transfer or non-viral strategy using polymer-based

nanoparticles or liposomes (Ben-Shushan *et al.*, 2014; Wang *et al.*, 2015). However, all of these strategies have some limitations, such as the reaction of immune system to the antagomiRs, occurrence of toxic manifestation, effectivity of the therapeutic delivery or assurance of sufficient absorption of the therapeutics into target cells.

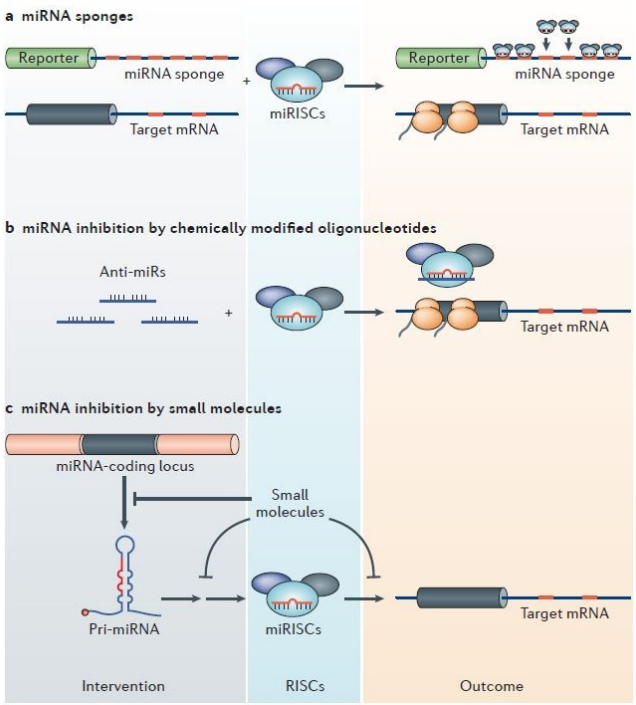

Figure 2-9: Main therapeutic strategies based on miRNAs (Li and Rana, 2014).

Despite these difficulties, the therapeutics based on antisense oligonucleotides are tested in preclinical and clinical trials. Only one miRNA therapeutic called Miravirsen entered clinical trial phase II (Janssen *et al.*, 2013). It is locked nucleic acid-modified (LNA-modified) antisense inhibitor of miR-122, which is liver-specific miRNA playing role in Hepatitis C Virus (HCV) infection (Gebert *et al.*, 2014). Another therapeutic in clinical trial is MRX34 functioning as a delivery system for miR-34 mimic and substituting the function of this tumor suppressor miRNA (Beg *et al.*, 2017). This therapeutic may be used in variety of tumors. However, the phase I of clinical trial was put to halt in 2016 because of serious immune responses (ClinicalTrials.gov; NCT number NCT01829971).

3. AIMS OF THE THESIS

The thesis focuses primarily on the analysis of miRNA expression in HPV-associated and HPV-independent head and neck tumors as a unique model of carcinomas with viral and non-viral etiology. The content of the submitted doctoral thesis is a result of many years research on human papillomaviruses performed by the group led by RNDr. Ruth Tachezy, Ph.D., now working in the Laboratory of Molecular and Tumor Virology in BIOCEV in Vestec. The laboratory closely cooperates with clinical departments, mainly those in Motol University Hospital, Prague, which allows for broadening of the studies and inclusion of clinical samples as well.

When I joined the laboratory as a doctoral student, the research of miRNAs was at its beginning without much experience in this field. The research had been supported by the grant from Czech Science Foundation (GACR) in 2012-2015, together with the grant supported by Charles University (GAUK) in 2014-2015, on which I participated as the principal researcher.

The main objectives of the thesis were postulated as follows:

- preparation and detailed characterization of samples for miRNA analysis
- detailed characterization of the studied tumors including the analysis of the status of HPV genome
- characterization of miRNA expression profiles in HPV-positive and HPV-negative tonsillar tumors and in cervical carcinomas
- characterization of miRNA expression profiles in the model system of human keratinocyte clones
- analysis of the received data and their evaluation
- technical validation of the expression of selected miRNAs
- confirmation of the differentially expressed miRNAs on the enlarged group of samples
- introduction of the *in situ* hybridization as a method for the demonstration of the miRNA localization in the cell

4. MATERIAL AND METHODS

The project was realized in cooperation with several clinical departments, namely Department of Otolaryngology and Head and Neck Surgery, 1st Medical Faculty Charles University and Motol University Hospital in Prague, and Department of Obstetrics and Gynecology, 2nd Medical Faculty Charles University and Motol University Hospital in Prague, both of which provided the clinical samples of tonsillar and cervical tumors and also healthy tissues. The primary and immortalized human keratinocyte clones were provided in cooperation with the University of Iowa, Iowa City, USA. The rest of the material was provided by the archives of Department of Experimental Virology in the Institute of Hematology and Blood Transfusion, Prague, which also provided most of the instrumental equipment. Moreover, we also cooperated with the Institute of Pathological Physiology, 1st Medical Faculty Charles University, Prague, with the International Agency for Research on Cancer in Lyon in France, which gave us the opportunity to use their equipment for miRNA expression analysis, and with the Ruđer Bošković Institute in Zagreb in Croatia.

During the work on the thesis, following methods were used at most:

- isolation of DNA and total RNA from fresh frozen samples, formalin-fixed paraffin-embedded samples, and cell lines
- chip electrophoresis, PCR/RT-PCR/RT-qPCR, gel extraction, sequencing
- reverse line blot (RLB) hybridization
- mapping of E2 integration breakpoint
- Amplification of Papillomavirus Oncogene Transcripts (APOT) assay, Southern blot
- TaqMan Low Density Array (TLDA) analysis
- use of miRNA analysis software (Expression Suite, GeneSpring, MeV, Genex)
- *in-situ* hybridization

5. RESULTS

5.1 List of publications related to the thesis

Analysis of the integration of human papillomaviruses in head and neck tumours in relation to patients´ prognosis

Zuzana Vojtechova, Ivan Sabol, Martina Salakova, Lubomir Turek, Marek Grega, Jana Smahelova, Ondrej Vencalek, Eva Lukesova, Jan Klozar and Ruth Tachezy

Int J Cancer. 2016 Jan 15;138(2):386-95. doi: 10.1002/ijc.29712.

IF_{2016} 6.513

In this study we focused on detailed characterization of the physical status of human papillomaviruses in a set of virus-associated tonsillar tumors, cervical carcinomas and clinically unaffected cervical tissues. Using the method based on the mapping of E2 integration breakpoint, APOT assay, and Southern blot, we determined the percentage of samples with integrated viral genome and with extrachromosomal form of the virus. Furthermore, we determined the type and the exact site of integration. Finally, we performed the survival analysis and revealed which factors influenced the disease specific survival and if it is affected also by the physical status of the viral genome in the cells. It represents one of the few projects studying the process of integration in the samples of head and neck tumors and, till then, it was the first study reporting the lack of influence of HPV integration status in head and neck cancer on disease specific survival of patients.

Contribution of the author: 60 %. I processed all samples and performed mapping of E2 integration breakpoint and APOT assay. Further, I carried out the evaluation of obtained data and wrote the original draft of the manuscript.

Comparison of the miRNA profiles in HPV-positive and HPV-negative tonsillar tumors and a model system of human keratinocyte clones

Zuzana Vojtechova, Ivan Sabol, Martina Salakova, Jana Smahelova, Jiri Zavadil, Lubomir Turek, Marek Grega, Jan Klozar, Bohumir Prochazka and Ruth Tachezy

BMC Cancer. 2016 Jul 4;16:382. doi: 10.1186/s12885-016-2430-y.

IF_{2016} 3.288

In this study we performed TLDA analysis to determine the miRNA expression profiles in HPV-associated and -independent tonsillar tumors together with non-malignant tissues and cervical carcinomas. Further, the miRNA expression profiles were evaluated also in primary and immortalized human keratinocyte clones as a model system and compared with the clinical samples. We defined miRNAs differentially expressed in each group of samples and identified "HPV core" miRNAs specific for only HPV-associated tumors. Finally, we determined several miRNAs as potential prognostic markers for patients with tonsillar tumors. Overall, our results contribute to the identification of useful and clinically relevant biomarkers of head and neck tumors with viral and non-viral etiology.

Contribution of the author: 60 %. I processed and characterized all samples and performed the TLDA analysis. Further, I contributed to the data analysis, performed the technical validation and confirmation of the data. Finally, I prepared the manuscript.

Comparison of the miRNA expression profiles in fresh frozen and formalin-fixed paraffin-embedded tonsillar tumors

Zuzana Vojtechova, Jiri Zavadil, Jan Klozar, Marek Grega, Ruth Tachezy

PLoS One. 2017 Jun 23;12(6):e0179645. doi: 10.1371/journal.pone.0179645.

IF_{2017} 2.806

In this study we performed the comparison of the miRNA expression profiles between FF and macrodissected FFPE tonsillar tumors using TLDA analysis. Further, we analyzed the influence of the used normalization method and software program on the results. We observed low overlap of differentially expressed miRNAs between the two

types of material and the appreciable variation between results evaluated by two types of normalization methods. Our study highlights the importance of using the same type of clinical material for the comparison of the miRNA expression profiles from published studies, and testing and selecting the best-performing normalization method for data analysis.

Contribution of the author: 80 %. I contributed to the design of the study, processed the samples and performed the TLDA analysis. Further, I analyzed the data and confirmed the results. Finally, I wrote the manuscript.

The role of miRNAs in virus-mediated oncogenesis

Zuzana Vojtechova and Ruth Tachezy

Int J Mol Sci. 2018 Apr 17;19(4). doi: 10.3390/ijms19041217.

IF_{2016} 3.226

The review focuses on the oncogenic viruses and summarizes the role of virus-encoded miRNAs by which viruses regulate their own expression or influence the host gene expression and thus contribute to carcinogenic processes. Further, it deals with the role of host´s miRNAs whose expression contribute to the regulation of tumor development. The review also discusses the question of miRNAs in some groups of oncoviruses, including HPV, where the existence of virus-encoded miRNAs is still controversial.

Contribution of the author: 80 %. I contributed to the layout of the review, prepared the original draft of the manuscript and edited the final version.

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RNDr. Ruth Tachezy, Ph.D., the supervisor

5.2 List of publications unrelated to the thesis

Detection of human polyomaviruses MCPyV, HPyV6, and HPyV7 in malignant and non-malignant tonsillar tissues

Martina Saláková, Eva Košlabová, Zuzana Vojtěchová, Ruth Tachezy, and Vojtěch Šroller

J Med Virol. 2016 Apr;88(4):695-702. doi: 10.1002/jmv.24385.

IF_{2016} 1.935

Morphological and proteomic analysis of early stage air-liquid interface biofilm formation in *Mycobacterium smegmatis*

Zuzana Sochorová, Denisa Petráčková, Barbora Sitařová, Karolína Buriánková, Silvia Bezoušková, Oldřich Benada, Olga Kofroňová, Jiří Janeček, Petr Halada, Jaroslav Weiser

Microbiology. 2014 Jul;160(Pt 7):1346-56. doi: 10.1099/mic.0.076174-0.

IF_{2014} 2.557

6. DISCUSSION

The thesis focuses on the miRNA analysis in HPV-associated and HPV-independent tumors. HPVs are related to almost 30 % of tumors associated with infectious agent (Plummer *et al.*, 2016). The most common HPV-associated tumor is cervical carcinoma, which is linked to the viral infection in almost 100 %. Among others HPV-related tumors are those developed in anogenital region, such as vaginal, vulvar, anal, or penile tumors, in which HPV-associated proportion varies between 25-90 % (de Martel *et al.*, 2017). HPV is also etiologically linked to the development of head and neck tumors, which make from 20 to 90 % depending on the geographical location. In my research I focused on the study of tonsillar tumors with double etiology, and cervical carcinomas as the most common HPV-related tumors.

The work started with a very detailed characterization of the samples, including not only the detection and typing of HPV DNA and detection of active infection, but also the determination of the status of the viral genome. The results of the analysis of integration of HPVs was published in 2016 in the International Journal of Cancer (Vojtechova *et al.*, 2016). There are several methodological approaches which might be used for the analysis of HPV genome status. We chose three different methods for our analysis - the mapping of E2 integration breakpoint using a set of specific primers to detect short reverse transcript amplicons that could be also used for more fragmented samples such as FFPE samples; a very sensitive and specific 3´-RACE-based (Rapid Amplification of cDNA Ends) method called APOT assay allowing us to analyze the exact site of integration, to identify the structure of HPV transcripts, and also, to detect the transcripts derived from both extrachromosomal and integrated genomes in the same sample, as well as in the excess of the extrachromosomal HPV copies (Klaes *et al.*, 1999; Vinokurova *et al.*, 2008); and finally the Southern blot, a standard method which allows detection of the samples with multiple tandem repeats of the viral genome (Lace *et al.*, 2011).

Until that time, limited information about HPV integration in HNC had been available. Several studies of integration of HPV in HNC (Koskinen *et al.*, 2003; Smeets *et al.*, 2006; Lace *et al.*, 2011; Deng *et al.*, 2013; Olthof *et al.*, 2014; Gao *et al.*, 2014) indicated that around 50 % of HPV-16 positive HNC samples contain integrated HPV genome. Our study brought a complex view of HPV integration analyzed with

different methods and on two types of HPV-associated tumors - tonsillar and cervical, as well as with two types of clinical material - FF and FFPE samples. For the analysis of FF samples we used all three chosen methods and, based on the most sensitive method - APOT assay, we revealed the HPV integration in 36 % of tonsillar tumors, the extrachromosomal form of the virus in 43 % of samples and both forms in 14 % of samples, which results agree with the data published previously (Koskinen *et al.*, 2003; Mooren *et al.*, 2013; Olthof *et al.*, 2014; Gao *et al.*, 2014), although, there are some studies revealing the integrated form in more than 60 % of HNC tumors (Hafkamp *et al.*, 2008; Lace *et al.*, 2011; Khoury *et al.*, 2013). The analysis performed on FFPE samples revealed the integrated form of the virus in 27 % of HNC samples. Due to the worse quality of isolated RNA from FFPE samples, APOT assay is not applicable for the analysis of genome status in this type of material, thus we used only the mapping of E2 integration breakpoint. This method does not distinguish between the mixed form of the virus and it may explain the different percentage of the integrated form detected in this way. The comparison of the status of the viral genome as revealed by E2 mapping on paired fresh frozen and FFPE samples showed 79% concordance between these two types of the material. It is important to note that the differences can be also explained by the fact that the samples from the two types of the material will never be exactly the same piece of the tumor and that the process of fixation of FFPE samples influences the compactness of extracted RNA. In the case of cervical carcinomas in our study, 60 % of samples harbored integrated form of virus and the HPV-positive clinically unaffected cervical tissues contained integrated virus in 50 % of samples.

The APOT assay also provided the opportunity to determine the type of integration (Lace *et al.*, 2011). In all tonsillar samples with integrated HPV genome we detected type A transcripts - spliced from splice donor (SD) 880 to the cellular splice acceptor (SA) site. In cervical samples we detected also the type B transcript which is spliced from SD 880 to viral SA 3358 and continued to the cellular sequence after nucleotide position 3496. Our results confirm the majority of the type A of integrated HPV, as published by Lace *et al*. in keratinocyte clones and tumors (Lace *et al.*, 2011).

The integration occurred on different human chromosomes without obvious predilection. Nevertheless, we identified some integration sites previously detected in cervical carcinomas, such as 17q23.1, 3p26.2, or 20p12.1 (Schmitz *et al.*, 2012), where the genes encoded in these loci play role in oncogenesis or gene silencing (Wieser, 2007;

Chen *et al.*, 2011). Similarly to other studies (Smeets *et al.*, 2006; Lace *et al.*, 2011) we identified the integration in loci 13q14 and 5q35. The integration in locus 3q28 encoding protein TP63 was detected in agreement with others (Khoury *et al.*, 2013; Olthof *et al.*, 2014). Moreover, in five samples HPV was integrated in the proximity of a common fragile sites that were previously presented as affected in cervical carcinomas and other solid tumors (Ragin *et al.*, 2004; Lagana *et al.*, 2010). Altogether, we have shown that for the oncogenesis initiated by HPVs, not only expression of viral oncoproteins, but also other changes in host genome raising from the viral integration are needed.

Our study was the first to report the influence of HPV genome status in HNC on disease specific survival (DSS) of patients. We analyzed a set of 186 samples where the HPV status was revealed by mapping of E2 integration breakpoint. We confirmed the HPV positivity as the strongest prognostic factor for patients with HNC, as published previously (Dahlstrand *et al.*, 2008; Rotnaglova *et al.*, 2011). However, our results show no statistically significant difference in DSS between patients with integrated form of the virus or the extrachromosomal/mixed one, but the patients with the extrachromosomal/mixed form of virus seem to have better prognosis and tend to live longer without disease recurrence. Similar results were previously published for patients with cervical carcinomas by Shin *et al*. (Shin *et al.*, 2014) who have revealed the HPV integration as a poor prognostic factor, and, further, Nambaru *et al*. (Nambaru *et al.*, 2009) have shown better disease-free survival for cervical carcinoma patients with extrachromosomal form of virus, however their results were not statistically significant. Recently, Nulton *et al*. have published a study based on TCGA data of 56 samples of HNC which revealed worse overall survival of patients with integrated form of HPV compared to those with extrachromosomal form (Nulton *et al.*, 2018).

Until now, there are not many published studies focusing on the differences in molecular profiles of HPV-associated and -independent tumors. For the proper understanding of mechanisms leading to the tumor development it is appropriate to determine whether the changes in molecular profiles are influenced by the presence of the virus or by the character of the cells in the region of the tumor development. In 2015, The Cancer Genome Atlas network published a study revealing the differences in molecular alterations between HPV-positive and HPV-negative head and neck squamous cell carcinomas (HNSCC) (Cancer Genome Atlas Network, 2015). Koncar *et al*. have

revealed similar molecular profiles in the set of HPV-positive tumors of vulva, anus, cervix, and oropharynx (Koncar *et al.*, 2017). Contrary, Tuna and Amos have detected similar changes in the genome of patients with HPV-associated tumors with the epigenomic and transcriptomic profiles differing between HPV-positive and HPV-negative tumors (Tuna and Amos, 2017). These data indicate that the accurate and precise determination of the etiology of the disease is necessary for the choice of the most appropriate and most effective medication leading to the more personalized treatment and prognostic advantage of the patients. Moreover, since the lesions of oropharyngeal tumors are most likely located in the tonsillar crypts, these tumors are often revealed in the late stage of the disease and the prognosis of patients is thus worse. The opportunity how to improve the patients´ prognosis is to find some specific, sensitive and clinically relevant biomarker for the earlier and precise diagnostic of the disease. Such biomarkers may be miRNAs whose signatures differ between cancer and clinically unaffected tissues, and their expression is specific for tumors of different origin.

In the paper published in 2016 in BMC Cancer (Vojtechova *et al.*, 2016) we focused on the evaluation of the miRNA profiles in tonsillar tumors of viral and non-viral etiology. Until that time, several studies analyzing miRNA profiles in HNC cell lines (Tran *et al.*, 2007; Chang S.S. *et al.*, 2008) and tumors (Childs *et al.*, 2009; Avissar *et al.*, 2009; Ramdas *et al.*, 2009; Hui *et al.*, 2010) have been published, however, these lacked comparability, due to the anatomical heterogeneity of the analyzed tumors, and due to the different methodological approaches. Furthermore, none of these studies considered the presence of HPV. Wald *et al*. analyzed the influence of HPV oncogene E6 on the miRNA profiles in HNC cell lines (Wald *et al.*, 2011). Lajer *et al*. in their first study analyzed nine HPV-positive oropharyngeal tumors (Lajer *et al.*, 2011), and, a year later, they have published a study where they enlarged the sample set with tonsillar and cervical tumors (Lajer *et al.*, 2012), which step allowed them to identify a group of "HPV-core" miRNAs that could have a role in HPV-related pathogenesis. Nevertheless, their studies lacked the confirmation of transcriptionally active HPV infection and they combined the analysis of FF and FFPE samples, which certainly effects the results, as discussed below. Recently, Miller *et al*. have identified a set of miRNAs deregulated in oropharyngeal carcinomas and validated the analysis with clinical data from The Cancer Genome Atlas (Miller *et al.*, 2015).

Our study has been so far the only one to compare the miRNA data obtained from clinical samples with results obtained from the model system. As a model for HPV-associated and -independent tumors we analyzed HPV-16 immortalized keratinocyte clones and/or keratinocyte clones immortalized by the human telomerase gene. We identified groups of miRNAs specific for each group of samples, as well as those common for tumors/clones regardless of HPV. During the analysis of the data, we found out that the tumor sample homogeneity in terms of the percentage of the tumor cells is more important for the robustness of the miRNA expression study than the samples set size. Unfortunately, the overlap between clinical samples and model system was small and we identified concordant deregulation in only three miRNAs in each group. In the analysis of immortalized keratinocyte clones versus primary cells we identified three miRNAs (miR-135b, miR-146b-5p, miR-205-5p) whose deregulation in immortalized cells is in the agreement with other published studies (Al-Khalaf and Aboussekhra, 2014; Li Y. *et al.*, 2015; Li J. *et al.*, 2015). These miRNAs participate in signaling pathways, tumor invasion or cell migration pathways connected with EMT. The miRNAs identified as deregulated in clinical samples of tonsillar tumors were also reported to contribute to cancer development. Only miR-125b identified in the comparison of HPV-positive tumors versus non-malignant tissues was previously reported to play role in productive HPV infection (Nuovo *et al.*, 2010).

Due to the simultaneous analysis of miRNA expression in cervical carcinomas we were also able to identify a group of five miRNAs specific for HPV-induced malignancies (miR-141-3p, miR-15b-5p, miR-200a-3p, miR-302c-3p, and miR-9-5p). As mentioned above, the only study performing analogous analysis was published by Lajer *et al*. (Lajer *et al.*, 2012). However, there was no overlap with our results, mainly because of the heterogeneity of their set of HNC samples as mentioned previously. Namely, their sample set included tumors of tonsillar as well as pharyngeal location, and also, they used different type of clinical samples, such as fresh frozen and formalin-fixed paraffin-embedded ones. The importance of the right selection of the material type is the subject of my third publication which is discussed below. Our results agree with Lajer´s only in miR-21 as the most commonly deregulated miRNA in cancers and we similarly identified it upregulated in HPV-associated tonsillar tumors. Further, in agreement with Hui *et al*. (Hui *et al.*, 2013), we identified miR-9 as deregulated in HPV-related tumors since we determined its upregulation in both tonsillar as well as cervical tumor samples.

The HPV-induced activation of the miR-9 expression was studied by Liu *et al*., who showed that this miRNA downregulates genes involved in pathways of cell migration and thus influences the motility of the cells (Liu *et al.*, 2014). The authors observed the activation of miR-9 expression as a result of HPV E6 expression. MiR-9 has been considered as HPV-related also by Miller *et al*. (Miller *et al.*, 2015).

Further, our study was the first one which performed the miRNA analysis of HPV-positive tumors and keratinocyte clones based on the status of the viral genome. We utilized our results from the detailed characterization of samples and showed that miRNA expression profiles differ in tumor tissues as well as in model system with extrachromosomal, integrated, or mixed form of the virus. Even though there was no overlap in deregulated miRNAs between clinical samples and the model system, we found out that identified miRNAs target the same genetic pathways, such as p53 signaling, cell cycle regulation, or PI3K-Akt signaling. Moreover, using factor analysis we analyzed the miRNA expression in the relation to the patient prognosis and identified twelve miRNAs (miR-196b, miR-485-3p, miR-589, miR-324-3p, miR-342-3p, miR-92a-1#, miR-155, miR-146b, miR-142-3p, miR-1260, miR-143, and miR-142-5p) that may influence it. Many of them have already been published as prognostic markers of other cancers (Lin *et al.*, 2012; Ren *et al.*, 2017; Xie *et al.*, 2017), or directly in head and neck tumors of different locations (Xu *et al.*, 2015; Baba *et al.*, 2015; Jamali *et al.*, 2015; Bufalino *et al.*, 2015). Nevertheless, for obtaining more reliable results, the study should be confirmed on a larger set of samples.

Our further aim of research was the optimization and implementation of the method *in situ* hybridization of miRNA for determination of the miRNA location in the cell. Since the method performed on FF tissues requires extensive optimization of the process and sufficient number of prepared slices, we performed *in situ* hybridization on FFPE tissues. However, we had to confirm the miRNA expression profiles obtained from FF samples also on FFPE ones. Based on our experience from previous research about the importance of homogenous set of samples for the analyses, we performed the TLDA analysis on RNA isolated from macrodissected FFPE tumor samples. The results were compared with the miRNA expression profiles obtained from FF samples and our findings were published in 2017 in Plos One (Vojtechova *et al.*, 2017). Moreover, for comparison, we processed the data by different software programs and we used two types of normalization of data.

The possibility to utilize FFPE samples available in archives for different analysis offers the opportunity to perform retrospective studies, use the long-term follow-up data from patients and to extend the studies. The utilization of RNA from FFPE tissues is still challenging due to the RNA fragmentation caused by crosslinks between tissues and molecular components (Doleshal *et al.*, 2008). However, in the case of small RNAs, their stability is not influenced by formalin fixation, probably due to their small size and secondary structure (Xi *et al.*, 2007; Doleshal *et al.*, 2008; Jung *et al.*, 2010). Therefore, FFPE samples are usable for miRNA expression, as has been shown previously (Osawa *et al.*, 2011; Lee *et al.*, 2013; Ganci *et al.*, 2014; Leichter *et al.*, 2015), also in the cases when the RNA integrity number showing the quality of isolated RNA is low (Chatterjee *et al.*, 2015). In our study, we performed the analysis of miRNA expression profiles in a set of tonsillar FFPE tumors and clinically unaffected controls and compared them with miRNA profiles obtained from paired FF samples. We revealed that the miRNA expression profiles differ between FF and FFPE samples, and, also, depending on the character of the tissue (tumor vs. normal tissue). We observed good correlation of miRNA profiles between paired FF and FFPE samples, however, the overlap between differentially expressed miRNAs was only 27-38 %. Macrodissected FFPE samples revealed less differentially expressed miRNAs compared to paired FF samples. Since our previous study revealed that the tumor tissue homogeneity is important for miRNA expression studies, we evaluated the correlation of samples based on the number of tumor cells. Contrary to our hypothesis, we observed no relationship. Most of the earlier published studies comparing FFPE and FF samples were focused only on few miRNAs (Li *et al.*, 2007; Hoefig *et al.*, 2008; Mortarino *et al.*, 2010; Leite *et al.*, 2011; de Biase *et al.*, 2012). More recent studies utilized also microarray analyses (Hui *et al.*, 2009; Romero-Cordoba *et al.*, 2012) and NGS (Meng *et al.*, 2013). These projects have revealed higher correlation of miRNA profiles between paired FF and FFPE samples, however, the limitation of these studies was the small number of analyzed samples, or the lack of macrodissection of samples. Last, but not least, the studies were not done on HNC. On the other hand, the results of the study of How *et al*. made on cervical tumors agree with ours (How *et al.*, 2015). They identified the miRNA signature in a cohort of FF samples and were unsuccessful to validate it in a cohort of FFPE samples.

Finally, to establish how the choice of normalization method and software for data analysis influenced the results, we performed the comparison of two normalization

methods (50^{th} percentile shift normalization and global normalization) and three software programs (GeneSpring GX v13.1, GenEx v6.1, and MeV v4.9). The differentially expressed miRNAs based on each normalization method overlap in 58-67 % for FF or FFPE samples. On the other hand, the comparison of different software programs revealed the overlap in 90 % of differentially expressed miRNAs. Thus, the use of different type of the clinical material and normalization methods increased the variability of results between studies and partly explained the small overlaps in the obtained pattern of differentially expressed miRNAs.

7. SUMMARY

The submitted thesis focuses on the study of miRNA expression profiles in human tumors related to human papillomaviruses, such as head and neck cancer and cervical tumors. Because of their tissue and tumor specificity and their small size, miRNAs are suitable candidates for being clinical biomarkers and for improving the current diagnostic and therapeutic possibilities.

My initial paper, included into this thesis, focused on the detailed characterization of obtained samples from cooperating clinics. We performed the analysis of viral genome status in tumors, because the integration of the virus into the host genome is considered as a critical step in carcinogenic progression. Based on our analysis we have revealed that in tonsillar tumors the integrated form of virus is in 36 % of samples and specified and described the integration sites. Furthermore, we have shown that the integration influences the disease specific survival of patients but not statistically significantly.

Such characterized samples were used in the main part of my research focused on miRNA profiling of HPV-positive and HPV-negative tonsillar and cervical tumors. We characterized the miRNA expression profiles of virus-associated tumors and tumors of non-viral etiology and for comparison we analyzed miRNA expression profiles in the model system of human keratinocyte clones. During the research, several important observations have been made. The importance of homogeneity of analyzed samples and the standardization of screened material and methods of analyses of obtained data have been revealed. All these observations allow for improvement of the following experiments. Finally, the HPV-core miRNAs were identified and will be in the subsequent projects evaluated in a set of HPV-related and non-related tumors from other anatomical locations as well as analyzed functionally.

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9. PUBLICATIONS

Publication I

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Analysis of the integration of human papillomaviruses in head and neck tumours in relation to patients' prognosis

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Integration, which leads to the disruption of the circular HPV genome, is considered as a critical, albeit not obligatory, step in carcinogenic progression. Although cervical carcinomas with extrachromosomal HPV plasmid genomes have been described, the virus is integrated in 70% of HPV16-positive cervical tumours. Limited information is available about HPV integration in head and neck tumours (HNC). In this study, we have characterised the physical status of HPV in a set of tonsillar tumour samples using different methods—the mapping of E2 integration breakpoint at the mRNA level, the 3' RACE based Amplification of Papillomavirus Oncogene Transcripts (APOT) assay and Southern blot. Furthermore, the impact of HPV integration on patients' prognosis has been evaluated in a larger set of 186 patients with head and neck cancer. Based on the analysis of E2 mRNA, HPV was integrated in the host genome in 43% of the HPV-positive samples. Extrachromosomal or mixed form was present in 57%. In fresh frozen samples, the APOT and E2 mapping results were in agreement. The results were confirmed using Southern blotting. Furthermore, the type and exact site of integration were determined. The survival analysis of 186 patients revealed HPV positivity, tumour size and lymph node positivity as factors that influence disease specific survival. However, no statistically significant difference was found in disease specific survival between patients with HPV-positive integrated *vs.* extrachromosomal/mixed forms of the virus.

Head and neck cancer (HNC) is the seventh most common cancer in men and the 13th most common cancer in women worldwide and its incidence is on the rise in some anatomical sites[1]. HNC has at least two different etiologies. Most HNC cases are associated with smoking and alcohol consumption, which induce mutations in important pathways[2,3]. However, ~26% of all HNC and up to 50% of oropharyngeal tumours are associated with the presence and expression of mucosal high-risk human papillomaviruses (Alfa HR-HPV; HR-HPVs)[4–6]. Mucosal HR-HPVs also cause cervical cancer and other neoplasms of anogenital skin and mucosae. The most common mucosal HR-HPV is HPV16, which is found in almost 90% of HPV-positive HNC, other common types being HPV18, HPV33 and HPV52[6,7]. Patients with HPV-associated HNC have a better prognosis, overall survival, and response to treatment[4].

The mechanisms of Alfa HR-HPV-induced carcinogenesis, regardless of the anatomical site, rely on the functions of viral oncoproteins, E6 and E7, as they inactivate tumour

Key words: HPV, integration, head and neck tumours, prognosis, survival

Abbreviations: 3′-RACE: 3′-rapid amplification of cDNA ends; AIC: Akaike information criterion; APOT assay: amplification of papillomavirus oncogene transcripts assay; DIPS-PCR: detection of integrated papillomavirus sequences polymerase chain reaction; DSS: disease specific survival; FFPE: formalin fixed and paraffin embedded; HNC: head and neck cancer; HPV: human papillomavirus; HR-HPV: high risk human papillomavirus; ORF: open reading frame; OSCC: oral squamous cell carcinoma; SA: splice acceptor; SD: splice donor; TGF-β: growing factor β; TSCC: tonsillar squamous cell carcinoma

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What's new?

The integration of human papillomavirus (HPV) into host DNA allows for greater expression of the viral oncogenes E6 and E7. Hence, HPV integration favours oncogenesis. But it is not mandatory for that process. In the present study of tissues from head and neck cancer (HNC) patients, HPV was found to be integrated into the host genome in 43% of HPV-positive samples. In the remaining positive samples, HPV was present either in extrachromosomal or mixed form. Although patients with extrachromosomal or mixed virus had slightly better prognosis than patients with integrated HPV, integration was not associated with patient survival.

suppressor proteins p53 and pRb, respectively[8]. The early region genes E2 and E8^E2, transcriptional regulators of HPV early genes encoded by the E2 open-reading frame (ORF), are disrupted or lost upon HPV integration into the host genome[9–11]. Most authors agree that in the cervix, the loss of E2 increases E6 and E7 oncogene expression and thus favours oncogenesis[10,12]. Even though HPV integration is strongly associated with high-grade lesions and cancer[13–15], some studies have not observed increased expression of the viral oncoproteins from integrated HPV genomes[16,17]. Also, a significant number (~30%) of cervical carcinomas harbor extrachromosomal HPV plasmid genomes[11]. HPV integration is thought to be a random process which occurs in almost all chromosomes without specific hotspots[18]. However, it has been observed near or within common fragile sites[19,20], with the integration sites located in or near translocation breakpoints, transcriptionally active regions or growth control genes[21–23].

There are several methodological approaches to the study of HPV integration; however, all have some limitations. DIPS-PCR (Detection of Integrated Papillomavirus Sequences)[24] maps chimeric virus-cell DNA sequences with the aid of single-side-specific ligation-mediated PCR followed by sequencing, but it is a costly and labour intensive method that demands high quality DNA. Another DNA amplification method used in the past, E2/E6 DNA ratio real-time PCR[25], attempted to detect HPV genomes with defects in the E2 region. While easy to perform, this method suffers conceptually from the fact that it is unable to distinguish extrachromosomal plasmid HPV genomes from viral genomes integrated in tandem repeats. More recently, methods based on next-generation sequencing are also appearing[23,26,27]. A very sensitive and specific method is the APOT (Amplification of Papillomavirus Oncogene Transcripts) assay[11], a modified 3′-RACE (Rapid Amplification of cDNA Ends) technique. This method provides information about the site of integration within the host genome and also enables the identification of the structure of HPV transcripts. In addition, this technique detects the presence of transcripts from both integrated and extrachromosomal genomes in the same tissue samples and is not negatively influenced by large excess of extrachromosomal HPV copies[11,28]. However, this method requires high-quality RNA.

Several studies have focused on the detection of HPV integration in HNC[17,21,27,29–31]. The overall results indicate that around 50% of HPV16-positive HNC cases harbour integrated HPV DNA, slightly fewer than cervical cancer. Therefore, further analysis of HPV integration in HPV-associated HNC might bring new information regarding the viral etiology of this cancer. Furthermore, not many studies compared different methods for performance in this kind of samples.

The primary aim of our study was to characterise the physical status of HPV in a set of tonsillar tumour samples using different methodological approaches—the mapping of E2 integration breakpoint at the RNA level, the APOT assay and Southern blot. Frozen tissue samples (FF) eligible for comparative testing by the more sensitive APOT method and E2 mapping yielded concordant results. Therefore, the mRNA E2 mapping method was used as the only option in testing the formalin fixed paraffin embedded (FFPE) samples in the large study. In a subset of frozen tissue samples HPV integration frequency and pattern was evaluated and chromosomal loci where the virus was integrated were identified. The impact of HPV integration on patients' prognosis was evaluated in a larger set of 186 patients with head and neck cancer.

Material and Methods

Clinical samples

HNC samples were obtained from patients treated at the Department of Otolaryngology and Head and Neck Surgery, 1st Medical Faculty Charles University and Motol University Hospital, Prague in the period of 2001–2014. Inclusion criteria were the primary squamous cell carcinoma of the oral cavity or oropharynx (ICD-10: C01–C06, C9–10) and treatment done by surgery in combination with radiotherapy.

The selection criteria for FF samples were the localisation of the tumour in tonsils, availability of the fresh frozen material, presence of at least 10% of the tumour cells in the tissue and HPV association as revealed by the preceding analysis of the FFPE tissues taken from the same tumour. Fourteen patients with HPV associated and 10 with HPV negative tumours were analysed. HPV associated tumours were positive for HPV16 DNA, for the expression of viral HPV16 E6 mRNA and p16 protein. HPV non-associated tumours were negative for all three of the above mentioned parameters.

Cervical samples were collected from patients treated at the Department of Obstetrics and Gynecology, 2nd Medical Faculty Charles University and Motol University Hospital, Prague from 2012-2014. From the group of six cervical samples, we had two paired samples where the tumour and the clinically unaffected tissue were available from the same patient.

Tonsillar tumour samples were taken from patients during surgery and were sent on dry ice to pathology. One tumour piece was fixed with tissue freezing medium and snap frozen in liquid nitrogen and stored at −80°C. Sections were cut on cryostat and histopathologically confirmed. The second part of the tumour was fixed in 10% neutral formalin and paraffin embedded. From each paraffin block, the first and last sections were histologically analysed to confirm that the sections in between contained at least 10% tumour cells and were suitable for viral nucleic acids isolation and analysis.

Healthy tonsillar tissues were taken from patients during tonsillectomy, were snap frozen in liquid nitrogen immediately after removal and stored at −80°C. Cervical samples were transported in RNAlater (Life Technologies, USA) transport medium and processed within one week.

All study patients signed informed consent. The study has received official institutional and ethical approval from the Ethics Committee of the University Hospital Motol, Prague.

Processing of samples

DNA from fresh frozen tissues was isolated using the QIAamp DNA Mini kit (Qiagen, Germany) and total RNA was extracted by the miRVana kit (Life Technologies, USA). Both DNA and RNA of FFPE samples were simultaneously extracted from two 20-μm sections by the Ambion RecoverAll™ total nucleic acid isolation kit for FFPE (Applied Bioscience, USA) according to manufacturer's protocol. The detection and typing of HPV DNA was performed by PCR with broad spectrum primers BSGP5+/6+bio and reverse line blot hybridisation as previously described[32]. The cDNA was prepared by reverse transcription of 1 μg of DNase treated RNA (Promega, USA) with M-MLV reverse transcriptase (Promega, USA). The absence of contaminating DNA was confirmed on the RNA treated with DNAse by the absence of human beta-globin PCR amplicon as described previously[33]. The expression of HPV16 E6 mRNA was assessed by RT PCR which amplifies the most abundant splice variant E6*I with a length of 86 bp as described before[34].

Mapping of E2 integration breakpoint

E2 integration breakpoint was analysed on cDNA after reverse transcription. Primers spanned the E7, E2, E4 and E5 ORF (Supporting Information Table S2). The 25 μl PCR mixtures were composed of 0.5 units of AmpliTaq Gold® DNA polymerase (Life Technologies, USA), 1× PCR Gold Buffer II (Life Technologies, USA), 200 μM dNTPs, 1.5 mM $MgCl_2$, 12.5 μM of each forward and reverse primer, and 2 μl of cDNA. The reaction mixture was subjected to an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing for 2 min, elongation at 72°C for 2 min, and final extension at 72°C for 3 min. The annealing temperatures varied for each set of primers; 55°C for the E7 and E5 sets, 50°C for the E7-E4 set, 47°C for E2B set, and 45°C for the E2C and E2D sets. The cervical squamous cell carcinoma cell line SiHa containing integrated HPV16 was used as a positive control and the HaCaT cell line and H_2O as negative controls. Amplicons were visualised by 3% agarose gel electrophoresis.

APOT assay

Analysis of the HPV transcripts was modified from Klaes *et al.*[11]. Briefly, 1.1 μg of total RNA was reverse transcribed using 10 μM adaptor-linked oligo$(dT)_{17}$ primer ($(dT)_{17}$-P3; 5′-GACTCGAGTCGACATCGA-3′), 50 units of M-MLV reverse transcriptase (Promega), and 20 units of Rnasin (Promega) in 20 μl reaction mixture at 37°C for 1 hr. The cDNA was amplified by PCR using HPV16 E7-specific primer P1 and linker P3. The 50 μl reaction mixture contained of 1.5 units of Taq DNA polymerase (Thermo Scientific, USA), 1x $(NH_4)_2SO_4$ Taq buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.25 μM of each primer, and 4 μl of cDNA. The reaction mixture was subjected to initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 59°C for 30 sec, elongation at 72°C for 4 min and final extension at 72°C for 7 min. Nested PCR reaction using 4 μl of initial amplicons was done with HPV16 E7-specific primer P2 and $(dT)_{17}$-P3. The cycling conditions were the same, except for the annealing step which was changed to be performed at 67°C for 30 sec.

Amplicons from the second PCR were separated on 2% agarose gels, blotted on nylon membrane (PerkinElmer, USA) using alkaline transfer and hybridised either with an E7-specific probe H1 or E4-specific probe H2. Hybridisation was performed at 55°C. Probes were labelled by streptavidin-HPR and the detection was done by the ECL detection kit (GE Healthcare, UK) following the manufacturer's protocol.

Amplification products longer than 300 bp were excised from the gel, purified using the MinElute Gel Extraction kit (Qiagen, Germany) and sequenced using the BigDye Terminator Sequencing kit (Applied Biosystems, USA). Sequence data were analysed using BLAST software (http://blast.ncbi.nlm.nih.gov/).

Detection of integration by Southern blotting

Southern blotting was done according to Lace *et al.*[21]. Briefly, 2 μg of DNA from HPV16-positive tonsillar tumours and from HPV16-positive cervical tissues in uncut form as well as digested with BamHI endonuclease were resolved on 1% agarose gels, depurinated in 0.25 M HCl and blotted directly onto positively charged nylon membranes (Hybond-XL, Amersham Biosciences Corp., Piscataway, NJ, USA) by alkaline transfer with 0.4 N NaOH. Hybridisation was performed at 65°C with probes (1.5 x 10^6 cpm/ml hybridisation buffer) containing an equimolar cocktail of PCR-amplified segments of HPV16 [α-^{32}P]dATP/dCTP labelled by random priming (HotPrime kit; GenHunter Corp., Nashville, TN, USA). HPV16 plasmid, CaSki cell line and linearised HPV16 were used as controls.

Table 1. Summary of results of E2 integration breakpoint mapping

Sample no.	Type of sample[1]	E6	E7	E7-E4	E2B	E2C	E2D	E5	HPV16 status[2]
RNA17A[3]	Normal Cx	+	−	+	−	+	−	−	Integrated
RNA19A[4]	Normal Cx	+	+	+	−	+	+	+	Extrachromosomal
RNA22	Normal Cx	+	−	+	−	+	+	+	Extrachromosomal
RNA24	Normal Cx	+	+	+	−	+	+	+	Extrachromosomal
RNA8	CxCa	+	+	+	−	+	+	+	Extrachromosomal
RNA9	CxCa	+	−	+	−	−	−	−	Integrated
RNA17B[3]	CxCa	+	+	+	−	+	−	−	Integrated
RNA18B	CxCa	+	+	−	−	−	−	−	Integrated
RNA19B[4]	CxCa	+	+	+	−	+	+	+	Extrachromosomal
ORL104	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL111	Tonsillar tumour	+	+	−	−	−	−	−	Integrated
ORL116	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL125	Tonsillar tumour	+	+	+	−	+	−	−	Integrated
ORL126	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL128	Tonsillar tumour	+	+	−	−	−	−	−	Integrated
ORL133	Tonsillar tumour	+	+	−	−	−	−	−	Integrated
ORL136	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL154	Tonsillar tumour	+	+	+	−	+	−	−	Integrated
ORL155	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL160	Tonsillar tumour	+	+	−	−	−	−	−	Integrated
ORL161	Tonsillar tumour	+	+	−	−	+	+	+	Extrachromosomal
ORL181	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
ORL187	Tonsillar tumour	+	+	+	−	+	+	+	Extrachromosomal
SiHa	CxCa cell line	+	+	−	−	−	−	−	Integrated

[1]Type of sample – CxCa=cervical carcinoma, Cx=cervical tissue.
[2]Assessment of HPV genome status – extrachromosomal =extrachromosomal/mixed.
[3]Paired samples from the same patient.
[4]Paired samples from the same patient.

Statistics

The Cox proportional hazards model was used for the disease specific survival (DSS) analysis. The Akaike information criterion (AIC) was used for model selection. The following demographical and clinical pathological factors were included: gender, age, education (≤12 years, >12 years), smoking (nonsmoker; ex-smoker; smoker), alcohol (nondrinker; ex-drinker; drinker), location (oropharyngeal, oral), tumour size (T1-4), nodal status (N0-3), tumour stage (I, II, III, IV), histological tumour grade (G1-3), and HPV status (HPV16 E6 mRNA negative; HPV16 E6 mRNA positive with an extrachromosomal and/or mixed form; HPV16 E6 mRNA positive with an integrated form; or HPV16 DNA positive transcriptionally silent (no HPV16 E6 mRNA expression)). Tumour size, nodal status, tumour stage and histological grading were numeric measures.

Results

Demographic and clinical pathological characteristics

Fifty-two percent of fresh frozen tonsillar tumours were HPV16 DNA positive (14/27), 37% were HPV16 DNA negative (10/27) and 11% were positive for another HPV type (3/27). All unaffected tonsillar tissues were HPV-negative. All cervical carcinomas were HPV16 DNA positive except for one tumour positive for HPV82. Furthermore, 67% of clinically unaffected cervical tissues (4/6) meant as negative controls were HPV16 DNA positive. Two of these morphologically unaffected tissues were obtained from patients with cervical cancer also containing HPV16. The expression of E6 mRNA was detected in all HPV16 DNA-positive fresh frozen samples including apparently unaffected tissues.

Formalin-fixed paraffin embedded (FFPE) tumour tissues from 186 patients were analysed. The detailed characteristics of patients are summarised in Supporting Information Table S1. Most patients (80.1%) were males, 62.2% were current drinkers and 43.2% were current smokers. The most frequent HPV-positive tumours were localised in tonsils (ICD-10:C090, C099, C091) (68.6%; 72/105), base of tongue (ICD-10:C01) (10.5%;11/105), and unspecified oropharynx (ICD-10: C051, C052, C102) (5.7%; 6/105) while in other locations (soft palate, floor of the mouth (ICD-10: C04), and retromolar region

Table 2. Summary of APOT assay results

	Detection of HPV genome			
Type of sample	Extrachromosomal form	Mixed form	Integrated form	Inconclusive results
Tonsillar tumour	6/14 (43%)	2/14 (14%)	5/14 (36%)	1/14 (7%)
Cervical carcinoma	1/5 (20%)	1/5 (20%)	3/5 (60%)	–
Positive unaffected cervix	2/4 (50%)	0/4 (0%)	2/4 (50%)	–

(ICD-10: C062), no HPV was found and among the unspecifed oral cavity (ICD-10: C00.3, C00.4, C06.0, C06, C03.0, C03.1, C05.0) and tongue tumours (ICD-10: C02.0, C02.1, C02.2), one HPV-positive sample was present. Among HPV DNA, positive tumours, 13% were transcriptionally silent (14/105).

Analysis of HPV genome status in fresh frozen tissues

Mapping of the E2 gene at the mRNA level permitted us to analyse the transcriptionally active viral genome form. Using a set of primers targeting different positions of the E2 ORF, we predicted where the integration breakpoint might be. If the sample was positive for all tested parts of the E2 ORF, the transcripts were considered to stem from extrachromosomal HPV genomes. Transcripts of integrated HPV genomes were expected to lack E2 DNA sequences or to be positive only for the beginning of the analysed region. Because the HPV region between positions 880 and 3358 nt is not found among transcripts because of mRNA splicing, the absence of E2B amplicons served as a control of DNA contamination; it was negative in all samples.

Based on the E2 integration breakpoint mapping, 43% (6/14) of HPV16 DNA positive tonsillar samples contained only an integrated form of the viral genome, 57% (8/14) contained an extrachromosomal or mixed form (Table 1). Viral DNA was integrated in 60% (3/5) of cervical cancer samples and in 25% of HPV16 positive normal cervical tissue samples (1/4).

All HPV16 DNA positive samples were evaluated by the more sensitive and specific APOT assay. Representative APOT assay results of selected tonsillar and cervical samples are shown in Supporting Information Figure S1 in the supplement. The standard extrachromosomal transcript has a length of 1050 bp (E7-E1^E4-E5)[11]. All products with different lengths are derived from integrated fusion transcripts or are artifacts that can originate from the standard extrachromosomal transcript and may be formed because of mispriming during the APOT assay when the (dT)17-P3 primer binds to an A-rich region mostly around position 3821 nt. The summary of the APOT assay results is in Table 2.

The APOT assay revealed that two tonsillar samples contained the virus in a mixed form. One tonsillar sample (ORL128) where the virus seemed to be integrated based on E2 mapping was not resolved by the APOT assay: we found several fragments which we were not able to sequence neither directly nor after cloning to the TOPO cloning vector (TOPO TA cloning protocol; Life Technologies) and was therefore excluded from the comparison. Results of the APOT assay and E2 mapping in all evaluable ($N = 13$) tonsillar samples were in agreement. In cervical tumours, a mixed form was detected in one sample. A disagreement between the two methods was found for a single sample of clinically unaffected cervical tissue (RNA19A) where the APOT assay only revealed an integrated form of HPV. Overall, there was almost perfect agreement between the two methods (Kappa value = 0.9076).

The results of some tonsillar and cervical samples with an integrated or mixed form of HPV genome were confirmed using Southern blotting (Supporting Information Fig. S2). Southern blot was not performed in all samples with integrated viral genome because of the limited amount of available DNA. Similar to the CaSki cell line which harbours multiple tandem repeats of the HPV16 genome, we observed this pattern in tonsillar tumour samples ORL133 and ORL187. In contrast, in sample ORL126, Southern blot analysis revealed only extrachromosomal HPV16 genomes whereas APOT identified both plasmid and integrated HPV16 mRNA transcripts. The summary of comparison of all used methods is in Table 3.

By analysing the sequence data, we could also determine which type of integration occurs in samples and assess the splicing of HPV transcripts. All integrated viral genomes in tonsillar samples were spliced from splice donor (SD) 880 to the cellular splice acceptor (SA) sites (transcripts type A). Samples with extrachromosomal genomes, both from tonsillar and cervical location, had three different SA sites: SA 3358 in most samples (11/12), SA 3421 in 1/12 of samples, and SA 3391 in 1/12 of samples and in sample ORL161, there were two different SA sites. HPVs integrated in cervical samples had both transcript types. All of these samples (6/6) were spliced from SD 880 to a cellular SA site (transcripts type A) and two of them also had transcripts spliced to SA 3358 with prolongation into the cellular sequence (transcripts type B). Other types of transcripts were not found.

The APOT assay gave identical results in the paired cervical tumour and unaffected tissue samples from one patient (RNA17A and RNA17B), while in the other patient, HPV genome was integrated into different chromosomes in cervical cancer and unaffected cervical tissue (RNA19A and RNA19B), and the tumour sample contained additional extrachromosomal genome copies.

All 20 identified fusion transcripts containing viral and cellular sequences were analysed by a BLAST search in the GenBank database. Summary of results is in Table 4. In tonsillar as well as cervical samples, integration occurred on different human chromosomes with no obvious predilection

Table 3. Comparison of the results based on E2 integration breakpoint mapping, APOT assay and Southern blot

Sample no.	Type of sample[1]	HPV status (based on E2)[2]	HPV status (APOT assay)	Southern blot analysis
RNA17A[3]	Normal Cx	Integrated	Integrated	N/D[4]
RNA19A[5]	Normal Cx	Extrachromosomal	Integrated	Integrated
RNA22	Normal Cx	Extrachromosomal	Extrachromosomal	N/D
RNA24	Normal Cx	Extrachromosomal	Extrachromosomal	N/D
RNA8	CxCa	Extrachromosomal	Extrachromosomal	N/D
RNA9	CxCa	Integrated	Integrated	Integrated
RNA17B[3]	CxCa	Integrated	Integrated	Integrated
RNA18B	CxCa	Integrated	Integrated	Integrated
RNA19B[5]	CxCa	Extrachromosomal	Mixed	Integrated
ORL104	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL111	Tonsillar tumour	Integrated	Integrated	Integrated
ORL116	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL125	Tonsillar tumour	Integrated	Integrated	N/D
ORL126	Tonsillar tumour	Extrachromosomal	Mixed	Extrachromosomal
ORL128	Tonsillar tumour	Integrated	Nonspliced	N/D
ORL133	Tonsillar tumour	Integrated	Integrated	Integrated in tandem repeats
ORL136	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL154	Tonsillar tumour	Integrated	Integrated	N/D
ORL155	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL160	Tonsillar tumour	Integrated	Integrated	N/D
ORL161	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL181	Tonsillar tumour	Extrachromosomal	Extrachromosomal	N/D
ORL187	Tonsillar tumour	Extrachromosomal	Mixed	Integrated in tandem repeats
SiHa	CxCa cell line	Integrated	Integrated	N/D

[1]Type of sample – CxCa = cervical carcinoma, Cx=cervical tissue.
[2]HPV genome status – extrachromosomal=extrachromosomal/mixed.
[3]Paired samples from the same patient.
[4]N/D, not done.
[5]Paired samples from the same patient.

(chromosomes 1, 3, 4, 5, 8, 13, 17, 19, 20, and X). Chromosomes 1 and 3 appeared in both tonsillar and cervical samples. In paired samples of cervical tumours and clinically unaffected tissues, the HPV was integrated into the same chromosome (3q26.2) in one patient (RNA17A and RNA17B) with both transcript types. In the second patient, the cervical cancer and unaffected cervical tissue harbored HPV16 genomes integrated at two independent sites on chromosomes 1q21 and 19q13.3. Both were expressed as type A transcripts.

Six transcripts were spliced into the sequence of an intron, one transcript was only spliced into the exon sequence and one transcript was spliced into the 3′-untranslated region of the gene. The rest of transcripts were spliced into the intergenic region. In one tonsillar cancer sample, integration occurred in the proximity of transcriptional factor *TP63*, which plays a role in the development and maintenance of stratified epithelial tissues and was reported to function in oncogenesis. In a cervical tumour, integration occurred near the *MECOM* complex. This complex encodes transcriptional regulator and oncoprotein, involved in a number of genetic pathways. The cellular gene loci in our samples include also genes coding for tumour suppressors (*TSC22D1*, *CSMD1*). Products of other genes where HPV integration was detected act as deacetylase (*MACROD2*), molecular chaperones (*CANX*) or cytoskeletal protein (*TUBD1*). Five samples showed HPV integration near the known fragile sites FRA4B, FRA1F, FRA5G, FRA17B and FRA19A.

Validation of the E2 integration breakpoint mapping method

We have performed the validation of the E2 integration breakpoint mapping on a larger patient cohort with tonsillar tumours since this method offers an easy way how to identify the HPV status retrospectively in formalin fixed paraffin embedded tissues. Altogether, both FF and FFPE samples were available from 20 patients. One sample (ORL128) was

Table 4. Summary of integration sites

Sample no.	Type of sample	Location on chromosome	Gene code	Gene name	Region in genome	Fragile sites
RNA9	CxCa	Xp11.3	–	–	Intergenic	–
RNA17B	CxCa	3q26.2	*MECOM*	MDS1 and EVI1 comlex locus	Intron	–
RNA18B	CxCa	ch4	–	–	Intergenic	FRA4B
RNA19B	CxCa	19q13.3	*RPL23AP80*	Ribosomal protein L23a pseudogene	Intergenic	FRA19A
RNA17A	Normal Cx	3q26.2	*MECOM*	MDS1 and EVI1 complex locus	Intron	-
RNA19A	Normal Cx	1q21	–	–	Intergenic	FRA1F
ORL111	Tonsillar tumour	1q32	–	–	Intergenic	–
ORL125	Tonsillar tumour	20p12.1	*MACROD2*	MACRO domain containing 2 deacetylase	Intron	–
ORL126	Tonsillar tumour	5q35	*CANX*	calnexin	3′UTR	FRA5G
ORL133	Tonsillar tumour	8p23.2	*CSMD1*	CUB and Sushi multiple domains	Intron	–
ORL154	Tonsillar tumour	3q28	*TP63*	tumour protein p63	Intron	–
ORL160	Tonsillar tumour	17q23.1	*TUBD1*	Tubulin delta 1	Exon	FRA17B
ORL187	Tonsillar tumour	13q14	*TSC22D1*	TSC22 domain family	Intron	–

excluded because it was inclonclusive on the APOT assay (for details see above). In the remaining 19 samples (results are summarised in Supporting Information Table S3), perfect agreement between the APOT assay and the E2 mapping was obtained. Integrated form of HPV was present in four, extrachromosomal in 11 samples and in four samples mixed form was detected by APOT and extrachromosomal by E2 mapping. It is in agreement with the limitation of E2 method which can't distinguish between mixed and extrachromosomal forms of HPV, and with the fact that APOT has been shown to have a higher sensitivity for the detection of the integrated form of HPV.

The comparison of the results of E2 mapping on FF and FFPE revealed 15/19 samples concordant (79%). In 3 of 4 discrepant samples, HPV was present in an extrachromosomal form in FF and in integrated form in FFPE. In two of these samples, the mixed form of the virus has been found on APOT assay suggesting that both forms are present. The outcome is dependent on the proportion of tumour cells in the particular sample and possibly on the degradation of RNA and therefore either one of the forms is detected (ORL244, ORL227). The overall agreement was substantial (Kappa value=0.683).

Survival of patients according to HPV status

The analysis of HPV status in the set of FFPE samples was based on the presence/absence of amplicons generated by E2D primers, and amplifiable samples were marked as extrachromosomal. Integrated HPV genome was detected in 27.5% of E6 mRNA positive tumours (25/91), whereas an extrachromosomal/mixed form of HPV genome was detected in 72.5% (66/91). In 29.2% (21/72) of tonsillar and in 33.3% (2/6) of nonspecified oropharyngeal tumours integrated HPV genome was found while in tumours of the base of tongue only extrachromosomal form of the virus was detected. The survival analysis revealed HPV positivity (for the definition see Materials and methods), tumour size and lymph node positivity as factors with influence on disease specific survival (DSS) (Table 5, Fig. 1). Additionally, gender was also added in the Cox model because it improved the quality of the model. As expected, HPV positivity of the tumour was the strongest prognostic factor (HPV16 E6 mRNA positive *vs.* HPV16 E6 mRNA negative $p < 0.0001$). The difference in DSS was statistically significant both for the group of patients with HPV16 E6 mRNA positive tumours with an extrachromosomal and/or mixed form of the virus as well as for those with an integrated form only (HPV-negative *vs.* HPV extrachromosomal/mixed $p < 0.0001$; HPV-negative *vs.* HPV integrated $p = 0.0037$). The difference in DSS between the group of patients with HPV-positive tumours with an extrachromosomal/mixed *vs.* integrated form was not statistically significant ($p = 0.6742$). The group of patients with transcriptionally silent HPV survived better than HPV-negative patients but the difference was not statistically significant ($p = 0.2635$). When we divide this group of patients into HPV DNA positive/p16 positive and HPV DNA positive/p16 negative, these subgroups behaved, in terms of survival, as patients with HPV-positive and HPV-negative tumours, respectively (data not shown).

Discussion

Limited information has been available about high-risk mucosal (HR) HPV integration in HPV-associated HNC. HPV genomes replicate in the form of unintegrated plasmids

Table 5. Factors with impact on patients' disease specific survival according to the Cox model

Covariate	Hazard ratio (95% confidence interval)	*P*-value
HPV status		
Negative	Referent	<0.0001
Extrachromosomal	0.149 (0.059–0.380)	
Integrated	0.196 (0.066–0.589)	0.0037
Trascriptionally silent	0.503 (0.151–1.678)	0.2635
Tumour size	2.224 (1.545–3.201)	<0.0001
Nodal status	1.443 (1.005–2.072)	0.0472
Gender	2.786 (0.976–7.954)	0.0556

in viral persistence. Integration of HR HPV fragments in cancers results in a break in the E2 gene region at the 3' end of the HPV genome and in the expression of the viral E6 and E7 oncogenes from chimeric mRNAs terminating by cellular sequence. Integration has been considered a frequent, albeit not necessary, step in the progression of precancerous lesions to invasive carcinomas in the cervix[14].

In this study, we wished to assess HPV integration status in an extended sample set of HPV-associated HNC lesions to determine whether HPV integration had an effect on patient prognosis and survival. First, we have determined the physical status of HPV genomes and the structure of viral transcripts in a set of HPV16-positive fresh frozen tonsillar tumours in comparison to cervical carcinomas and apparently unaffected cervical tissue samples. All HPV 16 positive tumours were characterised by the 3'-RACE-based method referred to as analysis of papillomavirus oncogenic transcripts (APOT)[11]. Those samples with enough material were further analysed by the standard method of detection of HPV integration *vs.* plasmid persistence, that is, restriction enzyme digestion of tumour sample DNA followed by Southern blotting.

We then compared the APOT results with those of a method designed to detect the absence of 3' HPV E2 gene sequences from E6-E7 oncogene transcripts. As expected, the APOT assay was the more sensitive of the two methods tested but E2 transcript mapping showed excellent agreement with the APOT results.

The E2 transcript mapping method utilises short reverse transcript amplicons and is applicable to mRNA samples extracted from formalin fixed, paraffin embedded (FFPE) tissues. A limitation of this assay is that it cannot detect the presence of integrated (E2-) HPV E6-E7 mRNAs in mixed lesions that also express HPV E6-E7 transcripts from plasmid genomes; the E2+ mRNA-containing tumours are classified as extrachromosomal/mixed (see discussion below). We were able to analyse an extended set of clinical FFPE samples for the expression of integrated (E2-) *vs.* unintegrated/mixed HPV E6-E7 mRNA transcripts (E2+) to determine if HPV integration has an effect on patient prognosis and survival. To our knowledge, our study represents survival analysis in the largest sample set of patients with HPV-associated HNC. Our results show that HR-HPV integration in HNC does not influence the survival of patients even though those with an extrachromosomal/mixed form seem to have a slightly better prognosis in comparison to an integrated form of the virus.

In cervical carcinogenesis, integration of HPV genome into cell DNA is a characteristic step in the progression of precancerous lesions to invasive carcinomas[13,28,35]. In contrast, only a few studies have focused on the analysis of HPV genome status in HNC. In a previous study, we detected an integrated form of the virus in 64% of tonsillar tumours in FFPE samples by maping E2 mRNA in the region 3352 to 3535[36]. This proportion was comparable to the data reported by Koskinen *et al.* and Hafkamp *et al.*[29,37]. Mooren *et al.* have revealed integrated HPV in 42% of tonsillar squamous cell carcinoma (TSCC) samples by means of *in situ* hybridisation[38]. Recently, two studies based on next-generation sequencing of HNC have been published[23,27]. Khoury *et al.*[23] have found integrated HPV16 in 63% of HNC samples but Gao *et al.*[27] only in 16% of patients with oropharyngeal squamous cell carcinoma. The difference in the percentages of integration can be explained by the use of different methodological approaches, different study populations and sample size.

In our analysis, by means of E2 integration breakpoint mapping and APOT assay, we detected HPV integration in 36% of fresh frozen tonsillar tumours, an extrachromosomal form in 43% of those samples, and both forms in 14% of samples. The higher percentage of the extrachromosomal form of HPV in HNC in our study of fresh frozen samples is in agreement with the data previously published[17,27,29,38]. On the other hand, Lace *et al.* using the APOT assay have reported integrated HPV in 67% of HNC tumours[21].

As expected, the APOT assay identified integrated forms of HPV with higher sensitivity. Nevertheless, the excellent agreement between the E2 mapping and APOT assay justified the use of the E2 mapping method as a sensitive one for a large set of FFPE samples. However, it is important to note that the fresh frozen and formalin fixed paraffin embedded samples will never represent exactly the same piece of tumour, number of tumour cells and that because of the fixation and embedding procedures DNA and/or RNA extracted from FFPE samples might be partially degraded and therefore not amplifiable. We detected an integrated form of HPV in 27% of FFPE samples and extrachromosomal/mixed form in 73%. HPV integration was found more often in our previous study done on a smaller number of patients with only tonsillar tumours[36].

The structure of virus-cell transcripts expressed from integrated HPV genome corresponded with those previously identified in cervical carcinomas and HNC[11,21]. All mRNAs derived from integrated HPV genomes in tonsillar samples were type A transcripts, spliced from splice donor (SD) 880 to a cellular splice acceptor (SA). In cervical tissues, we also detected type B transcripts where the mRNA is spliced from

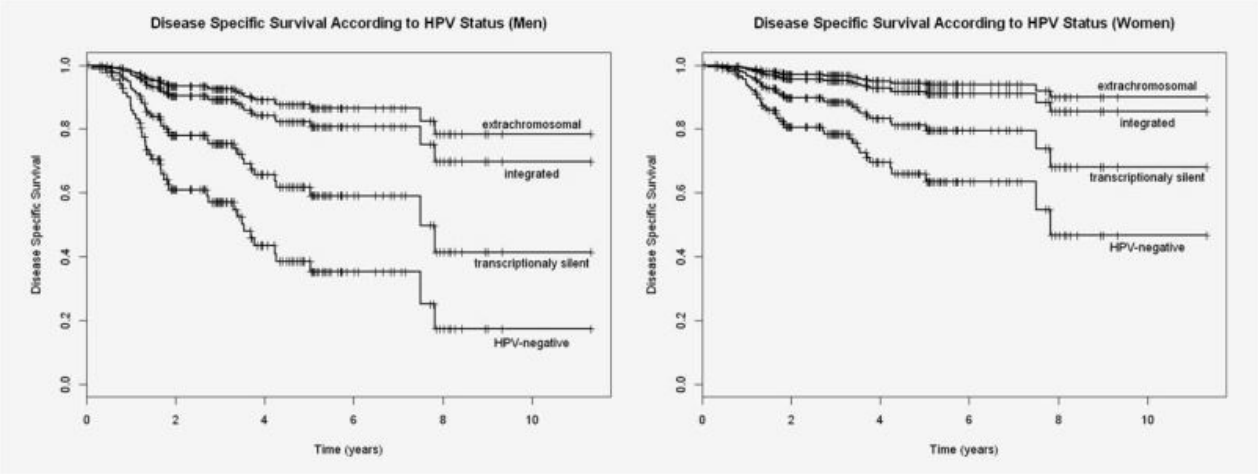

Figure 1. **Disease specific survival according to HPV status and gender.** HPV16 E6 mRNA negative; HPV16 E6 mRNA positive with extrachromosomal and/or mixed form; HPV16 E6 mRNA positive with integrated form; HPV16 DNA positive transcriptionally silent (no expression of HPV16 E6 mRNA). Survival curves are displayed for tumour size = T2 and nodal status = N2 which is the most common combination.

SD 880 to SA 3358. HPV sequences ended at position 3496 nt and continued to cellular DNA sequence. Other types of integrate-derived transcripts were not found. In extrachromosomal samples, we have revealed three different SA sites: SA 3358, as well as, SA 3421, and SA 3391 which have not been reported previously.

Integration events occurred on different chromosomes and some of the sites identified in our study have also been indicated for HPVs integrated in cervical carcinomas: 17q23.1, 3p26.2, and 20p12.1[22]. Locus 3p26.2 contains the *MDS1* and *EVI1* gene complex (MECOM). EVI1 is known as ecotropic virus integration site 1 and as a transcriptional factor and it plays an important role both in normal development and in oncogenesis[39,40]. Locus 20p12.1 encodes the *MACRO* domain containing 2 the deacetylase gene which may regulate gene silencing, life span, and other cellular processes[41]. Olthof *et al.* identified locus 17q23.1 also in oral squamous cell carcinoma (OSCC)[17].

In agreement with Lace *et al.* who used the APOT assay[21], and concordantly with Smeets *et al.*[30] who used high-resolution microarray comparative genomic hybridisation, we detected HPV integration in the 13q14 and 5q35 region. In our study, the integration in these regions influenced the *TSC22D1* gene encoding a protein that might play a critical role in tumour suppression through the induction of cancer cell apoptosis and the gene coding for molecular chaperone calnexin (CANX).

HPV integration into locus 3q28 identified in our study was previously reported in HNC by Olthof *et al.* and Khoury *et al.*[17,23] This region encodes protein TP63. This protein acts as a transcriptional factor which plays a role in the development and maintenance of stratified epithelial tissues and has been reported to function in oncogenesis.

In five of the samples, HPV was integrated near the common fragile sites (FRA1F, FRA4B, FRA5G, FRA17B, and FRA19A). HPV16 has been reported before to be integrated into cervical tumours close to four of these fragile sites (for review see Ragin *et al.*[42]). FRA1F, FRA5G and FRA19A have been mentioned as being frequently modified in other solid tumours and leukemias[43,44].

To our knowledge, our study is the first to report on the influence of HPV integration status in HNC tumours on disease specific survival (DSS) of patients. In agreement with our previous and other studies, we confirmed that HPV positivity is the strongest prognostic factor for patients with HNC[36,45]. However, the difference in DSS between patients with an integrated HPV form and extrachromosomal/mixed form was not statistically significant although patients with the latter form tend to live longer without disease recurrence. The applied method for determination of the integration status in FFPE samples should, however, be validated in future larger cohorts to allow for a definite statement on the influence of viral integration on patient survival.

Similar data as in our study have been reported for patients with cervical cancer. Nambaru *et al.*[46] have shown that patients with cervical tumours with an extrachromosomal form of HPV have better disease-free survival than those with integrated HPV16 but the difference was not statistically significant. However, Shin *et al.*[47] have reported HPV integration as a significant prognostic factor for poor disease-free survival in patients with cervical cancer.

In conclusion, we determined the HPV status in tonsillar tumours by two different methods. Furthermore, exact HPV integration sites were identified. The survival analysis in a large number of patients with HNC did not confirm HPV integrated genome status as a factor significantly influencing the patients' prognosis.

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Tab. S1 – Demographical and clinical pathological characteristics of 186 patient's enrolled in the survival analyses.

Characteristic		N [%]
Gender	male	149 [80.1]
	female	37 [19.9]
Age	mean	57.3
Education	≤12	112 [62.6]
	>12	67 [37.4]
Localization	oropharynx	148 [79.6]
	oral cavity	38 [20.4]
Tobacco status	Yes	80 [43.2]
	Ex-smoker	68 [36.8]
	No	37 [20]
Alcohol	Yes	115 [62.2]
	Ex-drinker	21 [11.3]
	No	49 [26.5]
Tumor size	T1+T2	152 [82.6]
	T3+T4	32 [17.4]
Nodal status	N0	63 [34.2]
	N1-3	121 [65.8]
Tumor stage	I+II	62 [33.7]
	III+IV	122 [66.3]
Tumor grade	1+2	131 [72.4]
	3	50 [27.6]
p16	positive	104 [59.1]
	negative	72 [40.9]
p53	positive	53 [30.8]
	negative	119 [69.2]
HPV DNA	positive	105 [56.5]
	negative	81 [43.5]
HPV 16 E6 mRNA	positive	91 [48.9]
	negative	95 [51.1]
Genome status	extrachromosomal	66 [62.9]
	integrated	25 [23.8]
	transcriptional silent	14 [13.3]

Tab. S2 - List of primers used for mapping of E2 integration breakpoint.

Amplicon	Primer Direction	Sequence (5´-3´)	Position
E7	forward	AGCACACACGTAGACATTCGTA	772-793
	reverse	AGATGGGGCACACAATTCCT	841-822
E7-E4	forward	AGCACACACGTAGACATTCGTA	772-793
	reverse	GGTGTCTGGCTCTGATCTTG	3496-3477
E2B	forward	ATGCATTATACAAACTGGA	3140-3158
	reverse	TGCACAAAATATGTTCGTATTCC	3264-3242
E2C	forward	ATTATTAGGCAGCACTTG	3383-3400
	reverse	GGTGTCTGGCTCTGATCTTG	3496-3477
E2D	forward	GATAGTGAATGGCAACGTGAC	3767-3787
	reverse	GATGCAGTATCAAGATTTG	3872-3854
E5	forward	ATACGTCCGCTGCTTTTGTCTG	3934-3955
	reverse	ACACCTAAACGCAGAGGCTGCCTGT	4026-4003

Tab. S3 - Detection of HPV status in FF and FFPE samples by APOT assay and E2 integration breakpoint mapping.

	Fresh frozen tissues (FF)		Formalin fixed paraffin embedded tissues (FFPE)
Sample no.	**APOT assay**	**E2 mapping**	**E2 mapping**
ORL104	extrachromosomal	extrachromosomal	integrated
ORL111	integrated	integrated	integrated
ORL133	integrated	integrated	integrated
ORL136	extrachromosomal	extrachromosomal	extrachromosomal
ORL154	integrated	integrated	integrated
ORL161	extrachromosomal	extrachromosomal	extrachromosomal
ORL181	extrachromosomal	extrachromosomal	extrachromosomal
ORL187	extrachromosomal/mixed	extrachromosomal	extrachromosomal
ORL263	extrachromosomal	extrachromosomal	extrachromosomal
ORL267	extrachromosomal	extrachromosomal	extrachromosomal
ORL269	integrated	integrated	extrachromosomal
ORL224	extrachromosomal	extrachromosomal	extrachromosomal
ORL227	extrachromosomal/mixed	extrachromosomal	integrated
ORL137	extrachromosomal	extrachromosomal	extrachromosomal
ORL257	extrachromosomal	extrachromosomal	extrachromosomal
ORL265	extrachromosomal	extrachromosomal	extrachromosomal
ORL243	extrachromosomal/mixed	extrachromosomal	extrachromosomal
ORL244	extrachromosomal/mixed	extrachromosomal	integrated
ORL280	extrachromosomal	extrachromosomal	extrachromosomal

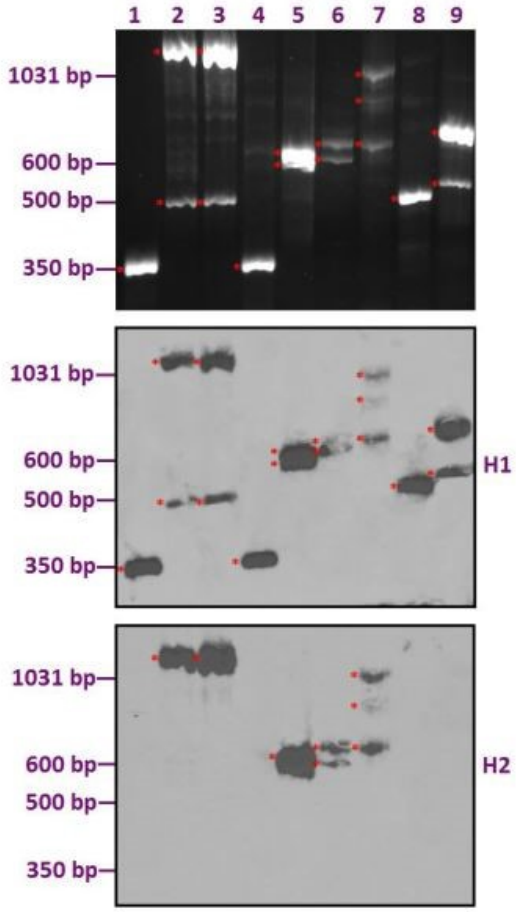

Fig. S1 Representative results of the APOT assay cervical carcinoma samples, cervical unaffected tissue and tonsillar tumor samples. Agarose gel electrophoresis (top), Southern blot hybridization with E7-specific probe H1 (middle) and E4-specific probe H2 (bottom). Product sizes are indicated on the left. All products visible on the electrophoresis gel were sequenced (labeled with red stars). All amplimers hybridized with the H1 probe, so they contain the amplified region from 789-813. Amplimers which hybridized with the H2 probe (3512-3535) were indicative for extrachromosomal HPV or for the viral-cellular transcript type B (based on sequencing). Viral-cellular transcripts type A didn´t hybridize with the H2 probe. List of samples: 1 - RNA9, 2 - RNA17A, 3 - RNA17B, 4 - RNA19A, 5 - RNA19B, 6 - ORL104, 7 - ORL181, 8 - ORL125, 9 - RNA154.

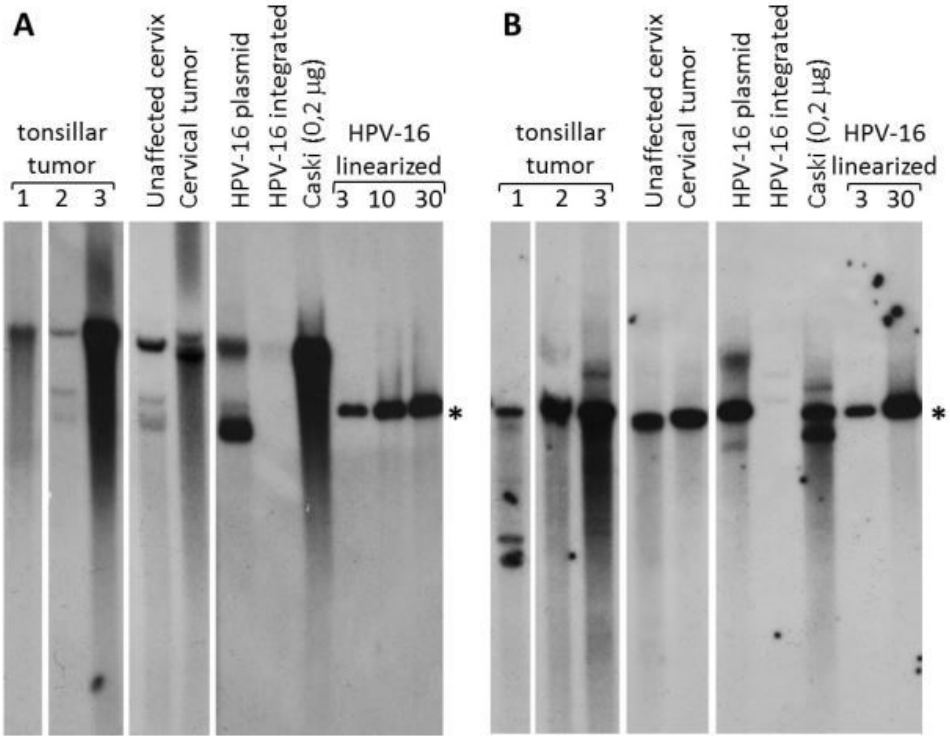

Fig. S2 - **Southern blots.** A - uncut DNA and B - DNA digested by a single cutting restriction enzyme BamHI (* 7,9 kb). Tonsillar tumor sample 1 (ORL133) – integrated form in tandem repeats (type II), tonsillar tumor sample 2 (ORL126) – extrachromosomal form, tonsillar tumor sample 3 (ORL187) - integrated form in tandem repeats. Unaffected cervix (RNA19A) harbors integrated form (type I) and cervical tumor (RNA19B) harbors mixed form of HPV based on APOT assay, but in the case of tumor sample it is integrated form type I based on Southern blot. Linearized HPV 16 DNA represents 1 (3 pg), 3 (10 pg) and 10 (30 pg) HPV genome copies/cell (2 µg).

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Comparison of the miRNA profiles in HPV-positive and HPV-negative tonsillar tumors and a model system of human keratinocyte clones

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Abstract

Background: Better insights into the molecular changes involved in virus-associated and -independent head and neck cancer may advance our knowledge of HNC carcinogenesis and identify critical disease biomarkers. Here we aimed to characterize the expression profiles in a matched set of well-characterized HPV-dependent and HPV-independent tonsillar tumors and equivalent immortalized keratinocyte clones to define potential and clinically relevant biomarkers of HNC of different etiology.

Methods: Fresh frozen tonsillar cancer tissues were analyzed together with non-malignant tonsillar tissues and compared with cervical tumors and normal cervical tissues. Furthermore, relative miRNAs abundance levels of primary and immortalized human keratinocyte clones were evaluated. The global quantitation of miRNA gene abundance was performed using a TaqMan Low Density Array system. The confirmation of differentially expressed miRNAs was performed on a set of formalin-fixed paraffin-embedded tumor samples enriched for the tumor cell fraction by macrodissection.

Results: We defined 46 upregulated and 31 downregulated miRNAs characteristic for the HPV-positive tonsillar tumors and 42 upregulated miRNAs and 42 downregulated miRNAs characteristic for HPV-independent tumors. In comparison with the expression profiles in cervical tumors, we defined miR-141-3p, miR-15b-5p, miR-200a-3p, miR-302c-3p, and miR-9-5p as specific for HPV induced malignancies. MiR-335-5p, miR-579-3p, and miR-126-5p were shared by the expression profiles of HPV-positive tonsillar tumors and of the HPV immortalized keratinocyte clones, whereas miR-328-3p, miR-34c-3p, and miR-885-5p were shared by the miRNA profiles of HPV-negative tonsillar tumors and the HPV-negative keratinocytes.

Conclusions: We identified the miRNAs characteristic for HPV-induced tumors and tonsillar tumors of different etiology, and the results were compared with those of the model system. Our report presents the basis for further investigations leading to the identification of clinically relevant diagnostic and/or therapeutic biomarkers for tumors of viral and non-viral etiology.

Keywords: Human papillomavirus, miRNA, Tonsillar tumor, Head and neck cancer

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Background

Head and neck cancer (HNC) mostly involves a group of squamous cell carcinomas (HNSCC) which arise from the epithelial cells of the mucosal lining in the head and neck region. HNSCC belong among the six most common cancers worldwide [1]. The most important risk factors are smoking and alcohol consumption; however, HPV infection is also recognized as another primary cause of HNC. The proportion of HPV-caused HNC varies around the world; in the USA about 40–80 % of oropharyngeal cancers are associated with the presence and expression of high-risk HPV (HR-HPV), whereas in Europe, this percentage ranges from 90 % in Sweden to less than 20 % in countries with high consumption of tobacco [2]. In the Czech Republic, 68 % of the oropharyngeal tumors are HR-HPV positive [3, 4]. Recently, an increasing incidence of oropharyngeal cancers associated with HPV has been reported in several countries [5–7]. The most prevalent mucosal HR-HPV in HNC is HPV16 occurring in up to 90 % of HPV-positive HNC [8, 9].

Although patients with HR-HPV associated HNC often present with more advanced disease, they have a remarkably better prognosis and overall survival [9, 10]. The prognostic advantage of HNC tumors etiologically linked to HPV could potentially lead to modified treatment regimens. Besides, oropharyngeal tumors are usually not detected in the early stages of the disease, most likely due to the location of the lesions in the tonsillar crypts, and the marker of early cancer formation might allow earlier treatment and improved prognosis. Meanwhile, patients start to be treated in the late stages of disease and their prognosis is relatively poor. Unlike cervical cancer which is nearly always associated with HR-HPV infection, virally and non-virally induced HNC represent a unique model to study cancer in the same localization that is caused by distinct molecular mechanisms.

MicroRNAs (miRNAs) are a class of short single-stranded non-coding RNAs. MiRNAs play an important role in post-transcriptional regulation of gene expression by inducing target mRNA degradation or by repressing the translation via binding to the 3′-untranslational region of their target mRNAs [11]. The regulation of miRNA expression has been demonstrated to play a key role in the development, cell growth, and differentiation processes in a variety of eukaryotic organisms. The expression of miRNAs is deregulated in human cancer and their abundance profiles are often specific for tumors of different origin where they can serve as oncogenes or tumor suppressors. It has been shown that both normal and cancer tissues have specific miRNA expression signatures and show differential expression across tumor types [12]. In previous studies, it has also been demonstrated that miRNAs are promising prognostic and diagnostic biomarkers of human cancers [13–16].

Several studies focused on miRNA profiling in HNC have been published in recent years. Distinct miRNA profiles were shown in head and neck cancer cell lines [17] as well as in tumor tissues compared with normal tissues [18–20]. However, to date only a few studies analyzing the miRNA profile in HNC with regard to HPV presence have been published. HPV-positive and HPV-negative HNSCC cell lines were studied by Wald et al. [21]. Lajer et al. profiled HPV-positive and HPV-negative oral, pharyngeal, and oropharyngeal (OSCC, PSCC, OPSCC) and cervical squamous cell carcinomas [22, 23] and identified the "HPV core" miRNAs.

The aim of our study was to characterize the expression profiles of miRNAs in well-characterized tonsillar tumors and define miRNAs characteristic for HPV-dependent and HPV-independent tonsillar tumors. Cervical tumors were also evaluated as a positive control of HPV-associated tumors, allowing us to identify miRNAs specific for HPV-induced tumors. Furthermore, we compared the results of miRNA expression profiles with the model system of isogenic primary human keratinocyte clones immortalized by HPV or human telomerase gene. We identified a group of miRNAs specific for HPV-induced tumors and a group of miRNAs specific for tonsillar carcinomas. The expression of the most differentially expressed miRNAs was confirmed on a large set of macrodissected tonsillar tumors by quantitative real-time PCR (RT qPCR). Finally, we have identified several miRNAs as potential prognostic markers but their significance has to be determined in a larger sample set. Our results serve as a starting point for the identification of useful and clinically relevant biomarkers of HNC tumors of HPV-related and HPV-independent etiologies.

Methods

Clinical samples

All tonsillar tumor samples were obtained in the scope of the ongoing study from patients treated at the Department of Otolaryngology and Head and Neck Surgery, 1st Faculty of Medicine, Charles University and Motol University Hospital, Prague in the period from 2005 to 2007. The study set, i.e. 23 fresh frozen (FF) tumor samples and 64 formalin-fixed paraffin-embedded (FFPE) tumor samples, was selected based on the virological and immunohistochemical characteristics of the FFPE tissue samples in our ongoing studies (p16, p53, HPV DNA, and HPV E6 mRNA) [24] and the availability of fresh frozen material. Normal tonsillar tissue samples ($N = 5$) were collected from patients who underwent tonsillectomy for non-malignant conditions. These patients were gender and age matched to the study patients with tonsillar tumors. Cervical samples, i.e. normal cervical tissues ($N = 2$) and cervical tumors ($N = 5$), were collected from patients treated at the Department of Obstetrics and Gynecology, 2nd Faculty of Medicine, Charles University and Motol

University Hospital, Prague in 2012. All patients enrolled in the study signed the informed consent form. The study received official institutional and ethical approval from the Motol University Hospital and Institute of Hematology and Blood Transfusion.

For patients from whom FF tonsillar tumor samples were obtained, data on demographics and clinical pathological characteristics were completed for each patient and collected by a questionnaire. All data are summarized in Additional file 1: Table S1. All patients were prospectively followed up.

The sampling and tissue handling for tonsillar tumor samples in the ongoing study has been described before [25]. Sections of fresh frozen tumor tissues were cut on a cryostat, and the number of tumor cells was determined by a pathologist. The samples of non-malignant tonsils were taken during the surgery and were snap frozen in liquid nitrogen immediately after removal and stored at −80 °C. Samples of normal cervical tissue and cervical tumors were taken during the surgery, stored and transported in RNAlater (Life Technologies, USA) transport medium, and processed within 1 week. FFPE samples were macrodissected. The area with the tumor was labelled by an expert pathologist on a hematoxylin stained slide and transported to the laboratory. The tissue from the labelled area was scraped off and used for immediate RNA extraction.

A set of nine isogenic primary human foreskin keratinocyte clones and the w12 cell line (derived from cervical carcinoma containing extrachromosomal HPV16) were kindly provided by the collaborating laboratories at the University of Iowa, USA. The preparation of the clones has been previously described [26, 27]. Five of the foreskin keratinocyte clones were immortalized by HPV16, two were immortalized by the telomerase gene, and the rest were primary keratinocyte cells. The cells were stored at -80 °C and used directly for the isolation of nucleic acids. The characteristics of analyzed clones and cell lines are summarized in Table 1.

Processing of samples

DNA from all FF tonsillar tissues and cervical tissues was isolated using the QIAamp DNA Mini kit (Qiagen, Germany) and total RNA was extracted by the miRVana kit (Life Technologies, USA) according to the manufacturer's protocol. For better yield, only RNA was isolated from the cell lines. Both DNA and RNA of FFPE samples were simultaneously extracted from four 10-μm sections enriched for the tumor cells by macrodissection using the Ambion RecoverAll™ total nucleic acid isolation kit for FFPE according to the manufacturer's protocol (Applied Biosystems, USA).

Table 1 Specification of keratinocyte clones and cell lines

Type of cells	Specification	Number
HPV16 immortalized human keratinocyte clones	integrated	3
	extrachromosomal	1
	mixed	1
HPV16-positive original w12 cell line	extrachromosomal	1
primary human keratinocytes	HPV-negative	2
hTERT immortalized human keratinocyte clones	HPV-negative	2

The detection and typing of HPV DNA was performed by a modified PCR method with broad spectrum primers BSGP5+/6 + bio specific for the L1 region and reverse line blot hybridization as previously described [28]. RNA concentration and quality were measured by Experion chip electrophoresis (Bio-Rad, USA). For the analysis of microarrays, only FF samples with a RIN (RNA integrity number) higher than seven were used. The expression of HPV16 E6 mRNA was assessed by RT PCR which amplifies the most abundant splice variant E6*I with a length of 86 bp as described before [29]. The status of the HPV genome in FF clinical samples as well as in the cell lines was evaluated by the mapping of E2 integration breakpoint and APOT assay as described recently [25].

TaqMan Low Density Array (TLDA) analysis

The global quantitation of miRNA gene expression was performed using the TaqMan® Array Human MicroRNA A + B Cards Set v3.0 (Life Technologies, USA) containing a total of 384 TaqMan® MicroRNA Assays and controls per card. Each array contains three assays of endogenous controls (RNU48, RNU44, and RNU6B) and one assay as a negative control.

Overall, we analyzed 35 clinical samples and 12 cell lines. First, 1000 ng of total RNA of each sample were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers specific for each card (both Life Technologies, USA) according to the manufacturer's instructions. The TLDA cards were analyzed on the Applied Biosystems 7900HT Real-Time PCR System.

Data analysis

For data processing and evaluation, we used the SDS 2.4 and the ExpressionSuite v1.0.1. software (Life Technologies, USA). Ct values for miRNAs identified by automated thresholding were exported separately for cards A and B. From the detected Ct values, the RQ (relative quantity) was calculated using the 2^-Ct formula. The data were further processed by the GeneSpring GX11 software (Agilent). Based on the per-sample measurement counts (hundreds), we selected the 50th percentile shift to perform within-sample normalization and applied it globally across the data sets. For comparison of two or three groups in the censored analyses, T-test or ANOVA, in combination with the Pavlidis Template Matching tool (TIGR TM4 suite) was used.

The *P*-value ($P < 0.05$) and fold-change (FC> 1.33) thresholds were set as exploratory-stage parameters (alpha setting only, no corrections for multiple testing were applied) in the comparison of differential abundance of miRNAs in different groups of samples. All data analysis was performed on a subset of samples ($N = 10$, six HPV-positive and four HPV-negative tonsillar tumors) with high tumor fraction (≥60 % of tumor cells). Only the miRNAs differentially expressed in at least 3/5 (60 %) samples with a valid (measured) result (except for groups with two samples where all samples had to have a measured result) were considered for further analyses.

In order to determine new variables reflecting information on the differentially expressed miRNAs in relation to patient prognosis, tumor size, patient age, and grading, we applied factor analysis allowing for the reduction of the number of variables, taking advantage of a relatively large set of miRNAs analyzed across ten patients with tonsillar tumor only. The set of data was reduced and the miRNAs not expressed in two or more of ten analyzed samples were excluded. Altogether, 368 miRNAs, prognosis, size of tumor, age, and grading were evaluated. Additional available characteristics, such as tumor stage, nodal status, smoking status, and alcohol consumption status, were identical in all or almost all subjects. This analysis was done using IBM SPSS v23.

Technical validation of microarray data

Selected miRNAs (miR-21, miR-205, let-7b, miR-24, miR-126, miR-378, miR-141, miR-200c, miR-146b, miR-191, and miR-484) which were found differentially expressed by the microarrays in tonsillar samples were further validated by the individual RT qPCR using the TaqMan® MicroRNA Assays (Life Technologies, USA) on the same set of samples. First, 10 ng of total RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, USA) and predesigned primers for each individual TaqMan® MicroRNA Assay according the manufacturer's instructions. RNU48 was used as the endogenous control. For each sample, qPCR reaction was done in triplicate and consisted of 2 μl cDNA, 1× TaqMan® MicroRNA Assay, 5 μl of TaqMan® Universal PCR Master Mix, no AmpErase UNG (Life Technologies, USA), and nuclease-free water. The cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The pool of unaffected tonsils was used as a calibrator. The $2^{-\Delta\Delta Ct}$ method was used for calculations of the fold change. The overall agreement between the microarray data and individual assay data was excellent with the exception of miR-378 where the directions of regulation were opposite.

Confirmation of microarray results

The confirmation of the differentially expressed miRNAs was performed on a large set of 64 macrodissected FFPE tumor samples (46 HPV-positive tumors and 18 HPV-negative tumors). The miRNA expression was assayed by individual RT qPCR using the TaqMan® MicroRNA Assays (Life Technologies, USA) as described above. As a calibrator, the pool of total RNA from unaffected tonsils were used. RNU48 was used as the endogenous control. The $2^{-\Delta\Delta Ct}$ method was used for calculations of the fold change. The cut-off fold change was set as for the arrays to +/− 1.33.

Results

Characterization of patients and samples

Demographic and clinical pathological parameters of patients with tonsillar tumors are summarized in Additional file 1: Table S1. The mean age of patients with tonsillar squamous cell carcinomas (TSCC) was 54.8 years and most of them were males (91.3 %). All HPV-positive tonsillar tumors contained HPV type 16 and expressed HPV16 E6 mRNA. The analysis of HPV genome status in HPV-positive tonsillar samples was done as published recently [25], with 43 % of the samples containing extrachromosomal HPV DNA, 14 % both extrachromosomal and integrated HPV DNA, and 36 % integrated HPV DNA. One sample yielded inconclusive results. All cervical tumors were HPV16-positive, whereas the non-malignant cervical tissues were HPV-negative. The majority of cervical tumors contained integrated HPV DNA.

Global miRNA profiling in the model system

MiRNA expression profiling was done in the model system of primary and immortalized human keratinocyte clones. As illustrated in Fig. 1, each group of the analyzed clones exhibited a very specific miRNA expression profile in principal component analysis (PCA).

The miRNA expression profiles of human keratinocyte clones were evaluated as a model system for clinical samples. As an analogy to the comparison of HPV-positive and/or HPV-negative tonsillar tumors with normal tissues, HPV16 immortalized keratinocyte clones and/or keratinocyte clones immortalized by the human telomerase gene and primary keratinocytes were used. In keratinocyte clones immortalized by HPV16, 39 miRNAs were differentially expressed whilst in keratinocyte clones immortalized by human telomerase gene, 30 miRNAs were found. MicroRNAs identified as differentially expressed with the *P*-value ($P < 0.05$) and fold-change (FC> 1.33) in the corresponding comparisons are listed in Additional files 2 and 3: Tables S2 and S3. MiRNAs miR-135b-3p, miR-146b-5p, miR-205-5p, miR-425-3p, miR-625-3p, and miR-485-3p which are involved in signaling and cell migration connected with epithelial to mesenchymal transition, tumor invasion, and metastasis, were detected in both the

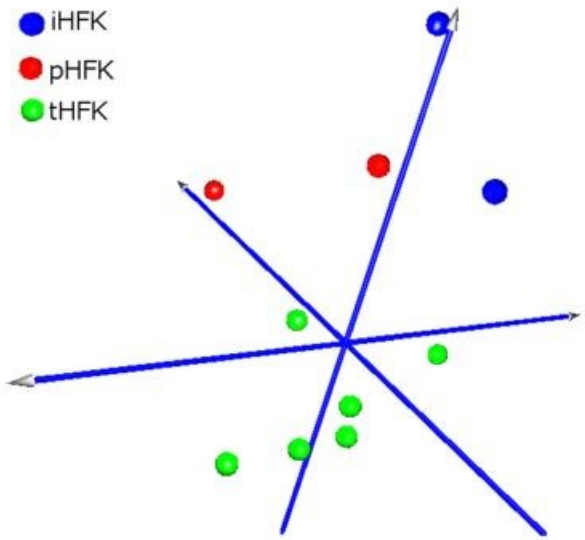

Fig. 1 Principal component analysis plot. Visualization of miRNA expression in model systems. Primary human keratinocytes (pHFK); human keratinocytes immortalized by human telomerase gene (iHFK); HPV immortalized keratinocytes (tHFK)

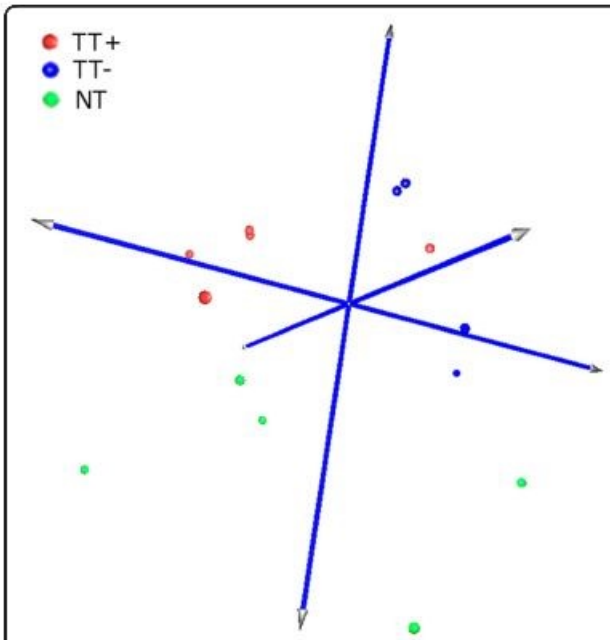

Fig. 2 Principal component analysis plot. Visualization of miRNA expression of tonsillar tumors and non-malignant tissues. HPV-positive tonsillar tumors (TT+); HPV-negative tonsillar tumors (TT-); non-malignant tonsillar tissue (NT)

comparison of HPV immortalized keratinocytes with primary keratinocytes and the comparison of telomerase immortalized clones with primary keratinocytes.

Global miRNA profiling in tumors and controls

In human tissues, non-censored PCA showed clear separation of the two groups of samples: normal HPV-negative cervical samples and non-malignant tonsillar tissue samples, suggesting the tissue specificity of the miRNA expression profile (Additional file 4: Figure S1). Clear group separation based on the miRNA expression profiles was also detected for the group of tumor samples and samples of normal tissue from the same anatomical location (Additional file 5: Figures S2A and B).

Figure 2 summarizes the miRNA expression of HPV-positive tonsillar tumors, HPV-negative tonsillar tumors, and non-malignant tonsillar tissues. We included only FF samples with more than 60 % of tumor cells which show much better separation in comparison to all samples. We found that tumor sample homogeneity (rather than sample size) is important for the robustness of miRNA expression analysis as separation is less clear if all samples irrespective of tumor cell content are visualized (data not shown).

The comparison of the miRNA expression profiles of tonsillar tumors and normal tonsillar tissues revealed 46 upregulated and 31 downregulated miRNAs in HPV-positive tumors (listed in Additional file 6: Table S4) and 42 upregulated and 42 downregulated miRNAs in HPV-negative tumors (listed in Additional file 7: Table S5).

To illustrate and identify the miRNAs specific for HPV-induced tumors and tonsillar tumors associated with or independent of HPV, a Venn diagram was constructed (Fig. 3). Altogether, we identified five miRNAs specific for HPV-induced malignancies, the so-called HPV-core miRNAs, common for HPV-positive TSCC and cervical tumors (listed in Table 2). One of these five miRNAs was inversely expressed in tonsillar and cervical tumors. The group of miRNAs specific for HPV-positive tonsillar tumors encompassed 30 miRNAs, and 38 miRNAs were exclusively specific for HPV-negative tonsillar tumors (all miRNAs listed in Table 3). Additionally, 35 miRNAs were common to both HPV-positive and HPV-negative tonsillar tumors which seem to be characteristic for the anatomical location of head and neck cancer (listed in Additional file 8: Table S6).

Comparison of miRNA profiles in clinical samples and the model system

The aim of our study was also the comparison of miRNA expression in clinical samples and in the model system of keratinocyte clones. HPV-positive tonsillar tumors and HPV-immortalized keratinocyte clones overlapped in three miRNAs (Table 4) specific to either group. MiR-335-5p and miR-579-3p were upregulated in both groups, whereas miR-126-5p was upregulated in clinical samples and downregulated in the model system. We also identified seven additional miRNAs common to these two groups, but they were significantly deregulated either in HPV-negative tonsillar tumors in comparison with normal tonsils or in keratinocyte clones immortalized by human

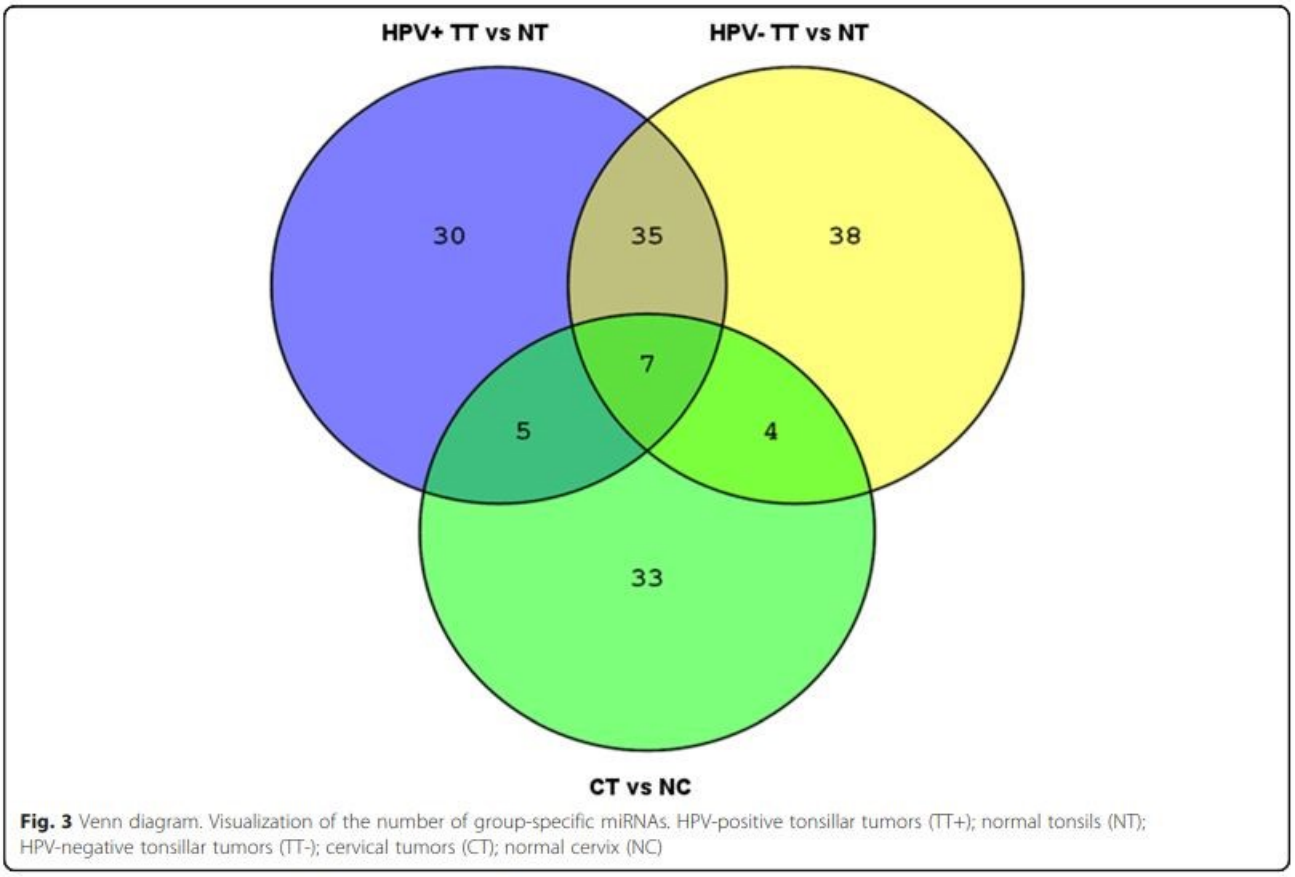

Fig. 3 Venn diagram. Visualization of the number of group-specific miRNAs. HPV-positive tonsillar tumors (TT+); normal tonsils (NT); HPV-negative tonsillar tumors (TT-); cervical tumors (CT); normal cervix (NC)

telomerase (hTERT) in comparison with primary keratinocytes, so they seemed not to be directly influenced by the presence of HPV. HPV-negative tumors and hTERT-immortalized keratinocyte clones overlap in three miRNAs specific to either group and nine additional miRNAs which were common also to other groups. We detected miRNA-328-3p which was upregulated in both groups. Inverse patterns of expression regulation of miR-34c-3p and miR-885-5p were found in the model system and clinical samples.

In the groups of HPV-positive tonsillar tumors and HPV-immortalized keratinocyte clones, we also evaluated the influence of HPV status on the changes in the miRNA expression profile. HPV-positive tumor tissues which contain extrachromosomal, integrated, or mixed HPV DNA differ in the miRNAs expression profiles. A similar situation was observed in the model system of keratinocyte clones. However, we found no overlap in the list of miRNAs differentially expressed in HPV-positive tumors and in HPV-immortalized keratinocytes with a particular form of HPV DNA, but the differentially expressed miRNAs were found to target the same genetic pathways.

Table 2 List of HPV core miRNAs

Probe name	miRNA family	TT+ versus NT[a]		CT versus NC[b]	
		Fold change	*p*-value	Fold change	*p*-value
miR-141-3p	mir-8	6.175	0.000197	17.039	0.002
miR-15b-5p	mir-15	3.072	0.034	10.659	0.045
miR-200a-3p	mir-8	8.173	0.000269	6.481	0.005
miR-302c-3p	mir-302	29.111	0.093	−1862.248	0.005
miR-9-5p	mir-9	24.460	0.001	177.296	0.018

[a]*TT+* HPV-positive tonsillar tumors, *NT* normal tonsils
[b]*CT* cervical tumors, *NC* normal cervix

Confirmation of microarray results

To confirm the results of differentially expressed miRNAs revealed by miRNA arrays, we selected nine specific miRNAs and confirmed their expression in a set of 64 FFPE samples by individual TaqMan miRNA assays. The miRNAs were selected based on the fold change and relevance in the literature. From the group of HPV core miRNAs, we selected miR-9, miR-141, and miR-200a which were upregulated in both groups of HPV-associated tumors (Additional file 9: Figure S3). From the group of the miRNAs specific for HPV-positive tonsillar tumors, the upregulated miR-125b-2*, miR-21, and miR-335 were selected (Additional file 10: Figure S4). MiR-335 was also upregulated in the model system of

Table 3 List of group-specific miRNAs derived from the Venn diagram

Exclusively TT+ specific[a]			Exclusively TT- specific[a]		
Probe name	miRNA family	FC[b]	Probe name	miRNA family	FC[b]
hsa-miR-125b-2-3p	mir-10	21.689	hsa-miR-431-5p	mir-431	37.879
hsa-miR-147b	mir-147	16.057	hsa-miR-517c-3p	mir-515	12.679
hsa-miR-523-3p	mir-515	10.983	hsa-miR-485-3p	mir-485	7.305
hsa-miR-542-3p	mir-542	9.273	hsa-miR-623	mir-623	6.535
hsa-miR-10a-5p	mir-10	4.513	hsa-miR-34c-5p	mir-34	4.851
hsa-miR-202-3p	mir-202	3.694	hsa-miR-221-3p	mir-221	4.505
hsa-miR-34a-3p	mir-34	2.854	hsa-miR-517a-3p	mir-515	4.327
hsa-miR-34a-5p	mir-34	2.574	hsa-miR-193b-3p	mir-193	2.966
hsa-miR-21-5p	mir-21	2.494	hsa-miR-193a-5p	mir-193	2.620
hsa-miR-335-5p	mir-335	2.378	hsa-miR-223-3p	mir-223	2.518
hsa-miR-18b-5p	mir-17	2.338	hsa-miR-323a-3p	mir-154	2.391
hsa-miR-130b-3p	mir-130	2.180	hsa-miR-1260a	mir-1260a	2.202
hsa-miR-197-3p	mir-197	2.077	hsa-miR-106b-3p	mir-17	2.072
hsa-miR-579-3p	mir-548	1.945	hsa-miR-151a-3p	mir-28	1.735
hsa-miR-132-3p	mir-132	1.699	hsa-let-7d-5p	let-7	−1.389
hsa-miR-214-5p	mir-214	−2.571	hsa-miR-30b-5p	mir-30	−1.461
hsa-miR-30e-3p	mir-30	−2.934	hsa-miR-487a-3p	mir-154	−1.474
hsa-miR-425-3p	mir-425	−3.147	hsa-let-7e-5p	let-7	−1.554
hsa-miR-500a-3p	mir-500	−3.333	hsa-let-7b-5p	let-7	−1.582
hsa-miR-126-5p	mir-95	−4.049	hsa-miR-564	mir-564	−1.590
hsa-miR-145-3p	mir-145	−4.805	hsa-miR-629-3p	mir-629	−1.616
hsa-miR-1247-5p	mir-1247	−5.233	hsa-miR-125b-5p	mir-10	−1.920
hsa-miR-505-5p	mir-505	−5.344	hsa-miR-100-5p	mir-10	−2.106
hsa-miR-30a-3p	mir-30	−5.981	hsa-miR-30d-5p	mir-30	−2.145
hsa-miR-136-3p	mir-136	−6.714	hsa-miR-26b-3p	mir-26	−2.369
hsa-miR-486-5p	mir-486	−7.824	hsa-miR-29a-3p	mir-29	−2.397
hsa-miR-1179	mir-1179	−8.498	hsa-miR-130b-5p	mir-130	−2.647
hsa-miR-1	mir-1	−10.729	hsa-miR-642a-5p	mir-642	−2.768
hsa-miR-575	mir-575	−11.791	hsa-miR-328-3p	mir-328	−2.806
hsa-miR-133a-3p	mir-133	−24.685	hsa-miR-744-3p	mir-744	−2.998
			hsa-miR-520d-3p	mir-515	−3.135
			hsa-miR-99a-5p	mir-10	−3.404
			hsa-miR-29c-3p	mir-29	−3.474
			hsa-miR-139-3p	mir-139	−3.666
			hsa-miR-885-5p	mir-885	−3.725
			hsa-miR-138-5p	mir-138	−12.185
			hsa-miR-142-3p	mir-142	−15.980
			hsa-miR-150-5p	mir-150	−17.261

[a]*TT+* HPV-positive tonsillar tumors, *TT-* HPV-negative tonsillar tumors
[b]*FC* fold change

HPV-immortalized keratinocyte clones. Next, miR-221 upregulated in a group of HPV-negative tonsillar tumors was analyzed (Additional file 11: Figure S5). Finally, miR-20b exemplified the miRNAs upregulated in HPV-positive tonsillar tumors and downregulated in HPV-negative tonsillar tumors, and miR-210 exemplified those upregulated in tonsillar tumors of either etiology (Additional file 12: Figure S6).

Table 4 List of significantly deregulated ($P < 0.05$) miRNAs in clinical samples vs. the model system

Probe name	miRNA family	tHFK vs. pHFK[a]	TT+ vs. NT[b]
		Fold change	Fold change
hsa-miR-335-5p	mir-335	8.361	2.378
hsa-miR-579-3p	mir-548	7.921	1.945
hsa-miR-126-5p	mir-95	2.580	−4.049
Probe name	miRNA family	iHFK vs. pHFK[a]	TT- vs. NT[b]
		Fold change	Fold change
hsa-miR-328-3p	mir-328	−22.020	−2.806
hsa-miR-34c-5p	mir-34	−16.419	4.851
hsa-miR-885-5p	mir-885	48.377	−3.725

[a]*tHFK* HPV-immortalized human foreskin keratinocyte clones, *pHFK* primary human foreskin keratinocyte clones, *iHFK* hTERT-immortalized human keratinocyte clones
[b]*TT+* HPV-positive tonsillar tumors, *TT-* HPV-negative tonsillar tumors

The expression of all above-mentioned miRNAs was confirmed in a set of FFPE macrodissected tumors. The values of fold changes based on individual assays were then compared with the values obtained with miRNA arrays (Fig. 4). The cut-off fold change for significantly deregulated miRNAs was set as for arrays to +/− 1.33. All individually analyzed miRNAs showed concordance of the expression with the results obtained with arrays, with the exception of miR-21. Although the trend in expression was maintained, the upregulated expression of miR-21 was confirmed only in 50 % of samples, and the median of absolute fold change in all samples was equal to 1.27. The deregulated expression of the other miRNAs was confirmed (miR-9 in 100 %, miR-141 in 59 %, miR-200a in 87 %, miR-125b-2* in 89 %, miR-335 in 54 %, miR-221 in 67 %, miR-20b in 93 % of HPV-positive tumors and in 87 % of HPV-negative tumors, and miR-210 in 100 % of tumors of both etiologies).

Prognostic miRNAs

The expression of 738 miRNAs was evaluated in ten tonsillar tumor samples. In order to evaluate if the analyzed miRNAs correspond with the clinical characteristics or patient prognosis, we performed the factor analysis. At first we compute the principal components and for them we made varimax rotation to obtain best view to data. The loading of obtained factor is displayed in Additional file 13: Figure S7, the components of the particular rotated factor are listed in Additional file 14: Table S7. For the analysis we used IBM SPSS v.23. One hundred percent of the variance can be explained by eight groups of factors and prognosis was the most strongly associated with miRNAs from group 5, which in total explains about 10.3 % of the variability. Twelve miRNAs in group 5 (miR-196b, miR-485-3p, miR-589, miR-324-3p, miR-342-3p, miR-92a1#, miR-155, miR-146b, miR-142-3p, miR-1260, miR-143, and miR-142-5p) have strong loading (absolute value ≥ 0.8).

Discussion

While the incidence of head and neck tumors of non-viral etiology is declining, the head and neck tumors associated with human papillomavirus infection are steadily increasing [5, 30]. The presence of HPV remains the strongest prognostic factor in HNC compared to HPV-negative tumors [9, 31]. Thus, the task of advancing knowledge on molecular pathogenesis of the disease and identifying diagnostic and prognostic biomarkers remains of key importance. Our aim was to characterize the expression profiles of miRNAs in well-characterized tonsillar tumors and define the miRNAs characteristic of HPV-associated or HPV-negative tonsillar tumors. Next, we wanted to compare the results from clinical samples with those obtained from the model system of isogenic primary human keratinocyte clones immortalized by HPV or human telomerase gene.

MicroRNA profiling in HNC has been reported in several studies, but the published data lack comparability, likely due to the anatomical heterogeneity of the studied tumors and differences in the methodological approaches. Tran et al. profiled miRNAs in a set of head and neck cancer cell lines [17], similarly to Chang et al. who have compared results from cell lines with the miRNA profiles from a small group of four tumor samples [32]. Afterwards, studies performed on tumor tissues from various locations have been published [18-20, 33]. However, none of these studies addressed a comparison between HPV-associated and HPV-independent tumors, although other studies analyzed the miRNA profiles in cell lines or clinical samples with regard to the HPV status. Wald et al. have studied the miRNA profiles in head and neck squamous cell carcinoma (HNSCC) cell lines [21], identifying a number of deregulated miRNAs and describing the influence of HPV oncogene E6 on the miRNA profiles. Lajer et al. analyzed a set of 51 oral and pharyngeal tumors in comparison to the normal tissues, reporting deregulation of 21 miRNAs in nine HPV-positive samples [22]. This study became extended to include a set of tonsillar and cervical tumors, and to identify miRNAs with roles in HPV-associated pathogenesis [23]. However, both these studies lacked confirmation of the transcriptionally active HPV infection. Hui et al. have identified several miRNAs associated with HPV status in a set of oropharyngeal carcinomas and suggested candidate miRNAs correlating with the patients' clinical outcome [34]. Recently, Miller et al. have identified a miRNA subset in oropharyngeal carcinomas, validating the analysis in the clinical data from The Cancer Genome Atlas [35].

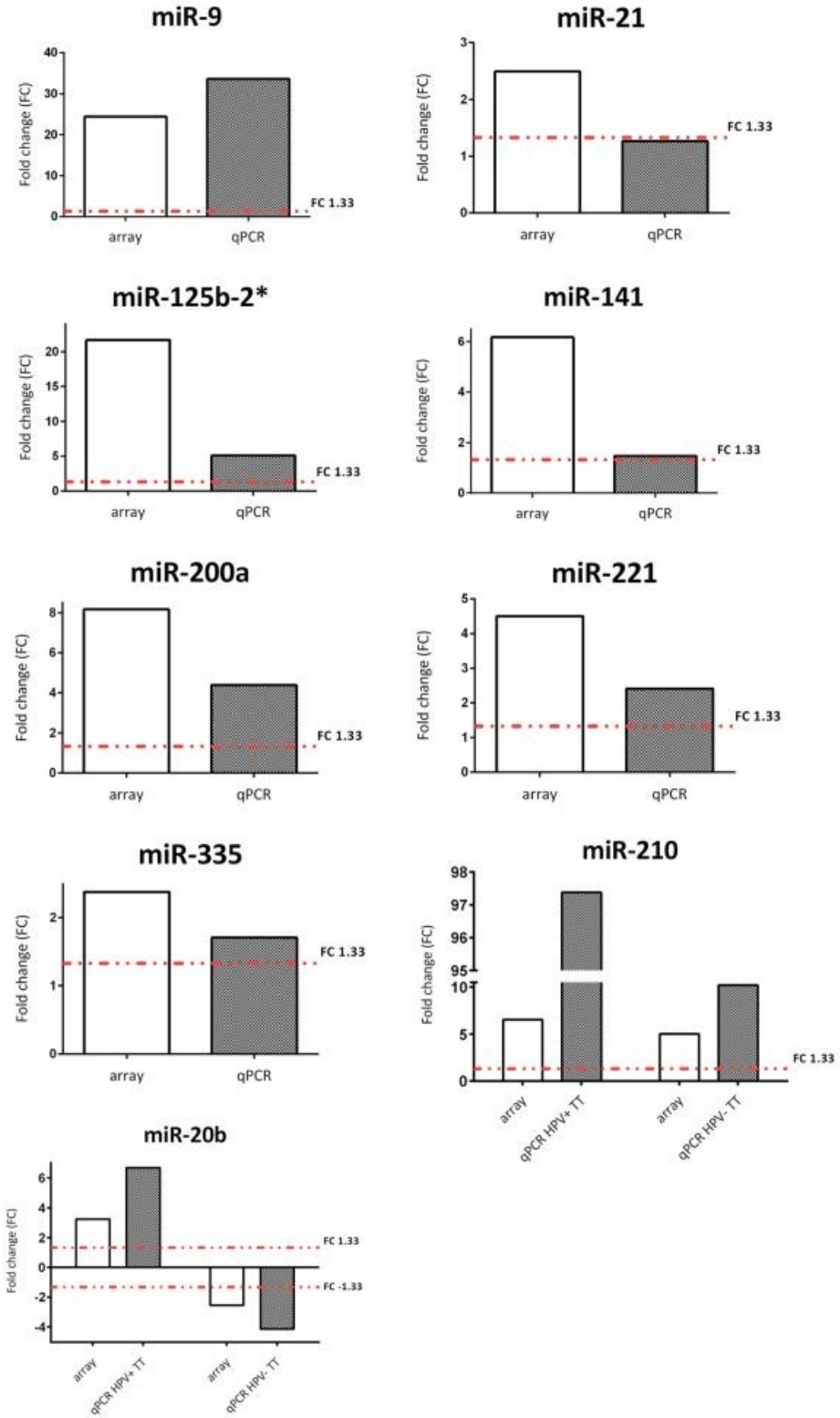

Fig. 4 Comparison of fold change for particular miRNAs between arrays and qPCR. HPV-positive tonsillar tumors (HPV+ TT), HPV-negative tonsillar tumors (HPV- TT). The fold change (FC) threshold was set to 1.33

Here we analyzed the miRNA expression profiles of well-characterized tonsillar tumors and in a set of keratinocyte clones, providing a first-of-its-kind comparison of the miRNA profiles in clinical samples with regard to the presence of HPV, with a model system of keratinocyte clones. Keratinocyte clones immortalized by HPV16 and primary keratinocytes were used as the model system for the HPV-positive tumors and/or normal tissues, whilst the keratinocyte clones immortalized by human telomerase gene mimicked HPV-independent tumors.

When comparing the two types of immortalized keratinocyte clones against primary cells, in order to identify the miRNAs characteristic for immortalized cells regardless of the mode of immortalization, miR-135b-3p and miR-146b-5p were downregulated while miR-205-5p was upregulated in both types of immortalized keratinocyte clones. This is in agreement with other miRNA profiling studies of different carcinomas and cell lines including HNC, and these miRNAs have been shown to participate in the signaling pathways and cell migration pathways connected with epithelial to mesenchymal cell transition (EMT), tumor invasion, and metastasis [36–38]. Next, we identified the miRNAs specific either for the HPV-immortalized keratinocyte clones or the hTERT-immortalized clones. The most upregulated miRNA in HPV-immortalized clones was miR-454-5p, reported previously as upregulated in human colorectal cancer cells [39]. Another highly upregulated miRNA was the tumor suppressor-like miR-335, linked to longer survival in cervical cancer patients [40, 41]. The most downregulated miRNAs in HPV-immortalized keratinocyte clones were miR-33a-5p and miR-133b. Wong et al. have demonstrated the tumor suppressor role of miR-133b in oral cell lines through the dysregulation of pyruvate kinase type M2 (PKM2) [42]. MiRNA-146a, here observed to be downregulated in HPV-immortalized keratinocytes, is also regulated by HPV oncoproteins, indirectly through the activation of c-*Myc* oncogene via viral E7 protein (reviewed by [43]).

The most upregulated miRNAs in hTERT-immortalized keratinocyte clones were less-studied miR-627-5p and miR-885-5p, although miR-885-5p has been reported to act as the post-transcriptional regulator of *CASP3* expression which has anti-apoptotic and carcinogenic effects [44]. MiR-199a-3p and miR-146b-5p were the most downregulated miRNAs in hTERT-immortalized clones. Several studies focused on the tumor type-dependent function of miR-199-3p in tumors and cell lines have found that its downregulation enhances proliferation, invasiveness, and adhesion [45] and it is a predictor of worse prognosis in patients with osteosarcoma [46].

In this study, we showed that each group of tumors has a specific miRNA profile. The most upregulated miRNAs in HPV-positive tonsillar tumors were miR-125b-2-3p and miR-147b while the most downregulated were miR-133a-3p and miR-575. MiR-125b-1 and miR-125b-2 originated from independent precursors located in different chromosomal loci, but their targets are identical. In contrast to our data, Nakanishi et al. have revealed the loss of miR-125b-1 to contribute to head and neck cancer development [47] and Henson et al. have reported decreased expression of miR-125b in oral cancer cells [48]. In the context of HPV infection, Nuovo et al. have observed that miR-125b has a role in productive HPV infection and that its upregulation leads to the reduction in viral DNA [49]. However, other miRNAs found to be upregulated in HPV-positive tumors in our study were also identified to play a role in cancer (e.g. miR-34a, miR-21, miR-10a, or some mir-30 family members). MiR-133a, tumor suppressor miRNA downregulated in several types of cancer including HNSCC, was also downregulated in our set of HPV-positive tumors [50–52]. MiR-133a has been shown to be involved in inhibition of cell proliferation, migration and invasion in HNSCC cell lines [53, 54].

In HPV-negative tumors, miR-431-5p and miR-517c-3p were the most upregulated and miR-150-5p and miR-142-3p the most downregulated miRNAs. Dysregulation of miR-150 has been demonstrated in a number of solid tumors (reviewed in [55]). Additionally, miR-485, miR-34c, miR-221, or miR-193a and miRNAs from the mir-10 or let-7 families identified as deregulated in HPV-negative tumors in our study participate in the regulation of proliferation, apoptosis, and invasion, and have been proposed as a prognostic indicators in patients with solid cancers [56–60].

As mentioned above, only three studies analyzed the miRNA profiles in head and neck tumors with regard to the HPV status. Lajer et al. addressed the miRNA profiles in HNC and compared the miRNA profiles of HPV-positive and HPV-negative HNSCC and cervical carcinomas, identifying a group of HPV-associated core miRNAs [22, 23]. Negligible overlap with their results was found in our study most likely due to the heterogeneity in the analyzed samples in Lajer´s study. Lajer et al. compared cervical tumors with a pool of HPV-positive tonsillar and pharyngeal carcinomas while in our study only well-characterized tonsillar tumors were evaluated. Our results do agree with theirs only in the identification of miR-21, the most commonly elevated miRNA in cancers. In agreement with the study of Hui et al. [34], we identified miR-9 as associated with the HPV status since it was upregulated in both tonsillar and cervical tumors. Recent studies have shown miR-9 to be involved in the pathways regulating metastasis [61]. Liu et al. [62] have shown that HPV-induced activation of miR-9 leads to the increase of cell motility through downregulation of genes involved in the pathways of cell migration. MiR-9 has also been considered as HPV-associated by Miller et al. using the bioinformatics analysis [35].

Despite the small overlap in the identified HPV core miRNAs with Lajer´s study the other HPV core miRNAs identified in our study have been previously mentioned as players in cancer development. Myklebust et al. have reported miR-15b to be strongly associated with the expression of several E2F-related genes [63] and its expression to be reduced after HPV16 E7 knockdown. MiR-141 has been found to be overexpressed in HPV-positive cervical carcinomas [64]. Members of the mir-8 family, which includes also miR-200a, are the major regulators of the EMT pathway, primarily targeting transcriptional factors ZEB1 and ZEB2 [65]. MiR-302c-3p inversely expressed in tonsillar and cervical tumors in our study has been shown before as miRNA inhibiting the tumor growth [66].

The aim of our study was to compare the miRNA expression profiles in clinical samples and in the model system of keratinocyte clones. The overlap between particular clinical samples and the model system was very small. The miRNA profiles of HPV-positive tonsillar tumors and HPV-immortalized clones overlap in three miRNAs while one was inversely expressed in clinical samples and in keratinocyte clones. HPV-negative tumors and hTERT-immortalized clones overlap also in three miRNAs. Two were expressed inversely in the two groups, miR-328-3p was downregulated in both groups, and decreased expression of this miRNA has also been reported in other carcinomas [67, 68].

Because we have done the detailed analysis of the HPV genome status in our HPV-positive tonsillar tissues and keratinocyte clones, we were able to evaluate the differences in the expression miRNA profiles in relation to the status of the viral genome. Tumor tissues which contain either extrachromosomal, integrated, or mixed form of HPV genome differ in their miRNA expression profiles, as was documented in the model system as well. Even though there is no overlap in the lists of miRNAs in tumors and HPV-immortalized keratinocytes regarding the genome status, the differentially expressed miRNAs were found to target the same pathways; pathways in cancer, p53 signaling, cell cycle regulation, and PI3K-Akt signaling pathways.

We confirmed the expression of nine specific miRNAs by individual TaqMan assays in a set of 64 FFPE samples. For some of these miRNAs the deregulated expression was not confirmed in all samples; miR-21 was deregulated in 50 % of FFPE samples, miR-335 in 54 %, miR-141 in 59 % and miR-221 in 67 %. This is most likely due to the difference in the number of tumor cells in the samples. For arrays FF samples with >60 % of tumor cells were used while for RT qPCR macrodissected FFPE samples containing only the tumor cells were analyzed. The expression profiles of less deregulated miRNAs can be easily influenced by the presence of non-malignant cells. The deregulation of all others miRNAs was confirmed in more than 80 % of FFPE samples.

Patients with tonsillar tumors included in our study were followed up for a long time, and we wanted to explore the relationship between the miRNA expression and patient prognosis. Since the number of variables was high and the number of samples with the completed analysis of 738 miRNAs was low, factor analysis was performed. Twelve miRNAs in this group (miR-196b, miR-485-3p, miR-589, miR-324-3p, miR-342-3p, miR-92a-1#, miR-155, miR-146b, miR-142-3p, miR-1260, miR-143, and miR-142-5p) seem to be influential on patient prognosis. The majority of these miRNAs have been reported as prognostic markers in different types of cancers, while miR-324-3p, miR-155, miR-142-3p, and miR-143 have been identified as prognostic indicators in head and neck tumors of different locations [69–73]. The expression levels of these miRNAs merit further validation in a larger set of tonsillar tumors.

Conclusions

In conclusion, we characterized the miRNA expression profiles of HPV-associated and HPV-independent tumors and compared the results with those obtained in the primary and immortalized human keratinocyte clones. Our data show that the miRNA profiles differ between tissues of different anatomical origin. Tumor tissues exhibited site-specific miRNA expression profiles, but the heterogeneity of the miRNA profiles in tonsillar tumors was much larger than in cervical tumors. We established that tumor sample homogeneity (rather than sample size) is important for the robustness of the miRNA expression analysis. We identified miRNAs specific for either tonsillar carcinomas or HPV-associated tumors and the results were compared with the data from the human keratinocyte clone model. The expression levels of the selected miRNAs were confirmed in a larger set of tonsillar tumor samples. Finally, we evaluated the differentially expressed miRNAs in relation to patient prognosis. Our results warrant further confirmation in a larger set of samples to allow future in-depth investigations of the role played by particular miRNAs in head and neck tumorigenesis.

Additional files

Additional file 1: Table S1. Demographic characteristics and clinical-pathological parameters of patients with tonsillar tumors (and available fresh frozen specimens). (XLS 39 kb)

Additional file 2: Table S2. Significant ($P < 0.05$) miRNAs differentially expressed in HPV16 immortalized keratinocytes (tHFK) in comparison to primary human keratinocytes (pHFK). (XLS 39 kb)

Additional file 3: Table S3. Significant ($P < 0.05$) miRNAs differentially expressed in keratinocytes immortalized by human telomerase gene (iHFK) in comparison to primary human keratinocytes (pHFK). (XLS 38 kb)

Additional file 4: Figure S1. Principal component analysis plot. Visualization of miRNA expression in normal HPV-negative cervical tissues

(NC, blue) and in non-malignant HPV-negative tonsillar tissues (NT, green). (TIF 1057 kb)

Additional file 5: Figure S2. Principal component analysis plot. Visualization of different miRNA expression in tumor and normal tissues of the same anatomical location. A - Tonsillar tumors (TT, red) versus non-malignant tissues (NT, green). B - Cervical tumors (CT, green) versus normal tissues (NC, blue). (TIF 1553 kb)

Additional file 6: Table S4. Significant ($P < 0.05$) miRNAs differentially expressed in HPV-positive tonsillar tumors in comparison to non-malignant tonsillar tissues. (XLS 43 kb)

Additional file 7: Table S5. Significant ($P < 0.05$) miRNAs differentially expressed in HPV-negative tonsillar tumors in comparison to non-malignant tonsillar tissues. (XLS 43 kb)

Additional file 8: Table S6. Significantly ($P < 0.05$) expressed miRNAs common to both HPV-positive and HPV-negative tonsillar tumors. (XLSX 12 kb)

Additional file 9: Figure S3. Expression values of HPV core miRNAs in miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), normal tonsils (NT), cervical tumors (CT), normal cervical tissues (NC). (DOC 325 kb)

Additional file 10: Figure S4. Expression values of miRNAs differentially expressed in HPV-positive tonsillar tumors on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), normal tonsils (NT), HPV-immortalized keratinocytes clones (tHFK), primary keratinocytes clones (pHFK). (DOC 166 kb)

Additional file 11: Figure S5. Expression values of miR-221 differentially expressed in HPV-negative tonsillar tumors on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-negative tonsillar tumors (HPV- TT), normal tonsils (NT). (DOC 123 kb)

Additional file 12: Figure S6. Expression values of miR-210 and miR-20b differentially expressed in tonsillar tumors of both etiologies on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), HPV-negative tonsillar tumors (HPV- TT), normal tonsils (NT). (DOC 519 kb)

Additional file 13: Figure S7. Screenplot showing the loading of the factors obtained by factor analysis. (TIF 920 kb)

Additional file 14: Table S7. Rotated Component Matrix computed by Rotation Method: Varimax with Kaiser Normalization showing the components of a particular factor sorted by absolute value of loading for each variable. (XLS 146 kb)

Abbreviations

APOT assay, Amplification of Papillomavirus Oncogene Transcripts assay; ARPC5, actin-related protein 2/3 complex subunit 5; CT, cervical tumor; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal cell transition; FC, fold change; FF, fresh frozen; FFPE, formalin fixed paraffin embedded; HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; hTERT, human telomerase reverse transcriptase; iHFK, hTERT-immortalized human foreskin keratinocytes; miRNA, microRNA; MSN, moesin; NC, normal cervix; NT, non-malignant tonsillar tissue; OPSCC, oropharyngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma; PCA, principal component analysis; pHFK, primary human foreskin keratinocytes; PKM2, pyruvate kinase type M2; PSCC, pharyngeal squamous cell carcinoma; RIN, RNA integrity number; RQ, relative quantity; RT qPCR, quantitative reverse transcription polymerase chain reaction; tHFK, HPV-immortalized human foreskin keratinocytes; TLDA, TaqMan Low Density Array; TSCC, tonsillar squamous cell carcinoma; TT-, HPV-negative tonsillar tumor; TT+, HPV-positive tonsillar tumor

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Availability of data and materials

All data are available in the enclosed supplementary files.

Authors' contributions

RT conceived of the study, and participated in its design and coordination. JK was responsible for the collection of tonsillar samples; follow up of patient and summarization of the clinical data. MG selected FFPE samples for molecular biological analyses and performed histological and immunohistochemical analyses. LT participated on the design of the experiment and was responsible for the selection of isogenic primary human keratinocyte clones. ZV, MS, JS performed molecular biological experiments. IS, RT, ZV, JZ, BP participated on the data analyses. All authors participated on the manuscript preparation. All authors read and approved the final manuscript.

Authors' information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All patients enrolled in the study signed the informed consent form. The study received official institutional and ethical approval from the Motol University Hospital and Institute of Hematology and Blood Transfusion.

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Table S1 Demographic characteristics and clinical-pathological parameters of patients with tonsillar tumors (and available fresh frozen specimens).

CHARACTERISTIC	Value	HPV positive cases	HPV negative cases
		N = 13 [%]	N = 10 [%]
Gender	male	12 [92]	9 [90]
	female	1 [8]	1 [10]
Age	mean	57.1	52.5
Education	≤12	9 [69]	9 [90]
	>12	4 [31]	1 [10]
Number of sex partners	≤6	5 [38]	2 [20]
	>6	8 [62]	7 [70]
Tobacco status	no	4 [31]	0 [0]
	yes	9 [69]	10 [100]
Alcohol	no	4 [31]	1 [10]
	yes	9 [69]	9 [90]
Tumor size	T1+T2	6 [46]	9 [90]
	T3+T4	7 [54]	1 [10]
Nodal status	N0	3 [23]	4 [40]
	N1-3	10 [77]	6 [60]
Tumor stage	I+II	1 [8]	4 [40]
	III+IV	12 [92]	6 [60]
Tumor grade	1+2	8 [62]	9 [90]
	3	5 [38]	1 [10]
Recurrence	no	9 [69]	8 [80]
	yes	3 [23]	2 [20]
p16	positive	12 [92]	0 [0]
	negative	1 [8]	10 [100]
p53	positive	0 [0]	6 [60]
	negative	13 [100]	4 [40]
HPV DNA	positive	13 [100]	0 [0]
	negative	0 [0]	10 [100]
HPV16 E6 mRNA	positive	13 [100]	0 [0]
	negative	0 [0]	10 [100]
Status of HPV genome	integrated	5 [38]	-
	extrachromosomal	4 [31]	-
	mixed	2 [15]	-

Table S2 Significant (P<0.05) miRNAs differentially expressed in HPV16 immortalized keratinocytes (tHFK) in comparison to primary human keratinocytes (pHFK).

Probe name	MiRNA family	Fold change
hsa-miR-454-5p	mir-454	18.778
hsa-miR-335-3p	mir-335	10.868
hsa-miR-335-5p	mir-335	8.361
hsa-miR-579-3p	mir-548	7.921
hsa-miR-345-5p	mir-345	6.738
hsa-miR-361-5p	mir-361	6.271
hsa-miR-128-3p	mir-128	4.749
hsa-miR-340-3p	mir-340	3.948
hsa-miR-501-5p	mir-500	3.068
hsa-miR-330-3p	mir-330	3.065
hsa-miR-18a-5p	mir-17	2.815
hsa-miR-126-5p	mir-95	2.580
hsa-miR-1180-3p	mir-1180	2.340
hsa-miR-18a-3p	mir-17	2.338
hsa-miR-425-3p	mir-425	2.301
hsa-miR-103a-3p	mir-103	2.273
hsa-miR-429	mir-8	2.257
hsa-miR-941	mir-941	2.204
hsa-miR-944	mir-944	1.992
hsa-miR-454-3p	mir-454	1.990
hsa-miR-126-3p	mir-126	1.858
hsa-miR-205-5p	mir-205	1.800
hsa-miR-30c-5p	mir-30	1.784
hsa-miR-221-3p	mir-221	1.574
hsa-miR-222-3p	mir-221	-1.457
hsa-miR-135b-3p	mir-135	-1.782
hsa-miR-539-5p	mir-154	-2.188
hsa-miR-146b-5p	mir-146	-2.514
hsa-miR-625-3p	mir-625	-2.617
hsa-miR-1290	mir-1290	-4.186
hsa-miR-518f-3p	mir-515	-6.219
hsa-miR-146a-5p	mir-146	-7.691
hsa-miR-370-3p	mir-370	-7.729
hsa-miR-376a-3p	mir-368	-9.021
hsa-miR-410-3p	mir-154	-14.708
hsa-miR-376c-3p	mir-368	-15.240
hsa-miR-485-3p	mir-485	-23.470

hsa-miR-133b	mir-133	-100.758
hsa-miR-33a-5p	mir-33	-60076.125

Table S3 - Significant (P <0.05) miRNAs differentially expressed in keratinocytes immortalized by human telomerase gene (iHFK) in comparison to primary human keratinocytes (pHFK).

Probe name	MiRNA family	Fold change
hsa-miR-627-5p	mir-627	30441.362
hsa-miR-885-5p	mir-885	48.377
hsa-miR-489-3p	mir-489	4.360
hsa-miR-625-3p	mir-625	2.845
hsa-miR-339-5p	mir-339	2.566
hsa-miR-205-5p	mir-205	2.009
hsa-miR-19a-3p	mir-19	1.967
hsa-miR-485-3p	mir-485	1.764
hsa-miR-28-3p	mir-28	1.756
hsa-miR-222-5p	mir-221	-1.445
hsa-miR-135b-3p	mir-135	-1.533
hsa-miR-200b-3p	mir-8	-1.760
hsa-miR-660-5p	mir-188	-2.499
hsa-miR-532-5p	mir-188	-2.791
hsa-miR-425-3p	mir-425	-2.882
hsa-miR-516b-3p	mir-515	-3.399
hsa-miR-140-3p	mir-140	-3.824
hsa-miR-210-3p	mir-210	-4.292
hsa-miR-22-3p	mir-22	-5.414
hsa-miR-224-5p	mir-224	-5.999
hsa-miR-452-5p	mir-452	-6.174
hsa-miR-650	mir-650	-7.069
hsa-let-7c-5p	let-7	-9.735
hsa-miR-362-3p	mir-362	-12.391
hsa-miR-218-5p	mir-218	-15.197
hsa-miR-34c-5p	mir-34	-16.419
hsa-miR-328-3p	mir-328	-22.020
hsa-miR-1271-5p	mir-1271	-53.427
hsa-miR-146b-5p	mir-146	-71.370
hsa-miR-199a-3p	mir-199	-1960.837

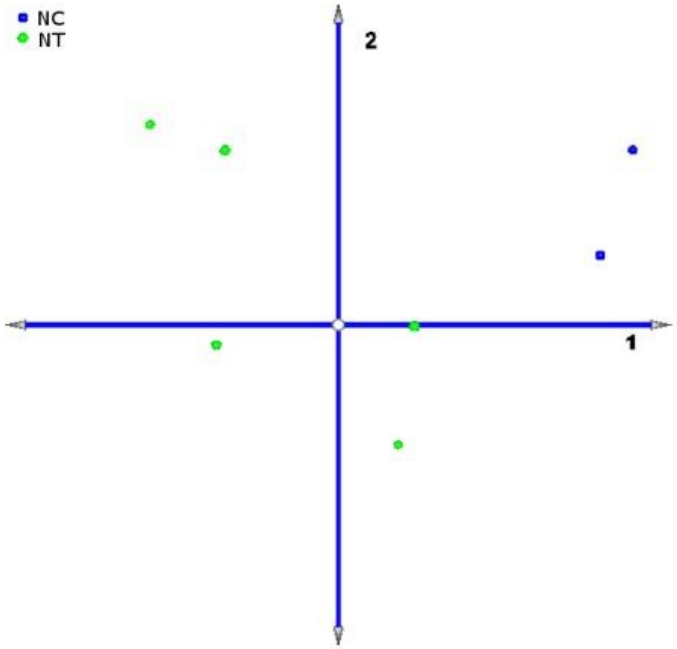

Figure S1 - Principal component analysis plot. Visualization of miRNA expression in normal HPV-negative cervical tissues (NC, blue) and in non-malignant HPV-negative tonsillar tissues (NT, green).

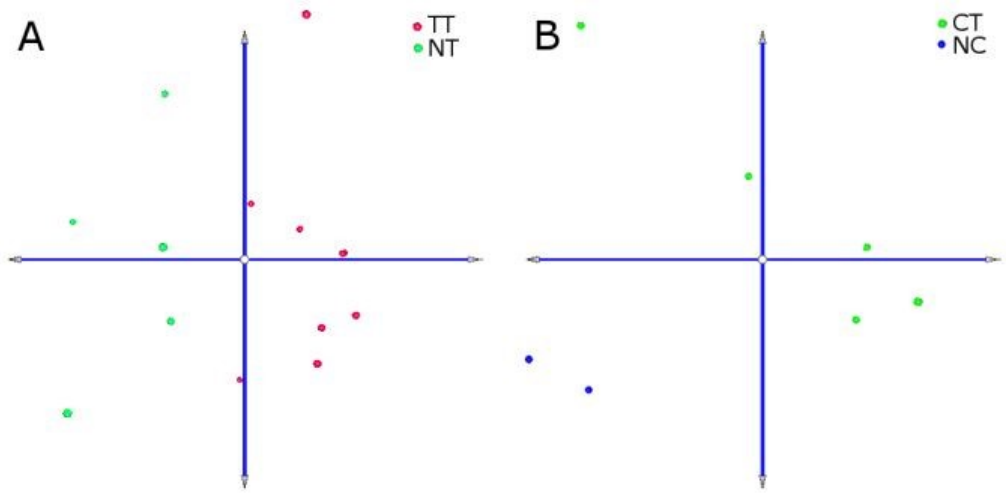

Figure S2 - Principal component analysis plot. Visualization of different miRNA expression in tumor and normal tissues of the same anatomical location. A - Tonsillar tumors (TT, red) versus non-malignant tissues (NT, green). B - Cervical tumors (CT, green) versus normal tissues (NC, blue).

Table S4 - Significant (P <0.05) miRNAs differentially expressed in HPV-positive tonsillar tumors in comparison to non-malignant tonsillar tissues.

Probe name	MiRNA family	Fold change
hsa-miR-302c-3p	mir-302	29.111
hsa-miR-9-5p	mir-9	24.460
hsa-miR-125b-2-3p	mir-10	21.689
hsa-miR-147b	mir-147	16.057
hsa-miR-523-3p	mir-515	10.983
hsa-miR-542-3p	mir-542	9.273
hsa-miR-196b-5p	mir-196	9.195
hsa-miR-224-5p	mir-224	9.071
hsa-miR-429	mir-8	9.043
hsa-miR-944	mir-944	8.487
hsa-miR-200a-3p	mir-8	8.173
hsa-miR-1825	mir-1825	8.016
hsa-miR-200b-3p	mir-8	7.837
hsa-miR-135b-3p	mir-135	6.766
hsa-miR-210-3p	mir-210	6.521
hsa-miR-141-3p	mir-8	6.175
hsa-miR-452-5p	mir-452	5.280
hsa-miR-1180-3p	mir-1180	4.695
hsa-miR-21-3p	mir-218	4.622
hsa-miR-10a-5p	mir-10	4.513
hsa-miR-205-5p	mir-205	4.443
hsa-miR-135b-5p	mir-135	4.425
hsa-miR-643	mir-643	4.232
hsa-miR-200c-3p	mir-8	3.698
hsa-miR-202-3p	mir-202	3.694
hsa-miR-335-3p	mir-335	3.623
hsa-miR-27a-5p	mir-27	3.351
hsa-miR-1244	mir-1244	3.253
hsa-miR-20b-5p	mir-17	3.253
hsa-miR-27b-5p	mir-27	3.154
hsa-miR-15b-5p	mir-15	3.072
hsa-miR-200a-5p	mir-8	2.924
hsa-miR-182-5p	mir-182	2.857
hsa-miR-34a-3p	mir-34	2.854
hsa-miR-183-5p	mir-183	2.598
hsa-miR-34a-5p	mir-34	2.573
hsa-miR-21-5p	mir-21	2.494

hsa-miR-222-5p	mir-221	2.403
hsa-miR-335-5p	mir-335	2.378
hsa-miR-18b-5p	mir-17	2.338
hsa-miR-130b-3p	mir-130	2.180
hsa-miR-197-3p	mir-197	2.077
hsa-miR-27a-3p	mir-27	1.988
hsa-miR-579-3p	mir-548	1.945
hsa-miR-132-3p	mir-132	1.699
hsa-miR-183-3p	mir-183	1.333
hsa-miR-26a-5p	mir-26	-1.623
hsa-miR-30a-5p	mir-30	-1.798
hsa-miR-577	mir-577	-1.813
hsa-miR-29b-3p	mir-29	-1.980
hsa-miR-29a-5p	mir-29	-1.997
hsa-miR-195-5p	mir-15	-2.173
hsa-miR-214-5p	mir-214	-2.571
hsa-miR-101-3p	mir-101	-2.612
hsa-miR-143-3p	mir-143	-2.786
hsa-miR-30e-3p	mir-30	-2.934
hsa-miR-425-3p	mir-425	-3.147
hsa-miR-486-3p	mir-486	-3.191
hsa-miR-500a-3p	mir-500	-3.333
hsa-miR-199b-5p	mir-199	-3.334
hsa-miR-140-3p	mir-140	-3.684
hsa-miR-204-5p	mir-204	-3.791
hsa-miR-145-5p	mir-145	-3.974
hsa-miR-126-5p	mir-95	-4.049
hsa-miR-145-3p	mir-145	-4.805
hsa-miR-1247-5p	mir-1247	-5.233
hsa-miR-505-5p	mir-505	-5.344
hsa-miR-30a-3p	mir-30	-5.981
hsa-miR-136-3p	mir-136	-6.714
hsa-miR-486-5p	mir-486	-7.824
hsa-miR-1179	mir-1179	-8.498
hsa-miR-139-5p	mir-139	-9.387
hsa-miR-1	mir-1	-10.729
hsa-miR-575	mir-575	-11.791
hsa-miR-144-5p	mir-144	-12.235
hsa-miR-184	mir-184	-16.585
hsa-miR-133a-3p	mir-133	-24.685

Table S5 - Significant (P <0.05) miRNAs differentially expressed in HPV-negative tonsillar tumors in comparison to non-malignant tonsillar tissues.

Probe name	MiRNA family	Fold change
hsa-miR-431-5p	mir-431	37.879
hsa-miR-944	mir-944	27.523
hsa-miR-196b-5p	mir-196	16.188
hsa-miR-517c-3p	mir-515	12.679
hsa-miR-135b-3p	mir-135	10.850
hsa-miR-224-5p	mir-224	9.384
hsa-miR-27a-5p	mir-27	7.995
hsa-miR-485-3p	mir-485	7.305
hsa-miR-135b-5p	mir-135	6.972
hsa-miR-623	mir-623	6.535
hsa-miR-1180-3p	mir-1180	6.484
hsa-miR-452-5p	mir-452	6.423
hsa-miR-1825	mir-1825	6.414
hsa-miR-205-5p	mir-205	5.170
hsa-miR-210-3p	mir-210	5.025
hsa-miR-429	mir-8	4.880
hsa-miR-34c-5p	mir-34	4.851
hsa-miR-221-3p	mir-221	4.505
hsa-miR-643	mir-643	4.429
hsa-miR-517a-3p	mir-515	4.327
hsa-miR-21-3p	mir-218	3.750
hsa-miR-200b-3p	mir-8	3.699
hsa-miR-183-5p	mir-183	3.475
hsa-miR-200a-5p	mir-8	3.441
hsa-miR-27a-3p	mir-27	3.236
hsa-miR-200c-3p	mir-8	3.212
hsa-miR-301a-3p	mir-130	3.121
hsa-miR-182-5p	mir-182	3.071
hsa-miR-193b-3p	mir-193	2.966
hsa-miR-183-3p	mir-183	2.928
hsa-miR-1244	mir-1244	2.789
hsa-miR-193a-5p	mir-193	2.620
hsa-miR-222-5p	mir-221	2.600
hsa-miR-223-3p	mir-223	2.518
hsa-miR-27b-5p	mir-27	2.481
hsa-miR-323a-3p	mir-154	2.391
hsa-miR-1260a	mir-1260a	2.202
hsa-miR-106b-3p	mir-17	2.072

hsa-miR-550a-3p	mir-550	1.939
hsa-miR-24-3p	mir-24	1.904
hsa-miR-151a-3p	mir-28	1.734
hsa-miR-335-3p	mir-335	1.489
hsa-let-7d-5p	let-7	-1.389
hsa-miR-30b-5p	mir-30	-1.461
hsa-miR-487a-3p	mir-154	-1.474
hsa-let-7e-5p	let-7	-1.554
hsa-let-7b-5p	let-7	-1.582
hsa-miR-564	mir-564	-1.590
hsa-miR-629-3p	mir-629	-1.616
hsa-miR-199b-5p	mir-199	-1.628
hsa-miR-143-3p	mir-143	-1.735
hsa-miR-125b-5p	mir-10	-1.920
hsa-miR-30a-5p	mir-30	-1.941
hsa-miR-145-5p	mir-145	-2.096
hsa-miR-100-5p	mir-10	-2.106
hsa-miR-30d-5p	mir-30	-2.145
hsa-miR-139-5p	mir-139	-2.325
hsa-miR-26b-3p	mir-26	-2.369
hsa-miR-29a-3p	mir-29	-2.397
hsa-miR-20b-5p	mir-17	-2.537
hsa-miR-486-3p	mir-486	-2.602
hsa-miR-130b-5p	mir-130	-2.647
hsa-miR-642a-5p	mir-642	-2.768
hsa-miR-328-3p	mir-328	-2.806
hsa-miR-744-3p	mir-744	-2.998
hsa-miR-520d-3p	mir-515	-3.135
hsa-let-7g-5p	let-7	-3.142
hsa-miR-195-5p	mir-15	-3.312
hsa-miR-99a-5p	mir-10	-3.404
hsa-miR-29c-3p	mir-29	-3.474
hsa-miR-26a-5p	mir-26	-3.522
hsa-miR-139-3p	mir-139	-3.666
hsa-miR-885-5p	mir-885	-3.725
hsa-miR-29b-3p	mir-29	-3.811
hsa-miR-577	mir-577	-4.452
hsa-miR-101-3p	mir-101	-4.642
hsa-miR-144-5p	mir-144	-4.964
hsa-miR-140-3p	mir-140	-5.448
hsa-miR-29a-5p	mir-29	-5.933
hsa-miR-138-5p	mir-138	-12.185

hsa-miR-142-3p	mir-142	-15.980
hsa-miR-150-5p	mir-150	-17.261
hsa-miR-204-5p	mir-204	-24.045
hsa-miR-184	mir-184	-51.691

Table S6 - Significantly (P<0.05) expressed miRNAs common to both HPV-positive and HPV-negative tonsillar tumors.

Probe name	MiRNA family	Fold change in TT+	Fold change in TT-
hsa-miR-224-5p	mir-224	9.071	9.384
hsa-miR-429	mir-8	9.043	4.88
hsa-miR-944	mir-944	8.487	27.523
hsa-miR-1825	mir-1825	8.016	6.414
hsa-miR-200b-3p	mir-8	7.837	3.699
hsa-miR-135b-3p	mir-135	6.766	10.85
hsa-miR-210-3p	mir-210	6.521	5.025
hsa-miR-1180-3p	mir-1180	4.695	6.484
hsa-miR-205-5p	mir-205	4.443	5.17
hsa-miR-643	mir-643	4.232	4.429
hsa-miR-200c-3p	mir-8	3.698	3.212
hsa-miR-335-3p	mir-335	3.623	1.489
hsa-miR-1244	mir-1244	3.253	2.789
hsa-miR-20b-5p	mir-17	3.253	-2.537
hsa-miR-27b-5p	mir-27	3.154	2.481
hsa-miR-183-5p	mir-183	2.598	3.475
hsa-miR-222-5p	mir-221	2.403	2.6
hsa-miR-27a-3p	mir-27	1.988	3.236
hsa-miR-183-3p	mir-183	1.333	2.928
hsa-miR-26a-5p	mir-26	-1.623	-3.522
hsa-miR-30a-5p	mir-30	-1.798	-1.941
hsa-miR-577	mir-577	-1.813	-4.452
hsa-miR-29b-3p	mir-29	-1.98	-3.811
hsa-miR-29a-5p	mir-29	-1.997	-5.933
hsa-miR-195-5p	mir-15	-2.173	-3.312
hsa-miR-101-3p	mir-101	-2.612	-4.642
hsa-miR-143-3p	mir-143	-2.786	-1.735
hsa-miR-486-3p	mir-486	-3.191	-2.602
hsa-miR-199b-5p	mir-199	-3.334	-1.628
hsa-miR-140-3p	mir-140	-3.684	-5.448
hsa-miR-204-5p	mir-204	-3.791	-24.045
hsa-miR-145-5p	mir-145	-3.974	-2.096
hsa-miR-139-5p	mir-139	-9.387	-2.325
hsa-miR-144-5p	mir-144	-12.235	-4.964
hsa-miR-184	mir-184	-16.585	-51.691

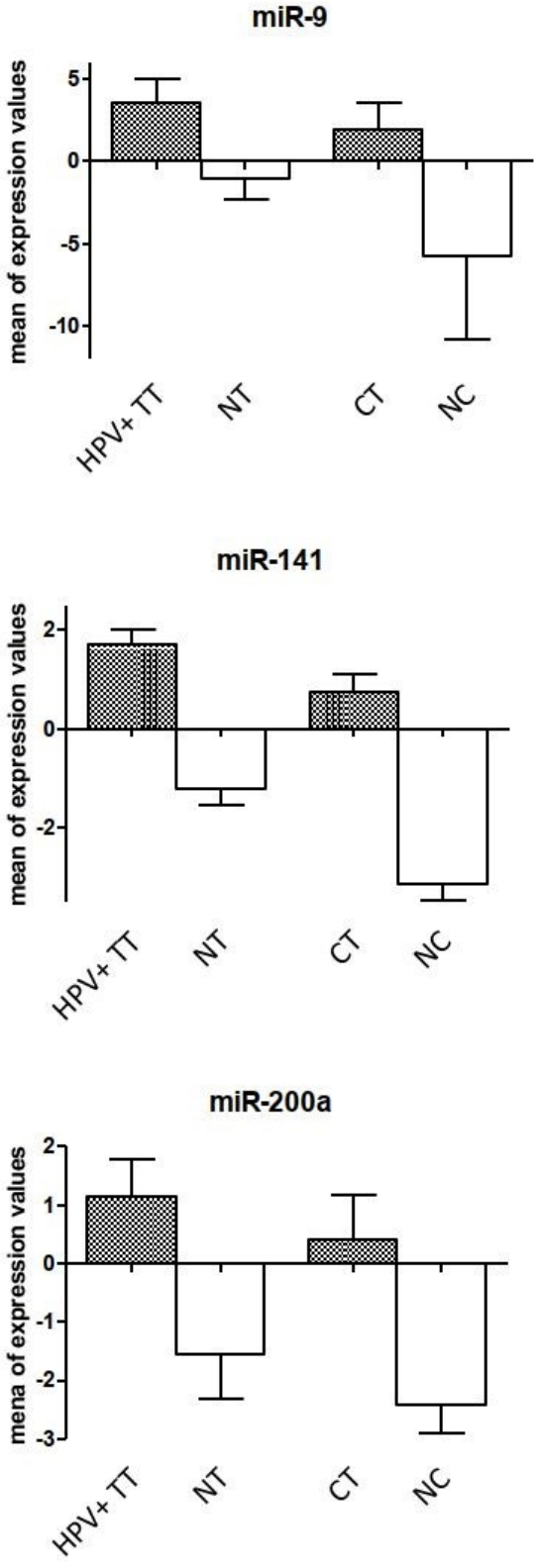

Figure S3 - Expression values of HPV core miRNAs in miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), normal tonsils (NT), cervical tumors (CT), normal cervical tissues (NC).

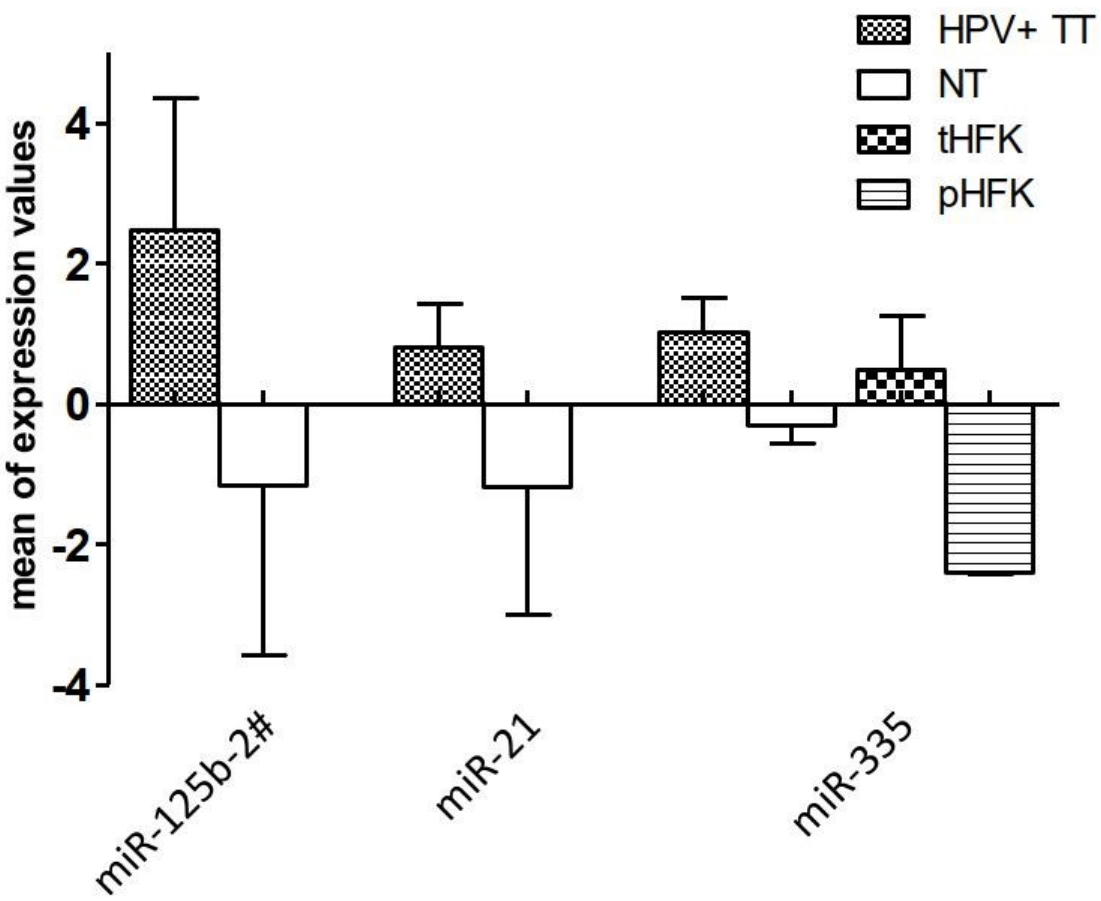

Figure S4 - Expression values of miRNAs differentially expressed in HPV-positive tonsillar tumors on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), normal tonsils (NT), HPV-immortalized keratinocyte clones (tHFK), primary keratinocytes clones (pHFK).

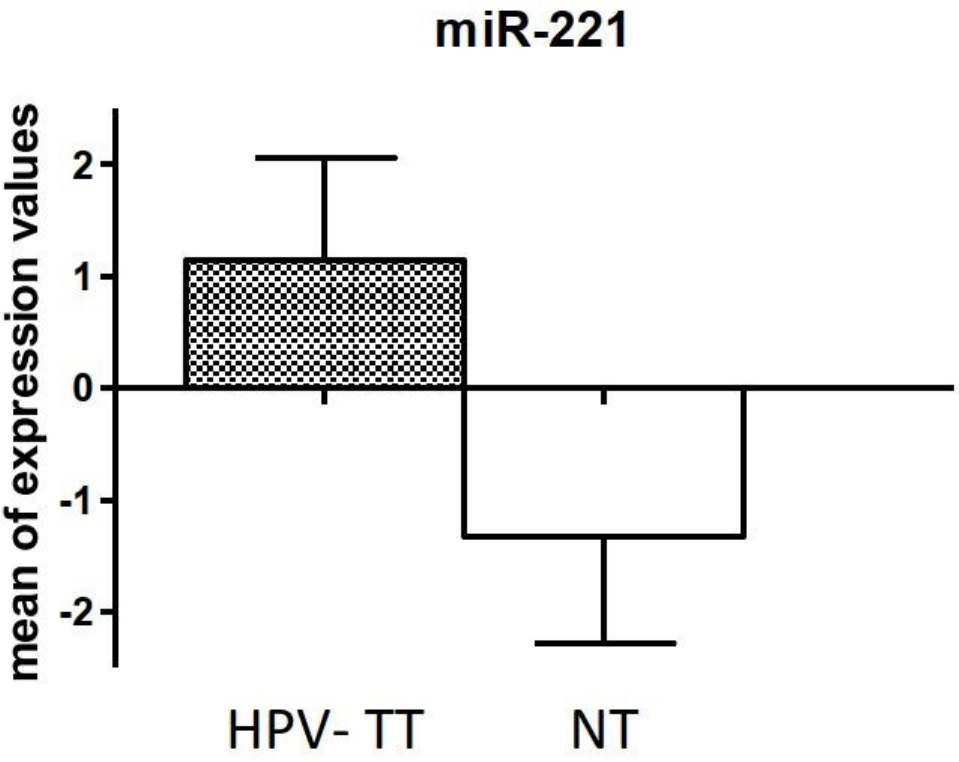

Figure S5 - Expression values of miR-221 differentially expressed in HPV-negative tonsillar tumors on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-negative tonsillar tumors (HPV- TT), normal tonsils (NT).

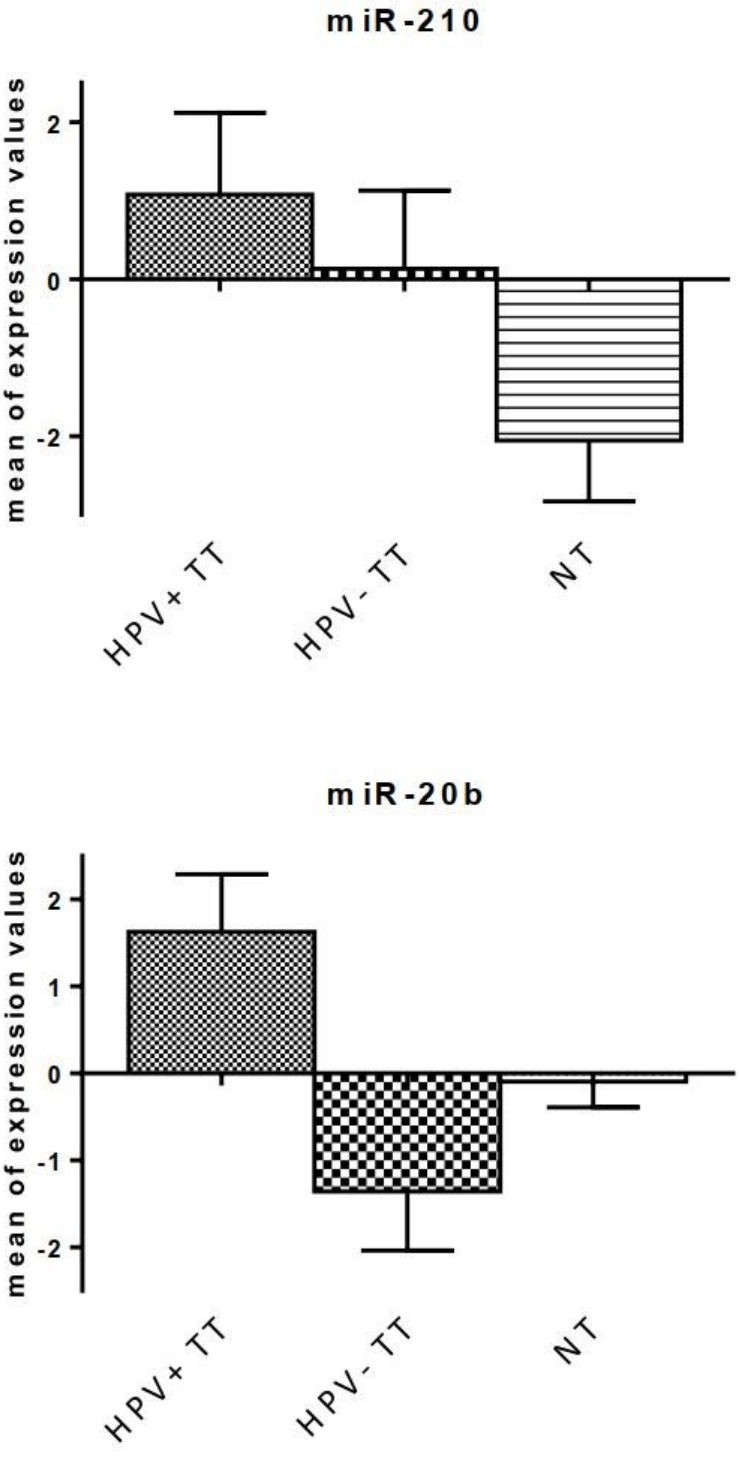

Figure S6 - Expression values of miR-210 and miR-20b differentially expressed in tonsillar tumors of both etiologies on miRNA arrays. Fold change >1.33, *P*-value <0.05. HPV-positive tonsillar tumors (HPV+ TT), HPV-negative tonsillar tumors (HPV- TT), normal tonsils (NT).

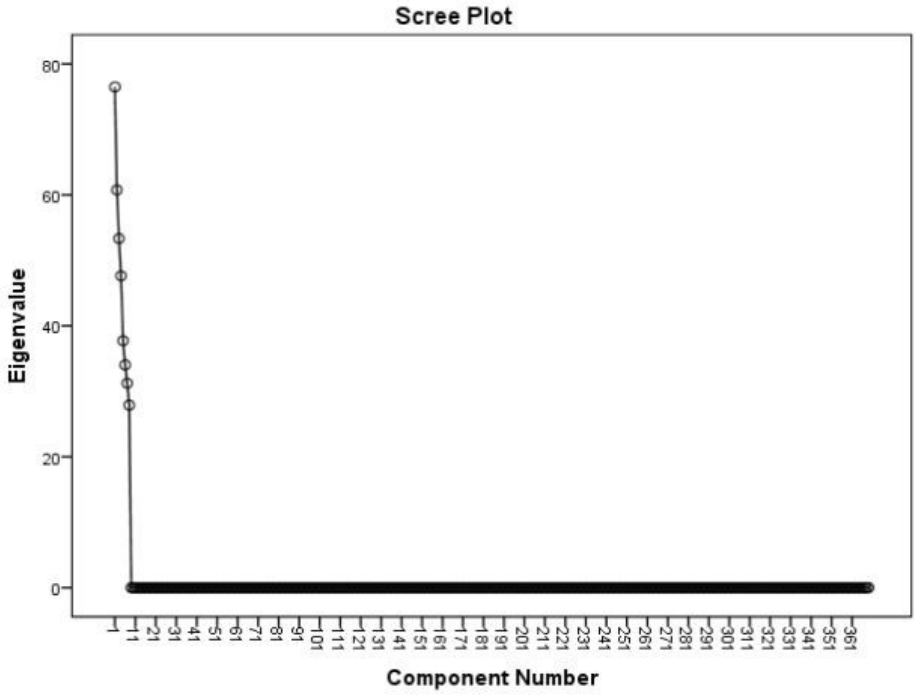

Figure S7 - Screenplot showing the loading of the factors obtained by factor analysis.

Table S7 - Rotated Component Matrix computed by Rotation Method: Varimax with Kaiser Normalization showing the components of a particular factor sorted by absolute value of loading for each variable.

	Component															
1		2		3		4		5		6		7		8	
Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading	Variable	Absolute value of loading
g1	-,969	g454	,930	g1267	-,986	g643	,917	g196b	,927	g5163p	,833	g148b#	-,928	g3613p	,884
g133a	-,968	gU8	,888	g378	-,977	g497	,892	g4853p	,922	g133b	-,819	g1291	,795	g5423p	,867
g200a	,921	g501	,879	g3385P	,952	g130b#	,852	g589	,922	g106b#	-,800	g660	,778	g5763p	-,860
g27b	,888	g374	,873	g596	,933	g382	,839	g3243p	,881	g3395p	,790	g361	,767	g548d5p	,853
g19b	,878	g320	,869	g766	,885	g301b	,826	g3423p	-,875	g10a	,784	g8863p	,761	g99b#	,837
g206	-,872	gU9	,863	g26a1#	,881	g618	,822	g92a1#	-,862	g424#	-,769	g564	-,746	g663B	,794
g200b	,871	g182	,851	g7#	,860	g1274B	,787	g155	-,860	g221	-,762	g30e3p	-,738	g424	,793
g33a#	,852	g328	-,849	g30a5p	,845	g655	,786	g146b	-,858	g20b	,748	g8865p	,726	g572	-,733
g4255p	,835	let7a	-,834	g1513p	,835	g1208	,786	g1423p	-,824	g208b	,747	g148b	,716	g181a2#	,680
g410	-,824	g186	,822	g335	,833	g138	-,773	g1260	,809	g146b3p	,728	g601	,701	g193#	,679
g106b	,816	g4835p	-,815	glet7i#	,825	g135b#	-,772	g143	,804	g181c#	,728	g579	,699	g650	-,660
g197	,813	g29c	,804	g141#	,816	g3245p	,771	g1425p	-,801	g720	,723	g15b	-,692	g7695p	,653
g141	,811	g629	,804	g144#	,807	g6285p	-,768	g152	,798	g283p	,722	g597	-,684	g573	,643
g25	,806	g3393p	,804	g520c3p	,802	g301	,761	g135a	,786	g194	,698	g6283p	,672	g12253p	,629
let7g	,790	g486	,797	g517a	-,795	g193a5p	,744	g8855p	-,772	g181a	,694	g185	,670	g1300	-,628
g19a	,788	g135b	,792	g26b#	,779	g539	,735	g145	,765	g15a	,683	g27a#	-,665	g363	,620
g23b	,787	g942	,780	g518f	-,767	g18a	,733	g425#	,756	g95	-,673	g4235p	,651	g22	,619
g106a	,785	g126	,771	g520g	-,761	g652	,730	g29a	-,737	g192	,670	g449	,648	g181c	-,613
g331	,782	g566	-,768	g195	,760	g18b	,730	prognosi	,721	g222#	-,657	g1298	-,645	g432#	-,603

g34a	,772	g511	-,763	g126#	,747	g520D3P	-,727	g370	,707	g4553p	-,657	g450a	-,641	g567	,591
g625#	-,762	g1395p	,753	g664	,731	g145#	,721	g29b	-,685	g365	-,655	g242#	-,623	g520g	-,581
g493	-,753	g892b	,743	g502	-,728	g449b	,711	g550	,684	g571	-,637	g1262	,623	g199b	,570
g20a	,736	g1244	-,742	g130b	,727	g320B	,708	gU7	-,675	g204	,634	g518b	,619	V158	,568
g200a#	,730	g433	-,735	g1305	-,725	g224	,699	g431	,636	let7b	,631	g346	-,618	g210	,554
g30c	,722	g148a	-,732	g191#	,721	g149#	,693	g150	-,631	let7e	,618	prognosi	-,612	g5323p	-,554
g376c	-,720	gU7	,710	g22#	,720	g25#	,691	g34b002	,629	gR1393p	,616	g494	,606	g1275	-,550
g128a	,716	g335#	,710	g345	,708	g19b1#	,677	g1247	-,622	g605	-,614	g1183	,602	g489	,538
glet7g#	,714	g107	-,694	g183#	,699	g5323p	,670	g708	,620	g1233	-,613	g522	,597	g31#	,532
g125b2#	,695	g192#	-,693	g144	,698	g190	,669	g5743p	,620	g130a	,601	g27a	,593	g518f	-,524
g592	,693	g146a	,691	g657	-,693	g1825	,667	g214#	,617	g21	,597	g744	,576	g627	-,514
g429	,692	g191	,687	g642	-,682	g103	,663	g95	-,608	g3425p	,595	g22	,575	g519a	,497
g99a	,690	g1243	,685	g888	-,681	g296	,654	g1274A	,598	g26b	,580	g622	,572	g5903P	,495
let7c	,687	g625	-,680	g375	-,673	g550002	,653	g622	-,584	g1247	-,580	g1290	,571	g150	-,474
g30b	,682	g575	-,676	g5825p	,672	g3315p	-,641	g99b	,581	g522	,567	g29c#	-,561	g1227	,465
g411	-,670	g452	,675	g27a#	,671	g378	,639	g411	,576	g1201	,565	g25#	-,558	g532	-,464
g9	,665	g517b	-,671	g93#	,664	g500	,638	g450a	-,572	g744	-,561	g1244	,551	g26a	-,460
g101	,657	g203	,668	g10b#	,660	g672	-,635	g1243	-,570	g15b	,557	g193a3p	,550	g639	,457
g223	-,655	g125a3p	,667	g939	,656	g98	,632	g376c	,569	g202	,551	g892b	-,541	g3315p	,456
g3623p	,653	g24	,666	g192#	,655	g627	-,623	g545	-,569	g1303	,547	g27b#	-,534	g708	,454
g199b	-,651	g567	-,665	g30a3p	,653	g1276	-,621	g214	,567	g1227	-,547	g320	,532	g661	,448
g598	,650	g193a3p	,665	g215	-,647	g204	-,619	g489	-,565	g601	-,544	V158	,532	g24	,447
g99a#	,641	g5123p	,657	g107	-,644	g656	,618	g132	-,563	g26a	,541	g99a	-,531	g29b	,445
g657	-,633	g9#	,653	g130a	,644	g20a	,610	g1403p	-,561	g148a	,537	g362	,528	g200c#	-,442
g200c#	-,629	g505#	-,646	g5903P	,639	g1403p	-,609	g639	,560	g550002	-,536	g449b	,526	g205	,439
g17	,618	g577	,644	g130a#	-,637	g6283p	-,607	g505	-,553	g454#	-,530	g30c	-,514	g362	-,439
g545#	-,617	g200c	,634	g523	,604	g30d	,590	g365	,550	g638	-,523	g452	,506	g1271	,438
g1275	,610	g449	,632	g210	,601	g1515P	,589	g605	-,550	g16	,522	g19b1#	-,504	g212	,437
g4093p	-,607	g212	,632	g27b#	,598	g597	,580	g15a	-,539	g27a	-,521	g3623p	,502	g571	,437

g3233p	-,606	g636	-,631	g34b002	,589	g185	,577	g199a	,537	g4863p	,521	g6285p	-,489	g161#	,434
g330	,605	g17	,631	g517c	-,585	g1285	-,565	g21	-,533	g92a	,517	g376a	,487	g149	-,432
g31	-,601	g5823p	-,627	g30d	,573	g218	-,560	g144	,533	g10b	,513	g125a5p	-,484	g1303	,426
g376a	-,600	g3233p	-,622	g4093p	,572	g222#	-,560	g34a#	-,531	g340	,506	g15a	-,479	g520c3p	-,424
g517c	-,593	g199a3p	-,622	g5823p	-,567	g29a	-,558	g376a	,526	g193a5p	-,500	g548c5p	,476	g505#	-,421
g10b	,591	g17#	-,609	g550	,563	g191#	-,554	g26b	-,518	g23a	-,495	g100	-,472	g548c5p	,420
g98	,585	g5905p0	,606	g1515P	,563	let7d	,552	g28	,517	g29a#	-,493	g636	-,469	g330	-,420
let7f	,584	g21#	-,595	g3315p	-,561	g455	,544	g1303	-,514	g320B	,489	g625	,468	g195	,417
g3425p	,582	let7d	-,595	g3383p	-,550	g573	,542	g222	-,513	g9	,486	g214	,464	g564	-,413
g15b	,582	g888	-,584	g484	,547	g532	,541	g500	,510	g523	,480	g664	-,454	g132	,411
g34c	-,579	gR1393p	-,579	g223#	,547	g362	,540	g454#	,509	g296	,478	g345	,453	g181a	-,402
g487a	,578	g598	,558	g4835p	-,544	g3383p	-,540	g9#	-,502	g616	,476	g5905p0	,445	g193b	,401
g4863p	-,570	g656	-,555	g8863p	-,543	g199a3p	,537	g29c	-,500	g511	-,469	g545#	-,442	g566	,399
g125b	,568	g29a#	,552	g1233	,542	g213	,537	g22	,499	g1290	-,452	g221	,439	g1825	-,395
g200c	,560	g214#	-,551	g577	,538	g223#	-,525	g5825p	-,493	g125b	,451	g296	-,437	g455	,393
g422a	,558	g642	-,545	g454#	,533	g575	-,525	g34c	,487	g17#	-,445	g10b#	-,434	g3245p	,390
g34a#	,556	g422a	,541	g200a#	,532	g340	,513	g340#	-,483	g101	,444	g532	,433	g545#	-,390
g100	,555	g592	,540	g1180	,528	g99a#	,510	g1290	,481	g455	-,438	g99b	-,430	let7e	-,385
g579	,555	g16	,533	g203	-,524	g30b	,497	g29a#	-,471	g650	,435	g31#	-,429	g605	,385
g213	-,554	g505	-,530	g31#	,519	let7f	,492	g126#	,470	g422a	,425	g125b	-,429	g502	-,385
g18b	,550	g205	,529	g125a5p	,519	let7e	,491	g144#	,458	g363	,424	g181c#	-,427	g636	,379
g378	,550	g192	,527	g455	-,519	g1271	,491	g375	,455	let7c	,421	g125a3p	,427	g31	,378
g15a	,550	g218	-,524	V158	,516	g494	,489	g518b	,454	g223	-,414	g1260	-,427	g30d#	,373
g205	,549	g1183	-,519	g638	-,514	g106b#	,488	g31	-,454	g1276	,407	g523	,425	g185	,371
g7695p	,548	g149#	-,518	g519a	-,510	g146a	-,486	g941	,451	g663B	-,404	g484	-,424	g29c#	,366
g320	,547	g149	,517	g136#	,509	g161#	,475	g661	,446	g519a	,403	g545	,416	g22#	,361
g222	,543	g4553p	-,511	g1300	,498	g19a	,472	g301	,445	g502	,402	g941	,406	g9#	-,360
g149	,542	g202	-,502	g34a#	,492	g181c	,466	g183#	,436	g661	-,400	g1271	-,403	g28	,359
g1201	,540	g320	,493	g199a	-,490	g629	-,465	g363	-,436	g487a	,397	g424	,402	g218	,357

g340#	,539	g638	-,490	g652	-,490	g425#	-,464	g223#	,428	g572	-,396	g375	-,399	g194	-,357
g161#	,536	g15a	,477	g3425p	-,485	g20a#	,463	g1274B	,426	g135a	-,396	g340#	,397	g431	,357
let7b	,534	g31	,468	g183	-,484	g1291	-,457	g212	-,418	g346	-,393	glet7g#	-,394	g191	-,353
g92a	,533	g199a	-,465	g941	,474	g155	-,456	g432#	,417	g433	-,391	g1285	,392	g505	-,353
g181c	,532	g34c	,462	g5123p	-,474	g194	-,448	g193b	,416	g378	-,389	g143	,391	glet7i#	-,349
g1180	,531	g214	-,462	g655	,469	g1180	,440	g494	,415	g545#	,387	g199a3p	,387	g34b002	,348
g136#	-,529	g519a	-,461	g27a	-,463	g125b	,437	g136#	,414	g598	-,385	g20a#	-,387	g4235p	,347
g190	,528	g1513p	,460	g545	-,457	g548c5p	-,432	g199b	,411	g195	,383	g93#	-,384	g126#	,347
g125a5p	,528	g223	,460	g197	,453	g8865p	-,432	let7g	-,411	let7g	,380	g18a	,382	g340	,345
g431	-,528	g23a	-,459	g433	-,446	g181a	,427	g4235p	,406	g5743p	,380	g181a	,381	g1305	,345
g20a#	,526	g744	,458	g191	,446	g92a	,424	g135b#	,406	g532	-,379	g30a3p	-,377	g183	-,340
g30d#	,526	g1227	,458	g30d#	,444	g29c#	-,423	g335	-,400	g500	-,375	glet7i#	-,372	g98	,338
g132	-,526	g1515P	,451	g1183	-,443	g660	,423	g3383p	,400	g432#	,372	g150	-,369	g941	,336
g103	,525	g10b	,450	g656	-,442	g99b	,420	g29c#	-,399	g30a5p	,369	g550	-,368	g21#	,336
g616	-,525	g99a#	,450	g571	-,438	g365	,420	g24	,399	g1275	,367	g501	-,357	g200c	-,335
g28	,519	g1298	,446	g522	,436	g487a	-,418	g145#	,398	g708	-,367	g130a#	,354	g145	-,331
g27b#	,519	g128a	-,445	g1274A	,435	g183	,416	g146a	-,393	g1262	-,365	g141#	-,354	g625	-,330
g484	,516	g213	-,443	g100	,434	g125a3p	-,407	g548c5p	,393	g18a	-,356	g625#	-,353	g146b3p	-,327
g518b	,514	g5743p	,443	g650	-,429	g625#	-,401	g125b2#	-,391	g138	,353	g24	,350	g106a	,326
g30a3p	-,505	g424#	-,441	g181a2#	,428	g150	-,399	g26b#	,390	g8855p	-,351	g193#	-,348	g100	,325
g5903P	,498	g672	-,439	g1825	-,428	g1201	,399	g340	-,390	g125b2#	,348	g204	-,346	g190	-,325
g193#	,497	g616	-,436	let7d	,424	g3393p	,398	g664	,389	g330	-,346	g1274A	-,341	g487a	,323
g1425p	,488	g1305	,434	g661	,424	g1298	,394	gU8	-,384	g449	,343	g222	-,340	g23a	,318
g486	-,487	g1262	-,433	g200c#	,423	g429	,394	g99b#	,381	g34b002	,337	g3245p	-,340	g135b#	-,316
g1395p	-,484	let7f	,431	g20a#	,419	g5825p	,392	g550002	,380	g30d#	-,337	g191#	,339	g4863p	,314
g21#	,482	g34a	-,431	g283p	,411	g361	-,391	g101	-,379	g15b	,335	g224	,336	g1403p	-,310
g202	,481	g183	,424	g30e3p	,410	g21	,384	g161#	-,376	g93#	-,334	g370	-,332	g450a	,307
g19b1#	,479	g545	,423	g1303	,410	g17	,383	g23a	,374	g200c#	,333	g34a#	-,329	g1276	,306
g218	-,475	g939	-,422	g15a	,410	g30d#	-,380	g16	-,371	glet7g#	,333	g650	,327	g15a	,304

g639	-,475	g361	,418	g9	,408	g517b	-,376	g130a#	,367	g132	,329	let7a	-,324	g629	-,302
g1285	-,474	g572	-,418	g101	-,405	g212	,376	g130b	,367	g141	,326	g596	,324	g214#	,302
g20b	,474	g138	,416	g99b	,401	g939	-,370	g5123p	-,364	g100	,325	g135b	,323	g672	,299
g214	-,471	g193b	,414	g636	-,398	g1262	,369	g15b	-,359	g1274B	-,323	g517b	-,318	g242#	-,299
g30e3p	-,468	g30d	,414	g505#	,398	g193b	-,368	g597	,358	g126	-,319	g25	-,316	g328	-,295
g517b	,467	g125b2#	,409	g15b	,397	g10b#	-,367	g487a	-,358	g1423p	,317	g5423p	,311	g130b#	-,294
g942	,466	let7c	-,408	g21#	-,397	g340#	,359	g3245p	,357	g92a1#	,317	g33a#	,309	g192	-,294
g12253p	,465	g720	,408	g26a	,394	g3623p	,354	g242#	,356	g222	-,317	g22#	-,307	g27a	,294
g224	,462	g618	-,407	g4255p	,394	g431	-,351	g128a	,355	g20a#	,312	g1305	-,306	g15a	,293
g23a	,461	g148b	-,404	g146b3p	-,391	g335#	,349	g627	,354	g215	,311	g672	-,304	g15b	,292
g215	,452	g93#	,396	g4553p	-,387	g148b	,348	g1180	,351	g370	,311	g616	,303	g5163p	,292
g672	-,449	g493	,391	g424	,384	g135b	,346	g484	,350	g1298	,310	g520c3p	-,301	g183#	,291
g5123p	-,448	g517a	,391	g10a	,382	g7#	-,345	g23b	,345	g10b#	,308	g592	,299	g125a3p	,291
g520D3P	,444	g1271	,388	g494	-,380	g8855p	,344	g616	-,343	g12253p	-,308	g283p	-,294	g335#	,290
g573	,438	g450a	-,388	g1271	,379	g130b	,339	g410	,342	let7f	-,307	g502	,294	g221	-,288
g152	,433	g28	-,384	g149#	-,379	g136#	,336	glet7g#	-,340	g3393p	,306	g181a2#	-,293	g622	,284
g5323p	,429	g449b	,382	g520D3P	,377	g16	-,331	g4093p	,339	g31#	,304	g26a	-,291	g8855p	-,282
g5905p0	,427	g346	-,379	g489	,373	g130a#	-,331	g939	,338	g29c#	-,304	g1208	-,289	g144	,278
g505#	-,424	g200a#	,368	g106b	,369	g126	-,330	g93#	,330	g1395p	-,302	g19a	,286	g16	,275
g335#	,422	g4863p	-,368	g242#	-,368	g181a2#	,330	g283p	,329	g941	-,301	g301b	-,286	g18b	,270
g183	,420	g208b	-,365	g346	,365	g1243	,329	g92a	-,327	g548c5p	,298	g663B	,283	g517c	-,269
g346	,417	g539	-,363	g202	-,364	g4255p	,328	g301b	,327	g1285	-,298	g21#	,282	g199a	,267
g26a	,417	g320B	-,362	g27b	-,362	g146b	-,328	g183	,325	g144#	,294	g489	,281	g643	,267
g191	,412	g1233	,362	g622	-,359	g22#	,326	g655	,324	g567	-,293	g1180	,281	g579	,266
g133b	-,411	g22#	-,356	g627	,357	g320	-,325	g1300	,319	g1291	-,290	g26b#	,281	g5905p0	,264
g505	-,404	g92a	-,354	g193a3p	,356	g26b	,323	g331	,315	g182	,288	g213	-,280	g494	,263
g126	-,397	g330	,352	g320	,356	g145	,322	g642	-,313	let7a	,287	g197	,280	g720	,262
g210	,397	g331	,352	g17#	,353	g639	-,319	g577	,313	g200c	,287	g429	,274	g370	,261
g345	,396	g12253p	,350	g149	,352	g331	-,318	g744	,311	g548d5p	,286	g639	,273	g106b	,261

g548c5p	,395	g190	-,349	g575	,351	g517c	-,318	g548d5p	,310	g28	,285	g411	,272	g99a	,261
g1276	-,393	g25	-,346	g4235p	,348	g17#	,316	g511	-,310	g374	,280	g376c	,271	g550	,259
g1274A	-,389	g106a	,346	g15b	-,348	g20b	-,306	g330	-,307	g141#	-,280	g708	-,270	g5823p	-,259
g1300	-,384	g26a1#	,345	g103	-,346	g4093p	,304	g135b	,306	g3233p	-,278	g3613p	-,270	g652	,257
g1423p	,383	g23b	-,344	g135a	-,344	g505	-,304	g1285	,306	gU9	-,274	g145#	-,269	g19b	,256
g1825	-,380	g222	,343	g425#	,344	g186	-,297	g564	-,304	g888	,272	g15a	,266	g9	-,256
g29b	,376	g340#	-,342	g34c	-,344	g5763p	-,296	g378	-,304	g193b	-,271	let7d	,260	g335	,256
g1247	-,375	g517c	,339	g3613p	,342	g152	,295	g10b#	,301	g103	,270	g125b2#	,258	g5825p	,255
g661	,374	g1260	-,339	g29a#	-,340	g1233	,294	g224	,300	g625	,269	g192	,258	g1201	,255
g148b	,374	g26b	,336	g1298	-,340	g181c#	-,294	g598	-,300	g1300	,268	g455	,257	g223#	,254
g26b	,374	g21	-,335	g370	-,339	g486	,291	g192	-,299	g892b	-,268	g92a1#	,255	g152	,253
g5823p	,373	g652	,333	g566	-,338	g4863p	-,291	g19b	-,296	g25#	-,265	g182	,255	g375	-,252
g660	,372	g522	-,332	g601	-,336	g222	-,290	g30d#	-,294	g199a3p	,262	g424#	,254	g500	-,246
g708	,367	g242#	-,331	g8865p	-,336	gR1393p	,290	g638	,294	g566	,261	g148a	,250	g664	,244
g193b	-,365	g340	,324	let7f	,336	g454#	-,290	g545#	,292	g99a#	,261	let7c	-,250	g200b	-,239
g3395p	,364	g18a	,323	g1208	,334	let7b	-,289	g517a	-,292	g345	,259	g4553p	,246	g136#	,238
g130a#	-,364	g1403p	,322	g429	,334	g15b	,287	g7695p	,291	g9#	,257	g1227	-,246	g30d	-,237
g17#	-,358	let7b	-,321	g7695p	,333	g30a5p	,284	g571	-,290	g520c3p	,257	g30b	-,242	g6285p	,236
g199a	-,357	g489	-,321	g205	-,327	g642	-,284	g429	,289	g592	,250	g9#	,240	g135a	-,236
g222#	,355	g301	,320	g222#	,327	g5423p	,283	g107	,289	g579	,245	g1425p	,240	g550002	-,233
g130b	,353	g29c#	-,316	g214#	,327	g141	-,282	g3233p	,288	g183#	,239	g410	,238	g15b	,232
g203	,353	g215	-,314	g564	,324	g203	,280	g573	,286	g489	,239	g136#	,237	g203	-,232
g186	,351	g1274A	-,313	g3395p	-,321	g518b	-,278	g199a3p	,285	g106a	,238	g454#	-,236	g520D3P	-,231
g363	-,351	g99b#	,312	g193b	-,317	g3423p	-,278	g382	,284	g148b	-,237	g141	,234	g320	-,231
g1208	,351	g532	,311	g452	,313	g493	-,277	g106b#	,283	g656	,235	g939	,233	g130a#	,231
g8865p	-,351	g206	-,309	g5743p	,311	g205	,276	g130a	,279	g4235p	,234	g3423p	-,233	g625#	-,230
g571	,349	g30c	,306	g30b	,309	g10b	,275	g888	,275	g125a5p	-,234	g200c	,232	gU9	-,230
g1227	,349	g194	-,303	g942	,303	g424#	,271	g148b#	,273	g223#	-,233	g1395p	-,231	g206	,229
g432#	,348	g497	-,301	g128a	,303	g663B	-,270	g181a2#	-,270	g146a	,230	g5903P	,229	g1233	,227

g145#	-,348	g130a	,297	g1276	-,300	g4553p	,269	g125a5p	,267	g146b	-,230	g1303	-,227	g410	,227
g146b3p	,348	g1201	-,295	g193#	,299	g589	,269	g26a	-,267	g24	-,228	g208b	-,226	g939	,227
g766	-,344	g161#	,293	g34a	-,298	g638	,269	g9	-,264	g5905p0	-,226	g193b	,226	g365	,223
g141#	,343	g30a3p	,290	g223	-,295	g432#	-,267	g181c#	,264	g484	,226	g23b	-,225	g146b	,223
g5163p	-,341	g132	,288	g296	,293	g30c	,265	g34a	-,264	g636	,225	g433	,225	g539	-,222
g1262	,341	g429	,285	g487a	-,291	g3395p	,264	g215	-,259	g493	,224	g183#	-,224	g130b	,221
g1183	-,341	g1276	,284	g376a	,290	g29b	-,264	g149	,259	g210	,222	g330	-,221	g95	-,220
g539	-,340	g4093p	-,282	let7b	,289	g1244	-,259	g1201	,256	g34a	,221	g202	-,217	g517b	-,220
g941	,340	g141	,282	g382	,286	g221	,259	g194	,254	g3383p	-,221	g577	,217	g1247	,220
g5743p	-,340	g3623p	-,280	let7e	,286	g200c#	,258	g1275	,254	g1183	-,220	g493	,215	glet7g#	-,220
g208b	,337	g183#	,279	g193a5p	-,286	g26b#	-,256	g362	,253	g29c	,220	g511	-,213	g10b	,219
g10a	,329	g382	-,279	g1275	-,284	g214#	,251	g518f	-,252	g301	-,216	g200c#	,211	g148b#	,217
g575	-,328	g210	,276	g5905p0	,283	g149	,248	g1271	,251	g320	,215	g422a	-,206	g3233p	-,216
g577	-,327	g106b	,276	g130b#	,282	g215	-,246	g12253p	-,251	g3623p	-,214	g29a#	,205	g3243p	-,212
g3383p	-,327	g181c#	,275	g23a	-,281	g133b	-,245	g208b	,251	g454	-,214	g223	,201	g493	-,211
g452	,323	g200b	,275	g135b#	,276	g125a5p	,243	g221	,249	g1403p	,213	g431	,201	gR1393p	,206
g31#	-,322	g26b#	,275	g95	-,274	g1274A	,243	g422a	,247	g5825p	-,213	g5743p	,200	g1290	,205
g4853p	-,316	g181c	,274	g539	,273	g363	-,241	g720	-,247	g200b	,212	g215	-,193	g34c	-,205
g143	-,314	g30b	,269	g3623p	,268	g370	-,241	g20b	-,244	g382	-,209	g618	-,192	g27b#	,198
g3243p	,314	g362	,268	g6283p	-,267	g146b3p	-,241	g452	,243	V158	-,206	g1274B	-,189	g1274B	,197
g517a	-,302	g20a#	,263	g4863p	-,264	g193a3p	,239	g17#	-,240	g145	-,205	g31	-,188	g213	,194
gR1393p	,301	g224	,263	g422a	,264	g8863p	-,237	g182	,239	g449b	-,202	g26a1#	-,187	g148a	-,194
g622	-,298	g27a#	-,260	g161#	,263	g566	-,235	g192#	,236	g199a	,202	g3233p	,184	g3383p	,193
g25#	,289	g223#	-,260	g625	,262	g410	-,235	g519a	,235	g657	-,200	g1825	-,183	g193a3p	,192
g149#	,288	g1290	-,258	g432#	,260	g523	-,234	let7a	,233	g1180	-,198	g331	-,182	g1285	,191
g18a	,285	g1285	-,256	g25	,259	g195	,233	g3393p	,233	g1271	-,195	g378	-,182	g3423p	-,188
g99b	,285	g1247	,255	g301b	,252	g501	,233	g539	,231	g766	,194	g539	-,179	g283p	,185
g283p	,283	g4235p	-,252	g363	,247	g1305	-,231	g213	,229	g642	-,193	g942	-,173	g33a#	,185
g242#	-,278	g103	,251	g374	,246	g328	,228	g328	-,226	g205	,193	g19b	-,171	g19a	,184

g7#	,278	g520g	,245	g5763p	-,245	g567	,227	g25	-,226	g425#	-,192	g1403p	-,169	g128a	-,184
g144	,277	g597	-,244	g1227	,243	g661	,226	g15b	-,223	g1243	-,190	gR1393p	,169	g133a	,183
g145	-,275	g145	-,240	g125b	,243	g1395p	,223	g206	,219	g136#	-,190	g1276	-,168	g145#	,182
g720	,273	g579	-,238	g204	,242	g15b	,223	g1233	-,219	g144	-,189	g92a	-,168	g25#	-,180
g340	,272	g639	-,237	gU8	-,241	g376c	,223	g523	-,219	g545	-,186	g500	-,166	g1515P	-,180
g523	-,271	g99a	-,236	let7c	,239	g548d5p	-,220	let7c	,219	g7#	-,185	g146a	-,163	g433	-,179
g375	,269	g564	-,234	g16	,239	g30a3p	-,219	g5323p	-,217	g3385P	,183	g548d5p	,162	g1208	,178
g605	,269	g29a	,234	g28	,238	g650	-,219	g10a	-,216	g3315p	,183	g12253p	,161	g320	,174
g196b	-,263	g33a#	,234	g33a#	,236	g572	-,219	g6283p	-,212	g242#	-,181	g7#	-,160	g197	-,173
g1290	,261	g627	-,233	g330	-,234	g28	,218	g3425p	-,204	g939	-,178	g374	,158	g191#	,173
g212	-,260	g10b#	,232	g1423p	-,233	g106a	,216	V158	-,203	g29b	,176	g146b	-,158	g34a#	,172
g30d	,258	g5423p	,231	g328	,233	g616	,215	g572	,202	g99a	,176	g103	,158	g484	,170
g5763p	,258	g3383p	-,230	g29b	-,232	g484	-,214	g5823p	,201	g21#	,175	g145	-,157	g577	-,169
let7e	,256	g500	,229	g186	,231	g519a	-,214	g5163p	-,200	g501	-,173	g1267	-,156	g1274A	,167
g16	,254	g941	,229	g335#	-,229	g143	-,209	g335#	-,199	g520D3P	,173	g135a	,156	g422a	-,165
g361	-,252	g1291	-,226	g618	-,228	g625	,209	gR1393p	-,198	g5763p	-,173	g27b	,154	g454	,165
g6285p	,251	g98	-,225	g99a	,227	g4235p	,209	g4255p	-,194	g17	,172	g223#	-,153	g29a	,163
g125a3p	-,251	g145#	-,224	g12253p	,226	gU9	-,208	g193#	-,192	g181c	,171	g193a5p	,152	g340#	,162
g21	,250	g411	-,222	g720	,226	g92a1#	-,208	g625#	-,191	g7695p	-,167	g638	,152	g301	,161
g193a5p	,242	g30e3p	,221	g133b	,225	g23b	,206	g1262	,191	g625#	,165	g10a	-,151	g616	,161
let7d	,241	g181a2#	-,219	g643	,225	g601	-,206	g942	-,190	glet7i#	-,165	g575	,150	g766	-,159
g362	,239	g661	-,217	g567	,224	g10a	,205	g449	,190	g27a#	-,165	g98	,150	g320B	-,159
g497	,238	let7e	-,214	g10b	,224	g605	-,204	g3395p	-,188	g31	,163	g766	-,149	g449b	,158
g135b#	-,231	g432#	-,213	g20a	-,222	g5905p0	-,204	g374	-,188	g340#	,163	g661	,149	g193a5p	,158
g532	,231	g7695p	-,206	g500	-,220	g335	-,204	g218	,187	g98	,162	g3243p	,148	g454#	,157
g29a	-,229	glet7g#	-,204	g1290	,219	g242#	-,203	g656	,184	g130b	,161	g1243	,147	g99b	-,156
g3423p	,226	g150	,204	g1244	-,219	V158	,202	g629	-,184	g486	,159	g214#	,146	g657	,156
g601	,226	g27a	,203	g493	,219	g208b	-,202	g30a3p	,184	g33a#	,159	g720	,146	g17	,154
g545	-,225	g199b	-,202	g892b	-,213	g345	-,198	g200c	,180	g143	-,156	g1300	,143	g30b	-,154

g138	,225	g195	-,197	g23b	-,210	g411	,197	g520D3P	,180	g130b#	,156	g378	-,142	g204	,151
prognosi	,223	g200a	,196	g518b	-,209	g592	,196	g320B	-,180	g8863p	,156	g1513p	-,141	let7b	-,150
g30a5p	,217	g657	,194	g145#	,209	g511	-,195	g1227	-,179	g199b	-,155	g572	,141	g101	,148
g9#	,213	g8855p	-,193	let7g	,208	g130a	,194	g660	-,177	g652	,154	g5825p	-,141	g411	,147
g130b#	,213	g605	,193	g20b	,207	g5823p	-,192	g148a	,173	g517a	-,153	g34c	,139	g642	-,145
g185	-,211	g18b	-,192	g3243p	,207	g708	,192	g27b#	-,173	g328	-,153	let7f	-,138	g29a#	,144
g26a1#	,209	g125b	-,186	g362	,207	g941	-,191	g18b	,171	g200a	,148	gU9	-,137	g301b	-,143
g424#	-,208	g335	,185	g99a#	-,206	g99a	,188	g1515P	,171	let7d	,148	g518f	-,137	g374	,142
g518f	,205	g29b	,184	g125a3p	,201	g106b	,187	g8865p	,171	g185	,147	g8855p	-,136	g1260	-,141
g1515P	,205	g370	,184	g92a1#	-,197	g99b#	-,186	g186	-,169	g505#	,145	g432#	-,135	g21	,141
g425#	-,204	g196b	,184	g200b	,196	g31#	-,186	g106a	,166	g224	-,145	g1247	-,134	g146a	-,139
g29a#	,203	g143	-,181	g208b	-,194	g720	,185	g100	,166	g1515P	,144	g3425p	,134	g744	,136
g301	,202	g27b	-,178	g132	-,185	g193#	-,185	gU9	-,164	prognosi	,143	g144	,134	g135b	-,135
g296	,201	g664	-,177	g135b	,183	g128a	,184	g31#	-,164	g214#	-,142	g126#	-,133	g523	,133
g320B	-,201	g523	,173	g376c	,181	g545#	,181	g21#	-,164	g411	-,142	g550002	-,133	g181c#	,131
g183#	,199	g133b	-,171	g550002	,179	g4853p	-,178	g517b	-,163	g126#	-,138	g656	-,132	g618	-,130
g1403p	-,196	g365	,170	g98	,174	g1275	-,175	g618	-,163	g213	,138	let7e	-,131	g215	-,130
g148a	,194	g5903P	,170	g331	,174	g505#	,175	g650	-,156	g375	,136	g30d	-,128	g1425p	-,130
g15a	,193	g6283p	,170	g3393p	-,172	g12253p	,175	g30b	-,155	g672	,135	g497	,127	g182	,129
g589	-,189	g136#	-,170	g454	-,172	g5163p	-,175	g320	,153	g643	,134	g3393p	,126	g452	,128
g150	,189	g660	,168	g497	,172	g223	-,174	g190	-,149	g20a	,133	g335#	,126	g149#	-,127
g655	-,186	g6285p	-,168	g182	,171	g502	-,172	g181a	,149	g3613p	,133	g186	-,125	let7c	-,127
g126#	,183	g130a#	-,168	g589	,167	g545	-,172	g27a	,148	g193a3p	-,131	g99b#	-,124	g425#	-,127
g181a2#	-,181	g30d#	,166	g30c	,166	g34c	,170	g200a	,147	g655	,130	g106a	,124	g200a	-,120
g223#	-,180	g301b	,164	g616	-,164	g200a	-,168	g3315p	,145	g99b	-,129	g30d#	-,122	g144#	,119
g638	-,176	g345	,163	g505	,163	g34a#	,167	g126	,142	g22	,129	g138	,122	g10a	,119
g8863p	-,171	g125a5p	,162	gU9	-,159	g424	-,166	g26a1#	,141	g320	,128	g519a	,120	g192#	,117
g146a	,170	g155	,157	g1243	-,158	let7a	,165	g4835p	-,139	g206	,127	g99a#	-,119	prognosi	-,116
g95	,170	g550002	-,157	g320B	,155	g191	-,158	let7b	,137	g518b	-,126	g888	-,117	g424#	,116

g627	-,163	g296	,150	g19a	-,155	g192	-,158	let7e	,135	g1825	-,126	g106b	,116	g223	-,116
g888	,154	g1208	,150	g592	,154	g3385P	,157	g138	-,135	g1513p	-,126	g517c	,116	g3385P	,114
glet7i#	,153	g135a	,149	g19b1#	,153	g1513p	,157	g223	,133	g577	-,124	g210	-,115	let7d	,110
g146b	-,150	g375	,147	g26b	,153	g202	-,156	g502	-,131	g125a3p	-,124	g144#	-,114	let7a	-,106
g8855p	,149	g126#	,146	g663B	,152	g200b	,153	g1513p	,131	g1260	-,123	g212	-,114	g26b#	-,105
let7a	,147	g1825	-,145	g214	-,151	g29c	-,151	g200a#	,130	g8865p	-,122	g320	,113	g93#	,103
g1303	-,145	g3395p	,145	g340	,151	g892b	,150	g25#	-,129	g190	-,122	g133b	,113	g942	-,101
g625	,145	g100	-,142	g181c#	-,149	g144#	,149	g30c	-,127	g6283p	-,118	g17#	-,113	g522	,100
g636	,144	g99b	-,136	g5163p	-,149	g206	-,148	g8863p	,125	g19a	-,118	g4863p	,112	g27b	,100
g4553p	-,141	g663B	-,136	g222	,148	g93#	,146	g130b#	-,123	g30b	,116	g29c	-,111	g26b	-,099
g1244	-,141	g20b	-,134	g598	-,148	g210	-,146	g892b	,123	g155	,114	g101	,111	g30a3p	,098
g502	,139	g376a	,134	g19b	,147	g197	-,144	g146b3p	-,122	g627	,114	g222#	,111	g511	,097
g1305	-,139	g708	-,134	g29c#	,146	g100	,143	g210	,120	g186	,113	g4853p	,110	g92a	-,096
g24	,137	g19b	-,131	g213	-,144	g376a	-,139	g493	,120	g596	,112	g21	,110	g30a5p	,096
g424	,137	g622	-,129	g196b	,142	g9	-,138	g6285p	-,119	g410	-,110	g23a	-,108	g429	-,095
g1291	-,136	g20a	,128	g199a3p	-,142	g1	,136	g433	,118	g301b	-,110	g1201	-,108	g125b2#	,095
g6283p	-,136	g27b#	-,125	g340#	,142	g1425p	-,136	g98	,117	g130a#	,106	g1423p	,107	g138	-,095
g15b	,136	g146b3p	,124	g152	,141	g101	,134	g592	-,117	g19b1#	,103	g95	-,107	g5743p	-,094
g130a	-,135	g1	-,124	g605	,141	g330	,130	g652	-,117	g212	-,103	g199a	,107	g638	,094
g618	-,135	g191#	-,122	g106b#	,141	g182	-,129	g20a#	,115	g5123p	-,102	g130b	,107	g589	,094
g370	,135	g130b#	,121	g639	,139	g378	-,126	g27b	,111	g1425p	,099	g30a5p	-,106	g141#	,093
gU7	,134	g144	,121	g218	,139	g449	-,125	g454	,109	g30e3p	,099	g194	-,105	g196b	,092
g181c#	,129	g1303	,117	g200a	,138	g564	-,123	g203	,109	g107	-,098	g199b	-,104	g17#	-,092
g22#	,125	g1275	-,115	g1285	,136	g200a#	-,122	g567	,107	g191#	-,098	g132	-,104	g888	,092
g34b002	,125	g19b1#	,113	g410	,134	g15a	-,122	g193a3p	-,106	g193#	,098	g3395p	,104	g1244	-,091
g572	-,123	g148b#	,107	g431	,134	let7g	,121	g505#	-,103	g452	-,092	g1233	,103	g133b	-,089
g194	-,120	g550	,106	g1425p	-,131	g1290	-,121	g222#	,102	g200a#	-,092	g181c	,103	g449	-,087
g374	,119	g424	,106	g18b	,131	g422a	-,121	g19b1#	,101	g22#	,091	g573	-,103	g200a#	-,087
g1513p	,119	g130b	-,105	g141	-,127	g622	-,119	g663B	,097	g203	-,091	g566	,101	g575	,087

g455	-,119	g101	-,105	g361	,127	g664	-,119	g1	-,097	g183	,090	g10b	-,098	g3425p	-,086
g214#	-,118	g5163p	-,104	g424#	-,126	g5743p	,119	g17	,095	g30d	,089	g1	,097	g4835p	,085
g192#	,118	g650	,102	g1274B	,125	g454	-,115	g497	,094	gU7	-,089	g1515P	-,094	g29c	,082
g1243	,118	g766	,102	g411	,123	g1260	-,113	g5905p0	-,094	g618	,088	g487a	-,093	g4093p	-,079
g450a	,116	g518f	,102	g660	-,118	g5903P	-,112	g195	-,094	g181a2#	-,083	g3315p	,093	g26a1#	-,075
g550002	-,116	g410	,099	g9#	,118	g183#	-,108	g33a#	-,094	g5423p	-,082	g340	,093	g1	,074
g106b#	,115	g625#	,096	prognosi	,116	g34a	,107	g193a5p	-,091	g26b#	-,082	g520D3P	,092	g1423p	-,074
g5825p	-,114	g518b	-,095	g138	-,113	g517a	-,105	g15a	-,090	g494	-,081	g130b#	-,091	g125a5p	,074
g22	-,114	g1300	-,094	g708	,112	g95	,102	g501	-,090	g429	-,080	g5763p	-,090	g501	,068
gU9	,113	g455	,093	g744	,112	g29a#	-,101	g346	,087	g135b#	,080	g18b	,088	g202	-,068
g744	-,110	g5825p	,092	g517b	-,112	g9#	,095	g1208	-,086	g573	-,079	g200a	,088	g23b	,068
g652	-,108	g487a	-,091	g133a	,110	g433	-,095	g657	-,086	g34c	-,077	g3385P	,087	g361	,066
g27a	,107	g502	,091	g486	-,109	g520g	-,094	g575	,080	g942	-,077	g126	,087	g8865p	-,063
g522	-,105	g520D3P	,086	gU7	-,109	g126#	-,093	g579	-,079	g30c	,075	g629	-,086	g25	,063
g1298	-,105	g3613p	-,086	g3423p	-,108	g489	,091	g191	-,079	g505	,074	g183	-,086	g655	,062
g643	-,105	g26a	-,081	g378	-,106	prognosi	-,091	g520c3p	,078	g149#	,073	g4093p	-,085	g92a1#	-,061
g328	,102	g5763p	,079	g221	,104	g3243p	-,091	g133a	,077	g19b	-,072	g320B	-,081	g30e3p	,054
g204	,100	g10a	-,078	glet7g#	,104	g133a	-,091	g106b	,076	g4853p	-,070	g657	,081	g20a#	,054
g382	-,099	g200c#	-,076	g5323p	-,103	glet7g#	,087	g636	,075	g152	,067	g7695p	,080	g3395p	,053
g5423p	,099	g484	-,076	g185	-,101	glet7i#	-,081	g30d	-,073	g564	,067	g643	,077	g1298	-,053
g567	,098	g31#	-,074	g106a	,101	g1300	,079	g532	-,073	g424	,067	g106b#	,076	g103	-,053
g3385P	,095	g3243p	,073	g548d5p	,099	g144	-,079	g1183	-,070	g3245p	,067	g505#	-,076	g222	,051
g1271	,094	g146b	,073	g6285p	-,098	g21#	,078	g202	-,068	g520g	,067	g196b	,071	g208b	-,051
g520c3p	-,094	g601	,071	g29c	-,097	g25	,073	g625	,068	g197	,064	let7b	,070	g517a	-,050
g4835p	-,094	g15b	,070	g365	,094	g598	-,070	g27a#	,067	g99b#	,063	g192#	-,070	g497	-,050
g454#	-,092	g548d5p	-,069	g181c	,093	g375	,069	g125b	,067	g214	,063	g26b	,069	g376a	-,050
g4235p	,089	g181a	-,069	g31	-,093	g374	,068	g141	,066	g335#	-,063	g17	,068	g345	-,049
g182	,087	g431	,069	g29a	,091	g4835p	-,067	let7f	-,065	g660	-,060	g190	-,066	g141	,047
g596	,087	g1274B	,067	g625#	,091	g744	-,066	g4553p	,064	g539	,060	g15b	,062	g346	,047

g107	-,085	g3245p	,067	g572	,090	g199a	-,065	g18a	-,064	g25	-,059	g335	-,062	g1395p	-,045
g454	,084	g3385P	,067	g126	,088	g579	,063	g125a3p	-,063	g431	,058	g571	-,062	g214	,045
g301b	,084	g3423p	,066	g25#	,088	g148a	-,059	g1298	,062	g450a	,058	g205	,062	g6283p	-,044
g193a3p	,083	g92a1#	,065	g548c5p	-,087	g30e3p	,059	g486	,058	g4093p	-,057	g34b002	,061	g30c	,044
g520g	,083	g520c3p	-,064	g17	,083	g27b#	,059	g596	,057	g589	-,056	g133a	-,060	g107	,042
g656	-,077	g454#	,064	g22	-,080	g196b	-,059	g296	-,056	g1208	,056	g301	-,058	g382	,041
g489	-,072	g15b	-,064	g155	,078	g26a	-,058	g517c	,056	g5903P	-,055	g365	-,058	g186	-,041
g378	,069	g1180	,062	g532	-,078	g27b	,056	g149#	,055	g622	-,054	g203	,056	g148b	,040
g155	-,067	g15a	,060	g511	,077	g19b	,052	let7d	-,052	g191	-,054	g29b	-,054	g7#	-,040
g199a3p	-,067	g3315p	-,059	g579	,076	g657	,051	g191#	,052	g128a	,052	g486	,051	g27a#	,040
g548d5p	-,066	g3425p	-,058	g1291	,074	g346	-,051	g185	-,051	g218	,051	g363	-,049	g155	-,039
g135a	,065	g204	,057	g190	-,072	g766	,051	g1244	,050	g597	,048	g206	,049	g3393p	,039
g500	,065	g5323p	-,055	g199b	,072	g452	-,050	g5763p	,050	g148b#	,048	g155	-,049	g125b	,039
g449b	,064	g106b#	,054	g21	,071	g27a#	,050	g424#	,050	g378	-,047	g29a	,048	gU7	,038
g29c	,063	let7g	,054	g148a	,069	g3233p	-,049	g4863p	,050	g192#	-,047	g1275	-,047	g126	,037
g26b#	-,058	g548c5p	-,053	g224	,067	g636	-,047	g141#	,048	g376a	-,046	g505	,046	g656	,037
gU8	-,058	g135b#	,053	g148b	-,066	g24	,046	g30e3p	-,047	g517b	-,044	g517a	,044	gU8	,036
g27a#	-,057	g34b002	,052	g8855p	,065	g518f	-,044	g520g	-,045	g550	-,043	let7g	,044	g1243	,036
g1233	,055	g95	-,052	g146a	,064	g3613p	-,044	g205	-,042	g135b	-,043	g655	,043	g130a	,035
g566	-,053	g7#	-,049	g597	,064	g1227	-,043	g99a#	-,040	g5823p	,041	g605	,040	g331	-,035
g192	,053	g1267	,047	g301	-,063	g15a	-,042	g19a	-,039	g29a	-,039	g20a	,039	g378	,034
g939	,052	g589	-,046	g629	,062	g520c3p	,042	g200b	,038	g664	,037	g218	,039	g143	-,034
g144#	,052	g8865p	,046	g192	-,062	g1183	,039	g320	,038	g517c	-,037	g5163p	,039	g1262	-,034
g519a	,052	g221	,045	g449	,062	g5123p	,037	g10b	-,038	g518f	,035	g28	,034	g378	-,033
g10b#	,049	g141#	-,043	g206	,062	g107	-,037	g1276	,037	g18b	-,035	g627	,034	g19b1#	-,033
g663B	,048	V158	-,041	g150	,061	g7695p	,036	g200c#	-,034	g15a	,034	gU7	-,033	g18a	,031
g511	-,047	g25#	-,040	g1247	-,059	g27a	,036	g204	,034	g145#	,034	g328	-,031	g1267	-,031
g3613p	-,045	g571	,039	g212	,058	g132	-,034	g1825	-,034	g3243p	,033	g4255p	,030	g20b	-,028
g664	,043	g376c	-,038	g545#	-,058	g596	,032	g566	-,031	g4255p	-,033	g200a#	-,029	g376c	,028

g3245p	-,043	g643	,037	g1	,058	g1247	-,032	glet7i#	,029	g30a3p	-,033	g146b3p	,029	g597	-,027
g433	-,042	g1423p	-,035	g320	-,057	g125b2#	-,030	g345	-,028	g1244	,027	g5823p	-,029	g518b	,026
g642	,041	g185	,035	g194	-,056	g199b	-,028	g5423p	,027	g23b	-,026	g652	,027	g545	,024
g92a1#	-,040	g30a5p	-,034	g200c	-,053	g141#	-,027	g643	-,026	g639	-,025	g161#	-,026	g598	-,024
V158	-,040	glet7i#	,033	g148b#	-,053	g1303	,026	g672	-,025	g196b	,024	gU8	-,022	let7g	-,024
g335	,040	g573	-,033	g501	,052	g888	,026	g148b	-,025	g1305	,024	g195	,022	g4255p	,023
g221	,039	prognosi	,033	g3233p	-,048	g22	,026	g5903P	-,025	g3423p	,020	g425#	,021	g892b	-,022
g365	-,039	g363	,031	let7a	,048	g200c	-,023	g378	-,023	g26a1#	,020	g191	,020	g1513p	,021
g181a	-,039	g19a	,031	g1262	-,045	g23a	-,021	g766	-,023	g4835p	-,020	g567	-,020	let7f	,021
g494	-,037	g197	-,029	g449b	,043	g571	,020	g601	-,022	g362	,019	g642	,019	g4853p	-,021
g93#	,034	g34a#	-,028	g24	-,042	g522	-,018	g3385P	-,022	g1	,019	g135b#	,018	g106b#	,018
g99b#	,033	g283p	-,028	g1403p	-,042	g577	,018	g3623p	-,021	g335	-,018	g152	-,018	g99a#	-,018
g3315p	,032	g545#	-,027	g99b#	,035	g192#	-,018	g181c	,018	g34a#	-,017	g149#	,018	g4553p	,017
g135b	,031	g1425p	,024	g1395p	,032	let7c	,017	g1305	-,018	g161#	-,016	g589	,018	g199a3p	-,016
g191#	-,029	g4853p	,024	g573	,031	g31	,017	g197	,016	g27b	-,015	g34a	,017	g10b#	,015
g3393p	,028	g133a	-,021	g1201	,029	g135a	,017	g522	,015	g106b	,014	g149	-,016	g8863p	-,013
g892b	-,027	g152	,021	g672	-,027	g1423p	,016	g449b	,015	g1274A	,014	g3383p	,016	g601	,013
g320	,025	g193#	-,020	g146b	-,026	g3425p	-,016	g103	,014	g27b#	-,014	g9	,016	g1291	-,012
g1274B	,025	g193a5p	,019	gR1393p	,025	g34b002	-,015	g1267	,014	g629	-,014	g454	-,015	g224	,012
g629	,021	g655	-,017	g4853p	-,023	g33a#	,015	g7#	,014	g575	,009	g200b	-,014	g486	,011
g148b#	,019	g22	-,017	g145	,021	g283p	-,011	g22#	-,013	g1267	-,009	g382	-,012	g1180	,008
g550	,018	g8863p	,015	g1260	,019	gU8	,011	g20a	-,012	gU8	-,008	g4835p	,012	g20a	-,006
g29c#	-,017	g494	,013	g15a	,019	g214	-,011	g30a5p	,010	g376c	-,007	g130a	-,011	g3623p	,005
g1260	,013	g596	,010	g92a	-,012	g450a	-,010	g1395p	-,009	g331	,005	g128a	,008	g592	-,005
g501	,013	g222#	-,009	g181a	-,009	g320	,009	g361	-,009	g497	-,005	g107	,007	g296	,004
g564	-,012	g4255p	-,009	g5423p	-,009	g148b#	,009	g455	,007	g6285p	-,004	g5123p	-,005	g1183	,003
g195	-,010	g144#	-,009	g450a	-,008	g26a1#	-,006	g424	,006	g133a	,003	g5323p	-,004	g5123p	-,002
g597	,009	g9	,003	g125b2#	-,007	g942	,005	g1291	,004	g5323p	-,002	g16	-,003	g34a	-,002
g1267	-,005	g425#	-,002	g3245p	,004	g1267	-,004	g133b	-,004	g150	-,002	g520g	-,002	g660	,002

g449	,005	g378	-,002	g18a	-,004	gU7	-,002	g3613p	,001	g361	,001	g598	-,001	g596	,001
g650	,000	g378	-,001	g143	,003	g550	,000	g99a	,000	g149	,000	g20b	,000	g222#	-,001

Publication III

PLOS ONE

RESEARCH ARTICLE

Comparison of the miRNA expression profiles in fresh frozen and formalin-fixed paraffin-embedded tonsillar tumors

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Abstract

MicroRNAs are considered as promising prognostic and diagnostic biomarkers of human cancer since their profiles differ between tumor types. Most of the tumor profiling studies were performed on rarely available fresh frozen (FF) samples. Alternatively, archived formalin-fixed paraffin-embedded (FFPE) tissue samples are also well applicable to larger-scale retrospective miRNA profiling studies. The aim of this study was to perform systematic comparison of the miRNA expression profiles between FF and macrodissected FFPE tonsillar tumors using the TaqMan Low Density Array system, with the data processed by different software programs and two types of normalization methods. We observed a marked correlation between the miRNA expression profiles of paired FF and FFPE samples; however, only 27-38% of the differentially deregulated miRNAs overlapped between the two source systems. The comparison of the results with regard to the distinct modes of data normalization revealed an overlap in 58–67% of differentially expressed miRNAs, with no influence of the choice of software platform. Our study highlights the fact that for an accurate comparison of the miRNA expression profiles from published studies, it is important to use the same type of clinical material and to test and select the best-performing normalization method for data analysis.

Introduction

MicroRNAs (miRNAs) are small non coding RNAs (~21 nucleotides) which play an important role in post-trancriptional regulation of gene expression. Their binding with perfect or imperfect complementarity to the 3′ untranslated region of the target mRNA leads either to mRNA cleavage and degradation or to inhibition of mRNA translation [1]. Of interest, Ørom et al. also reported an association of miRNA with the 5′ untranslated region which resulted in the activation of gene expression [2, 3]. MiRNAs are involved in many biological processes such as

collection and analysis, decision to publish, or preparation of the manuscript.

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cellular development, proliferation, differentiation, survival or regulation of apoptosis. The expression of miRNAs is deregulated in human cancer. It has been reported that they can act as tumor suppressors or oncogenes based on their targets. The miRNA expression profiles are specific for malignant and non-malignant tissues, and it has been shown the miRNA profiles differ across tumor types [4]. Many studies focused on the miRNA profiling of tumor types of different origin have been published since miRNAs are considered as promising prognostic and diagnostic biomarkers of human cancer [5–7].

Most of the miRNA profiling studies of tumors were at first performed on the fresh frozen samples (FF) where the RNA is well preserved. However, this type of clinical material is rarely available. The disadvantage of formalin fixation is that it preserves the tissue by creating cross-links between all tissue and molecular components, and these modifications can cause the fragmentation of RNA [8]. However, contrary to mRNA, the stability of miRNAs is not influenced by formalin fixation, probably due to their small size and secondary structure [8–10]. Archived material, formalin-fixed paraffin-embedded tissue samples (FFPE), has proven usable for miRNA analyses and allows for retrospective large studies where the results can be correlated with clinical parameters and prognosis of patients.

Numerous studies have shown the tissue specific expression profiles as well as the differences in the abundantly expressed miRNAs in different types of tumors. However, the overlap of the lists of tumor specific miRNAs is, for most cancer types, poor. Several reasons can explain the discrepant data. One of them can be the use of different types of clinical material or of different methodological approaches.

In our previously published study [11], we found that the tumor homogeneity is important for the robustness of miRNA expression studies, especially in head and neck tumors which are very heterogeneous. Therefore, macrodissection of the FFPE samples provides benefit by increasing the homogeneity of the analyzed samples. To evaluate our previous data on FF HNC samples, we analyzed paired FF and macrodissected FFPE samples for the abundantly expressed miRNAs and the level of concordance in the profiles.

Few studies comparing miRNA expression in FF and FFPE paired samples have been published [12–14]. In the majority of microarray studies, good correlation of results was revealed (correlation coefficient up to 0.95), but the studies were either based on small groups of samples or differed in the workflow. None of these comparative studies has been conducted in head and neck tumors.

The aims of our study were to perform the miRNA expression profiling in a set of macrodissected FFPE samples, to compare the results with the miRNA profiles of paired fresh frozen tumors, and to assess differences possibly resulting from the use of different normalization methods and software for data analysis. Finally, from the analyses of FFPE samples, several differentially expressed miRNAs were selected for confirmation in a larger set of macrodissected FFPE tissues.

Materials and methods

Clinical samples

Ten cases of tonsillar tumors and five non-malignant tonsillar tissues were selected for the comparison of the miRNA expression profiles in fresh frozen and formalin-fixed paraffin embedded samples. All samples were obtained from patients treated at the Department of Otolaryngology and Head and Neck Surgery, 1st Faculty of Medicine, Charles University and Motol University Hospital in Prague. Fresh-frozen samples were already used in our recent study [11]. The signed informed consent form was obtained from all patients enrolled in this study. The study received official institutional and ethical approval from the Motol University

Hospital and Institute of Hematology and Blood Transfusion. The study set was selected based on the presence of HPV DNA and HPV E6 mRNA we defined in our previous studies [11, 15].

The sampling and tissue handling for FF samples has been described before [11, 15]. Sections of FF tumor samples were cut on a cryostat, and the number of tumor cells was determined by a pathologist. All FF samples contained more than 50% of tumor cells. FFPE samples were macrodissected as described before [11].

Processing of samples

Total RNA from all FF tissues was isolated by the miRVana kit (Life Technologies, USA) according to the manufacturer's protocol. Total RNA from FFPE samples was isolated simultaneously with DNA from four 10-μm sections enriched for the tumor cells by macrodissection using the Ambion RecoverAll™ Total Nucleic Acid Isolation kit for FFPE according to the manufacturer's protocol (Applied Biosystems, USA). RNA concentration and quality was measured by a Nanodrop™ Spectrophotometer (Thermo Scientific, USA) and Experion chip electrophoresis (Bio-Rad, USA).

TaqMan Low Density Array (TLDA) analysis

The miRNA expression profiling was performed by the TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies, USA) containing a total of 384 miRNA probes and controls per card. Overall, we analyzed 15 FF samples and 15 FFPE samples (five HPV-positive tumor samples, five HPV-negative tumor samples, and five non-malignant tissues of each material). For the analysis of FF samples, 1000 ng of total RNA was used for reverse transcription. In the case of FFPE samples, the input was 350 ng due to the smaller concentration of extracted RNA from FFPE samples. Total RNA of each sample was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers specific for each card (both Life Technologies, USA) according to the manufacturer's protocol. The workflow without preamplification was chosen for both types of material, FF as well as FFPE samples. The TLDA cards were analyzed on the Applied Biosystems 7900HT Real-Time PCR System.

Data analysis

Each set of samples was evaluated separately, and the results were compared. For data processing and evaluation, we used the SDS 2.4 and the ExpressionSuite v1.0.1 software (Life Technologies, USA). Ct values identified by automated thresholding were exported separately for cards A and B, and the RQ (relative quantity) was calculated from the detected Ct values using the 2^{-Ct} formula. Data were further processed by the GeneSpring GX v13.1 software (Agilent), GenEx v6.1 (MultiD), and MeV v4.9 (TIGR TM4 suite) for comparison. We also applied two types of data normalization—50^{th} percentile shift and global normalization—to perform within-sample normalization, globally across the data sets. For comparison of two groups in censored analyses, the T-test in combination with Pavlidis Template Matching (PTM) algorithm were used. In the comparison of differential abundance of miRNAs in the groups, the *P*-value ($P<0.05$) and fold-change ($FC>1.33$) were set. Only the miRNAs differentially expressed in at least 3/5 or 6/10 (60%) samples with measured results were considered for further analyses. The correlation was evaluated in the GraphPad InStat 3.0 tool using the Spearman nonparametric correlation.

Confirmation of microarray results

Selected miRNAs which were found to be differentially expressed by the microarrays in FFPE tonsillar tumor samples in comparison to the non-malignant tonsillar tissues were confirmed

by the individual RT qPCR using the TaqMan® MicroRNA Assays (Life Technologies, USA) as described recently [11]. Total RNA from five FFPE samples of non-malignant tonsils were used as a calibrator, and RNU48 served as the endogenous control. The data were analyzed in the GenEx v6.1 (MultiD) software. The $2^{-\Delta\Delta Ct}$ method was used for calculations of the fold change. The cut-off fold change was set as for the arrays to +/- 1.33 (33% fold change). T-test or Mann Whitney nonparametric test was applied depending on the data distribution. All results were statistically significant (P-value $\leq$0.05).

Results

The quality and concentration of total RNA was analyzed by a Nanodrop™ Spectrophotometer and Experion chip electrophoresis. The RNA integrity number (RIN) was higher than seven in all FF samples. As documented in Fig 1, total RNA isolated from FFPE samples showed characteristic profiles for degraded RNA fragments (RIN in the range 1.9–2.6), but fragments with a length of around 200 bp were present allowing for a miRNA expression analyses.

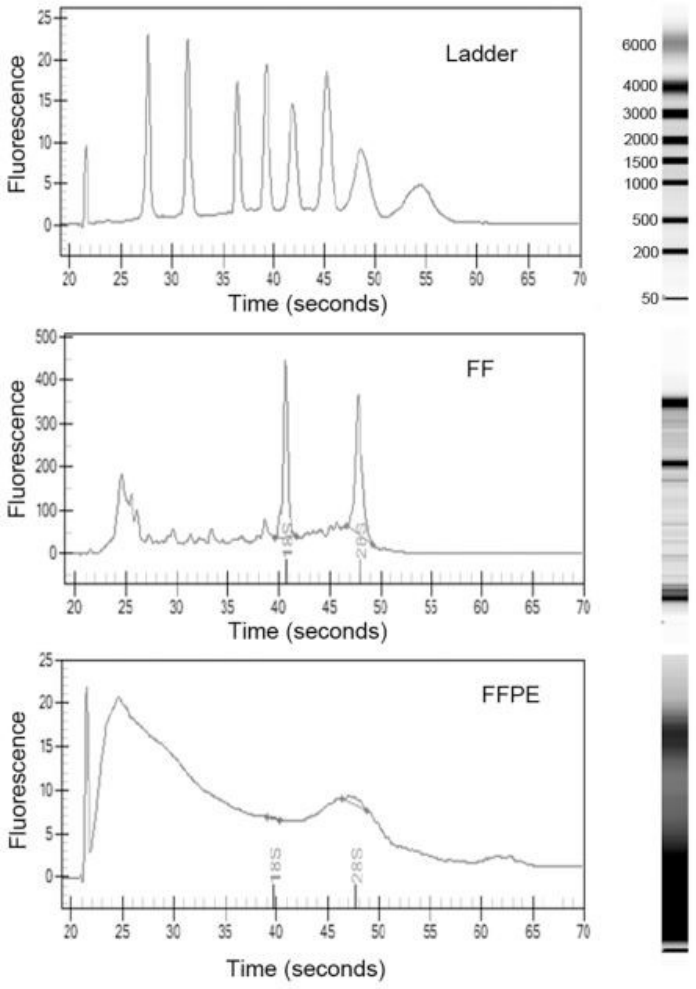

Fig 1. Comparison of electropherogram outputs. At the top—ladder, in the middle—fresh frozen sample, at the bottom—formalin-fixed paraffin-embedded sample. Right—lines from virtual gel.

https://doi.org/10.1371/journal.pone.0179645.g001

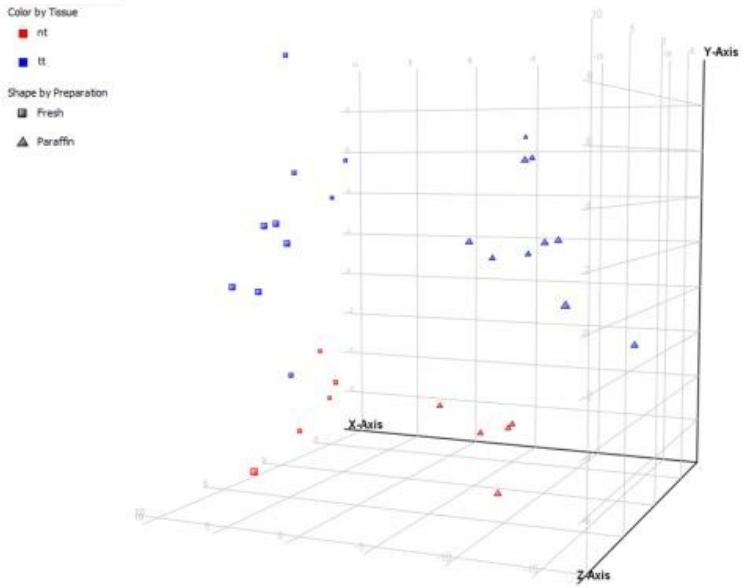

Fig 2. Principal component analysis plot. Visualization of the miRNA expression in fresh frozen (squares) and formalin-fixed paraffin-embedded (triangles) samples, as well as miRNA expression separation of tumor (blue) and non-malignant samples (red).

https://doi.org/10.1371/journal.pone.0179645.g002

As illustrated by the principal component analysis (PCA) (Fig 2), the miRNA expression profiles of all samples differ depending on the analyzed type of material (FF vs FFPE) and characteristics of the tissue, tumor vs normal tissue (TT vs NT). The correlation between the miRNA expression profiles of paired FF and FFPE samples was evaluated using the Spearman correlation test. The correlation of the expression level for the well characterized miRNAs located on card A was better (correlation coefficient $\geq$0.65 in 7/10 samples ($p<0.0001$)) in comparison to all analyzed miRNAs (miRNAs on cards A and B). The exact Spearman correlation values of each comparison and the confidence intervals are summarized in Table 1. The correlation of paired samples is also visualized in scatter plots (Fig 3). Our assumption that FF samples with higher percentage of tumor cells would be more likely to give better correlation of the expression miRNA profiles with FFPE macrodissected samples was not proven. The correlation coefficient of the miRNA expression profiles of FF and FFPE samples was not influenced by the number of tumor cells in the particular FF sample (Table 1).

Differentially expressed miRNAs were identified by the *P*-value ($P<0.05$) and fold-change >1.33 with the 50th percentile shift normalization method using the GeneSpring software. In our study, macrodissected FFPE samples revealed less differentially expressed miRNAs (N = 58) than paired fresh frozen tumors (N = 83 miRNAs). When comparing the lists of deregulated miRNAs between FF and FFPE samples, we detected overlaps in 27-38% (Table 2, on the left).

To establish how the method used for normalization influences the final results, we applied, besides the 50th percentile shift normalization, also global normalization for each group of the analyzed samples. As illustrated in Fig 4 and Table 3, the differentially expressed miRNAs based on each normalization method overlap in 58–67% for FF and/or FFPE samples. Using different software and the identical normalization method, almost 90% of the deregulated miRNAs were concordant between FF and FFPE samples (data not shown).

Table 1. Spearman correlation values between miRNA expression profiles of FF and FFPE paired samples.

Sample		R	CI	tumor cells in FF (%)
FF 102 vs FFPE 58	card A	0,6982	0,6199 to 0,7628	> 50%
	card A+B	0,5848	0,5088 to 0,6517	
FF 116 vs FFPE 1	card A	0,5194	0,4064 to 0,6167	> 70%
	card A+B	0,4642	0,3698 to 0,5491	
FF 148 vs FFPE 60	card A	0,7406	0,6757 to 0,7941	> 70%
	card A+B	0,6749	0,6150 to 0,7271	
FF 161 vs FFPE 13	card A	0,8302	0,7829 to 0,8680	> 60%
	card A+B	0,7174	0,6632 to 0,7641	
FF 174 vs FFPE 49	card A	0,6378	0,5500 to 0,7117	> 50%
	card A+B	0,5748	0,4999 to 0,6412	
FF 183 vs FFPE 62	card A	0,6905	0,6098 to 0,7570	70%
	card A+B	0,6373	0,5664 to 0,6989	
FF 187 vs FFPE 30	card A	0,5879	0,4883 to 0,6724	> 70%
	card A+B	0,5152	0,4289 to 0,5922	
FF 191 vs FFPE 63	card A	0,7332	0,6648 to 0,7894	> 80%
	card A+B	0,6593	0,5959 to 0,7145	
FF 137 vs FFPE 6	card A	0,7914	0,7363 to 0,8360	90%
	card A+B	0,7361	0,6828 to 0,7816	
FF 224 vs FFPE 22	card A	0,7371	0,6697 to 0,7925	80%
	card A+B	0,7124	0,6571 to 0,7601	

https://doi.org/10.1371/journal.pone.0179645.t001

We performed additional analysis and identified 16 miRNAs commonly detected in both type of material and using both type of normalization (Table 4). These miRNAs were screened in database miRSearch V3.0 (Exiqon). Most of them have experimentally proved target genes participating in signaling pathways and cellular processes including not only tumor development. Noteworthy, target genes are transcription factors E2F, MAP kinases, members of RAS protein family, tumor suppressor PTEN, transforming growth factors TGFs, tumor suppressor TP53, proteins participating in cell cycle (cyclins, cyclin-dependent kinases) or regulating cell death.

Finally, from the deregulated miRNAs in FFPE samples, we have selected 11 tumor-specific miRNAs, also with regard to viral or non-viral etiology of the tumor. The expression of these miRNAs was evaluated in a larger set of 64 macrodissected tumor samples by individual TaqMan assays (Fig 5). MiR-106b# and miR-9 were selected as specific for HPV-positive tonsillar tumors, miR-16, miR-34a, miR-193b, miR-31, miR-221, and miR-21 as specific for HPV-negative tumors, and miR-155, miR-126, and miR-205 as specific for tonsillar tumors of any etiology. The differential expression of six miRNAs (miR-106b#, miR-9, miR-16, miR-34a, miR-155, and miR-126) ($P<0.05$; $FC>1.33$) was confirmed in a larger set of 64 tumor samples, the fold change of miR-193b was equal to 1.23, but the trend of expression was also maintained. The trend of expression of four miRNAs (miR-31, miR-221, miR-21, and miR-205) analyzed in the large set of samples was opposite to that derived from the results revealed by arrays.

Discussion

The archives of formalin-fixed paraffin-embedded material in pathology laboratories offer the possibility to analyze the samples retrospectively and provide the option for clinical cancer research to extend the studies, and, moreover, to correlate the results with clinical data

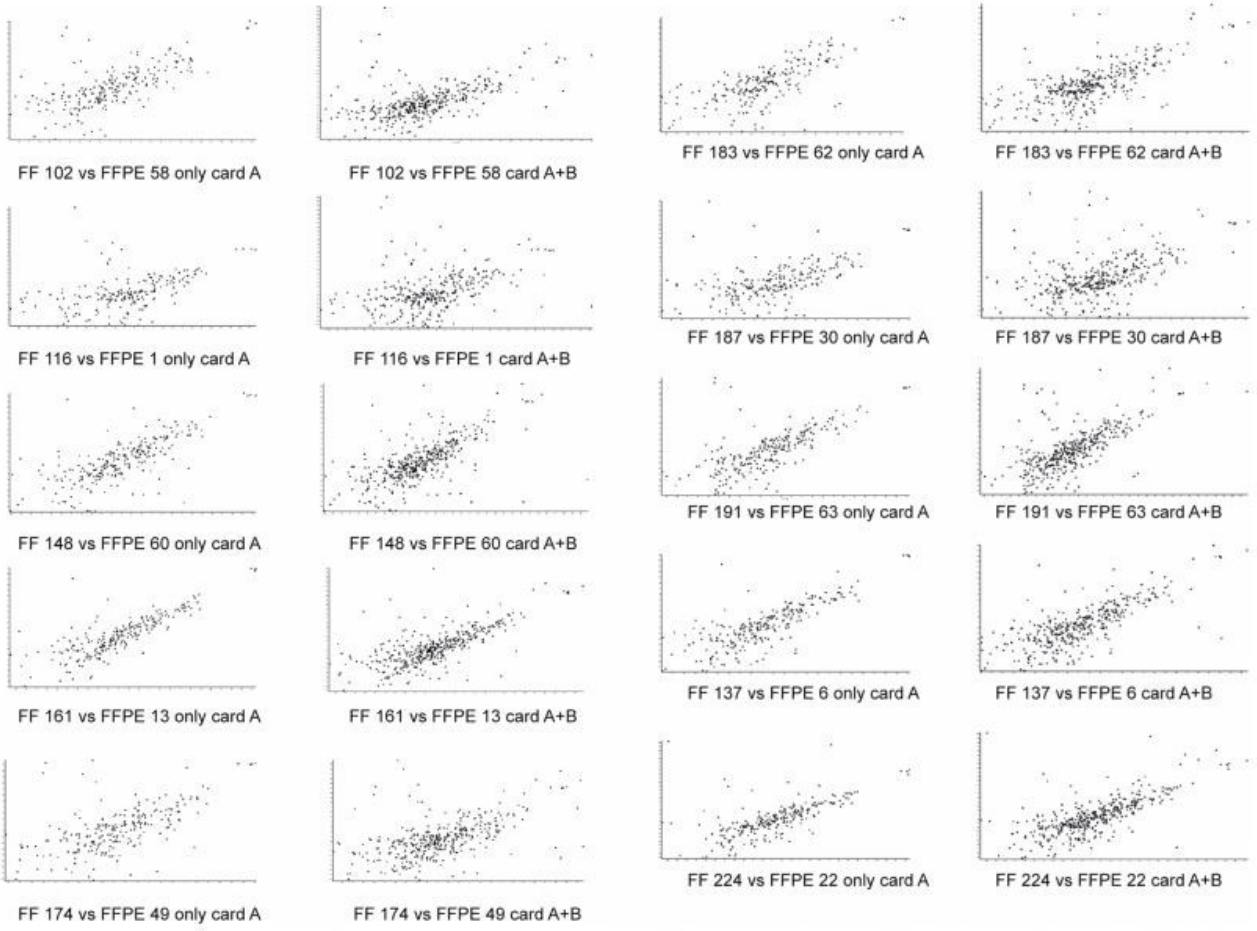

Fig 3. Correlation of paired samples visualized on scatterplots. Scatter plots of the normalized RQ values in pair-wise comparison from fresh-frozen (FF) and formalin-fixed paraffin embedded (FFPE) samples.

https://doi.org/10.1371/journal.pone.0179645.g003

obtained during long-term follow-up of patients. The utilization of FFPE material for RNA based studies is still challenging, especially due to RNA fragmentation. However, the small size of miRNAs contributes to their stability during the processing of FFPE samples [9] so that FFPE tissues are applicable for miRNA expression analysis studies as previously shown [16–19]. Chatterjee et al. have presented a cross comparison of miRNA detection technologies in FFPE samples and revealed that it is possible to obtain high quality sequence reads for miRNA profiling in FFPE samples with an RNA integrity number around 2 [20].

In our study, we performed the miRNA expression profiling by TLDA analysis in a set of macrodissected FFPE samples and compared the results with the miRNA expression profiles of paired fresh frozen samples. Most studies comparing the miRNA expression between FF and FFPE samples were performed using only several selected miRNAs [21–25].

A comparison of the miRNA expression profiles in FF and FFPE samples obtained by the microarray analysis has also been published. Romero-Cordoba et al. have performed a comparative analysis of paired samples of breast cancer using the TaqMan Low Density Array platform, similarly to Hui et al. [12, 14]. They revealed a high correlation; however, both studies analyzed small groups of paired samples and did not use dissected samples. Goswami et al. have also observed a high correlation between the samples; however, their workflow differed

Table 2. Differentially expressed miRNAs detected in tonsillar tumors versus non-malignant tonsillar tissue. The overlap between FF vs FFPE material is highlighted in green. On the left—results using 50th percentile shift normalization for data analysis. On the right—results using global normalization for data analysis. FC—fold change.

50th percentile shift normalization				global normalization			
FF		FFPE		FF		FFPE	
TTall vs NT				TTall vs NT			
miRNA	FC	miRNA	FC	miRNA	FC	miRNA	FC
hsa-miR-101-002253	-2.87	hsa-miR-101-002253	-8.70	hsa-let-7g-002282	-1.85	hsa-let-7g-002282	-2.57
hsa-miR-126#-000451	-2.29	hsa-miR-126#-000451	-8.27	hsa-miR-126#-000451	-1.85	hsa-miR-126#-000451	-4.28
hsa-miR-130b-000456	1.86	hsa-miR-130b-000456	3.42	hsa-miR-140-3p-002234	-2.10	hsa-miR-140-3p-002234	-4.50
hsa-miR-140-3p-002234	-2.42	hsa-miR-140-3p-002234	-4.15	hsa-miR-141-000463	4.71	hsa-miR-141-000463	3.85
hsa-miR-141-000463	4.04	hsa-miR-141-000463	2.67	hsa-miR-143-002249	-2.14	hsa-miR-143-002249	-1.68
hsa-miR-142-3p-000464	-3.85	hsa-miR-142-3p-000464	-8.79	hsa-miR-144#-002148	-4.52	hsa-miR-144#-002148	-2.95
hsa-miR-143-002249	-2.02	hsa-miR-143-002249	-2.89	hsa-miR-184-000485	-6.15	hsa-miR-184-000485	-5.46
hsa-miR-195-000494	-1.92	hsa-miR-195-000494	-2.89	hsa-miR-196b-002215	7.00	hsa-miR-196b-002215	2.88
hsa-miR-196b-002215	8.26	hsa-miR-196b-002215	4.42	hsa-miR-200a-000502	3.52	hsa-miR-200a-000502	3.32
hsa-miR-200b-002251	3.86	hsa-miR-200b-002251	2.60	hsa-miR-200b-002251	3.04	hsa-miR-200b-002251	2.89
hsa-miR-200c-002300	3.45	hsa-miR-200c-002300	3.00	hsa-miR-200c-002300	2.83	hsa-miR-200c-002300	3.33
hsa-miR-205-000509	4.22	hsa-miR-205-000509	5.12	hsa-miR-205-000509	4.58	hsa-miR-205-000509	6.71
hsa-miR-21#-002438	2.99	hsa-miR-21#-002438	2.53	hsa-miR-210-000512	3.74	hsa-miR-210-000512	3.26
hsa-miR-210-000512	4.10	hsa-miR-210-000512	2.70	hsa-miR-224-002099	5.62	hsa-miR-224-002099	5.78
hsa-miR-221-000524	2.69	hsa-miR-221-000524	2.70	hsa-miR-27a#-002445	4.10	hsa-miR-27a#-002445	2.79
hsa-miR-224-002099	6.01	hsa-miR-224-002099	6.03	hsa-miR-27a-000408	2.19	hsa-miR-27a-000408	1.80
hsa-miR-27a#-002445	3.12	hsa-miR-27a#-002445	4.47	hsa-miR-29a-002112	-1.61	hsa-miR-29a-002112	-2.57
hsa-miR-27a-000408	1.81	hsa-miR-27a-000408	1.92	hsa-miR-429-001024	3.25	hsa-miR-429-001024	2.37
hsa-miR-29a-002112	-1.83	hsa-miR-29a-002112	-3.26	hsa-miR-452-002329	2.79	hsa-miR-452-002329	1.89
hsa-miR-452-002329	3.00	hsa-miR-452-002329	3.84	hsa-miR-484-001821	2.02	hsa-miR-484-001821	2.40
hsa-miR-486-3p-002093	-3.79	hsa-miR-486-3p-002093	-4.97	hsa-miR-886-3p-002194	3.04	hsa-miR-886-3p-002194	2.94
hsa-miR-886-3p-002194	3.00	hsa-miR-886-3p-002194	2.59	hsa-let-7i#-002172	-2.59	hsa-miR-106b-000442	1.94
hsa-miR-100-000437	-1.62	hsa-let-7b#-002404	-6.10	hsa-miR-100-000437	-1.88	hsa-miR-1253-002894	11.36
hsa-miR-1180-002847	3.95	hsa-let-7g-002282	-3.04	hsa-miR-101-002253	-2.43	hsa-miR-1260-002896	1.58
hsa-miR-1227-002769	4.37	hsa-miR-126-002228	-3.28	hsa-miR-1244-002791	2.49	hsa-miR-126-002228	-2.95
hsa-miR-125b-000449	-1.93	hsa-miR-1262-002852	-10500.89	hsa-miR-125b-000449	-2.44	hsa-miR-127-000452	3.13
hsa-miR-132-000457	1.66	hsa-miR-127-000452	2.01	hsa-miR-132-000457	1.75	hsa-miR-1282-002803	1.35
hsa-miR-135b#-002159	5.18	hsa-miR-138-002284	-3.48	hsa-miR-135b-002261	4.30	hsa-miR-130b-000456	3.51
hsa-miR-135b-002261	4.25	hsa-miR-146a-000468	-3.45	hsa-miR-138-002284	-3.48	hsa-miR-142-3p-000464	-5.87
hsa-miR-136#-002100	-3.36	hsa-miR-146b-001097	-2.48	hsa-miR-139-5p-002289	-3.48	hsa-miR-142-5p-002248	-2.40
hsa-miR-139-3p-002313	-2.80	hsa-miR-150-000473	-10.82	hsa-miR-141#-002145	2.64	hsa-miR-146a-000468	-2.75
hsa-miR-139-5p-002289	-2.97	hsa-miR-155-002623	-3.39	hsa-miR-145#-002149	-2.09	hsa-miR-146b-001097	-2.25
hsa-miR-144#-002148	-5.50	hsa-miR-15a-000389	-2.18	hsa-miR-145-002278	-2.28	hsa-miR-150-000473	-8.31
hsa-miR-145#-002149	-2.55	hsa-miR-16-000391	-2.58	hsa-miR-146b-3p-002361	1.29	hsa-miR-152-000475	2.37
hsa-miR-145-002278	-2.60	hsa-miR-188-3p-002106	-15.67	hsa-miR-151-3p-002254	1.69	hsa-miR-155-002623	-3.37
hsa-miR-181a-000480	-1.50	hsa-miR-19a-000395	-2.14	hsa-miR-151-5P-002642	-1.96	hsa-miR-16-000391	-2.23
hsa-miR-182-002334	3.36	hsa-miR-19b-000396	-2.64	hsa-miR-15b#-002173	4.90	hsa-miR-183#-002270	1.77
hsa-miR-1825-002907	8.50	hsa-miR-20a#-002437	-4.90	hsa-miR-15b-000390	1.89	hsa-miR-18a#-002423	1.84
hsa-miR-183-002269	2.77	hsa-miR-21-000397	1.49	hsa-miR-181a-000480	-1.64	hsa-miR-19b-000396	-2.58
hsa-miR-184-000485	-22.46	hsa-miR-213-000516	-4.21	hsa-miR-183-002269	2.49	hsa-miR-20a#-002437	-2.15
hsa-miR-191#-002678	4.11	hsa-miR-219-2-3p-002390	26.64	hsa-miR-195-000494	-2.33	hsa-miR-21#-002438	2.58
hsa-miR-197-000497	1.72	hsa-miR-323-3p-002227	5.88	hsa-miR-199a-3p-002304	-1.59	hsa-miR-213-000516	-3.40
hsa-miR-200a#-001011	3.42	hsa-miR-342-3p-002260	-3.35	hsa-miR-200a#-001011	4.35	hsa-miR-221-000524	2.86

(*Continued*)

Table 2. (*Continued*)

50th percentile shift normalization				global normalization			
FF		FFPE		FF		FFPE	
TTall vs NT				TTall vs NT			
miRNA	FC	miRNA	FC	miRNA	FC	miRNA	FC
hsa-miR-200a-000502	3.64	hsa-miR-342-5p-002147	-3.96	hsa-miR-21#-002438	3.76	hsa-miR-223#-002098	-2.63
hsa-miR-204-000508	-5.28	hsa-miR-362-001273	2.43	hsa-miR-21-000397	2.68	hsa-miR-299-5p-000600	4.75
hsa-miR-211-000514	34.58	hsa-miR-374-000563	-2.24	hsa-miR-214#-002293	-1.74	hsa-miR-320-002277	1.92
hsa-miR-222#-002097	2.09	hsa-miR-423-5p-002340	-5.05	hsa-miR-222#-002097	2.38	hsa-miR-323-3p-002227	4.17
hsa-miR-24-000402	1.59	hsa-miR-455-3p-002244	7.08	hsa-miR-222-002276	2.04	hsa-miR-342-3p-002260	-3.14
hsa-miR-26a-000405	-2.12	hsa-miR-485-3p-001277	3.99	hsa-miR-24-000402	1.73	hsa-miR-362-001273	2.07
hsa-miR-26b#-002444	-5.11	hsa-miR-490-001037	3.37	hsa-miR-26a-000405	-1.84	hsa-miR-455-3p-002244	8.08
hsa-miR-26b-000407	-2.22	hsa-miR-516-3p-001149	-12.45	hsa-miR-26b#-002444	-3.16	hsa-miR-485-3p-001277	2.33
hsa-miR-27b#-002174	1.88	hsa-miR-566-001533	-4.10	hsa-miR-26b-000407	-2.85	hsa-miR-532-001518	-2.24
hsa-miR-29a#-002447	-2.62	hsa-miR-583-001623	12.93	hsa-miR-29a#-002447	-1.98	hsa-miR-550-002410	-2.05
hsa-miR-29b-000413	-2.17	hsa-miR-590-5p-001984	-6.36	hsa-miR-29b-000413	-2.45	hsa-miR-590-5p-001984	-2.27
hsa-miR-29c-000587	-2.39	hsa-miR-640-001584	-4.53	hsa-miR-29c-000587	-2.64	hsa-miR-629-001562	-2.19
hsa-miR-302a-000529	222.12	hsa-miR-660-001515	-1.96	hsa-miR-302c-000533	18.15	hsa-miR-663B-002857	1.78
hsa-miR-30a-3p-000416	-3.27	hsa-miR-663B-002857	2.55	hsa-miR-30b-000602	-1.76	hsa-miR-888-002212	-551.31
hsa-miR-30a-5p-000417	-1.93	hsa-miR-942-002187	-3.76	hsa-miR-30d-000420	-1.95		
hsa-miR-30d-000420	-1.94			hsa-miR-31-002279	6.09		
hsa-miR-30e-3p-000422	-2.39			hsa-miR-32-002109	-2.47		
hsa-miR-31-002279	5.72			hsa-miR-335#-002185	2.58		
hsa-miR-335#-002185	2.04			hsa-miR-342-5p-002147	-7.89		
hsa-miR-335-000546	2.33			hsa-miR-34b-002102	2.35		
hsa-miR-34a#-002316	1.91			hsa-miR-505#-002087	-1.68		
hsa-miR-378-000567	-1.87			hsa-miR-511-001111	2.16		
hsa-miR-429-001024	3.66			hsa-miR-520g-001121	3.52		
hsa-miR-431-001979	16.66			hsa-miR-577-002675	-3.38		
hsa-miR-484-001821	2.01			hsa-miR-596-001550	-2.00		
hsa-miR-486-001278	-3.26			hsa-miR-649-001602	3.05		
hsa-miR-497-001043	-4.12			hsa-miR-708-002341	2.14		
hsa-miR-505#-002087	-2.84			hsa-miR-944-002189	5.27		
hsa-miR-511-001111	3.25			hsa-miR-9-000583	7.17		
hsa-miR-517a-002402	3.23						
hsa-miR-517c-001153	3.66						
hsa-miR-523-002386	10.93						
hsa-miR-576-3p-002351	8.52						
hsa-miR-627-001560	29.22						
hsa-miR-643-001594	2.71						
hsa-miR-658-001513	2.12						
hsa-miR-659-001514	6.10						
hsa-miR-708-002341	2.26						
hsa-miR-944-002189	6.72						
hsa-miR-99b#-002196	-4.26						
22/83 (27%)		**22/58 (38%)**		**21/72 (29%)**		**21/57 (37%)**	

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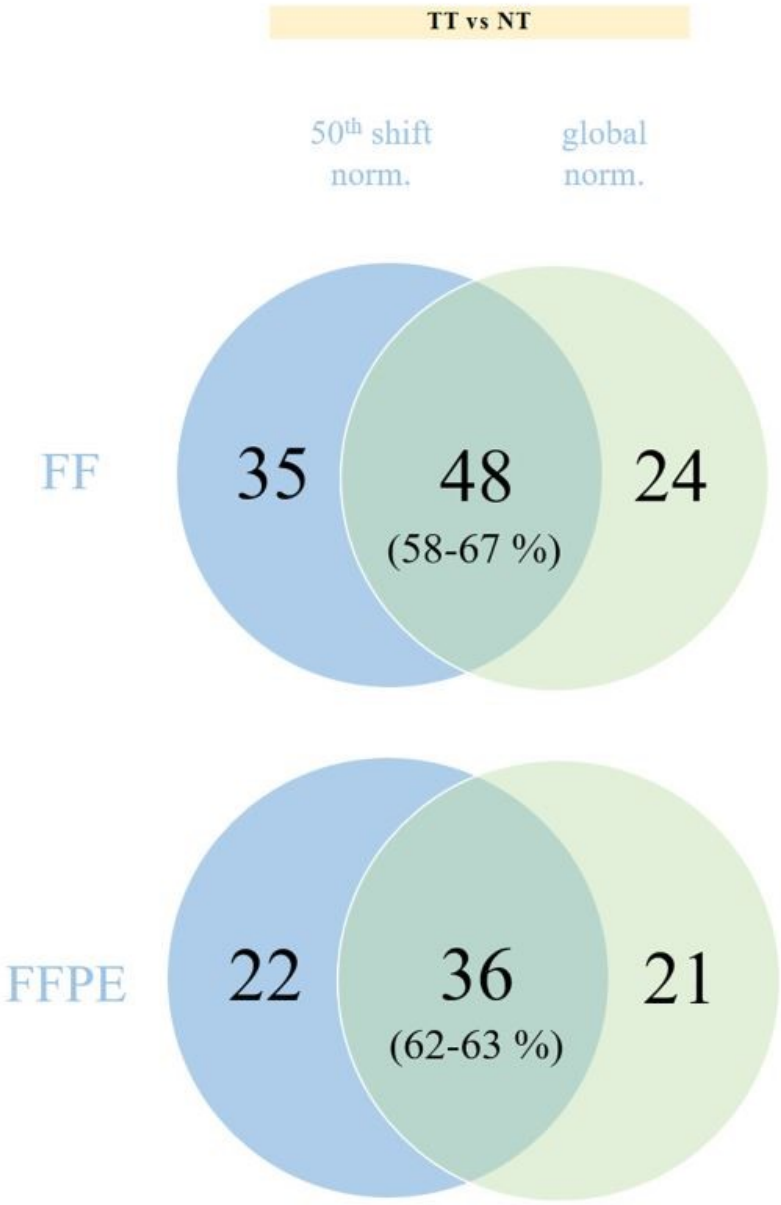

Fig 4. Comparison of differentially expressed miRNAs. Comparison of differentially expressed miRNAs identified using 50th percentile shift normalization and global normalization for each clinical material.

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in several aspects (assessment of extraction method, platform optimization, etc.) [13]. Additional studies were performed using other microarray platforms [9, 26, 27] or next generation sequencing platforms [28]. None of this comparative studies has been performed in head and neck tumors.

Since our recent study has shown that the tumor tissue homogeneity is important for the robustness of the miRNA profiling studies, we performed macrodissection of FFPE samples to enrich them for tumor cells. To our knowledge, only one study dealt with the issue of FFPE dissection. It tested the possibility of a non-satisfactory correlation of the expression of several miRNAs between FF and dissected FFPE samples due to the enrichment of the tumor cells.

Table 3. Differentially expressed miRNAs detected tonsillar tumors versus non-malignant tonsillar tissue. Overlap between results using 50th percentile shift normalization and global normalization is highlighted in green. On the left—results from FF samples, on the right—results from FFPE samples. FC—fold change.

FF				FFPE			
TTall vs NT				TTall vs NT			
50th percentile shift normalization		global normalization		50th percentile shift normalization		global normalization	
miRNA	FC	miRNA	FC	miRNA	FC	miRNA	FC
hsa-miR-100-000437	-1.62	hsa-miR-100-000437	-1.88	hsa-let-7g-002282	-3.04	hsa-let-7g-002282	-2.57
hsa-miR-101-002253	-2.87	hsa-miR-101-002253	-2.43	hsa-miR-126#-000451	-8.27	hsa-miR-126#-000451	-4.28
hsa-miR-125b-000449	-1.93	hsa-miR-125b-000449	-2.44	hsa-miR-126-002228	-3.28	hsa-miR-126-002228	-2.95
hsa-miR-126#-000451	-2.29	hsa-miR-126#-000451	-1.85	hsa-miR-127-000452	2.01	hsa-miR-127-000452	3.13
hsa-miR-132-000457	1.66	hsa-miR-132-000457	1.75	hsa-miR-130b-000456	3.42	hsa-miR-130b-000456	3.51
hsa-miR-135b-002261	4.25	hsa-miR-135b-002261	4.30	hsa-miR-140-3p-002234	-4.15	hsa-miR-140-3p-002234	-4.50
hsa-miR-139-5p-002289	-2.97	hsa-miR-139-5p-002289	-3.48	hsa-miR-141-000463	2.67	hsa-miR-141-000463	3.85
hsa-miR-140-3p-002234	-2.42	hsa-miR-140-3p-002234	-2.10	hsa-miR-142-3p-000464	-8.79	hsa-miR-142-3p-000464	-5.87
hsa-miR-141-000463	4.04	hsa-miR-141-000463	4.71	hsa-miR-143-002249	-2.89	hsa-miR-143-002249	-1.68
hsa-miR-143-002249	-2.02	hsa-miR-143-002249	-2.14	hsa-miR-146a-000468	-3.45	hsa-miR-146a-000468	-2.75
hsa-miR-144#-002148	-5.50	hsa-miR-144#-002148	-4.52	hsa-miR-146b-001097	-2.48	hsa-miR-146b-001097	-2.25
hsa-miR-145#-002149	-2.55	hsa-miR-145#-002149	-2.09	hsa-miR-150-000473	-10.82	hsa-miR-150-000473	-8.31
hsa-miR-145-002278	-2.60	hsa-miR-145-002278	-2.28	hsa-miR-155-002623	-3.39	hsa-miR-155-002623	-3.37
hsa-miR-181a-000480	-1.50	hsa-miR-181a-000480	-1.64	hsa-miR-16-000391	-2.58	hsa-miR-16-000391	-2.23
hsa-miR-183-002269	2.77	hsa-miR-183-002269	2.49	hsa-miR-196b-002215	4.42	hsa-miR-196b-002215	2.88
hsa-miR-184-000485	-22.46	hsa-miR-184-000485	-6.15	hsa-miR-19b-000396	-2.64	hsa-miR-19b-000396	-2.58
hsa-miR-195-000494	-1.92	hsa-miR-195-000494	-2.33	hsa-miR-200b-002251	2.60	hsa-miR-200b-002251	2.89
hsa-miR-196b-002215	8.26	hsa-miR-196b-002215	7.00	hsa-miR-200c-002300	3.00	hsa-miR-200c-002300	3.33
hsa-miR-200a#-001011	3.42	hsa-miR-200a#-001011	4.35	hsa-miR-205-000509	5.12	hsa-miR-205-000509	6.71
hsa-miR-200a-000502	3.64	hsa-miR-200a-000502	3.52	hsa-miR-20a#-002437	-4.90	hsa-miR-20a#-002437	-2.15
hsa-miR-200b-002251	3.86	hsa-miR-200b-002251	3.04	hsa-miR-21#-002438	2.53	hsa-miR-21#-002438	2.58
hsa-miR-200c-002300	3.45	hsa-miR-200c-002300	2.83	hsa-miR-210-000512	2.70	hsa-miR-210-000512	3.26
hsa-miR-205-000509	4.22	hsa-miR-205-000509	4.58	hsa-miR-213-000516	-4.21	hsa-miR-213-000516	-3.40
hsa-miR-21#-002438	2.99	hsa-miR-21#-002438	3.76	hsa-miR-221-000524	2.90	hsa-miR-221-000524	2.86
hsa-miR-210-000512	4.10	hsa-miR-210-000512	3.74	hsa-miR-224-002099	6.03	hsa-miR-224-002099	5.78
hsa-miR-222#-002097	2.09	hsa-miR-222#-002097	2.38	hsa-miR-27a#-002445	4.47	hsa-miR-27a#-002445	2.79
hsa-miR-224-002099	6.01	hsa-miR-224-002099	5.62	hsa-miR-27a-000408	1.92	hsa-miR-27a-000408	1.80
hsa-miR-24-000402	1.59	hsa-miR-24-000402	1.73	hsa-miR-29a-002112	-3.26	hsa-miR-29a-002112	-2.57
hsa-miR-26a-000405	-2.12	hsa-miR-26a-000405	-1.84	hsa-miR-323-3p-002227	5.88	hsa-miR-323-3p-002227	4.17
hsa-miR-26b#-002444	-5.11	hsa-miR-26b#-002444	-3.16	hsa-miR-342-3p-002260	-3.35	hsa-miR-342-3p-002260	-3.14
hsa-miR-26b-000407	-2.22	hsa-miR-26b-000407	-2.85	hsa-miR-362-001273	2.43	hsa-miR-362-001273	2.07
hsa-miR-27a#-002445	3.12	hsa-miR-27a#-002445	4.10	hsa-miR-452-002329	3.84	hsa-miR-452-002329	1.90
hsa-miR-27a-000408	1.81	hsa-miR-27a-000408	2.19	hsa-miR-455-3p-002244	7.08	hsa-miR-455-3p-002244	8.08
hsa-miR-29a#-002447	-2.62	hsa-miR-29a#-002447	-1.98	hsa-miR-485-3p-001277	3.99	hsa-miR-485-3p-001277	2.33
hsa-miR-29a-002112	-1.83	hsa-miR-29a-002112	-1.61	hsa-miR-590-5p-001984	-6.36	hsa-miR-590-5p-001984	-2.27
hsa-miR-29b-000413	-2.17	hsa-miR-29b-000413	-2.45	hsa-miR-886-3p-002194	2.59	hsa-miR-886-3p-002194	2.94
hsa-miR-29c-000587	-2.39	hsa-miR-29c-000587	-2.64	hsa-let-7b#-002404	-6.10	hsa-miR-106b-000442	1.94
hsa-miR-30d-000420	-1.94	hsa-miR-30d-000420	-1.95	hsa-miR-101-002253	-8.70	hsa-miR-1253-002894	11.36
hsa-miR-31-002279	5.72	hsa-miR-31-002279	6.09	hsa-miR-1262-002852	-10500.89	hsa-miR-1260-002896	1.58
hsa-miR-335#-002185	2.04	hsa-miR-335#-002185	2.58	hsa-miR-138-002284	-3.48	hsa-miR-1282-002803	1.35
hsa-miR-429-001024	3.66	hsa-miR-429-001024	3.25	hsa-miR-15a-000389	-2.18	hsa-miR-142-5p-002248	-2.40
hsa-miR-452-002329	3.00	hsa-miR-452-002329	2.79	hsa-miR-188-3p-002106	-15.67	hsa-miR-144#-002148	-2.95

(*Continued*)

Table 3. (*Continued*)

FF				FFPE			
TTall vs NT				TTall vs NT			
50th percentile shift normalization		global normalization		50th percentile shift normalization		global normalization	
miRNA	FC	miRNA	FC	miRNA	FC	miRNA	FC
hsa-miR-484-001821	2.01	hsa-miR-484-001821	2.02	hsa-miR-195-000494	-2.89	hsa-miR-152-000475	2.3
hsa-miR-505#-002087	-2.84	hsa-miR-505#-002087	-1.68	hsa-miR-19a-000395	-2.14	hsa-miR-183#-002270	1.77
hsa-miR-511-001111	3.25	hsa-miR-511-001111	2.16	hsa-miR-21-000397	1.49	hsa-miR-184-000485	-5.46
hsa-miR-708-002341	2.26	hsa-miR-708-002341	2.14	hsa-miR-219-2-3p-002390	26.64	hsa-miR-18a#-002423	1.84
hsa-miR-886-3p-002194	3.00	hsa-miR-886-3p-002194	3.04	hsa-miR-342-5p-002147	-3.96	hsa-miR-200a-000502	3.32
hsa-miR-944-002189	6.72	hsa-miR-944-002189	7.17	hsa-miR-374-000563	-2.24	hsa-miR-223#-002098	-2.63
hsa-miR-1180-002847	3.95	hsa-let-7g-002282	-1.85	hsa-miR-423-5p-002340	-5.05	hsa-miR-299-5p-000600	4.75
hsa-miR-1227-002769	4.37	hsa-let-7i#-002172	-2.59	hsa-miR-486-3p-002093	-4.97	hsa-miR-320-002277	1.92
hsa-miR-130b-000456	1.86	hsa-miR-1244-002791	2.49	hsa-miR-490-001037	3.37	hsa-miR-429-001024	2.37
hsa-miR-135b#-002159	5.18	hsa-miR-138-002284	-3.48	hsa-miR-516-3p-001149	-12.45	hsa-miR-484-001821	2.40
hsa-miR-136#-002100	-3.36	hsa-miR-141#-002145	2.64	hsa-miR-566-001533	-4.10	hsa-miR-532-001518	-2.24
hsa-miR-139-3p-002313	-2.80	hsa-miR-146b-3p-002361	1.29	hsa-miR-583-001623	12.93	hsa-miR-550-002410	-2.05
hsa-miR-142-3p-000464	-3.85	hsa-miR-151-3p-002254	1.69	hsa-miR-640-001584	-4.53	hsa-miR-629-001562	-2.19
hsa-miR-182-002334	3.36	hsa-miR-151-5P-002642	-1.96	hsa-miR-660-001515	-1.96	hsa-miR-663B-002857	1.78
hsa-miR-1825-002907	8.50	hsa-miR-15b#-002173	4.90	hsa-miR-663B-002857	2.55	hsa-miR-888-002212	-551.31
hsa-miR-191#-002678	4.11	hsa-miR-15b-000390	1.89	hsa-miR-942-002187	-3.76		
hsa-miR-197-000497	1.72	hsa-miR-199a-3p-002304	-1.59				
hsa-miR-204-000508	-5.28	hsa-miR-21-000397	2.68				
hsa-miR-211-000514	34.58	hsa-miR-214#-002293	-1.74				
hsa-miR-221-000524	2.69	hsa-miR-222-002276	2.04				
hsa-miR-27b#-002174	1.88	hsa-miR-302c-000533	18.15				
hsa-miR-302a-000529	222.12	hsa-miR-30b-000602	-1.76				
hsa-miR-30a-3p-000416	-3.27	hsa-miR-32-002109	-2.47				
hsa-miR-30a-5p-000417	-1.93	hsa-miR-342-5p-002147	-7.89				
hsa-miR-30e-3p-000422	-2.39	hsa-miR-34b-002102	2.35				
hsa-miR-335-000546	2.33	hsa-miR-520g-001121	3.52				
hsa-miR-34a#-002316	1.91	hsa-miR-577-002675	-3.38				
hsa-miR-378-000567	-1.87	hsa-miR-596-001550	-2.00				
hsa-miR-431-001979	16.66	hsa-miR-649-001602	3.05				
hsa-miR-486-001278	-3.26	hsa-miR-9-000583	5.27				
hsa-miR-486-3p-002093	-3.79						
hsa-miR-497-001043	-4.12						
hsa-miR-517a-002402	3.23						
hsa-miR-517c-001153	3.66						
hsa-miR-523-002386	10.93						
hsa-miR-576-3p-002351	8.52						
hsa-miR-627-001560	29.22						
hsa-miR-643-001594	2.71						
hsa-miR-658-001513	2.12						
hsa-miR-659-001514	6.10						
hsa-miR-99b#-002196	-4.26						

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Table 4. Differentially expressed miRNA in both types of clinical material by using two different normalization methods.

hsa-miR-126#-000451	hsa-miR-196b-002215	hsa-miR-21#-002438	hsa-miR-27a-000408
hsa-miR-140-3p-002234	hsa-miR-200b-002251	hsa-miR-210-000512	hsa-miR-29a-002112
hsa-miR-141-000463	hsa-miR-200c-002300	hsa-miR-224-002099	hsa-miR-452-002329
hsa-miR-143-002249	hsa-miR-205-000509	hsa-miR-27a#-002445	hsa-miR-886-3p-002194

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However, the improvement in the correlation when using non-dissected FFPE samples was due to a low number of samples which was not convincing [25]. In our analysis, we compared FF tumor samples with more than 50% of tumor cells and macrodissected FFPE tumor cells. Although, based on the PCA, the miRNA expression profiles differ between FF and FFPE, we performed the Spearman correlation test which showed a good correlation (Spearman correlation coefficient in the range from 0.65 to 0.84) in 70% of samples. Based on the study of de Biase et al. [25] who have pointed to the fact that dissection can influence the expression results which is necessary to take into account if the miRNA analysis is performed with or without dissection, we evaluated the correlation of the differentially expressed miRNAs in FF and FFPE based on the number of tumor cells in FF samples. However, no relationship between the number of tumor cells and correlation level was observed.

We revealed higher numbers of differentially regulated miRNAs in FF tonsillar tissues compared to the FFPE material which we attributed to the higher amount of RNA used for the miRNA expression analysis in FF samples. Therefore, some less expressed miRNAs could have been missed in FFPE samples. The overlap of differentially expressed miRNAs between FF and FFPE samples was around 30% which should be explained by the fact that unlike the tumor samples, the control non-malignant tissues were not paired between FF and FFPE and could influence the miRNA expression profiles more than we expected. Nevertheless, the analysis of both groups of tested material revealed the deregulation of the known tumor associated miRNAs such as miR-205, miR-210, or miRNAs from the family mir-8, as has been demonstrated in a number of solid tumors [29–32].

Further, when comparing the results obtained using two different types of data normalization, we revealed an overlap of between 58–67%. Therefore, the comparison between studies is obviously difficult because of the use of different methods of data analysis. In our analysis, both tissue types and both normalization methods overlap in 16 common miRNAs. All of these miRNAs were shown to be cancer related, nevertheless their role in head and neck cancer has not been elucidated. The role of miR-27a was mentioned by Venkatesh et al. as it regulates the expression of tumor suppressors in oral squamous cell carcinomas [33]. The role of miR-126# was found in endothelial proliferation [34, 35]. For miR-140-3p it was shown that it contributes to reduction of proliferation and migration of cancer cells in breast and lung cancer [36, 37] and in spinal chordoma, in contradictory, to be a marker of poor prognosis [38]. MiR-141 plays role in AKT signaling and inhibits prometastatic mesenchymal characteristics [39]. MiR-143 is a part of miR cluster 143/145 expressed in many tissues and regarded as tumor suppressing [40]. It´s reduced expression was found also in cervical squamous cell carcinomas [41] and low expression of this miRNA contributes to poor prognosis in oral squamous cell carcinomas [42]. As recently discovered the expression of miR-196b promotes cell migration and invasion in oral cancer [43, 44]. Members of mir-200 family, which includes miR-200b, miR-200c and miR-205, are the major regulators of EMT pathway, primarily targeting transcriptional factors ZEB1 and SP1 [45], and play a prognostic role in various malignancies [46]. Deregulated expression of hypoxia-induced miR-210 was found in various tumors and influences cancer cell proliferation, apoptosis, angiogenesis and other cellular processes involved in

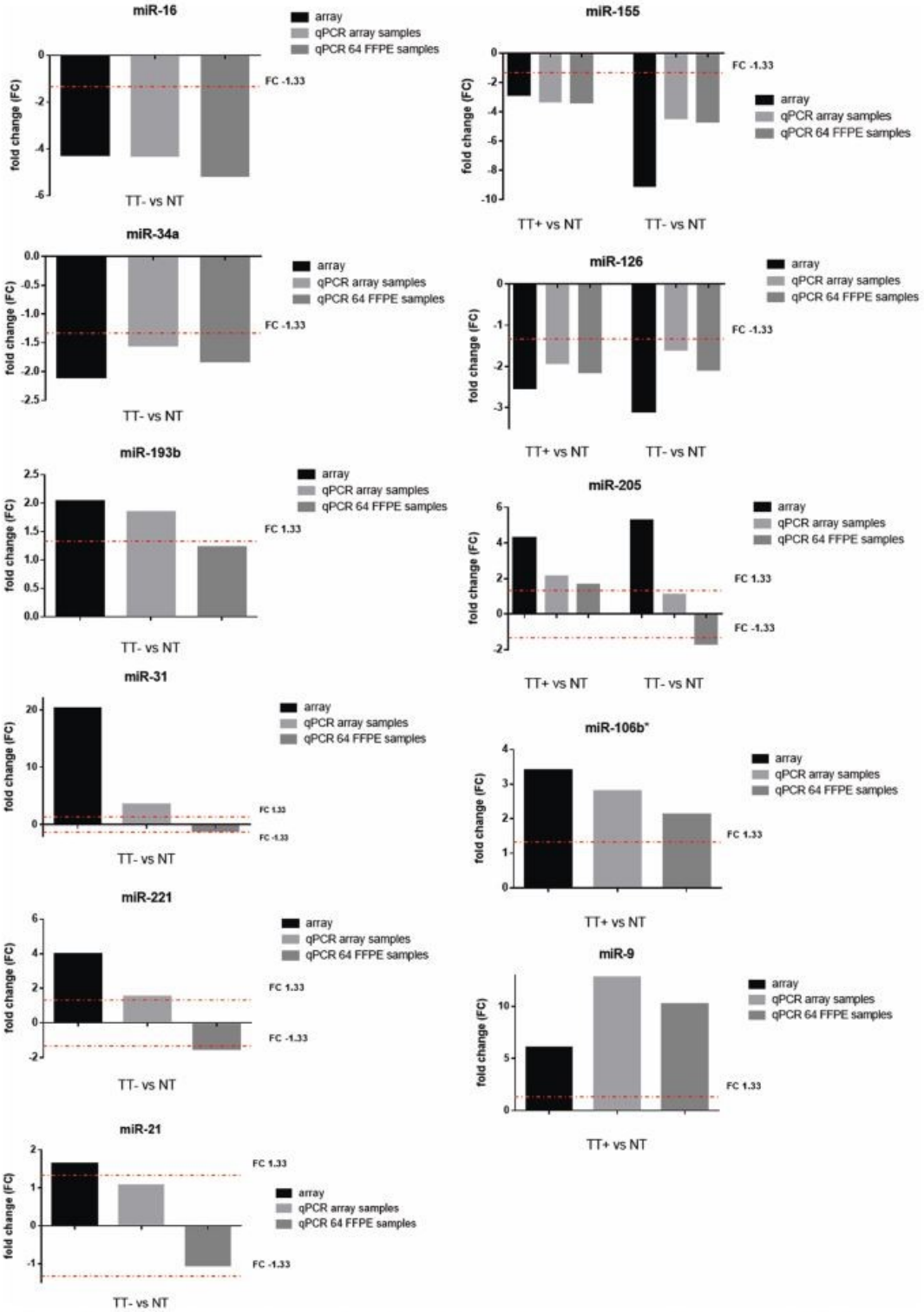

Fig 5. Comparison on fold-change for selected miRNAs between arrays and qPCR. The treshold of FC was set to 1.33. T-test or Mann Whitney nonparametric test was applied depending on the data distribution. All results were statistically significant (P-value ≤ 0.05).

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tumor development [47]. The group of Seki et al. have been interested in the functional significance of mir-29 family, including miR-29a, in cervical cancer as well as in head and neck cancer. They found out that this tumor-suppressive miRNA inhibits cancer cell migration and invasion [48, 49]. Although some studies focused on the functional impact of identified miRNAs in HNC have been published, more extensive research is necessary to more accurately explain the relevance in HNC tumor development.

From the groups of deregulated miRNAs identified in FFPE tumors of either viral or non-viral etiology, we selected 11 miRNAs based on the results and relevance in the literature, the expression of which was evaluated in a larger set of 64 FFPE samples. From the group of miRNAs specific for HPV-positive tonsillar tumors, we choose upregulated miR-9 and miR-106b# whose deregulation was previously reported in HNC [50–52]. Furthermore, miR-9 has been shown to be activated by HPV leading to increased cell motility [53] and to be involved in the pathways regulating metastasis [54]. Upregulated miR-31, miR-221, and miR-21 were selected for confirmation from the group of miRNAs specific for HPV-independent tumors including HNC as playing a role in increasing cell proliferation, invasion, and migration [55–58], participating in regulation of epithelial-mesenchymal transition (EMT), or having a prognostic impact [59, 60]. Further choices were miR-34a, specific for HPV-negative tumors, a tumor suppressor whose downregulation has been shown in a number of tumor types including HNC [61, 62], as well as miR-16, the inhibition of which promotes cell proliferation, migration, invasion, and EMT and contributes to tumor progression [63]. The last miRNA selected for confirmation from the group of HPV-negative tumors was miR-193b whose high expression in tissue was identified as an independent prognostic risk factor in patients with ovarian cancer [64]. From the group of tonsillar specific miRNAs regardless of the virus presence, we selected miR-205, miR-155, and miR-126 which participate in oncogenic pathways including cell proliferation, migration, or invasion and serve as prognostic predictors of patients with HNC [31, 65, 66].

We were able to confirm deregulated expression in the same set of samples as run on array. In this subset, deregulated expression was confirmed for all miRNAs except miR-21 and miR-205. However, their trend of expression was maintained and the fold change was close to the cut-off value. In the larger set of samples, deregulated expression was confirmed in 64% of comparisons. The fold change of miR-193b was equal to 1.23, and the trend of expression of four additional selected miRNAs was opposite to that revealed by arrays. The results suggest possible variability between samples and show that not all deregulated miRNAs detected in a smaller tested cohort might be representative of a broader spectrum of samples.

In conclusion, our study compares the miRNA expression profiles in tonsillar tumors as identified in fresh frozen and formalin-fixed paraffin-embedded samples. Although the correlation between the defined groups was relatively good for all miRNAs, the overlap of the selected differentially expressed miRNAs was rather suboptimal. We concluded that for accurate comparison between studies, the key factors are the use of the same type of clinical material and selection of the normalization method for data analysis. Additionally, we suggest that combining multiple analytical methods rather than a single test alone is advisable for more robust, reliable detection of differential abundance of miRNAs and interpretation of results.

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Visualization: ZV RT.

Writing – original draft: ZV JZ RT.

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Publication IV

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Review

The Role of miRNAs in Virus-Mediated Oncogenesis

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Abstract: To date, viruses are reported to be responsible for more than 15% of all tumors worldwide. The oncogenesis could be influenced directly by the activity of viral oncoproteins or by the chronic infection or inflammation. The group of human oncoviruses includes Epstein–Barr virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), human herpesvirus 8 (HHV-8) or polyomaviruses, and transregulating retroviruses such as HIV or HTLV-1. Most of these viruses express short noncoding RNAs called miRNAs to regulate their own gene expression or to influence host gene expression and thus contribute to the carcinogenic processes. In this review, we will focus on oncogenic viruses and summarize the role of both types of miRNAs, viral as well as host's, in the oncogenesis.

Keywords: microRNA; virus-mediated oncogenesis; viral miRNA; EBV; HHV-8; HBV; HPV; MCPyV; HCV; retroviruses

1. Introduction

More than 14 million cancer cases are diagnosed each year worldwide, with more than 15% attributable to the carcinogenic infections [1]. Oncoviruses are responsible for more than 63% of cases, the bacterium *Helicobacter pylori* accounts for 35%, and the remaining 2% of cases are associated with three parasites (*Schistosoma haematobium*, *Opisthorchis viverrini* and *Clonorchis sinensis*). In 2000, Hanahan and Weinberg identified six hallmarks of cancer—evading antigrowth signals, tissue invasion and metastasis, enabling replicative immortality, sustained angiogenesis, evading apoptosis, and maintaining proliferative signaling [2]. In 2011, these principles were updated by adding another four, including deregulated cellular energetics, avoiding of immunological destruction, tumor-promoting inflammation, and genomic instability and mutations [3]. Oncoviruses, as infectious agents, can induce all of these hallmarks and thus contribute to tumor development through multiple pathways. However, it is important to mention that in viral oncogenesis, viruses are necessary but not sufficient to cause cancer, so the incidence of cancer is much lower than the prevalence of the causative viruses [4].

The oncogenic viruses evoke and maintain persistent infection during which they are hidden from the immune system, which is compatible with carcinogenic processes. Most oncogenic viruses have mechanisms through which they are equally segregated into the daughter cells during cell division, and thus their genome is maintained in the host cells during proliferation. The virus-mediated cell immortalization is then influenced either directly or indirectly. The direct mechanisms include the deregulated expression of cellular oncogenes/tumor-suppressor genes, influenced by integration of the viral genome into the host genome (e.g., retroviruses, human papillomaviruses, hepatitis B viruses), or the expression of viral oncogenes (e.g., herpesviruses) which inactivate major regulators of genome stability and cell cycle leading to DNA damage and transformation of the host cell. The indirect mechanisms of transformation comprise the tissue damage caused by immune cells and chronic

inflammation, or establishment of immunosuppression due to viral infection, resulting in the inhibition of antitumor surveillance mechanisms.

Oncogenic viruses are represented in all groups of viruses. The most relevant are DNA viruses, of which we should mention Epstein–Barr Virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), human herpesvirus 8 (HHV-8), or polyomaviruses. The group of RNA viruses is represented by hepatitis C virus (HCV), and finally, the group of retroviruses includes transregulating viruses such as human immunodeficiency virus (HIV) or human T-lymphotropic virus (HTLV-1).

MicroRNAs (miRNAs) are short noncoding RNAs of ~21 nucleotides in length which post-transcriptionally regulate gene expression and have an important role in the development, cell growth, differentiation processes, survival, or regulation of apoptosis in a variety of eukaryotic organisms [5]. It is suggested that the expression of at least one third of human genes is influenced by miRNAs [6], and the deregulated expression of miRNAs has been observed in many types of tumors [7,8]. However, most of the oncogenic viruses also express miRNAs to regulate their own gene expression or to influence host gene expression, and thus contribute to the carcinogenic processes. Even though many new virus-encoded miRNAs were discovered in the last 15 years, only a minority of them were shown to fulfill the criteria for authentic viral miRNAs. These criteria are very important when deep-sequencing techniques are applied, since many small non-viral RNAs can be detected. Besides the number of miRNA copies per cell, identification of the region in the viral genome from which the miRNA is derived, the size of the miRNA within the range of 19–25 bases, the specificity of the 5′ end, and identification of the hairpin structure of pre-miRNA should be specified for an authentic viral miRNA [9]. In this review, we will more closely focus on the role of viral miRNAs in oncogenesis and on the impact of their expression on the hosts. Moreover, the role of the host's miRNA expression during oncogenesis will be discussed.

2. Epstein–Barr Virus

Epstein–Barr Virus (EBV, HHV-4), the first oncovirus discovered, is classified as a DNA virus from the family *Herpesviridae*. It was detected first in Burkitt lymphoma (BL) cells in 1964 [10], and this discovery started the research on the virus-mediated oncogenesis. The primary infection of EBV is most common in childhood, and then EBV persists in latent form mostly in resting memory B-cells and less commonly in T-cells, NK-cells, or epithelial cells [11]. Besides Burkitt lymphoma, EBV is associated with other B-cell lymphoproliferative disorders such as Hodgkin lymphoma (HL) or post-transplant lymphoproliferative disorder (PTLD), with T-cell lymphoproliferative disorders or epithelial malignancies such as gastric carcinoma and nasopharyngeal carcinoma (NPC) [12–14].

The mechanism of EBV-mediated carcinogenesis is the coding of the viral oncoproteins. The main oncoprotein is latent membrane protein 1 (LMP1), which is a transmembrane protein functionally mimicking CD40, a member of the tumor necrosis factor receptor (TNFR) superfamily. The activation of this receptor leads to the initiation of the signaling pathways such as phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), mitogen-activated protein kinase (MAPK), Janus kinase/signal transducer and activator of transcription proteins (JAK/STAT) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to B-cell differentiation towards memory B-cells, expression of anti-apoptotic proteins, and increased cell proliferation [15]. Oncoprotein LMP2 is structurally and functionally similar to the B-cell receptors (BCRs) which, after phosphorylation, activate the non-receptor tyrosine kinase (Src) and spleen tyrosine kinase (Syk) signaling pathways and thus increases the survival of latently infected B-cells by production of cytokines such as interleukin 10 (IL-10) and by expression of anti-apoptotic factors [16–18]. EBV nuclear antigen 1 (EBNA1) is a multifunctional protein which influences viral replication, transcription and latency. As the only one of the nuclear proteins, it is expressed in both lytic and latent phases of the viral life cycle [19]. EBNA1 suppresses the function of the promyelocytic leukemia (PML) protein, which is a tumor suppressor protein regulating p53 activation. Thus, EBNA1 inhibits p21 activation and signaling, leading to inhibition of apoptosis and cell survival [20]. EBNA-LP (EBNA leader protein) functions

in cooperation with EBNA2, and both proteins participate in initiating the transcription of viral and cellular proteins responsible for B-cell immortalization and transformation, for example, cellular gene *c-myc* [21]. Finally, EBNA-3 proteins associate with many cellular proteins from different signaling pathways such as recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ) and thus contribute to increased proliferation and transformation of B-cells [22].

EBV was the first virus for which the viral-encoded miRNAs were described. Pfeffer et al. have published the report describing viral-encoded miRNAs from cell lines infected with EBV [23]. These five miRNAs were encoded in two clusters, BHRF1 (miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3) and BART (miR-BART1 and miR-BART2). Nowadays, according to the miRBase database, EBV encodes 25 pre-miRNAs producing at least 44 mature miRNAs [24]. The benefit of viral-encoded miRNAs, not only for EBV but for all viruses coding their own miRNAs, is the ability to regulate viral as well as host gene expression without the production of viral proteins, allowing for the immune invisibility of the infected cells.

As mentioned above, EBV miRNAs have been reported to regulate their own expression. BART miRNAs (miR-BART16, -17-5p, -1-5p) target viral transcripts for protein LMP1 and thus contribute to cell transformation [25]. MiR-BART22 negatively regulates translation of LMP2A in NPC and thus helps EBV-infected cells to escape the host immune surveillance [26]. EBV also uses the miRNAs for indirect regulation of the switch between the lytic and latent life phases. For example, miR-BART2 regulates DNA polymerase BALF5 and helps to replicate the viral genome in the lytic phase, and thus its negative regulation promotes the latent stage of the virus [27]. Iizasa et al. have reported miR-BART6-5p to regulate the viral replication and latency through suppressing the EBNA2 viral oncogene [28].

EBV miRNAs also regulate the translation of host mRNAs and thus may influence cancer development. MiR-BART5-5p and miR-BART19-5p negatively regulate the translation of p53 upregulated mediator of apoptosis (PUMA) protein, a factor that positively influences cellular apoptosis and was found at significantly lower levels in NPC cells infected by EBV compared to noninfected ones [29]. Nasopharyngeal carcinoma cells were analyzed also by Cai et al. [30], and they revealed that miR-BART7-3p promotes the epithelial–mesenchymal transition (EMT) and metastasis through suppressing the major human tumor suppressor PTEN, which modulates PI3K/Akt/GSK-3β signaling. Vereide et al. revealed that miR-BART1 and miR-BART16 help to block apoptosis in BL cells in the absence of other viral oncogenes through their targeting of caspase 3 (Casp3) [31]. Infected cells thus survive, and the virus may replicate. Further, the EBV miRNA miR-BART3 has been reported to negatively regulate the expression of the cellular tumor suppressor DICE1 (determination of interleukin 4 commitment 1), which was found downregulated in NPC cells, leading to increased proliferation and transformation of the cells [32]. EBV also uses its own miRNAs for the immune evasion strategy. MiR-BHRF1-3 targets the mRNA of host interferon-inducible cytokine CXCL11 [33]. Targeted suppression of this cytokine may serve as an immunomodulatory mechanism in EBV-associated tumors. In-vitro studies have demonstrated that the action of miR-BHRF1 enhances the B-cell transformation and decreases the antigen loading of cells [34]; however, in-vivo studies have revealed that miR-BHRF1 facilitates the development of acute infection but does not enhance the oncogenic potential [35]. The evasion of the host immune system is also mediated by miR-BART2-5p, which negatively regulates the expression of major histocompatibility complex (MHC) class I chain-related protein B (MICB) molecules upregulated on the infected cells' surface and recognized by NK cells [36]. The expression of EBV-encoded miRNAs may also influence the survival of patients with EBV-associated cancer. A high level of miR-BART20-5p was associated with worse survival of patients with EBV-related gastric cancer [37]. Moreover, the expression of viral miR-BART7 predicted the responsiveness of NPC cells to radiation treatment because of its targeting of the glutamine fructose-6-phosphate transaminase 1/transforming growth factor beta 1 (GFPT1/TGFβ1) signaling, regulating the DNA damage repair machinery [38].

The deregulation of cellular miRNA expression is a feature detectable in many human tumors including EBV-associated tumors. It has been reported that EBV infection of primary B-cells results in downregulation of cellular miRNA expression [39]. Iizasa et al. have revealed that EBV miR-BART6-5p suppresses the expression of Dicer and thus influences expression of many miRNAs as well as miR-BART6-5p itself by a negative feedback loop [28]. The tumor suppressor miR-31 is consistently inactivated in NPC, since it targets the MCM2 protein and inhibits the growth of NPC cells [40]. Nevertheless, many cellular miRNAs are upregulated due to the infection, such as miR-155, which promotes the proliferation and migration of NPC cells [41] or B-cell immortalization [42]. The aberrant expression of miR-155 is driven by EBV LMP1 and LMP2A [43,44], and its upregulated expression in EBV-associated tumors was also confirmed by the study of Sakamoto et al. [45], who used a next-generation sequencing approach. The miRNA related to EBV infection in BL is miR-127, which was not found in EBV-negative BL, and it contributes to the development of lymphoma through the blocking of BLIMP-1 or XBP-1, regulators of B-cell differentiation [46]. MiR-21, a very important oncomiR in tumor development, was found to be positively regulated by EBV EBNA2 protein in B-cell lymphoma [47], and in tumors has anti-apoptotic and prometastatic roles. The EBNA2 protein further negatively regulates the expression of miR-146a, a miRNA involved in the innate immune response [47]. Oussaief et al. [48] have shown that LMP1 protein expression triggered downregulation of the miR-183-96-182 cluster, whose expression is downregulated in BL cell lines and has a key role in EBV-mediated transformation. Finally, Chen et al. [49] have revealed that the expression of miR-1 in NPC is downregulated by LMP1. This miRNA functions as a tumor suppressor by regulating *K-ras* (Kirsten rat sarcoma) gene expression with pro-apoptotic effect and inhibition of the angiogenesis during tumor development.

3. HHV-8

Another oncovirus from the family *Herpesviridae* is human herpesvirus 8 (HHV-8). Sometimes it is called Kaposi's sarcoma-associated herpesvirus (KSHV) since it is a causal agent of Kaposi's sarcoma (KS), a proliferative disease of vascular and lymphatic endothelial cells [50]. HHV-8 infection mainly manifests in immunocompromised patients, such as those with AIDS and those after transplantation or chemotherapy. Apart from KS, HHV-8 causes primary effusion lymphoma (PEL) [51] or multicentric Castleman's disease [52], with both affecting B-cells. HHV-8 influences the proliferation and cell cycle of the infected cells due to the sequence homology with host genes. In the latent phase of the viral infection, viral cyclin D (v-cyclin D) and LANA1 regulating the cell cycle, viral FLICE inhibition protein (v-FLIP)—inhibitor of apoptosis, kaposins, or viral interferon response factors (vIRF) modulating the immune system and influencing the proliferation—are expressed.

KSHV encodes 12 viral pre-miRNAs, which evolve into 25 mature miRNAs. All miRNA genes are clustered together and are under the control of latent kaposin promoter (LTd). Most of the pre-miRNA genes are intronic, located between the sequence for *kaposin* and open reading frame (ORF) 71, except for miR-K10, which is located within the ORF of *kaposin*, and miR-K12 located on the 3′ end of the *kaposin* gene [53]. It is important to mention that viral latency is critical for tumor development. KSHV miRNAs participate in the regulation of the viral life cycle targeting directly key viral genes as well as indirectly, through cellular genes regulating viral replication and thus contributing to the oncogenesis. The viral miRNAs miR-K9-5p and miR-K7-5p are modulators of the latent–lytic switch as they target the viral RTA (R transactivator) protein, the regulator of lytic induction [54,55]. The viral life cycle is also regulated by miR-K3, which targets cellular nuclear factor I/B and thus negatively influences the expression of RTA [56], or by G-protein-coupled receptor kinase 2 (GRK2), enhancing in this way the viral latency [57]. The expression of RTA could also be restrained by the activity of miR-K12-11 which targets cellular myeloblastosis transcription factor (MYB) [58], previously reported to be an activator of the RTA promoter [59]. Moreover, miR-K12-11 modulates interferon signaling through targeting I-kappa-B kinase epsilon (IKKε), contributing to the maintenance of viral latency [60]. The latent phase of the viral infection is also maintained by methylation of the *RTA* promoter, which is ensured by

DNA methyl transferase 1 (DNMT1) [61]. The activity of this methyltransferase is regulated by KSHV miR-K12-4-5p, which inactivates its suppressor retinoblastoma-like protein 2 (Rbl2), affecting cell cycle and cellular differentiation control.

KSHV miRNAs play roles also in the dissemination and angiogenesis of KS. Several miRNAs, such as miR-K12-1, miR-K3-3p, miR-K6-3p, or miR-K12-11, negatively regulate the expression of thrombospondin 1 (THBS1), which is an antagonist of angiogenesis, and its downregulation leads to abnormal angiogenesis and proliferation of KSHV-infected cells [62]. Another miRNA promoting dissemination and angiogenesis is miR-K6-3p, whose activity stimulates the STAT3 pathway leading to cell migration and the invasion of KS cells [63]. As has been shown by Guo et al. [64], KSHV miRNAs regulate matrix metalloproteinases (MMPs) and expression of pro-angiogenic factors, and thus play roles in KSHV-induced cell motility and angiogenesis. KSHV miRNAs help to promote the development of KS and other KSHV-associated malignancies through the cell cycle arrest, cell survival, and cell transformation. Zhu et al. have reported that KSHV promotes these processes by suppressing the aerobic glycolysis and oxidative phosphorylation under nutrient stress [65]. They have revealed that KSHV regulates the key metabolic pathways of the cells by miRNAs to adapt to the tumor microenvironment. MiR-K12-1 is an anti-apoptotic miRNA, which downregulates the expression of protein p21, the inhibitor of cyclin-dependent kinases and a key inducer of cell cycle arrest [66], and in this way, contributes to the survival of viral-transformed cells. Also, miR-K12-1, miR-K12-3, and miR-K12-4-3p, which inactivate the critical inducer of apoptosis, caspase 3 (Casp3), participate in inhibition of apoptosis and thus play a role in KSHV-induced oncogenesis [67].

Like EBV, KSHV influences the expression and function of cellular miRNAs. Viral miR-K12-11, playing a role in PEL development, shares the seed sequence with the cellular miR-155, whose targets affect B-cell differentiation, and thus regulates a set of common mRNA targets [68,69]. Tsai et al. have revealed that KSHV protein K15 via the viral SH2-binding motif contributes to KSHV-associated tumor metastasis and angiogenesis by regulation of cellular miR-21 and miR-31 [70]. The KSHV-encoded protein vFLIP K13 activates the NF-κB pathway, suppressing the expression of cytokine C-X-C chemokine receptor type 4 (CXCR4) through upregulation of cellular miR-146a [71]. CXCR4 plays a key role in the retention of immature endothelial cells in the marrow, and its downregulation contributes to premature release of these cells into the circulation and to KS development. Only one study analyzing the miRNA expression profiles in KSHV-infected B-cells has been published so far. Hussein and Akula performed the analysis of the early stages of KSHV infection of human B-cells and have revealed 32 known and 28 novel differentially expressed miRNAs [72]. The potential biological implications of the known differentially expressed miRNAs included promoting cell survival and latent infection, inhibiting the host immune response, or inducing critical cell signaling.

4. Hepatitis B Virus

Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae* and causes an acute liver disease—viral hepatitis. Most infected adults recover completely in a few months, but about 5% of the adult patients proceed to chronic infection [11], which can consequently develop into hepatocellular carcinoma (HCC). In patients infected perinatally or in childhood, the percentage of chronic infection is higher (90% and 20% of cases, respectively). HBV is the third most frequent infectious agent contributing to cancer development, with 420,000 of new cancer cases reported in 2012 [1]. Apart from HCC, HBV infection is associated with B-cell non-Hodgkin lymphoma (B-NHL) [73] and nasopharyngeal carcinoma (NPC) [74], but the exact virus-associated pathogenesis is still unclear.

The HBV genome encodes four ORFs, which consist of genes for surface proteins (*preS1*, *preS2* and *S*), genes for core (*core* and *precore*) proteins, genes for polymerase, and genes for protein HBx. The oncogenesis is influenced by the direct mechanisms of the virus such as activity of viral oncoprotein HBx or surface proteins in the transcriptional regulation, regulation of DNA repair or expression of miRNAs [75–78], and/or the integration of the viral DNA into the host genome in the proximity

of the fragile sites [79,80]. Further, HBV contributes to the development of the tumors by indirect mechanisms, such as chronic inflammation and activation of protumorigenic signaling pathways [81].

The presence and the function of HBV-encoded miRNAs has so far not been proven, but at least two published studies suggested existence of HBV-specific miRNAs, and we discuss them below. On the other hand, numerous studies focused on the cellular miRNA profiles and their deregulation during HBV-associated tumorigenesis have been described. Highly specific to hepatocytes is miR-122, which maintains the differentiated phenotype of cells, and its downregulation was observed in HCC cell lines as well as in clinical samples. MiR-122 negatively regulates the expression of a tumor promoter, N-myc downstream-regulated gene 3 (*NDRG3*) [82], or pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF) whose upregulation, because of the loss of miR-122 expression, leads to the cell growth and invasion of the HCC tumor [83]. The tumor cell growth is also regulated by cyclin G1, whose expression increases depending on the suppression of miR-122 [84]. Song et al. [85] have revealed that the reduction of the miR-122 level in HCC cells is due to the protein–protein activity of viral HBx protein with peroxisome proliferator activated receptor-gamma (PPARγ), which normally enhances the miR-122 transcription by binding to its promoter. Besides miR-122, protein HBx further influences the expression of cellular miR-29a, which is upregulated in HBx-transfected hepatoma cells and whose upregulation positively correlates with the metastatic potential [86]. MiR-29a increases the migration ability of cells through targeting tumor suppressor phosphatase and tensin homolog (PTEN) and activation of the Akt signaling pathway. MiR-29a/b also targets MHC class I chain-related protein A (MICA) or MICB, whose decreased expression results in a limited activity of natural killer (NK) cells and promotion of chronic infection [87]. Moreover, HBx decreases the level of miR-101, enhancing tumorigenesis through epigenetic silencing of tumor suppressor genes (*TSGs*) [88]. This miRNA targets DNA methyltransferase 3A (DNMT3A), which catalyzes the methylation of *TSG* promoter regions and thus inhibits their expression. DNA methylation is also influenced through HBx downregulation of miR-152, which targets DNA methyltransferase 1 (DNMT1) and epigenetically regulates the expression of *TSGs* [89]. Not only miRNAs mediate epigenetic modifications of DNA, but also the protein HBx itself induces DNA methylation of promoter. Wei et al. [90] have revealed that HBx induces DNA hypermethylation of the miR-132 promoter and thus promotes cell proliferation through the Akt signaling pathway. Moreover, they have found that the serum levels of miR-132 correlate with that in the tumor tissues, implicating that it might be a noninvasive candidate for a diagnostic biomarker of HBV-related HCC. Protein HBx also negatively regulates the level of miRNA let-7a [91]. These tumor suppressor miRNAs are involved in cell differentiation and proliferation through the STAT signaling pathway, and are often downregulated in HCC. Also, a decreased level of let-7a leads to the activation of proliferation factors, such as Ras [92] or Myc [93]. Let-7 expression might also be regulated by HBx indirectly through the upregulation of the let-7 inhibitors LIN28A and LIN28B [94,95].

Profiling studies of virus-related HCC search for miRNAs specific for the early stages of the tumors or progression of the disease. Mizuguchi et al. [96] have used next-generation sequencing and bioinformatics to reveal the miRNA transcriptome of HBV-related HCC. The global profiling of miRNAs in HBV-related HCC was also performed by Wang et al. [97], who analyzed 12 pairs of HCC and matched nonmalignant tissues from HBV-positive and HBV-negative patients. They have revealed eight miRNAs involved in HCC unrelated to virus, a further five miRNAs involved in HBV infection, and finally, seven miRNAs specifically altered in HBV-associated HCC. The possible role of these miRNAs (miR-150, miR-342-3p, miR-663, miR-20b, miR-92a-3p, miR-376c-3p, and miR-92b) in HBV-related HCC development must be further investigated.

Not only viruses can influence the expression of cellular miRNAs and thus the development of the tumor, but also host miRNAs regulate the viral life cycle and replication and thus affect the progression of chronic hepatitis to HCC. Direct targeting of HBV mRNA, by transcript of gene *HBx*, was observed by Wang et al. [98]. They have found that the tumor suppressors miR-15a/miR-16-1 target viral mRNA and thus reprogram the expression of multiple cellular miRNAs including these miRNAs themselves, leading to the HCC development. A similar negative feedback suppression

was observed by Jung et al. [99] for cluster miR-17-92. Finally, Chen et al. [100] have revealed that miR-122 downregulates HBV replication by binding to the viral target sequence contributing to chronic HBV infection.

5. Human Papillomavirus

The human papillomaviruses (HPVs), double-stranded DNA tumor viruses, belong to the family *Papillomaviridae*. HPVs infect epithelial cells of the skin or mucosa and may cause benign proliferations such as papillomas or warts. Further, some types of HPVs also have an oncogenic potential and are the main etiologic factor or cofactor in a variety of carcinomas. HPVs are the most frequent viral agents involved in oncogenesis attributable to infectious agents, with almost 640,000 new cases in 2012 [1]. HPVs cause almost 100% of cervical cancers. HPVs participate in oncogenesis in other anogenital regions, such as vulvar, penile, or anal area, and in the development of head and neck cancer; however, the global burden of HPV-associated cancer in these anatomical locations is substantially lower [1].

The genome of HPV is composed of early-region genes *E1–E7*, a late region with two capsid proteins L1 and L2, a long control region (LCR) with regulatory sequences, and a viral origin of replication. Some types of HPV also express the E8^E2C fusion protein [101–103]. Viral early-region proteins E6 and E7 are the main oncoproteins in HPVs, and their overexpression contributes to tumor development. The E5 viral protein has a high transformation potential in bovine papillomavirus 1 (BPV1) [104]. In HPVs, the presence of *E5* in the viral genome correlates with the risk of cancer, and E5 cooperates with the main viral oncoproteins E6 and E7 [105,106]. Protein E6 inactivates the function of tumor suppressor p53 [107,108], while protein E7 binds the retinoblastoma protein Rb and thus activates cell cycle progression [109]. The virus might incorporate into the host genome and thus enhance the malignant progression. The integration is an important event in HPV-related carcinogenesis, whose frequency depends on the stage of disease, HPV type, and type of HPV-associated tumor [110], but it is not obligatory for cell transformation.

Profiling of miRNA expression in HPV-associated malignancies has been done in numerous studies with the aim to define new biomarkers for the detection of premalignant stages of the disease as well as markers for selection of patients for modified treatments [111]. Since HPV-associated tumors are etiologically distinct from viral-unrelated tumors of the same anatomical location, for the interpretation of the data, it is important whether the studies also specify the viral status. For cervical cancer, numerous such studies have been published [112–115], while for head and neck tumors, the information about the viral status has not been commonly evaluated [111,116–121]. The knowledge about tumor viral status is specifically very important for head and neck tumors where only 20–90% of them are HPV-associated [122,123].

The process of how the HPV oncoproteins affect the expression of cellular miRNAs was studied by Harden et al. [124], who have assumed that the modulation of the cellular miRNA expression is the main oncogenic activity of these proteins and have suggested several mRNA–miRNA pairs as potential drivers of HPV carcinogenesis. The miR-106b~25 cluster is one of those regulated by transcription factors of the E2F family (transcription factors of higher eukaryotes) and thus by HPV E7 [125]. Furthermore, the expression of the miR-15b~16-2 cluster or the miR-34 family is regulated by HPV oncoproteins [126,127] leading to cell cycle progression and contributing to tumor development. The expression of miR-23b has been shown to be downregulated by the action of the viral E6 protein, resulting in an increase in a direct miRNA target, urinary plasminogen activator (uPA), and thus promoting tumor cell migration [128]. The expression of miR-9 has been found upregulated in both cervical and tonsillar tumors [119,121]. The activation of miR-9 increases cell motility and has been shown to be involved in the pathways regulating metastasis [129,130]. Downregulation of miR-218, identified in cervical cancer as well as in head and neck cancers, has been shown to promote cell migration and invasion [131,132]. While the expression of miR-375 was also found downregulated in cervical tumors [133], it functions as a tumor suppressor by targeting HPV transcripts. This leads to

the repression of the expression of E6/E7, cell cycle arrest, and reduced proliferation of HPV-positive cervical cancer cells.

6. Merkel Cell Polyomavirus

Polyomaviruses are nonenveloped DNA viruses belonging to the family *Polyomaviridae* which infect a number of hosts, such as birds or mammals. It was long considered that humans could only be infected by JC and BK polyomaviruses; nevertheless, up to now, at least 11 more human polyomaviruses have been identified, including Merkel cell polyomavirus (MCPyV). MCPyV was discovered to be associated with human cancer in 2008 [134], and since then, the interest in polyomavirus research has increased. Whereas MCPyV was detected in more than 90% of Merkel cell carcinomas (MCCs) [135,136], the oncogenic properties of JCPyV and BKPyV have only been documented in cell cultures and animal models [137]. Only scarce studies suggest their oncogenic potential in humans [138–140].

The oncoproteins that drive the virus-mediated oncogenesis are early-coded large T (LT) antigen and small T (ST) antigen [141]. Besides these early antigens, MCPyV encodes two more early proteins, the 57kT antigen and alternative LT open reading frame (ALTO), and three late-coded proteins, VP1-3. The first polyomaviral miRNA was described in polyomavirus SV40 [142]; it is encoded on the 3′ end of the late transcript and is complementary to early viral mRNAs. The authors have shown that it reduces the cytotoxic T-lymphocyte-mediated cell lysis and interferon gamma (INF-γ) release. Later, JCV-miR-J1 has been identified in JCPyV and BKV-miR-B1 in BKPyV [143]. These two miRNAs function as inhibitors of NK-cell response [144]. In 2009, MCV-miR-M1 was discovered in MCPyV [145]. All these viral miRNAs influence the expression of LT antigen and thus regulate the viral life cycle and contribute to the host immune evasion.

As documented in previous chapters, miRNAs encoded by oncogenic viruses contribute to the tumorigenesis; however, no human polyomaviral miRNAs have yet been implicated in this process. In tumors, MCPyV miRNAs are not detected or are detected in very low levels (<0.025%) [146,147], because MCPyV DNA is integrated into the host genome in most tumors [134,148]. Therefore, only early genes are expressed, whilst PyV miRNAs are encoded from the late transcripts that are expressed during lytic infection. However, this is in contrast with the study of Chen et al. [149], who chose phylogenetically-related raccoon PyV (RacPyV) to investigate the function of PyV miRNA in tumors. Despite the genomic and sequence similarities of RacPyV miRNA with MCPyV miRNA, high levels of early gene transcripts and miRNA levels have been detected in RacPyV-associated tumors [149,150]. Thus, their observations suggest that these PyV-associated tumors arise via different mechanisms, and MCPyV miRNA probably is not involved in MCC tumorigenesis. Therefore, only future research will reveal the role of PyV miRNAs in virus-mediated carcinogenesis.

The effect of LT antigen on the cellular miRNA expression has not yet been investigated; however, the control of host miRNA expression by the LT antigen is assumed, since this protein is involved, among others, in transcriptional regulation of cellular genes, affects RNA polymerase II-dependent transcription, and thus, might influence the production of pri-miRNAs [137]. The only study to analyze the cellular miRNA expression profiles was that of Xie et al. [151], who compared the miRNA profiles in MCPyV-positive and MCPyV-negative MCC. As expected, they have revealed distinct patterns and have found miR-203, miR-30a, miR-769-5p, miR-34a, and miR-375 to be significantly deregulated. In addition, the authors tested the functional consequences of the overexpression of miR-203 in MCC and have revealed that it functions as a tumor suppressor since it inhibits the cell growth, induces cell cycle arrest, and regulates survivin expression in MCC cells non-associated with MCPyV [151].

7. Hepatitis C Virus

Hepatitis C virus (HCV) belongs to the family *Flaviviridae* and is the only representative of the RNA viruses group that is associated with the development of tumors. HCV affects hepatocytes and causes acute infection, which progresses to chronic disease in 75–80% of cases, thus increasing the

risk of cirrhosis and/or hepatocellular carcinoma (HCC) [152]. HCV might also contribute to the development of several other malignancies, such as pancreatic or renal cancer and B-cell non-Hodgkin lymphoma [153]. HCV is the fourth leading infectious agent contributing to carcinogenesis, with almost 8% of new cases of cancer being attributable to infection in 2012 [1]. The HCV genome is a positive RNA strand which encodes structural proteins (Core, E1 and E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). HCV-associated oncogenesis is promoted by both direct and indirect mechanisms [154]. The direct mechanisms involve primarily the activity of viral proteins, such as core and nonstructural proteins, that promote tumorigenesis through interaction with cell factors leading to the activation of tumorigenic pathways and cell transformation, and the indirect mechanisms then include the promotion of inflammation and oxidative stress.

There is no evidence that HCV encodes its own miRNAs. This is probable because of the separation of the first step of the miRNA biogenesis in the nucleus and replication of the RNA viruses in the cytoplasm where they are physically separated from the nuclear Drosha enzyme and thus cannot undergo the splicing process to generate pre-miRNAs. Still, some groups searched for HCV-encoded miRNAs, but with no success [155]. Nevertheless, HCV infection is associated with changes in cellular miRNA expression, and also, cellular miRNAs influence the HCV life cycle.

An important role in HCV infection and life cycle is played by liver-specific cellular miR-122, whose downregulation results in a decrease in the HCV RNA level [156–158]. This miRNA has a binding site in the 5′ UTR of the HCV genome and, interestingly, its binding to the target site does not lead to the repression of HCV genes, but miR-122 protects the HCV genome from nucleolytic degradation and, in this way, promotes viral RNA stability. This miRNA is a very promising candidate for the anti-HCV treatment. Its inhibition by antisense oligonucleotides has been tested in animals [159], and it is now in clinical trials for the treatment of HCV infection [160]. Apart from miR-122, other miRNAs were found to inhibit HCV replication and therefore are potential targets for anti-HCV therapy. MiR-199a targets the 5′ UTR of the HCV genome [161] and appears to be decreased in HCC [162]. MiRNA let-7b binds to two conserved regions in the HCV genome, 5′ UTR and NS5B and besides the inhibition of viral replication, it acts synergistically with IFNα [163]. Mukherjee et al. [164] have shown that miR-181c binds to the E1 and NS5A sites in the HCV genome and reduces viral replication. However, still more research is needed, since the efficiency of miR-181c binding differs between HCV genotypes. The HCV life cycle can also be regulated by host miRNAs that target host mRNA. MiR-373 facilitates HCV RNA replication through the regulation of the JAK/STAT signaling pathway [165], and miR-27a influences the production of HCV particles by inhibiting genes related to the lipid metabolism signaling pathways [166].

There are many studies reporting deregulated expression of cellular miRNAs in HCV-associated tumors. Zhang et al. [167] have observed the upregulation of miR-155 during HCV infection. This miRNA promotes the proliferation of hepatocytes by the activation of Wnt signaling and inhibits apoptosis. Moreover, the growth, proliferation and tumorigenesis of hepatocytes are facilitated by the downregulation of miR-152 [168], miR-181c [164], or miR-491 [169]. The direct role of miR-141 has been shown by Banaudha et al. [170], who found miR-141, required for HCV replication, to inhibit tumor suppressor gene *DCL-1* (Dicer-like 1) which encodes a Rho GTPase-activating protein, and thus promotes the cell proliferation. Varnholt et al. [171] have examined the miRNA expression profiles in a set of liver tumors and dysplastic samples. They have revealed 10 upregulated and 19 downregulated miRNAs in tumors compared to normal tissues. Moreover, they validated five of these miRNAs on a larger set of samples and found the well-known miR-122 to be overexpressed, as was also the case with miR-100 and miR-10a, whereas the expression of miR-198 and miR-145, considered as tumor suppressors, was significantly decreased in tumors. Ura et al. [172] performed a study comparing miRNAs in HBV- and HCV-associated HCC and have revealed that in HCV-related HCC, the differentially deregulated miRNAs are linked to the regulation of the immune response, antigen presentation, cell cycle, or lipid metabolism, while in HBV-associated HCC, they are rather involved in the pathways regulating cell death, DNA damage or signal transduction. Bandiera et al. [173]

performed a profiling analysis and have revealed 72 miRNAs to be deregulated more than two-fold in HCC. They further focused on miR-146a-5p, which positively influences the HCV replication and increases HCV infection. Finally, a comprehensive study was done by Pineau et al. [174], who analyzed 104 HCC cases, 90 cirrhotic livers, 21 normal tissues and 35 HCC cell lines, and have identified 12 miRNAs (miR-106b, miR-21, miR-210, miR-221, miR-222, miR-224, miR-34a, miR-425, miR-519a, miR-93, miR-96 and let-7c) as linked to disease progression and tumorigenesis, with four of them (miR-21, miR-221, miR-222 and miR-224) being previously reported as deregulated in HCC.

8. Retroviruses

Retroviruses are a group of single-stranded RNA viruses that use their own encoded reverse transcriptase to produce DNA intermediate from their RNA genome, and then the virus integrates into the genome of the host and is transcribed and translated along the cellular genes. Retroviruses include tumor-associated viruses infecting animals, such as Rous sarcoma virus or mouse mammary tumor virus (MMTV). The malignant transformation is caused by viral proto-oncogenes or through the disruption or activation of cellular proto-oncogenes. The most important tumorigenic retrovirus of humans is human T-cell lymphotropic virus 1 (HTLV-1) from the genus *Deltavirus*, which is linked to the type of lymphocytic leukemia and non-Hodgkin lymphoma called adult T-cell leukemia/lymphoma (ATL). HTLV-1 was responsible for only 0.1% of new cancer cases attributable to infectious agents in 2012, with around 3000 new cases reported worldwide [1]. HTLV-1 encodes the oncogenic protein Tax, which interacts with more than one hundred cellular proteins and prevents apoptosis, enhances cell signaling, induces cell cycle dysregulation, or activates cellular proto-oncogenes. The second important oncogenic protein is HTLV-1 bZIP factor (HBZ), which is present in 100% of ATL cells, enhances T-cell proliferation, and contributes to the prevention of apoptosis [175]. The human immunodeficiency virus (HIV), a member of the genus *Lentivirus*, causes acquired immune deficiency syndrome (AIDS). This virus does not appear to induce cancer directly, but increases the risk of the development of other viruses-related tumors, such as Kaposi's sarcoma, cervical cancer, or non-Hodgkin lymphoma.

There are several studies focused on cellular miRNA targets in the retroviral genome and the influence of their binding on viral replication, gene expression, or infectivity. Huang et al. [176] have shown that the 3′ end of the HIV mRNA in resting CD4+ T-cells is targeted by cellular miR-28, miR-125b, miR-150, miR-223 or miR-382, and contributes to HIV-1 latency. Two computational studies were conducted to find potential target sites of cellular miRNAs in HTLV-1 [177,178]. Bai and Nicot [179] have found that miR-28-3p inhibits HTLV-1 replication and expression by targeting a specific site within gag/pol in viral mRNA, and have identified a mechanism of how cellular miRNA prevents viral transmission. Besides the direct targeting of the viral genome, viral replication can also be influenced by cellular miRNAs indirectly through host-dependency factors (HDFs) [180]. For example, miR-20a and miR-17-5p target the p300/CBP-associated factor important for long terminal repeat (LTR) activation, and the upregulation of these miRNAs reduces HIV-1 replication [181]. Further, the overexpression of miR-198 and miR-27b leads to the repression of cyclin-T1, a cofactor of Tat, and thus to HIV inhibition [182,183]. On the other hand, miR-217 and miR-34a target sirtuin 1 (SIRT1), which disrupts LTR activation by Tat, leading to HIV transactivation [184].

Similarly to other oncoviruses, retroviruses also dysregulate the expression of cellular miRNAs. Van Duyne et al. [185] have shown that HTLV-1 infection significantly downregulates Drosha protein expression, which is required for the cleavage of pri-miRNA, by direct interaction with the Tax protein, and thus deregulates the miRNA biogenesis pathway. Several studies have characterized the miRNA expression profiles in HTLV-1-transformed cell lines or ATL patients. Pichler et al. [186] have demonstrated deregulation of five miRNAs (miR-21, miR-24, miR-146a, miR-155 and miR-223) in HTLV-1-transformed T-cells. Moreover, they have found that miR-146a is transactivated directly by the Tax protein via NF-κB signaling, and its upregulation promotes HTLV-1 T-cell proliferation. The analysis of miRNA expression of HTLV-1 transformed T-cell lines and ATL patients has revealed six miRNAs to be consistently upregulated, with two of them (miR-93 and miR-130b) targeting the 3′

UTR of tumor suppressor protein TP53INP1 (TP53-induced nuclear protein 1), impacting proliferation and survival of HTLV-1-transformed/infected cells [187]. Bellon et al. [188] analyzed the miRNA profiles of ATL patients with microarrays, reporting deregulation of several miRNAs. However, they have also observed that two miRNAs (miR-150 and miR-223) are differentially expressed, both in vitro and ex vivo. Similarly, Yamagishi et al. [189] performed microarray analysis of ATL cells from patients and, interestingly, have revealed downregulation of 59 miRNAs out of 61, with miR-31 being the most repressed. MiR-31 is reported as a tumor suppressor regulating the NF-κB signaling pathway, and its deregulation leads to resistance of cells to apoptosis. The cellular miRNA expression can also be influenced by another retroviral oncoprotein, HBZ, which activates miR-17 and miR-21 in CD4+ T-cells [190]. These miRNAs target DNA-damage factor OBFC2A and thus promote cell proliferation and genomic instability.

9. Controversial Oncoviral-Encoded miRNAs

Besides approved and validated oncoviral-encoded miRNAs, the existence of viral-specific miRNAs from other groups of human oncoviruses has been reported in the literature. However, for these miRNAs, the evidence of their miRNA biogenesis as well as their function and clinical significance is not in the literature adequately supported. Therefore, further research is needed.

The existence of HBV-encoded miRNAs was not experimentally confirmed until recently. There was only one study, that of Jin et al. [191], who analyzed candidates for viral-encoded miRNAs in silico. They found only one pre-miRNA candidate for which one target viral mRNA was revealed but was not confirmed in vivo. No cellular mRNA was found as a target of this predicted viral miRNA. Recently, Yang et al. [192] performed a study of HBV-encoded miRNAs by deep sequencing and Northern blotting. Although they did not find the previously computationally predicted miRNA [191], they identified five small noncoding RNAs (snRNAs) aligning to HBV transcripts, and validated a novel HBV-miR-3, which they suggested, is involved in the viral replication process and represses HBV protein expression and virion production, probably contributing to the establishment of persistent infection. However, the number of reads obtained by deep sequencing in their study is not compelling, since it makes up only <0.0004% of the standard number of NGS reads. Moreover, the predicted secondary structure of pre-miRNA also does not fulfil the criteria for authentic viral miRNAs and natural substrate for enzymes of miRNAs biogenesis. Furthermore, they showed that the biogenesis of this HBV-encoded snRNA proceeds via the classical Dicer and Drosha route [192]. This is contradictory to results published by Wang et al. [193], who demonstrated that HBV-derived snRNAs are not processed by Dicer. Additionally, several studies analyzed the expression of snRNAs in hepatocellular carcinomas associated with HBV by deep sequencing [162,194–196]. Even though these studies were focused primarily on cellular miRNAs, we assume that any HBV viral miRNAs markedly differentially expressed would have been noticed in these studies. Thus, these controversial data highlight the fact that further research on HBV-encoded miRNAs is needed.

The second group of oncoviruses with not-definitely-approved viral-encoded miRNAs are HPVs. It is generally accepted that HPVs do not encode their own miRNAs; nevertheless, results of two research groups have suggested the existence of HPV-encoded miRNAs. First, the group of Auvinen has reported the identification and validation of the first papillomavirus-encoded miRNAs in human cervical lesions and in cell lines using SOLiD sequencing, quantitative RT–PCR, and in-situ hybridization [197]. They have successfully validated four miRNAs, and based on the prediction of target genes, they have suggested their role in cell cycle regulation, immune functions, cell adhesion and migration, development, and cancer. Several years later, the same group performed the analysis of the reported miRNAs in cervical samples by quantitative RT–PCR, and they detected low levels of expression of these miRNAs in all cases [198]. Finally, Weng et al. [199] have developed a systematic method for viral miRNA identification and regulatory network construction based on genome-wide sequence analysis, and have predicted other putative miRNAs by bioinformatics approaches. The target genes of these predicted miRNAs play roles in virus infection and carcinogenesis and

might be possible targets for antiviral drugs. However, no studies of in-vivo function of the predicted HPV-specific miRNAs have been published so far.

The topic of miRNAs encoded by retroviruses is also still quite controversial. One of the reasons is the risk of cleavage of the viral RNA genome with Rnase Drosha during the biogenesis of miRNAs. However, two groups have reported studies of in-vitro Dicer processing of ~50-nt long HIV-1 TAR RNA, which forms a stem–loop structure similar to pre-miRNA [200,201]. Moreover, Rouha et al. [202] have revealed that miRNAs might be produced by RNA viruses replicating in the cytoplasm without impairing viral RNA replication. Recently, Harwig et al. [203] suggested that the HIV-1 TAR hairpin structure could be a source of miRNAs without cleavage of the RNA genome, however, the biogenesis differ from the canonical miRNA pathway. Currently, the miRNA database (available online: http://mirbase.org) indicates that HIV-1 encodes three precursors of miRNAs and four mature miRNAs, however, they should be referred to as small noncoding RNAs (snRNAs) since there is a lack of significant experimental support demonstrating that these are functional miRNAs that arise from a stable hairpin RNA structure and are processed by the classical miRNA biogenesis pathway. Bernard et al. [204] have reported that HIV-1 produces the viral miRNAs vmiR-88, vmiR-99 and vmiR-TAR, with the first two stimulating human macrophage TNFα release and contributing to chronic immune activation; however, in-vivo confirmation of their in-vitro results remains to be determined. Moreover, Li et al. [205] have detected vmiR-TAR-3p in primary macrophages infected by HIV, and have shown its inhibitory effect on the viral replication. Klase et al. [206] and Ouellet et al. [207] have reported the anti-apoptotic role of vmiR-TAR-5p and vmiR-TAR-3p. Further, hiv1-miR-N367, described within the *nef* gene, targets the Nef protein for degradation, which may play a role in the establishment of viral latency [208,209]. Finally, hiv1-miR-H1, identified in 3′ LTR, targets the gene for apoptosis antagonizing transcription factor (AATF), leading to an increase in cell apoptosis [210], and hiv1-miR-H3 has been reported to target 5′ LTR within the TATA box increasing the promoter activity and positively regulating the viral replication [211]. Unlike for HIV, miRNAs encoded by HTLV-1 have not yet been identified. Despite these findings, various reports have been published where the authors failed to detect HIV-1 miRNAs, and/or the candidate miRNAs failed to satisfy all defined criteria for authentic viral miRNAs [155,212,213]. Lin et al. [212] demonstrated that neither HIV-1 nor HTLV-1 express significant levels of small interfering RNAs (siRNAs) or miRNAs, as well as that they do not repress cellular RNA interference machinery in the infected cells. This was supported by Whisnant et al. [213], who performed deep sequencing analysis of HIV-1 infected cell cultures and revealed only very few reads aligning to the viral genome. In contrast to reads aligning to the human genome that peak at ~22 nt, viral reads showed more reads at smaller size. Moreover, matching of HIV-1-derived snRNAs to the proviral genome was scattered over the proviral sequence, and their 5′ ends did not share the parameters characteristic for the authentic miRNAs. The authors also refuted the TAR-derived miRNAs that are mentioned above [206,207], since the TAR stem–loop differs from the authentic pre-miRNAs. Finally, they demonstrated [213] that HIV-1-derived snRNAs were not loaded into RNA-induced silencing complex (RISC), and further, they failed to detect a significant level of small RNA reads derived from the HIV-1 antisense strand, which was previously reported by Schopman et al. [214]. Similarly, Vongrad et al. [215], using high-throughput methods, failed to detect the incorporation of HIV-1-derived sncRNA or HIV-1 target sequences into the AGO2-RISC complex of RNAi pathway. However, it is worth mentioning the observation made by Kincaid et al. [216], who found out that bovine leukemia virus (BLV) from the family *Retroviridae* encodes a conserved cluster of miRNAs. They further show that one of these viral miRNAs shares a partial sequence with cellular miR-29, whose overexpression is associated with B-cell neoplasm. This type of malignancy resembles tumors initiated by BLV, and therefore, their findings suggest the possible way how this BLV-associated miRNA can contribute to tumorigenesis. Therefore, despite the numerous studies that support the nonexistence of HIV-1-specific miRNAs, the findings of bovine leukemia virus-specific miRNAs pointed to the importance of further research in this area.

10. Concluding Remarks

Carcinogenic infections are the cause of 15% of tumors worldwide. Tumors related to oncoviruses are studied extensively with the aim to detect new diagnostic and prognostic markers and treatment targets. This review provides a comprehensive overview on the mechanisms of the contribution of human oncoviruses to tumor development, and describes how oncoviruses utilize their own encoded sequences, miRNAs, for the regulation of their gene expression, as well as to influence the gene expression of their hosts (summarized in Table 1 and Figure 1). Besides the expression and function of miRNAs encoded by some groups of oncoviruses, the existence of virally coded miRNAs in several groups of human oncoviruses remains controversial. However, the miRNA regulation is reciprocal and the viral life cycle can also be influenced by cellular miRNAs. In this review, we summarize the accumulated body of knowledge in this area and focus on the oncogenesis mediated by EBV, HHV-8, HBV, HPV, MCPyV, HCV, HIV and HTLV-1. The research into miRNA profiling to set a panel of diagnostic miRNAs for specific tumors is of high relevance, and there have been already developed cancer focus panels with differentially expressed miRNAs for breast cancer, prostate cancer or lung cancer. MiRNAs could be a promising tool for early diagnosis of virus-related tumors and their noninvasive treatment. Currently, some miRNA-based therapies are being tested in preclinical and clinical trials, for example, miR-122 in HCV infection, miR-34 in liver cancer, or miR-208 in cardiometabolic disease [217]. Despite these achievements, additional research is needed for a more precise understanding of the miRNA pathways, including those that are virus associated.

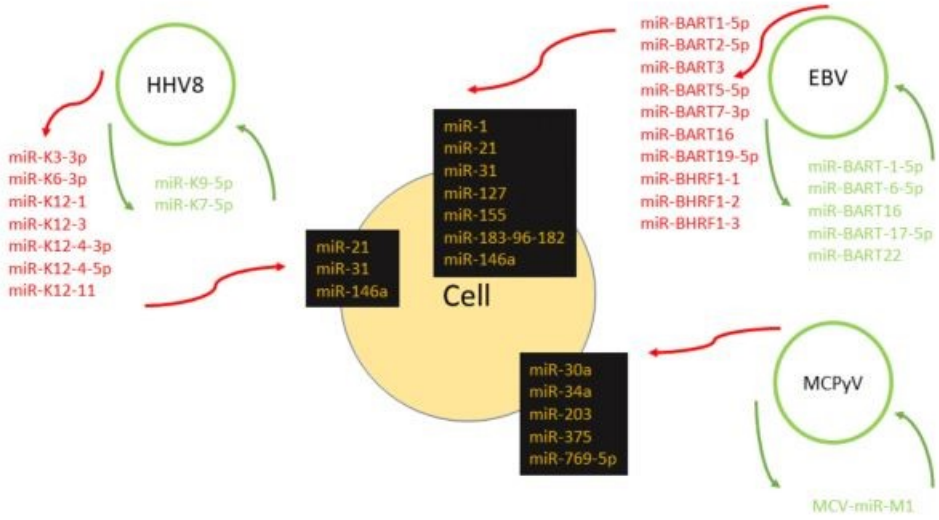

Figure 1. Viral miRNAs of Epstein–Barr virus (EBV), human herpesvirus 8 (HHV8) and Merkel cell polyomavirus (MCPyV), and cell miRNAs whose expression is influenced by the viral infection. The deregulation of these miRNAs contributes to the transformation of the cell and to tumor development. Green = viral miRNAs that target viral mRNAs, red = viral miRNAs that target cellular mRNAs, and yellow = cellular miRNAs influenced by the viral infection.

Table 1. Summary of authentic viral-encoded miRNAs mentioned in the review, and their viral and cellular targets.

Virus Family	Virus Species	Mature miRNAs (According to miRBase, Updated 2014)	MiRNAs Mentioned in This Review	Proposed Function	Target	Reference
Herpesviridae	Epstein–Barr virus (EBV)	44	miR-BART17-5p	cell transformation	LMP1	[25]
			miR-BART16	cell transformation, anti-apoptotic role	LMP1, Casp3	[25,31]
			miR-BART1-5p		LMP1, Casp3	[25,31]
			miR-BART5-5p		PUMA	[29]
			miR-BART19-5p		PUMA	[29]
			miR-BART22	escape from host immune surveillance	LMP2A	[26]
			miR-BART2-5p	regulation of latent–lytic switch, evasion of the host's immune system	BALF5, MICB	[27,36]
			miR-BART6-5p	regulation of viral replication	EBNA2	[28]
			miR-BART7-3p	promotion of EMT and metastasis, regulation of radiation sensitivity	PTEN, GFPT1	[30,38]
			miR-BART3	proliferation and cell transformation	DICE1	[32]
			miR-BHRF1-1	immunomodulatory function	CXCL11	[33]
			miR-BHRF1-2		CXCL11	
			miR-BHRF1-3		CXCL11	
	Herpesvirus-8 (HHV-8)/Kaposi's sarcoma herpesvirus (KSHV)	25	miR-K9-5p	regulation of lytic induction	RTA	[54,55]
			miR-K7-5p			
			miR-K3	regulation of viral latency and angiogenesis	nuclear factor I/B, GRK2, THBS1	[56,62,63]
			miR-K12-11		MYB, IKKε, THBS1	[58,60,62]
			miR-K12-4	regulation of viral latency, anti-apoptotic role	Rbl2, Casp3	[61,67]
			miR-K6-3p	regulation of angiogenesis	THBS1, SH3BGR	[62,63]
			miR-K12-1	anti-apoptotic role, regulation of angiogenesis	p21, Casp3, THBS1	[62,66,67]
Polyomaviridae	Merkel cell polyomavirus (MCPyV)	1	MCV-miR-M1	regulation of viral lifecycle	early viral transcripts	[145]

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Glossary

Immortalization of the host cell	continual proliferation of the cell mostly caused by mutation or by the activity of the viral oncogenes
Transformation of the host cell	morphologic, physiologic and genetic changes of the cell initiating a process of tumor development and progression

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