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Clinical Biochemistry

Epigenetic Alternations in Head and Neck Squamous Cell Carcinoma: Potential Biomarkers

Epigenetické alterace u dlaždicobuněčných nádorů hlavy a krku: potenciální biomarkery

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Hradec Králové, 2019

Author ‘s Declaration

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I declare hereby that this dissertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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1 Summary CZE

Dlaždicobuněčné nádory hlavy a krku jsou skupinou heterogenních tumorů vznikajících v epiteliálních buňkách aerodigestivního traktu a jsou charakteristické složitou diagnostikou, léčbou a prognózou onemocnění. Konzumace alkoholu, kouření a infekce vysoce rizikovými lidskými papillomaviry jsou velmi dobře popsané rizikové faktory výskytu nádorů hlavy a krku. Sinonasální karcinomy jsou skupinou nádorů vznikajících v dutině nosní a vedlejších dutinách nosních. Karcinomy orofaryngu jsou nádory vznikající v oblasti krku za ústní dutinou.

mikroRNA (miRNA) jsou krátké (~23 nukleotidů) nekódující molekuly RNA účastnící se regulace genové exprese. Primární rolí mikroRNA molekul je negativní regulace translace jako součást komplexu RISC (komplex umlčující geny indukovaný RNA) pomocí represe translace a mRNA degradace. miRNA se účastní regulace procesů u mnoha onemocnění, jako jsou neurodegenerativní choroby, metabolické poruchy a malignity. Kromě toho jsou miRNA rozeznávány jako klíčové molekuly účastnící se kancerogeneze a progrese u různých typů nádorových onemocnění.

Ve své výzkumné práci v rámci doktorského studia jsem se zaměřila na výzkum relativní exprese několika dříve vytipovaných mikroRNA s využitím real-time PCR. A dále jsme zkoumali korelaci jejich expresních profilů se zaznamenanými klinickopatologickými daty pacientů se sinonasálním dlaždicobuněčným karcinomem a orofaryngeálním dlaždicobuněčným karcinomem. Zkoumané mikroRNA byly vybrány na základě literární rešerše zaměřené na ostatní typy dlaždicobuněčných karcinomů hlavy a krku (v případě studie u sinonasálních karcinomů) a na základě výsledků dvou vysoce-výkonných metod (miRNA microarray a sekvenování nové generace malých RNA) ve fázi I studie zaměřené na karcinom orofaryngu. Na základě výsledků exprese miRNA a následné statistické analýzy, jsem došla k závěru, že miR-9 (mimo dalších miRNA) by mohla být dobrým potenciálním biomarkerem sinonasálního dlaždicobuněčného karcinomu a miR-150-5p, mimo dalších miRNA, by mohly být dobrým potenciálním biomarkerem orofaryngeálního dlaždicobuněčného karcinomu.

2 Summary ENG

Head and neck squamous cell carcinomas are a group of heterogenic tumors arising from epithelial tissue of aerodigestive tract characterized by difficult diagnosis, treatment and prognosis. Alcohol consumption, smoking and high-risk human papillomavirus infection are very well described risk factors of head and neck cancer development. Sinonasal carcinomas are group of malignancies developing in nasal and paranasal sinuses. Oropharyngeal carcinomas are malignancies developing in the throat area downstream to oral cavity.

microRNAs (miRNAs) are short (~23 nucleotides) non-coding RNA molecules participating in regulation of gene expression. Primary function of miRNAs is negative translation regulation as part of RISC (RNA-induced silencing complex) by translational repression and mRNA degradation. miRNAs are involved in various disease pathologies such as neurodegenerative diseases, metabolic disorders and cancer. Moreover, microRNAs have been recognized as key molecules in cancer development and progression in various types of tumors.

My doctoral study research activities were focused on investigation of relative expression of several preselected miRNAs (using real-time PCR) and the relationship between their expression and clinicopathological characteristics of the squamous cell carcinoma and oropharyngeal carcinoma patients. miRNAs of interest for this study were selected based on literary review focused on other types of squamous cell head and neck cancer (sinonasal cancer study) and two high-throughput miRNA expression methods (miRNA microarray and small RNA Next-generation sequencing) in the phase I of the oropharyngeal cancer study. Based on miRNA expression results and statistical analysis, I have concluded that (among others) miR-9 might be the best potential biomarkers for sinonasal squamous cell carcinoma and miR-150-5p (among others) may be the best potential biomarker for oropharyngeal squamous cell carcinoma.

3 Used abbreviations

AC	Adenocarcinoma
ac-pre-mir-451	Ago-cleaved pre-mir-451
AdCC	Adenoid cystic carcinoma
Ago2	Argonaute2
AUC	Area under the Receiver operating characteristics curve
BCL	B-cell lymphoma
C00-C14	Malignant neoplasm of lip, base of tongue, other and unspecified parts of tongue, gum, floor of mouth, palate, other and unspecified parts of mouth, parotid gland, other and unspecified major salivary glands, tonsil, oropharynx, nasopharynx, pyriform sinus, hypopharynx, other and ill-defined sites in the lip, oral cavity and pharynx
C30 – C32	Malignant neoplasm of nasal cavity and middle ear, accessory sinuses and larynx
C30.0	Malignant neoplasm of nasal cavity
C31	Malignant neoplasm of accessory sinuses
CDDP	Cis-diaminodichlorplatine
CDK6	Cyclin-D kinase-6
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CpG	Cytosine-guanine dinucleotide
CSCs	Cancer stem cells
DE	Differential expression
DGCR8	DiGeorge Syndrome Critical Region 8 protein
DMSO	Dimethylsulfoxid
DNMT	DNA methyltransferase
dsDNA	Double-stranded DNA molecule
EBV	Epstein Barr virus
EMT	Epithelial-mesenchymal transition

epi-miRNAs	miRNAs targeting epigenetic machinery
ER	Error rate
Exp5	Exportin-5
HNC	Head and Neck Cancer
HNSCC	Head and neck squamous cell carcinoma
HPV-	HPV negative
HPV	Human papillomavirus
HPV+	HPV positive
HR-HPV	High-risk Human Papillomavirus
IMRT	Intensity-modulated radiation therapy
ITAC	Intestinal-type adenocarcinoma
LEC	Lymphoepithelial carcinoma
miRBase	microRNA Registry
miRNA	microRNAs
miRNome	microRNA expression signature profile
moRs	microRNA-offset RNAs
mRNA	Messenger RNA
MS-HRM	Methylation-sensitive high-resolution melting
NGS	Next-generation sequencing
NTC	No template control
NUT	Nuclear protein in testis
NUT	Nuclear protein in testis gene
oncomiRs	Oncogene miRNAs
OPSCC	Oropharyngeal squamous cell carcinoma
PARN	Poly(A)-specific ribonuclease
precursor miRNA	pre-miRNA
pri-miRNA	Primary miRNA transcript
pRNA	Piwi-interacting RNA
Rb	Retinoblastoma
RIN	RNA integrity number
RISC	RNA-induced silencing complex

ROC	Receiver operating characteristics
rRNA	Ribosomal RNA
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine;
SCC	Squamous cell carcinoma
SCNEC	Small cell neuroendocrine carcinoma
SE	Sensitivity
shRNA	Short hairpin RNAs
SMARCB1/INI1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1/integrase interactor 1
SNAC	Non-intestinal type adenocarcinoma
SNC	Sinonasal Carcinoma
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SNUC	Sinonasal undifferentiated carcinoma
SP	Specificity
sRNA	Small RNA
SSCC	Sinonasal squamous cell carcinoma
TC	Typical carcinoid
TCGA	Cancer Genome Atlas
TE	Transposable element
TRBP	Transactivating response RNA-binding protein
tRNA	Transfer RNA
VMAT	Volumetric modulated arc therapy
WHO	World Health Organization

4 Background

4.1 Head and Neck Cancer

Head and neck cancer (HNC) is a group of heterogenic tumors arising from epithelial tissue of aerodigestive tract including cancers of the lip, tongue oral cavity and gum, nasal cavity, nasal sinuses, salivary gland, nasopharynx, oropharynx, hypopharynx and larynx (Figure 1). Head and neck cancers are predominantly squamous cell carcinomas (HNSCC), which make up to 90 % of head and neck cancers worldwide. Squamous cell carcinoma (SCC) is also the most commonly researched subtype of HNC [1].

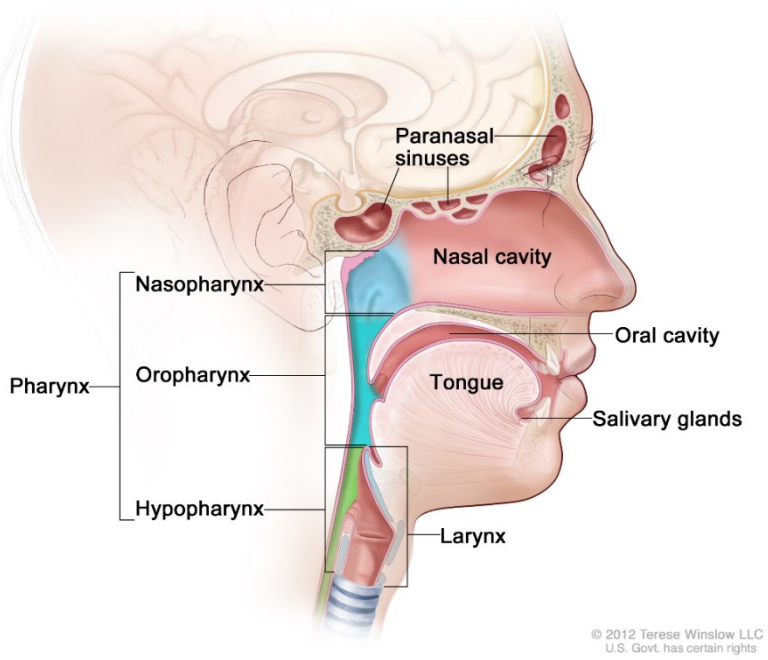

Figure 1. Anatomical subsites of Head and Neck Cancer [2].

Alcohol consumption and smoking are risk factors for developing head and neck cancer especially in oral cavity and oropharynx. Human papillomaviral (HPV) infection dramatically increases the risk of oropharyngeal carcinoma and oral cancer [3]. The first choice of treatment of HNC in early stages is usually a surgery usually combined with radiotherapy. Whereas, in later stages of the disease combination

of surgery with post-operational radiotherapy and radical chemotherapy using cis-platin derivate is recommended [4].

Worldwide, there are more than 550,000 of new cases of head and neck cancer diagnosed annually and 380,000 HNC patients die every year [2]. HNCs more commonly occur in men than in women in 2:1 to 4:1 ratio [5]. The highest prevalence of HNC is in developing countries especially in South-East Asia, which has been linked to popularity of chewing betel nuts in the region [6]. Five-year survival of HNC patients is 40 % and it is dependent upon the original anatomical site affected, stage and HPV status of the tumor [7; 8]. The incidence of these tumors is in the Czech Republic 21.27/100,000 and mortality rate is 10.38/100,000 (the latest data available is from 2016) (we included following diagnosis to the analysis: C00 – C14, C30 – C32) [9].

4.1.1 Sinonasal cancer

Sinonasal carcinomas (SNC) are group of malignancies developing in nasal and paranasal sinuses which make up 3 % to 5 % of all head and neck cancers and 1 % of all kinds of tumors [10; 11]. Half of the cases are located in a nasal cavity, while other tumors originate in maxillary and ethmoid sinuses. Finally, tumors in frontal and sphenoid sinuses are rare (Figure 2). The most common subtype of sinonasal cancer is sinonasal squamous cell carcinoma (SSCC), although it represents smaller proportion of cases than in other types of head and neck cancers (65 – 75%). SSCC are further classified as non-keratinizing or keratinizing, when non-keratinizing tumors are more likely to be linked to high-risk HPV (HR-HPV) infection. Other squamous cell tumors of this site can be classified as basaloid SCC, papillary SCC or spindle cell carcinoma. Lymphoepithelial carcinoma has been linked to Epstein Barr virus (EBV) infection of the tissue [12].

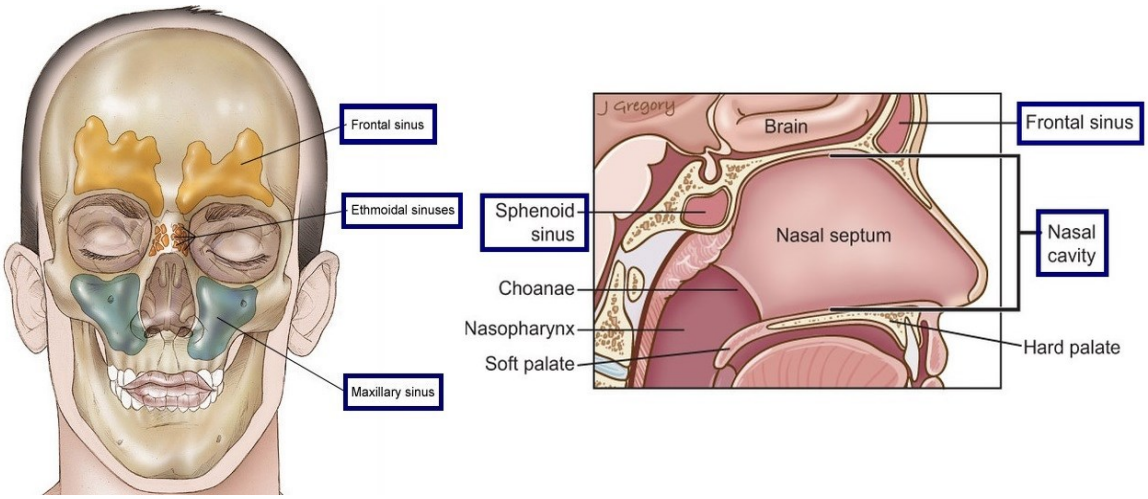

Figure 2. Sinonasal carcinoma regions [13], adjusted.
Areas, where sinonasal cancer can arise are highlighted by blue boxes.

Other types of carcinomas are intestinal-type adenocarcinomas (ITACs) and non-intestinal sinonasal adenocarcinomas (SNACs), which has been strongly associated with wood-dust exposure. Sinonasal undifferentiated carcinoma (SNUC) is not a very common sinonasal tumor, which is diagnosed by exclusion of neuroendocrine carcinomas including small cell carcinoma and large cell neuroendocrine tumors. In the newest version of 2017 WHO classification system of tumors new categories of tumors were described. NUT midline carcinoma (NMC) is defined by rearrangement of the *NUT* (nuclear protein in testis) gene and in many cases, it arises from sinonasal tract mediastinum [14; 15]. SMARCB1 (INI-1) deficient sinonasal carcinoma is characterized by loss of expression of SMARCB1/(INI-1) protein [16] (Table 1).

Table 1. Summarization of SNC subtypes and their characteristics.

Tumor type	Subtype	Characterization	Association
Squamous cell carcinoma	Keratinizing		
	Non-keratinizing		HR-HPV
	Basaloid		HR-HPV
	Papillary		HR-HPV
	Spindle cell carcinoma		
	Lymphoepithelial carcinoma		EBV
	Intestinal-type adenocarcinoma		Wood-dust exposure
Adenocarcinomas	Non-intestinal type adenocarcinoma	Low-grade (papillary, tubular)	
		High-grade (Sinonasal renal cell-like adenocarcinoma)	
Neuroendocrine carcinomas	Small cell carcinoma		HR-HPV
	Large cell neuroendocrine carcinoma		HR-HPV
Sinonasal undifferentiated carcinoma		*RB-1* gene mutation	
NUT carcinoma		Rearrangement of the NUT gene	
SMARCB1/INI-1 carcinoma		Loss of expression of SMARCB1/INI-1	

EBV, Epstein Barr virus; HR-HPV, High-risk Human Papilloma Virus; *NUT,* nuclear protein in testis.

Incidence of SNC (diagnosis codes C30.0 and C31) is less than 5 % in a million. Specifically, in the Czech Republic in 2016 the incidence was 0.5/100,000 and mortality was 0,23/100,000. The tumors are more common in men than women, which is caused by increased risk of sinonasal cancer development due to risk of professional exposure in predominantly male occupations. The tumors usually appear in advanced age (mean; approximately 62 years of age) [17]. Even though aggressive combined treatment is usually used, survival median is less than 18 months and 5-year survival rate is only 20 % — 30 % [18]. Early symptoms of the disease are rhinorrhea, epistaxis, epiphora, and nasal obstruction. Advanced lesion symptoms include blurred vision, diplopia or proptosis [19].

Thanks to non-specific symptoms, patients often develop highly advanced tumors with extensive invasion to neighboring tissues such as eyes, optic nerves and chiasm.

Due to complexity of the region and proximity of several important structures treatment of the tumors is challenging and it is often necessary to utilize multimodality therapeutic approach. The treatment is usually based on radical surgical resection with postoperative radiotherapy. Treatment effects have improved due to new endoscopic surgical approaches and involvement of new radiation techniques such as IMRT, volumetric modulated arc therapy (VMAT) and proton or heavy ion therapy that allow targeted high dose of radiation. Neo-adjuvant (induction) chemotherapy with cis-platin derivate can be used to reduce the tumor size prior to surgery [20].

One of the risk factors involved in SNC carcinogenesis is cigarette smoking. Specifically, cigarette smoke inhalation has the largest impact on squamous cell sinonasal carcinoma [21]. The influence of HPV infection on SNC carcinogenesis has been studied recently. Approximately 20 – 30 % of SNC tumors harbor transcriptionally active HPV infection, which is a lower proportion in comparison to other head and neck tumors [22]. High risk HPV type 16 is the most aggressive type which has been found in about 90 % of HPV+ cases of SSCC. Patients with HPV+ tumors have much better prognosis than patients with HPV- tumors [23-25].

Professional exposure to specific cancerogenous substances (such as wood-dust, leather-dust and formaldehyde) is another established risk factor for SNC development [26]. Dangerous professions include wood workers (cabinetmakers, carvers or carpenters), who often inhale wood-dust. Mechanism of SNC development due to wood-dust (especially from hard wood types) have been clarified studying ITACs. Hard wood-dust inhalation causes chronic inflammation in sinonasal area, which leads to *TP53* mutagenesis and subsequently to carcinogenesis in the area [27]. Moreover, in danger of SNC development are leather industry workers (shoe manufacturers and tanner workers) due to leather-dust inhalation, individuals exposed to nickel compounds

in electrolytic nickel refining and alkaline battery manufacturers or employees exposed to formaldehyde [21; 28].

4.1.2 Oropharyngeal cancer

Malignancies developing in the throat area downstream to oral cavity are called oropharyngeal cancers. Oropharynx is located between the soft palate and hyoid bone and it consists of base of the tongue, tonsillar region, soft palate and uvula and posterior and lateral pharyngeal walls (Figure 3). Tonsils are the most common subsites of the disease followed by base of the tongue. Patients with oropharyngeal cancer might manifest symptoms such as persistent sore throat, odynophagia, otalgia, dysphagia or globus sensation [29; 30]. Almost all cases of oropharyngeal cancer are squamous cell carcinomas (OPSCC), other subtypes such as adenocarcinomas are rare.

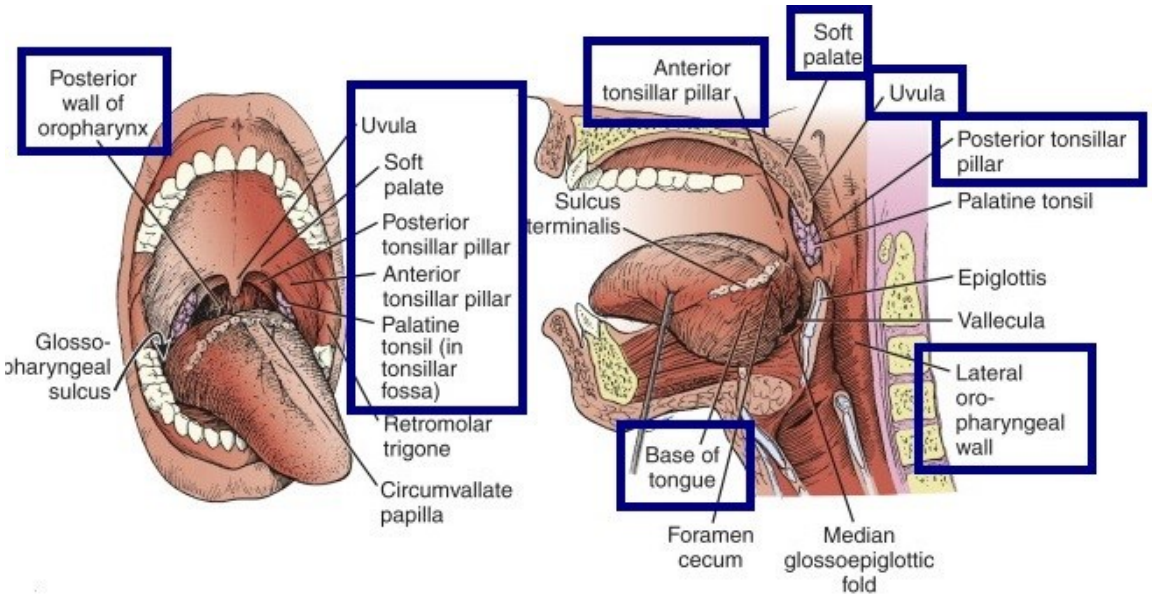

Figure 3. Anatomy subsites of oropharyngeal cancer [31], adjusted. Oropharyngeal cancer sites of origin are highlighted by blue boxes.

Most of the oropharyngeal squamous cell carcinoma tumors develop from premalignant lesions such as leukoplakia, erythroplakia and oral submucous fibrosis that can be often asymptomatic [32]. Prevalence of OPSCC is on the rise

globally (especially in the US and Western countries), while incidences of other types of head and neck cancer are decreasing. Traditional risk factors include excessive tobacco smoke and alcohol exposure (with multiplicative effect) and low socioeconomic status [32; 33]. In the "high tobacco consumption era" the incidence of oropharyngeal cancer was similar to other malignancies of upper aerodigestive tract and the occurrence of comorbidities was high within the patients [34]. Recently oncogenic human papillomavirus, especially p-16, has emerged as another factor of oropharyngeal carcinogenesis. Particularly tumors developing in the base of the tongue and tonsils are mostly HPV+, which is caused by microanatomy of the reticulated epithelium lining that contains immune system component. Consequently, it can harbor HPV antigens without causing immune inflammatory response [34].

The incidence of oropharyngeal cancer (diagnosis codes C09, C10) in the Czech Republic (estimated data for 2019) is 5.2/100,000 with rising tendency and mortality 1.8/100,000, which is above European average [35]. Patients with HPV negative tumors are usually older (mean; 66 years of age) and have a history of tobacco and alcohol abuse. On the other hand, patients with virally induced HPV (p16) tumors tend to be younger (mean; 59 years of age), have small primary tumors with extensive nodal involvement and may have history of dangerous sexual behavior [34]. However, they have better prognosis than patients with HPV negative tumors. Frequently, neck nodal metastases without clinically obvious primary lesions are present. Nodal metastases are frequently large with pushing borders and expansion of the lymph nodes rather than extensive infiltration of the surrounding tissue [14].

Decline in number of head and neck cancers has been attributed to decline in alcohol and tobacco use in society. Conversely, it has been concluded that increasing number of OPSCC cases might correlate with rising incidence of virally induced HPV cancers and changes in sexual trends (especially oral sex popularity) [36]. Since HPV positive tumors show good response to treatment regardless what kind of therapy is used, it has been suggested that older versions of TNM classification are no longer sufficient for oropharyngeal tumor classification.

Therefore, HPV positive and HPV negative tumors are currently recognized as two clinically different entities [37-41]. Therapy of OPSCC in majority of the cases used to be limited to surgery with subsequent radiation therapy. Recently, new treatment approaches, such as immune therapy, have emerged. Advanced radiotherapy techniques and intensity-modulated radiation therapy (IMRT) allow more accurate dose delivery focused specifically on the tumor. HPV+ tumor treatment approaches have been de-intensified in response to better understanding of their biology. Surgical therapy can nowadays utilize state-of-the-art techniques such as robotic or laser surgery [34].

4.2 Epigenetic modifications

Epigenetic changes are defined as heritable changes in gene expression, which are not accompanied by DNA sequence modifications. They comprise of DNA methylation, covalent histone modification and posttranscriptional modifications by microRNAs (miRNAs).

Histone modifications are alterations in chromatin structure and they are caused by posttranslational modifications of histone proteins such as acetylation, methylation and phosphorylation. DNA in cell nucleus is packed in chromatin resulting in forming a dynamic structure consisting of nucleosomes. Nucleosomes are composed of 147-base pair segments of DNA wrapped around octamer of four core histone proteins (H3, H4, H2A and H2B) [42]. Acetylation of lysine residues in histone molecules is catalyzed by histone-acetyltransferases. This process leads to structural changes in nucleosomes, which enables transcription factors to access promotors of gene locuses, subsequently, leading to transcription initiation. Acetylation is a reversible process which is catalyzed by enzymes called histone-deacetylases. Proper function of posttranslational histone modifications is necessary for proper function of developmental processes [43].

4.2.1 microRNAs

microRNAs are short (approximately 23 nucleotides long) non-coding RNA molecules participating in regulation of gene expression. The first evidence of their existence was published by Lee et al. in 1993 [44] as a result of experiments with *Caenorhabditis elegans*. All information about annotated mature miRNAs and their hairpin sequences are summarized in searchable online reference database miRbase ("microRNA registry"). The newest version (Release 22.1, October 2018) of miRbase contains 38,589 entries in total, from which 1,917 are human miRNA precursors and 2,654 human mature miRNA entries [45; 46]. Primary function of miRNAs is negative translation regulation as a part of RISC (RNA-induced silencing complex) by translational repression and mRNA degradation. mRNA decay is induced by endonucleolytic cleavage by RISC in case of perfect or nearly perfect base pairing of miRNAs to the targeted mRNA. However, mammalian miRNAs more often recognize the target mRNA by partial complementarity within the so called "seed region", which are nucleotides 2 – 7 or 2 – 8 of the miRNA. In this case of imperfect complementarity, mRNA is silenced by translational repression of additional effector proteins [47].

About half of the known miRNAs have been identified as intragenic, meaning they are embedded in the introns of non-coding genes and some exons of protein coding genes. The rest of the miRNAs are intergenic. They are transcribed independently of a host gene and transcription regulation is done by their own promoter. Some cluster miRNAs are transcribed as one long transcript prior to cleavage considered as a miRNA family. microRNAs can target up to several hundred mRNAs, which makes them powerful regulators in various pathways. They have been established as key molecular components of the cell in both normal and pathologic states [48]. Target mRNAs of known miRNAs were either experimentally verified (small fraction) or predicted by various algorithms. Algorithms based on conservation criteria are used by following tools: PicTar [49], Target Scan [50]. However, DIANA-microT [51], PITA [52] and RNA22 [53] use

other parameters such as free energy of secondary structures of 3' UTRs promoting or preventing miRNA binding [54].

4.2.1.1 microRNA biogenesis

The canonical pathway of miRNA biogenesis (Figure 4) starts with transcription of the appropriate sequence by RNA polymerase II resulting in creation of primary miRNA transcript (pri-miRNA) located in nucleus. Pri-miRNA is then cleaved by microprocessor complex of RNA binding protein DGCR8 (DiGeorge Syndrome Critical Region 8) and ribonuclease III enzyme Drosha forming a characteristic 70 nt long loop structure of precursor miRNA (pre-miRNA). This structure is transported by export factor Exportin-5 (Exp5) into cytoplasm, where second cleavage (loop removal) is performed by another ribonuclease II enzyme Dicer resulting in a mature miRNA duplex formation. Directionality of the processed miRNA strand determinates its mature name; 5p miRNAs originate from 5' end and 3p miRNAs are created from 3' strand. One of the duplex strands is joined to the RISC complex compiling of Argonaute2 protein (Ago2), Dicer and TRBP (transactivating response RNA-binding protein). In this complex miRNA is the key to mRNA target recognition during RNA interference. The other strand of mature miRNA is called the passenger strand, in the past labeled with *, and it is subsequently cleaved by Ago2 and degraded by the cell [55; 56].

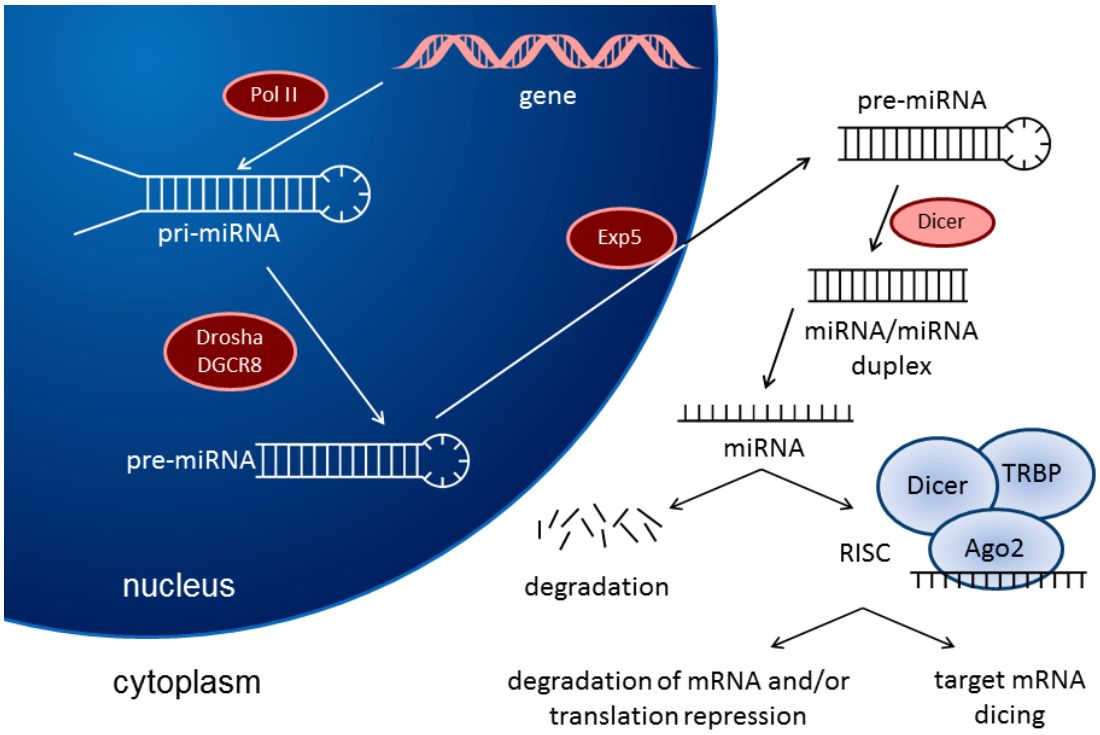

Figure 4. The canonical pathway of miRNA biogenesis.
Pol II, RNA polymerase II; pri-miRNA, primary miRNA transcript; DGCR8, DiGeorge Syndrome Critical Region 8 protein; pre-miRNA, precursor miRNA loop structure; Exp5, Exportin-5; RISC, RNA-induced silencing complex; TRBP, transactivating response RNA-binding protein; Ago2, Argonaute2 protein.

Furthermore, about 1 % of conserved miRNAs are produced by different non-canonical pathways (Figure 5), which have been recently described. Their structural and functional resemblance to canonical miRNAs was discovered thanks to development of deep sequencing techniques [57; 58]. Non-canonical miRNAs can have various origins such as mirtrons, snoRNAs (small nucleolar RNAs), endogenous shRNAs (short hairpin RNAs) and tRNAs and can be produced by Microprocessor-independent or Dicer-independent way. The first describe non-canonical pathway is mirtrons production. Mirtrons are produced from introns of mRNA genes [55]. In this pathway Drosha and DGCR8 mediated step is skipped and miRNA precursor is processed by mRNA splicing. After splicing of host mRNA, the assembly refolds into a short stem-loop pre-miRNA like structure.

Some mirtrons are characterized by having extra sequences at the 5' or 3' end, which are ultimately trimmed by exonucleases.

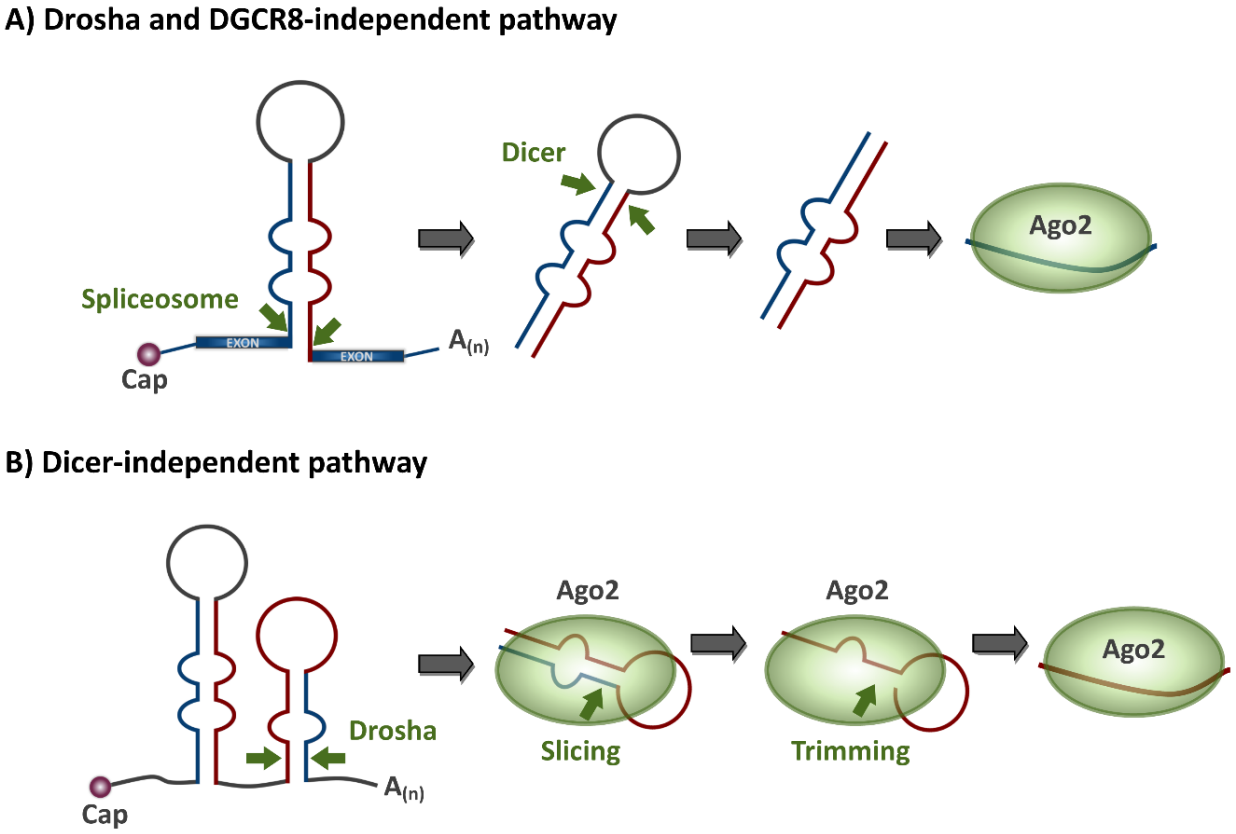

Figure 5. Non-canonical biogenesis of miRNAs.
A) Drosha and DGCR8-independent pathway, where mirtrons are first transcribed in the introns of genes and then spliced. Finally, pre-miRNAs are processed by Dicer and joined to the Ago2 protein ultimately forming RISC. B) Dicer-independent pathway of miRNA biogenesis. Pri-miRNAs are firstly cleaved by Drosha and then attached to Ago2, where they are cleaved and ultimately trimmed by PARN.
Cap, 5' Cap RNA modification; A(n), 3' Polyadenylation RNA modification; Ago2, Argonaute 2 protein; RISC, RNA induced silencing complex; PARN, Poly(A)-specific ribonuclease.

Small RNAs (sRNA) derived from shRNAs are generated through direct transcription and not cleaved by Drosha. However, they still depend on Dicer processing. In the other case of non-canonical (erythropoietic miR-451) biogenesis is independent on Dicer and it relies on catalytic activity of Ago2. Drosha-mediated

processing of pri-miR-451 creates short hairpin with stem of ~18 bp that is too short to be processed by Dicer. Consequently, pre-miR-451 is immediately attached to Ago2 and cut in the middle of its 3' strand leading to 30-nucleotide long Ago-cleaved pre-mir-451 (ac-pre-mir-451). Mature miR-451 (~23 nt in length) is produced by Poly(A)-specific ribonuclease PARN by trimming the 3' end of ac-pre-mir-451 (Figure 5) [57]. Non-canonical miRNAs have been linked to various pathological processes such as psoriasis, ischemic disease, diabetes mellitus or epithelial malignancies [58].

4.2.1.2 microRNA nomenclature

Continual improvement of deep sequencing techniques has brought increase in number of published miRNA reads obtained from next generation sequencing experiments. Some of the captured miRNAs and miRNA genes are indeed new, some of them have been previously described in other than human species. New sequencing approaches also put focus on isomiRs. They are variants of the same miRNA sequences derived from the same gene, but they vary in sequence due to posttranscriptional processing and may have different functions in cells. NGS has also exposed sequences called moRs (microRNA-offset RNAs), which are considered as byproducts of miRNA biosynthesis without any biological significance. Sequencing experiments have also revealed single strand RNA fragments originating in the loopRNA portion of pre-miRNA hairpin called loop-origin miRNAs (loRs). However, biological relevance of these variants is not fully understood yet [59; 60].

Nomenclature guidelines for novel miRNAs to be included in miRBase require experimentally confirmation by cloning or verification with evidence of expression and processing. New miRNAs always have sequential identifiers assigned. Information about the species are coded in three letter prefixes ("hsa" is code for human miRNA, "mmu" is mouse, etc.). The mature miRNA sequences are coded as "miR" and precursor miRNAs are labelled with "mir". The number codes for the same miRNAs in different species tend to be in preserved, as a result hsa-miR-101

in human and mmu-miR-101 in mouse are orthologues. Sequences with 1 or two nucleotide difference are called paralogues and are given letter suffixes (for example a, b and c). Mature miRNAs arising from the same hairpin loci are differentiated by number suffixes (-1 or -2). Moreover, two different mature miRNAs are named after the opposite arms of the same hairpin precursor (-5p suffix marking miRNAs originating from 5' end and -3p suffix a code for miRNAs originating at the 3' arm of the hairpin) [61; 62].

4.2.1.3 microRNAs and cancer

miRNAs are involved in various diseases such as neurodegenerative diseases, metabolic disorders and cancer. Connection between microRNA and cancer was first described by Calin et al. in 2002 [63]. They discovered, that *miR-15* and *miR-16* genes are localized on chromosome 13q14, which is an area frequently deleted (65 %) in patients with chronic lymphocytic leukemia (CLL). Deletion of the two miRNAs causes form of the disease non-responsive to treatment. Lu et. al [64] were first to describe that miRNA expression profile varies in different types of cancerous tissue. Moreover, they observed deregulation of some miRNAs when comparing tumor samples and control samples (today whole miRNA expression signature is known as miRNome). Finally, they discovered that expression of miRNAs varies even within the patients' group, so researchers might be able to predict progression of the disease and site of origin of the tumor. microRNAs regulate several cancer-linked processes such as cell proliferation, apoptosis, migration and invasion, angiogenesis, immune evasion, cell differentiation, formation of cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT) phenotype gain. miRNA profiles might able to distinguish not only between normal and cancerous tissue, but also between different subgroups of tumors, predict outcome and patient's survival or response to therapy or specific drug resistance.

microRNA expression mimics the expression of other cancer associated genes. Therefore, it can be altered by chromosomal amplification/deletion, promoter methylation and transcription factor activation. miRNAs in cancer can work

as oncomiRs (their upregulation is associated with cancer development) or tumor-suppressors (their negative regulation promotes carcinogenesis) [48]. Information about miRNAs involved in various types of cancer development are included in the Cancer Genome Atlas (TCGA) [65].

Recently, different expression of circulating miRNAs in biological fluids (such as plasma or serum, saliva, urine and amniotic fluid) of the patients was discovered, which suggests that miRNAs could be utilized as potential clinical biomarkers for diagnostics, treatment response prediction, remission and relapse prediction, monitoring and prognostic purposes. Using circulating miRNAs for diagnosis might precede conventional methods. Unprotected miRNAs are sensitive to degradation by RNases naturally occurring in blood. Therefore, circulating miRNAs can be bound to RNA-binding proteins, high density lipoproteins or encapsulated within extracellular vesicles (EVs) [66]. Ultimately microRNAs might be useful in cancer therapy by interfering with molecular mechanisms of tumorigenesis [67]. Various pre-clinical trials have shown that miRNAs have great potential as cancer therapeutics, however, no miRNAs have been approved for clinical use yet. After several pre-clinical studies were concluded as a success, miR-34a mimic replacement therapy development for clinical use started. In April 2013 miRNA mimic bounded in liposomes (MRX34) was the first miRNA-based drug to enter phase I clinical trial. In 2016 the trial was terminated due to immune-related severe side effects of the treatment in five patients [68].

4.2.1.4 microRNA and head and neck cancer

Current research on epigenetics and miRNA involvement in head and neck cancer biogenesis is mainly focused on the most prevalent tumor subtypes and subsites: squamous cell carcinoma of oral cavity, larynx and oropharynx. Most of the differentially expressed miRNAs in HNSCC could be divided into following categories: miRNAs related to tumor invasiveness and metastases development (for example miR-200 family), oncogenic miRNAs (oncomiRs) (miR-21 is the most studied and wildly deregulated miRNA in this category) and miRNAs

associated with HPV status or smoking (for example miR-9 and miR-145) [69]. Many teams investigate differentially expressed miRNAs in HNSCC compared to normal epithelial tissue. Deregulation of several miRNAs was consistently reported in several studies including: miR-21, miR-31, miR-106b-25 cluster, miR-155, miR-363 and let-7, which were upregulated, and miR-125 and miR-375, which were downregulated [70].

Ogawa et al. [71] studied correlation between downregulation of miR-34a and resistance to cis-diaminodichlorplatine (CDDP) treatment in sinonasal cancer. Authors concluded that miR-34a and other miRNA species might be involved in regulation of tumor chemosensitivity to CDDP treatment. Zhao and Wang [72] confirmed downregulation miR-34a in sinonasal squamous cell carcinoma and its association with poor prognosis of the patients. They suggested that miR-34a limits migration and invasiveness of SSCC cells by targeting BCL-2. Tomasetti et al. reported potential role of miR-126 as a diagnostic biomarker to differentiate malignant Sinonasal Intestinal-type Adenocarcinoma [73].

Research on miRNA expression levels in OPSCC is nowadays mainly focused on miRNome differences in HPV positive and HPV negative tumors. It has been also discovered that HPV genomes encode their own miRNAs. The role of these miRNAs has not been experimentally verified, however, target prediction algorithms suggest regulation of HPV genome itself (within HPV genes *E5*, *E1*, *L1* and LCR region) as well as host genome targets [74]. The most studied miRNAs in OPSCC include miR-31, which might play oncogenic role in tumor development. Then, if miR-24 is upregulated, enhances cancer cell proliferation and reduction in apoptosis occurs. On the other hand, miR-146a functions as tumor-suppressor and it is associated with favorable prognosis of the disease [75].

4.2.2 DNA methylation

DNA methylation is an epigenetic modification, in which methyl group is added to C5 position of cytosine in cytosine-guanine (CpG) dinucleotide. Methylation is catalyzed by enzyme DNA methyltransferase, which transfers methyl group

from S-adenosyl-methionine. DNA methylation results in formation of 5'methyl-cytosine (Figure 6).

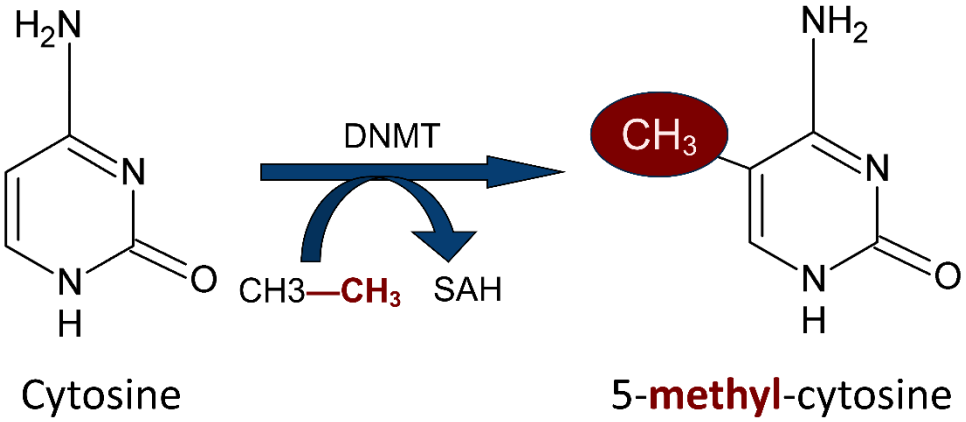

Figure 6. DNA methylation.
Methyl group is added to C5 cytosine. DNMT, DNA methyltransferase; SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine.

CpG dinucleotides are not equally distributed throughout the genome, but they are focused on areas with higher density of CpG dinucleotides called CpG islands. These areas are usually localized on promotor/first exon regions of the genes [76; 77]. The methylation of cytosine's is catalyzed by class of enzymes called DNA methyltransferases (DNMTs). Their primary purpose is to catalyze transfer of the methyl group from S-adenosyl-methionine to cytosine. Three DNMT families have been identified in mammals. DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT3a a DNMT3b work as *de novo* methylation enzymes, whereas, DNMT1 serve as maintenance enzymes of parental DNA pattern restoration. The rest of the DNA methyltransferases does not work as cytosine methyltransferases [78]. DNA methylation is important for 1) proper embryonic development, 2) chromosome X deactivation, 3) imprinting and 4) silencing transposable elements (TEs) [79].

Cancer cells are characterized by different methylation patterns from normal cells. However, both hypomethylation and hypermethylation changes can be observed in cancer. Global decrease of methylated CpGs results in genomic instability and alternatively activation of silenced oncogenes. Hypermethylation

of CpG islands in promotor regions of specific genes is a common characteristic of many cancer cells acting as tumor-suppressors in healthy tissue.

4.2.2.1 DNA methylation and microRNAs

About 50 % of miRNA genes are surrounded by CpGs, so they can be regulated by hypermethylation or hypomethylation. Expression of miRNAs located in introns of the host genes is usually regulated by one shared promotor (Figure 7) [80]. miRNAs acting as tumor-suppressor genes might be silenced by hypermethylation of CpG islands in their own promoter regions [78]. It has been discovered that DNA hypermethylation silencing of miR-124a leads to Cyclin-D kinase-6 (CDK6) overexpression and higher phosphorylation of retinoblastoma (Rb). When Rb is phosphorylated by CDK6, it loses its anti-proliferative activity. This miRNA hypermethylation has been also observed in glioblastoma multiforme, gastric cancer, hematopoietic malignancies, cervical cancer and hepatocellular carcinoma. One of the well-known tumor-suppressor miRNAs is miR-145 and its deregulation by promoter DNA methylation was reported in many human cancers to lead to pluripotency reacquisition. Other miRNAs regulated by DNA methylation include: miR-199a, miR-34a, miR-200 family or miR-9 family [81].

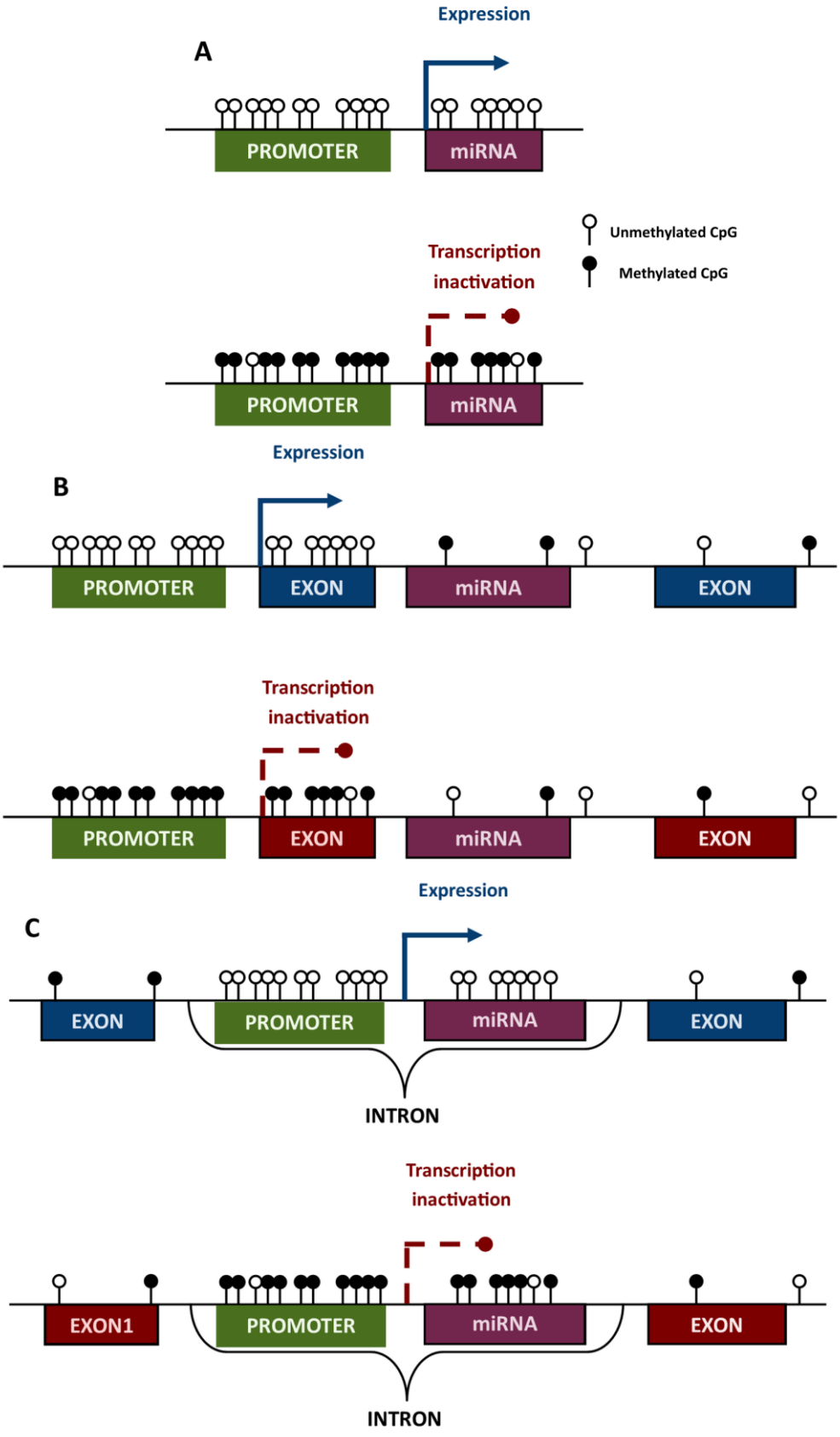

Figure 7. Regulation of tumor-suppressor miRNAs by DNA methylation.

In normal cells regulatory (promoter) regions are unmethylated, coded miRNAs are expressed and may target mRNAs. However, in cancer cells miRNA expression is suppressed by their promoter methylation and cannot inhibit mRNA translation. Therefore, oncogenes normally silenced by miRNAs can be expressed.

A) Some of miRNAs are regulated by their own promotor, so methylation of CpGs in the promotor region leads to loss of expression.

B) miRNAs localized in the introns of host genes can be regulated by host gene methylation.

C) Even miRNAs located in the host genes can have their own promoter, which can be methylated.

miRNAs targeting directly or indirectly effectors of epigenetic machinery such as DNMTs are called epi-miRNAs. miR-29 was the first experimentally verified epi-miRNA. Epigenetic targets of miR-29a, b and c are DNMT3A, DNMT3B and DNMT1. Other miRNAs such as miR-143 also regulate methylation (targeting DNMT3A), miR-148a,b (targeting DNMT1 and DNMT3), miR-185 (targeting DNMT3B and DNMT1), miR-152 (targeting DNMT1) and miR-301 targeting DNMT1 [80].

5 Objectives

Following objectives were defined for this study:

1. Use multiple high-throughput and low-throughput methods to investigate relative expression of microRNAs in two types of head and neck cancer samples: sinonasal and oropharyngeal carcinoma.

2. Determine, if other epigenetic modifications such as DNA methylation are involved in regulation of studied microRNAs.

3. Determine possible correlations between deregulated miRNAs and clinicopathological data of the patients by performing statistical analysis.

4. Propose which miRNAs might be potentially utilized in the future as head and neck squamous cell cancer biomarkers.

6 Materials and Methods

6.1 Sinonasal cancer patients and samples

A total of 63 formalin-fixed, paraffin-embedded tissue samples from SSCC patients and normal control sinonasal tissue were analyzed in this study: 46 cancer cases samples and 17 control samples. Only tumors primarily originating from the nasal cavity, maxillary sinuses, and ethmoid complex were included, while no tumors were found in the frontal or sphenoid sinuses. The samples used as controls were 8 mucosal specimens from the nasal cavity and 9 from the maxillary sinus which were obtained from patients treated for a non-malignant diagnosis such as chronic rhinitis and sinusitis.

Paraffin samples used in the study were obtained from Departments of Pathology of three University Hospitals in the Czech Republic (Hradec Kralove, Prague, Olomouc) and all malignancies were diagnosed between August 1995 and August 2014. Carcinoma cases were classified according to the current World Health Organization (WHO) classification [82] and all the samples were reviewed by experienced head and neck pathologist. The study was approved by the Ethics Committee of University Hospital Hradec Kralove (201511 S27P).

The tumors included exclusively squamous cell carcinomas (conventional, verrucous, papillary, basaloid, spindle cell, acantholytic, and adenosquamous). Vascular invasion, perineural spread, and microscopic findings in the surrounding mucosa were described.

Data such as gender, age at the time of diagnosis, smoking history (non-smoker vs. ex-smoker vs. current smoker), occupation (high vs. low risk), tumor localization (nasal cavity, maxillary sinus and ethmoid complex), clinicopathological TNM were recorded for every patient. During the follow-up period (until February 2016) local recurrence, regional recurrence, distant recurrence, death and tumor-related death staging were documented (Table 2). The patients were treated with radical surgery, radiotherapy, and chemotherapy in various combinations.

Table 2. Clinicopathological data of sinonasal cancer patients

Clinicopathological Characteristics[a]			SSCC	
		N	No. of patients	Proportion of N
Gender (46)	Male	46	31	67 %
	Female		15	33 %
Age (46)	≤ 50	46	5	11 %
	> 50		41	89 %
Smoking status (39)	Smoker	39	22	56 %
	Non-smoker		17	44 %
Occupation (39)	High risk	39	3	8 %
	Low risk		36	92 %
Localization (45)	Nasal cavity	45	21	47 %
	Maxillary sinus		22	49 %
	Ethmoid complex		2	4 %
pT (37)	T1, T2	37	10	27 %
	T3, T4		27	73 %
pN (45)	N0	45	36	80 %
	N1, N2, N3		9	20 %
cM (45)	M0	45	44	98 %
	M1		1	2 %
Resection margin (46)	Positive	46	38	83 %
	Negative		8	17 %
Grading (43)	G1	43	38	88 %
	G2, G3		5	12 %
Vascular invasion (46)	Yes	46	6	13 %
	No		40	87 %
Perineural invasion (46)	Yes	46	1	2 %
	No		45	98 %
Local recurrence (41)	Yes	41	15	37 %
	No		26	63 %
Regional recurrence (40)	Yes	40	3	8 %
	No		37	92 %
Distant recurrence (39)	Yes	39	3	8 %
	No		36	92 %
HPV status (46)	Negative	46	30	65 %
	Positive		16	35 %

[a] Due to a few missing clinical data, sums do not always add up to the total number of patients.

HPV, Human papilloma virus; pT, pathological characterization of primary tumor; pN, pathological classification of spread to regional lymph nodes; cM, presence of distant metastases.

HPV status was analyzed using HPV DNA in situ hybridization (ISH), HPV E6/E7 mRNA in situ hybridization (ISH), HPV DNA polymerase chain reaction (PCR) and typing, and HPV E6/E7 mRNA reverse transcription and polymerase chain reaction (RT-PCR). For the purpose of statistical analysis, a case was considered HPV positive if it was positive for HPV DNA ISH/PCR and/or HPV E6/E7 mRNA ISH/PCR. For detailed information about HPV analysis see manuscript by Laco et al. [25]. Methods used to study miRNA expression and subsequent DNA methylation of miRNA genes in sinonasal cancer sample set are summed up in Figure 8 and Figure 9.

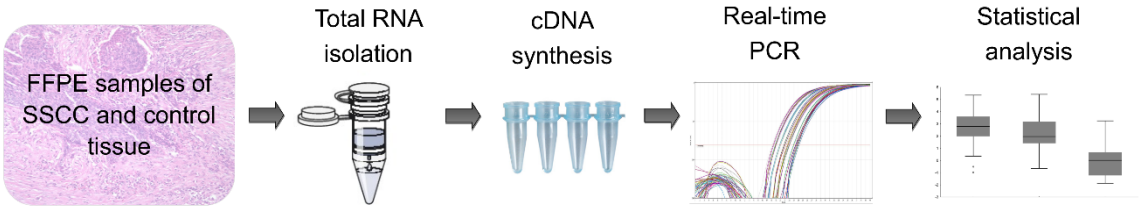

Figure 8. Methodology used for squamous cell sinonasal cancer samples analysis.
Total RNA was isolated from histologically verified FFPE samples of sinonasal cancer with miRNeasy FFPE Kit. cDNA synthesis was performed with TaqMan™ Advanced miRNA cDNA Synthesis Kit and subsequent real-time PCR reaction with TaqMan™ Fast Advanced Master Mix and selected TaqMan™ Advanced miRNA Assays. Final data analysis was performed in STATISTICA software.

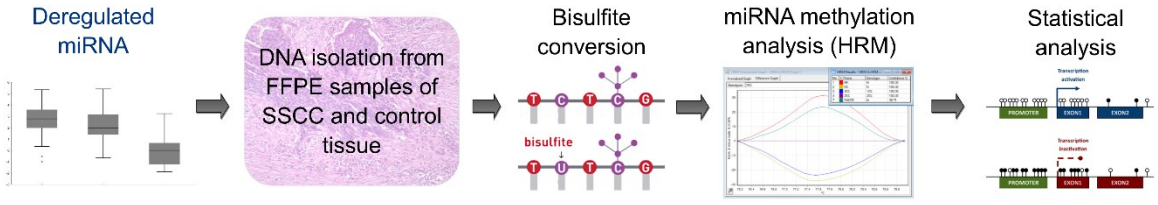

Figure 9. Schematics of methodology used for DNA methylation analysis of *miR-143* and *miR-145* genes.
DNA from samples was purified and bisulfite converted for subsequent methylation-specific high-resolution melting analysis (HRM). All data were statistically analyzed at the end of the experiment.

6.2 Oropharyngeal cancer patients

Due to unique characteristics of the samples (tumors with corresponding metastases and healthy control tissue) different approaches to miRNA expression analysis as in SNC samples were used. Firstly, high-throughput analysis of miRNA expression such as miRNA microarray analysis (Figure 10) and small RNA sequencing (Figure 11) was used to select significantly dysregulated miRNAs within the sample set. Relative expression of pre-selected miRNAs was ultimately verified using real-time PCR analysis.

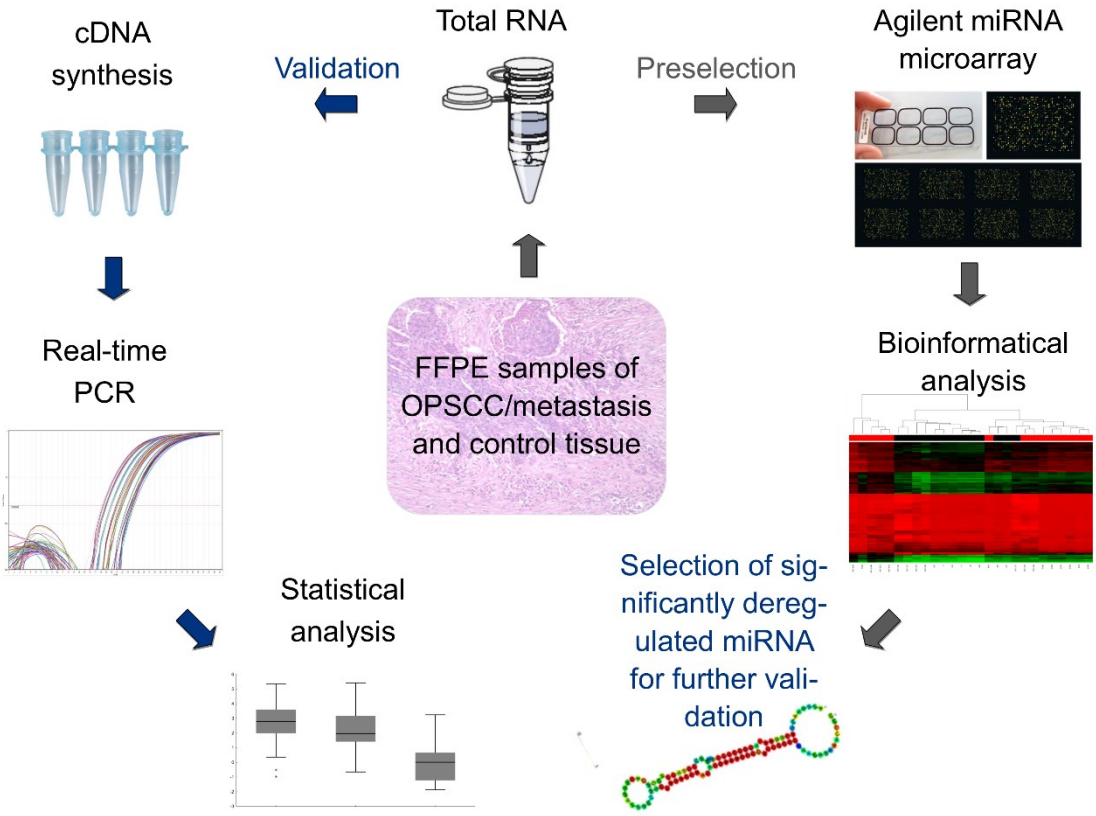

Figure 10. Methodology used for miRNA microarray analysis and subsequent verification by RT-qPCR in OPSCC sample set.
First, miRNA expression microarray from Agilent and subsequent bioinformatical analysis was used for preselection of significantly deregulated miRNAs. The results were further validated by real-time PCR with preselected miRNA assays.

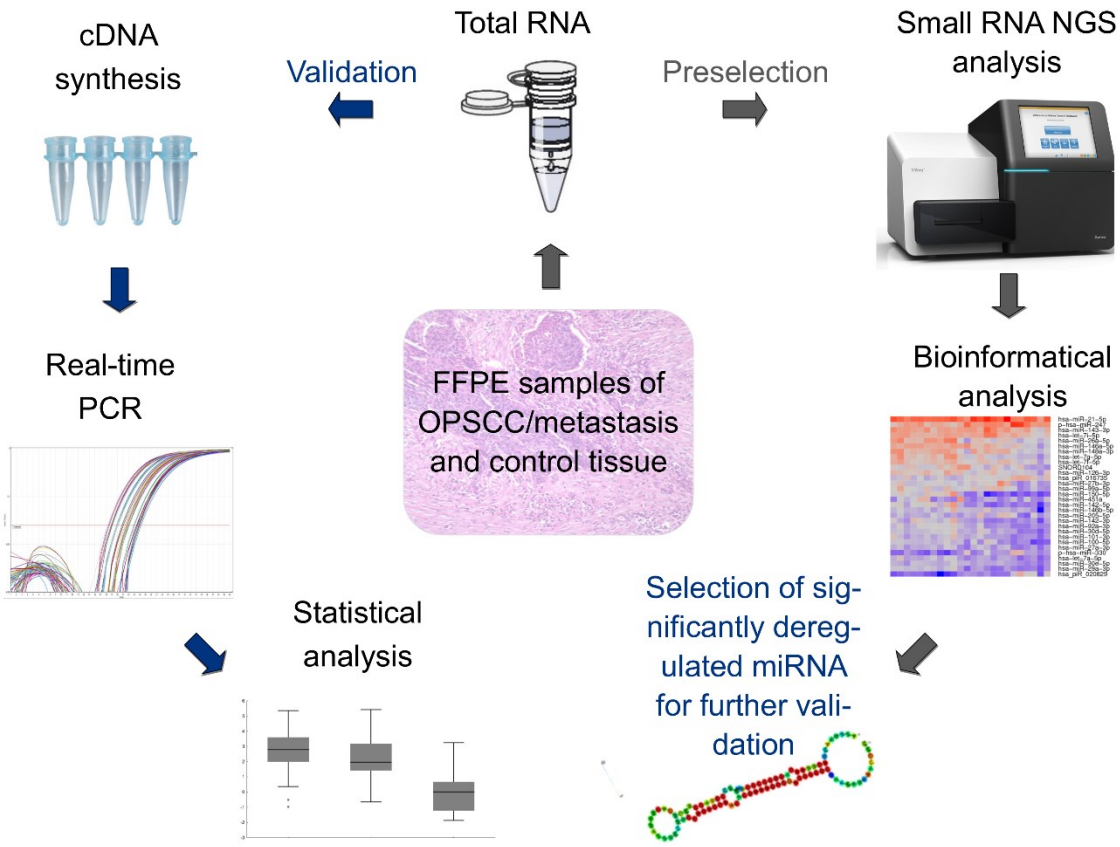

Figure 11. Methodology used for sRNA NGS analysis and subsequent verification by RT-qPCR in OPSCC sample set.
First, sRNA NGS analysis on Illumina platform subsequent bioinformatical analysis was used for preselection of significantly deregulated miRNAs. The results were further validated by real-time PCR with preselected miRNA assays.

As the primary aim of the study was the comparison of miRNA expression profiles between primary tumors, corresponding lymph node metastasis and control samples, only surgically treated oropharyngeal carcinoma patients with positive cervical lymph node metastases were enrolled in the study. A review of the surgical pathology files at The Fingerland Department of Pathology (University Hospital, Hradec Kralove, Czech Republic) identified a total of 73 patients who were diagnosed with metastasizing squamous cell carcinoma of the oropharynx between January 2000 and December 2014. Sufficient follow-up data were known for these patients and paraffin blocks were available for the analysis. All cases were reclassified by experienced head and neck pathologist according to the current WHO

Classification of Head and Neck Tumours [82]. The study was approved by the Ethics Committee of University Hospital Hradec Kralove (201611 S059 and 201711 S18P).

For each patient, gender, age at the time of diagnosis, tumor localization (palatine tonsils and arches, root of the tongue, soft palate, and posterior wall of the oropharynx), tumor size, and pathological TNM staging (at the time of the study, 7th edition was valid in the Czech Republic) [83] were recorded (Table 3). During the follow-up period (until March 2019) data on local recurrence, regional recurrence, distant recurrence, death, and tumor-related death were collected. All patients were treated by radical surgery with radiotherapy; chemotherapy was added in 22 patients. HPV status was examined at the Fingerland Department of pathology by p16 immunohistochemistry and HPV DNA detection by PCR for further information see manuscript Laco et al. [25].

All squamous cell carcinomas were further subclassified as non-keratinizing, non-keratinizing with maturation, or keratinizing according to the recently proposed criteria [84]. Only keratinizing carcinomas were graded as well, moderately, or poorly differentiated [82]. Vascular invasion, perineural spread, and status of resection margins (microscopically positive (R1) or negative (R0) were also noted. As we did not differentiate between blood and lymphatic vessel invasion, vascular invasion was present in all patients due to the presence of lymph node metastases (inclusion criterion). Apart from parameters necessary for pN staging, extranodal extension of metastasizing tumor cells was evaluated as well.

As controls we used 44 samples of palatine tonsils resected due to chronic tonsillitis. Thus, a total of 190 formalin-fixed, paraffin-embedded samples of oropharyngeal tissue were analyzed in this study.

Table 3. Clinicopathological data of oropharyngeal cancer patients

Clinicopathological Characteristics			OPSCC		
			N	No. of patients	Proportion of N
Gender	Male		73	55	75 %
	Female			18	25 %
Age	≤ 55		73	25	34 %
	> 55			48	66 %
Smoking	Smoker		73	22	30 %
	Non-smoker			26	36 %
	Former smoker			25	34 %
Alcohol	Yes		73	21	29 %
	No			52	71 %
HPV status	Negative		73	10	14 %
	Positive			63	86 %
HPV p16 status	Negative		73	14	19 %
	Positive			59	81 %
Localization	Tonsils		73	46	63 %
	Oropharynx			20	27 %
	Tonsillar fossa			4	6 %
	Base of tongue			3	4 %
Typing	Keratinizing	Grade 2	73	6	9 %
		Grade 3		1	1 %
	Non-keratinizing			46	63 %
	Non-keratinizing with maturation			20	27 %
pT	T1		73	19	26 %
	T2			36	49 %
	T3			13	18 %
	T4	T4a		2	3 %
		T4b		3	4 %
pN	N0		73	0	0 %
	N1			17	23 %
	N2	N2a		27	37 %
		N2b		25	34 %
		N2c		1	1 %
	N3			3	4 %
Vascular invasion	Yes		73	73	100 %
	No			0	0 %
Perineural invasion	Yes		73	10	14 %
	No			63	86 %
Extracapsular invasion	Yes		73	10	14 %
	No			63	86 %
Recurrence	Yes		73	11	15 %
	No			62	85 %
Local recurrence	Yes		73	1	1 %
	No			72	99 %
Regional recurrence	Yes		73	4	5 %

Distant recurrence	No	73	69	95 %
	Yes		8	11 %
	No		65	89 %

HPV, Human papilloma virus; pT, pathological characterization of primary tumor; pN, pathological classification of spread to regional lymph nodes.

6.3 Relative expression of microRNAs

6.3.1 Total RNA isolation

Two to four 5 µm thick sections were cut from FFPE tissue samples and deparaffinized using xylene and ethanol. Total RNA including miRNAs was isolated from FFPE tissue samples using two commercially available kits based on total RNA purification on silica columns purchased from Qiagen company (Hilden, Germany). miRNeasy FFPE Kit is designed to purify total RNA (including miRNAs) from FFPE samples and AllPrep DNA/RNA FFPE kit is designed to purify DNA and RNA (including short RNAs) from the same sample. All total RNA isolations were performed according to manufacturer's protocol with small changes. The extracted RNA was ultimately eluted in 25 µl of RNase-free water. After the isolation, the samples were immediately processed or stored at -80 °C.

6.3.2 Total RNA quality control

Concentration and purity of isolated RNA was first assessed by NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the optical density at 260 nm and 280 nm, A260/280 ratio and A260/230 ratio. Some of the samples were further analyzed on Qubit Fluorimeter (Thermo Fisher Scientific) using Qubit RNA HS Kit (Thermo Fisher Scientific), which provides accurate and selective method for the quantitation of low-abundance RNA samples. Total RNA quality (RIN) for subsequent miRNA microarray analysis was checked on Agilent Bioanalyzer (Agilent, Santa Clara, CA,

USA) using RNA 6000 Nano assay kit (Agilent). For the analysis 1 µl of each sample was used.

6.3.3 miRNA microarray

G3 Human miRNA Microarray, Release 21, 8×60K (Agilent) was used for high-throughput miRNome expression analysis of 40 selected samples of OPSCC (tumors, related metastases and controls). On one glass chip eight microarrays are printed with 60,000 oligonucleotide probes giving information about expression of 2,549 miRNAs based on miRbase release 21.0. Two glass arrays were hybridized at our workplace (Department of clinical biochemistry and diagnostics, University Hospital Hradec Králové, Molecular biology section) and then scanned on Agilent SureScan Microarray Scanner at HPST, s.r.o. (Písnická 372/20, Prague 4, Czech Republic). Analysis on following 3 glass arrays was performed at Central European Biosystems, s. r. o. (Pekařská 603/12, Prague 5, Czech Republic), where subsequent bioinformatics analysis of the scanned data was performed.

All steps were performed according to manufacturer's protocol with 100 ng of total RNA in the reaction. Briefly, Labeling Spike-In and Hybridization Spike-In controls were prepared to be added to the reaction as processing controls. Secondly, the samples were dephosphorylated by adding Calf Intestinal Phosphatase to the mixture and incubated at 37 °C. Thirdly, the samples were denatured by adding DMSO (dimethylsulfoxid) and incubation at 100 °C. Fourthly, Cyanine 3-pCp dye was ligated to the sample by T4 RNA Ligase followed by purifying the labeled RNA using Micro Bio-Spin P-6 Gel column to remove DMSO and excessive free non-ligated Cyanine 3-pCp dye. After labeling reaction, the samples had to be completely dried up using vacuum concentrator with heater. Lastly, hybridization of the prepared samples to the glass array using the hybridization chamber and the hybridization gasket was performed. Hybridization oven was set to 55 °C for 20 hours. After hybridization the slides were washed with appropriate wash buffers and scanned on Agilent SureScan microarray scanner and unpacked by Feature extraction software.

Expression data were bioinformatically analyzed and divided into three groups (tumors × metastases × controls). The quantile normalization between arrays without background correction was performed at first. As the second step, the probe signal summarization to the level of individual miRNA genes was applied [85]. Both above steps were performed by the function rmaMicroRna from R package AgiMicroRna to all individual runs. The differentially expressed genes were identified by limma package. The batch effects were modeled as random effects during differential expression analysis. Differentially expressed miRNAs between these three groups were identified for further validation by qPCR.

6.3.4 Small RNA Next generation sequencing

For sRNA NGS library preparation NEBNext Small RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used. This kit contains the adaptors, primers, enzymes and buffers required to convert sRNAs (including miRNAs) into indexed libraries for next-generation sequencing on the Illumina platform. For NGS library preparation 40 samples of OPSCC were used (tumors, metastases and controls). Starting amount of total RNA in the reaction was approximately 1 μg. All steps from the procedure were performed according to manufacturer's protocol with minor modifications.

Briefly, the first step of the procedure is to ligate 3' SR Adaptor with addition of 3' Ligation Enzyme Mix to the mixture. The second step of the workflow is to hybridize the Reverse Transcription primer, which is important to prevent adaptor-dimer formation. The SR RT Primer hybridizes to the excess of 3′ SR Adaptor (that remains free after the 3′ ligation reaction) and transforms the single stranded DNA adaptor into a double-stranded DNA (dsDNA) molecule, which are not substrates for ligation mediated by T4 RNA Ligase 1 and therefore do not ligate to the 5′ SR Adaptor in the subsequent ligation step. This step needs to be immediately followed by Reverse transcription reaction using ProtoScript II Reverse Transcriptase. Next, PCR Amplification is performed using unique Index Primers (1-4) for Illumina to differentiate each sample after pooling.

At this step there are several different methods for performing size selection and pooling of the samples. Our NGS libraries were pooled based on concentrations of each sample determined by Qubit 3.0 using Qubit™ dsDNA HS Assay Kit (Invitrogen). Ultimately, three libraries based on concentration of the sample were prepared and concentrated using MinElute PCR Purification Kit. Each pooled library was run on 3% Agarose gel. The appropriate gel band (at approximately 140 bp, Figure 12) was cut out of the gel and the cDNA purified by NucleoSpin® Gel and PCR Clean-up Kit. Molarity of the prepared library was quantified using the KAPA library quantification assay (Kapa Biosystems, Wilmington, MA, USA) and a 2 nM or 4 nM library was prepared.

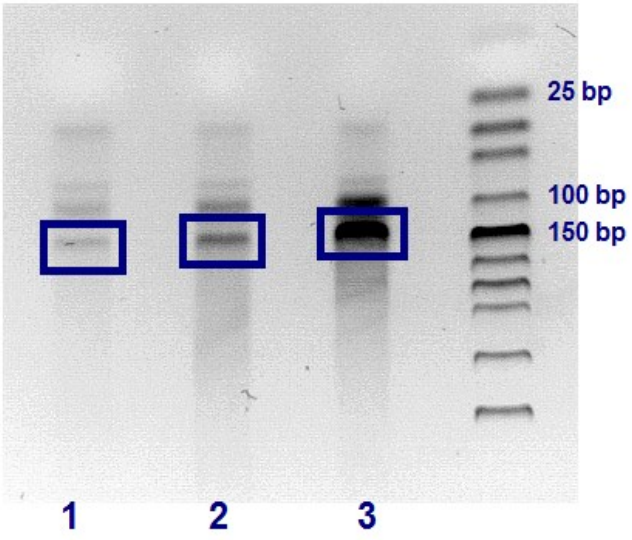

Figure 12. Visualization of 3% agarose gel used for purification and size selection of pooled NGS libraries.
Selected bands containing miRNAs (approximately 140 bp) are highlighted by blue boxes.

Three runs of 1 x 50 single read NGS were performed on Illumina MiSeq instrument using MiSeq Reagent Kit v2 (50-cycles). The FASTAQ files were analyzed using Oasis 2.0 detection software [86]. sRNA detection module was used to obtain each miRNA counts by comparing the FASTAQ files to the reference genome and to perform quality control on NGS data. Differential expression (DE) analysis module was used to determine differentially expressed sRNAs (with elimination

of data outliers and low-quality samples) by comparing tumor samples with related metastasis samples and control samples. sRNA Classification module was used to detect novel sRNA biomarkers, their RNA targets and test for their functional enrichment.

6.3.5 Quantitative real-time PCR

The synthesis of cDNA was performed using TaqMan™ Advanced miRNA cDNA Synthesis Kit with universal reverse transcription primers (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol with 8 – 10 ng of total RNA in the reaction. Real-time PCR was done with TaqMan™ Fast Advanced Master Mix (Applied Biosystems) and specific TaqMan™ Advanced miRNA Assays (Applied Biosystems) on Rotor-Gene Q (Qiagen). Hsa-miR-361-5p was used as endogenous control, because any other control miRNAs (such as Hsa-miR-939-5p were not stably expressed throughout all tissues). All steps were performed following the manufacturer's protocol. Studied miRNAs were selected based on: 1) literary review dealing with deregulation of miRNAs in various types of head and neck cancer, 2) previous analysis by high-throughput methods such as Agilent miRNA microarray analysis and sRNA next-generation sequencing (Table 4).

All reactions were performed in triplicates and each run contained no template control (NTC), reverse transcription control (without reverse-transcription enzyme) a calibrator (cDNA mixture for between runs normalization) prepared during cDNA synthesis reaction. Reaction volume was 10 µl with adding 2.5 µl of the sample to 7.5 µl of TaqMan™ Fast Advanced Master Mix TaqMan™ Advanced miRNA Assay mixture. The reaction conditions were set according to the manufacturer's protocol as follows: enzyme activation at 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Captured fluorescence data were analyzed in the Rotor-Gene Q Series Software. Relative expression of each miRNA was determined using the $2^{-\Delta\Delta Ct}$ method [87] using in-house prepared Microsoft Excel template with expression levels of miR-361 used as endogenous control for data normalization. This workflow was chosen based on literature review

and manufacturer's recommendation for endogenous controls listed in the user guide for TaqMan™ Advanced miRNA Assays.

Table 4. Information about TaqMan™ Advanced miRNA Assays used for real-time PCR analysis.

TaqMan™ Advanced miRNA Assay Name	Accession number	Mature miRNA sequence	Based on	
			In SSCC samples	In OPSCC samples
Hsa-let-7a-5p	478341_mir	UGGAAGACUAGUGAUUUUGUUGU	Literature	
Hsa-let-7d-5p	478439_mir	AGAGGUAGUAGGUUGCAUAGUU	Literature	
Hsa-miR-124-3p	477879_mir	UAAGGCACGCGGUGAAUGCC		Literature
Hsa-miR-137-3p	477904_mir	UUAUUGCUUAAGAAUACGCGUAG	Literature	
Hsa-miR-141-5p	478712_mir	CAUCUUCCAGUACAGUGUUGGA	Literature	
Hsa-miR-142-5p	477911_mir	CAUAAAGUAGAAAGCACUACU		miRNA microarray
Hsa-miR-143-3p	477912_mir	UGAGAUGAAGCACUGUAGCUC	Literature	
Hsa-miR-145-5p	477916_mir	GUCCAGUUUUCCCAGGAAUCCCU	Literature	
Hsa-miR-146a-5p	478399_mir	UGAGAACUGAAUUCCAUGGGUU	Literature	
Hsa-miR-150-5p	477918_mir	UCUCCCAACCCUUGUACCAGUG		Small RNA NGS
Hsa-miR-155-5p	483064_mir	UUAAUGCUAAUCGUGAUAGGGGUU	Literature	
Hsa-miR-196a-5p	478230_mir	UAGGUAGUUUCAUGUUGUUGGG	Literature	
Hsa-miR-200c-3p	478351_mir	UAAUACUGCCGGGUAAUGAUGGA	Literature	
Hsa-miR-200c-5p	478754_mir	CGUCUUACCCAGCAGUGUUUGG	Literature	
Hsa-miR-206	477968_mir	UGGAAUGUAAGGAAGUGUGUGG		miRNA microarray
Hsa-miR-21-5p	477975_mir	UAGCUUAUCAGACUGAUGUUGA	Literature	
Hsa-miR-223-3p	477983_mir	UGUCAGUUUGUCAAAUACCCCA	Literature	
Hss-miR-345-5p	478366_mir	GCUGACUCCUAGUCCAGGGCUC	Agilent miRNA microarray	
*Hsa-miR-361-5p**	478056_mir	UUAUCAGAAUCUCCAGGGGUAC	Literature Control	Literature Control
Hsa-miR-363-5p	478840_mir	CGGGUGGAUCACGAUGCAAUUU	Literature	
Hsa-miR-3656	480800_mir	GGCGGGUGCGGGGGUGG		miRNA microarray
Hsa-miR-375-3p	478074_mir	UUUGUUCGUUCGGCUCGCGUGA	Literature	Small RNA NGS
Hsa-miR-4530	478918_mir	CCCAGCAGGACGGGAGCG		miRNA microarray
Hsa-miR-484	478308_mir	UCAGGCUCAGUCCCCUCCCGAU	Literature	
hsa-miR-7-5p	478341_mir	UGGAAGACUAGUGAUUUUGUUGU	Literature	
*Hsa-miR-939-5p**	478245_mir	UGGAAGACUAGUGAUUUUGUUGU	Literature	
Hsa-miR-9-3p	478211_mir	AUAAAGCUAGAUAACCGAAAGU	Literature	
Hsa-miR-9-5p	478214_mir	UCUUUGGUUAUCUAGCUGUAUGA	Literature	
Hsa-miR-99a-5p	478519_mir	AACCCGUAGAUCCGAUCUUGUG	Literature	

* Used as endogenous control assay

6.3.6 Statistical analysis

All statistical analyses were performed using STATISTICA (data analysis software system) version 13 (TIBCO Software Inc., Tulsa, OK, USA). miRNA expression values were log-transformed to a normal distribution of data for parametric tests. Student's t-test was used to compare level of expression of miRNAs in tumor and non-tumor samples. The null hypothesis was based on theory that there was no difference between expression levels of studied microRNAs between tumor samples and control samples. For best diagnostic marker selection two web-based interactive tools were used: PanelComposer and CombiROC. CombiROC is a tool for validating biomarker candidates based on ROC (receiver operating characteristics) curves and the area under the ROC curve (AUC) using a logistic regression model. It is available online at: http://panelcomposer.proteomix.org/ [88]. CombiROC can determine optimal markers combination from different methods using sensitivity/specificity filters. It is freely available at: http://CombiROC.eu [89]. For the analysis data was log transformed.

One-way analysis of variance (ANOVA) and regression analysis were used to analyze the correlation between expression levels of miRNAs and various clinicopathological features. The Kaplan-Maier method and Logrank test were used to determine overall survival rate and significance. All tests were two-tailed and $P < 0.05$ results were considered statistically significant.

6.4 DNA methylation analysis

6.4.1 DNA extraction and bisulfite conversion

Genomic DNA was isolated from FFPE tissue samples of SSCC and control samples using QIAamp Genomic DNA Mini Kit or by QIAamp DNA FFPE Tissue Kit both supplied by Qiagen following manufacture's protocol. Quality and purity

of extracted DNA was assessed by NanoDrop ND 1000 spectrophotometer and Qubit Fluorimeter (both supplied by Thermo Fisher Scientific). Sodium bisulfite treatment changes unmethylated cytosines into uracils, whereas 5-methylcytosines are resistant to this modification [90]. 500 ng of genomic DNA was treated with bisulfite using the EZ DNA Methylation-Gold™ Kit according to the manufacturer's protocol (Zymo Research Corporation, Irvine, CA, USA).

6.4.2 Methylation-sensitive high-resolution melting analysis

HRM analysis is based on the fact that methylated and unmethylated, PCR products have different melting profiles when subjected to thermal denaturation. The methylation-sensitive high-resolution melting (MS-HRM) analysis procedure is based on the comparison of the melting profiles of PCR products from unknown samples with profiles specific for PCR products derived from methylated and unmethylated control DNAs. The procedure consists of PCR amplification of bisulfite-modified DNA with primers designed to proportionally amplify both methylated and unmethylated templates and subsequent high-resolution melting analysis of the PCR product [90].

Methylation analysis of miR-145 and miR-143 genes was performed to investigate, whether statistically significant downregulation of miR-145-5p might be linked to DNA methylation of the coding sequence. miR-145 and miR-145 are microRNAs that belong to the same cluster, so they are located very close to each other on the genome. Primers for MS-HRM were designed in MethPrimer [91] to the promotor region of *miR-143* and *miR-145* genes and their mutual miR-143-3p and miR-145-5p host gene (*CARMIN*) (Table 5). The PCR products were designed to be shorter than 150 bp considering fragmentation of DNA obtained from FFPE samples. PCR amplification of all regions and subsequent HRM analysis was performed on Rotor-Gene Q (Qiagen).

Table 5. MS-HRM primer design information.

Gene	Primer sequence	Amplicon size (bp)	Annealing Temp. (°C)	CpG sites	Polymerase
miR-145-1	F_GTTATAGATGGGGTTGGATGTAGAA	123	60	5	Gold DNA polymerase
	R_AAAAAATTCCTAAAAAAACTAAACC				
miR-145-2	F_TAGATAGTAGAGGGTAGTTTTGGGG	141	55	6	HotStarTaq *Plus* DNA Polymerase
	R_TAACAACCAAAATAAAATACCACAC				
miR-143	F_GTTAGAGTTGGAGAGGTGGAGTTTA	123	60	4	Gold DNA polymerase
	R_TACACCTCAAACTAAAAAACAAAAC				
miR-143/145	F_TTTTAGGGGGTAAAAGTAATATTTAGTA	145	57	5	HotStarTaq *Plus* DNA Polymerase
	R_TATATAAACTAAAAAAACAAACCCC				

PCR was carried out in final volume of 10 µl. Volumes of each reactant for PCR of miR-145-1 and miR-143 amplicons were as follows: 1 µl of Reaction Buffer without $MgCl_2$ (10×), 1 µl of $MgCl_2$, 0.8 µl of dNTP mix for PCR (Takara Bio Inc., Kusatsu, Japan), 0.5 µl of each primer (Forward and Reverse, 10µM), 0.15 µl of SYTO™ 9 Dye (0.05 mM, Invitrogen), 0.125 µl of AmpliTaq Gold™ DNA Polymerase (All Applied Biosystems), 1 µl of bisulfite converted DNA sample and 4.93 µl of water. Cycling conditions are described in Table 6. Volumes of each reactant for PCR of miR-145-2 and miR143/145 amplicon were as follows: 2.5 µl of 2× EpiTect HRM PCR Master Mix (Qiagen), 0.75 µl of each primer (Forward and Reverse, 10µM), 1 µl of bisulfite converted DNA sample and 2.5 µl of water. Cycling conditions are described in Table 6. Each reaction run included an NTC and, a bisulfite-converted universal methylated and unmethylated DNA (Qiagen) and prepared standards containing 10 % and 25 % of universal methylated DNA which served as a cut-off for methylation status. HRM data were analyzed using Rotor-Gene Q software (Qiagen).

Table 6. Cycling conditions for HRM analysis.

Stage		Mir-145-1 and miR-143 amplicon		miR-145-2 and miR-143/145 amplicon	
		Temperature profile	Time	Temperature profile	Time
Initial denaturation		95°C	5 min	95°C	5 min
Cycling 40×	*Denaturation*	95°C	20 s	95°C	10 s
	Annealing	60°C	30s	55°C	30 s
	Extension	72°C	30s	72°C	10 s
Final Extension		72°C	5 min	-	-
HRM analysis		68-85°C ramping by 0.1°C	2 s hold	64-84°C ramping by 0.1°C	2s hold
Cool down		40°C	1 min	40°C	1 min

6.4.3 Statistical analysis

All statistical analyses were performed using STATISTICA (data analysis software system) version 13 (TIBCO Software Inc., Tulsa, OK, USA). The null hypothesis was based on theory 1) that there is no correlation between impaired miRNA expression deregulation and DNA methylation of promotor regions of miRNA genes.

Basic descriptive statistics were adopted for the analysis: median, mean, and 95% confidence interval for continuous data, and absolute and relative frequencies for categorical data. The relationship between gene methylation and other independent factors was analyzed using the chi-square test, Fisher's exact test, or Logistic regression analysis. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

7 Results

7.1 Differentially expressed miRNAs in sinonasal carcinoma

7.1.1 Real-time PCR relative expression analysis

In this study relative expression of 22 miRNAs (Table 4) was studied using TaqMan™ Advanced real-time PCR approach based on literary review of miRNAs deregulated in squamous cell head and neck cancer in 46 SSCC samples and 17 controls. As statistically significant upregulation was considered result with $P < 0.05$ and Fold change (FC) > 2.0 and as downregulation was considered statistically significant, if P was < 0.05 and FC was < 2.0 compared to control sample expression.

We detected significant upregulation of 6 miRNAs (Table 7): let-7a-5p, let-7d-5p, miR-196a-5p, miR-21-5p, miR-9-3-p, miR-9-5p (Figure 13) and significant down-regulation of 1 miRNA: miR-145-5p (Figure 14).

Some of the miRNAs examined were deregulated, however the results were not statistically significant (such as miR-143-3p, miR-146a-5p, miR-155-5p, miR-137-3p, miR-223-3p, miR-454 and miR-99a-5p and miR-200c-3p).

Expression levels of miR-141-5p, miR-200c-5p, miR-363-5p and miR-7-5p were below detectable level of the used method (real-time PCR with TaqMan™ Advanced Assays). As a result, no reliable conclusions can be made regarding their expression in sinonasal squamous cell carcinoma.

Table 7. Relative expression of selected miRNAs in squamous cell sinonasal carcinoma detected by real-time PCR.

microRNA	P value	FC
Upregulated (Tumor versus Control)		
let-7a-5p	**< 0.001**	**5.65**
let-7d-5p	**< 0.001**	**3.93**
miR-143-3p	0.17	1.46
miR-146a-5p	0.76	1.12
miR-155-5p	0.27	1.46
miR-196a-5p	**0.0086**	**4.39**
miR-200c-3p	0.22	1.52
miR-21-5p	**< 0.001**	**6.41**
miR-9-3p	**0.017**	**3.07**
miR-9-5p	**< 0.001**	**6.80**
Downregulated (Tumor versus Control)		
miR-137-3p	0.98	-1.04
miR-145-5p	**< 0.001**	**-2.78**
miR-223-3p	0.18	-1.71
miR-484	0.33	-1.39
miR-99a-5p	0.92	-1.33

Bold numbers show statistically significant results.

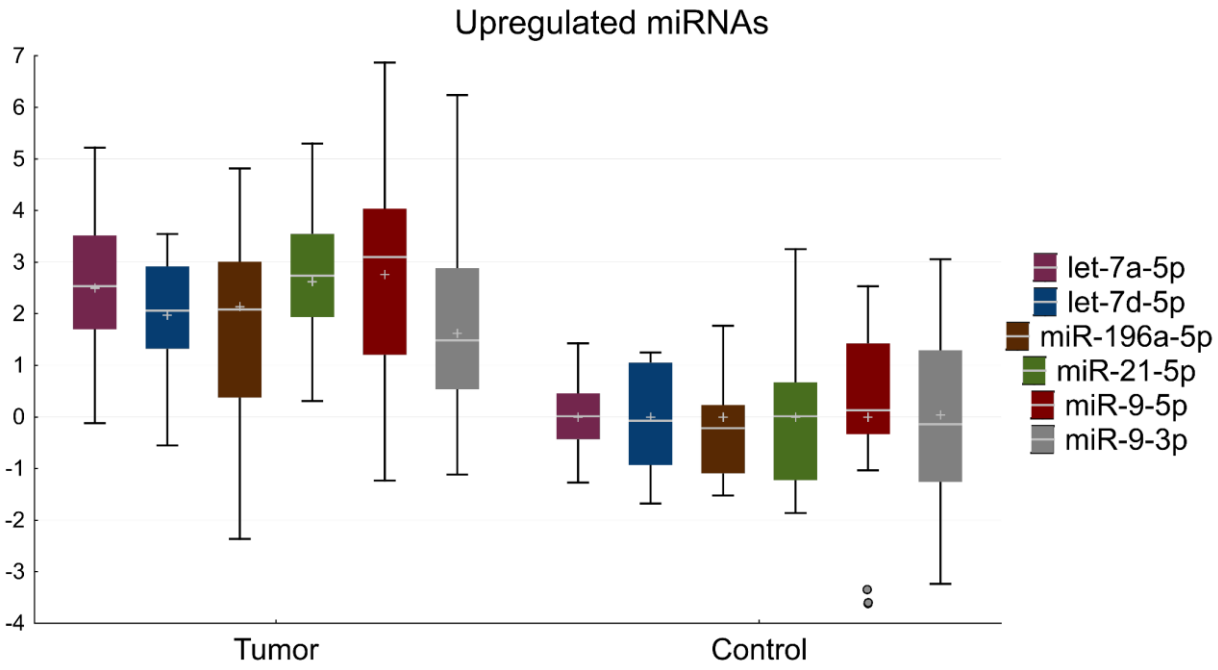

Figure 13. Box plot of significantly upregulated miRNAs in SSCC samples. Horizontal line in the box marks median value and plus sign marks mean value. The box bounds Interquartile Range. Whiskers show Minimum and maximum values with outliers symbolized as dots.

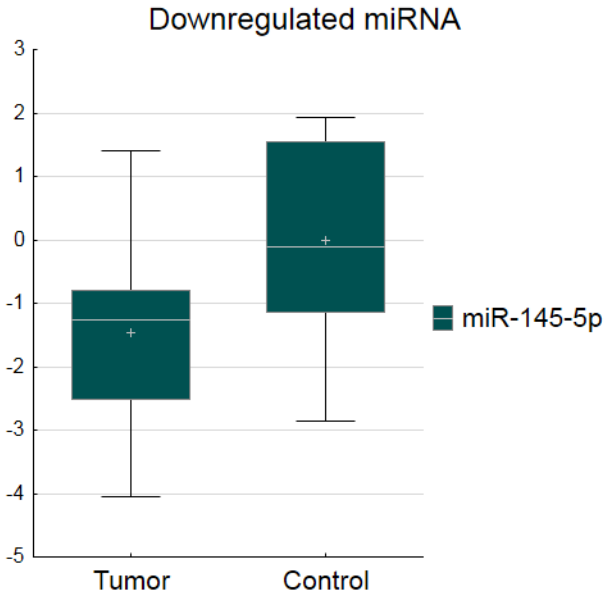

Figure 14. Box plot of significantly downregulated miRNA in SSCC samples. Horizontal line in the box marks median value and plus sign marks mean value. The box bounds Interquartile Range. Whiskers show Minimum and maximum values with outliers symbolized as dots.

All detected miRNA expression values were further analyzed to determine their diagnostic biomarker potential by web-based tools for evaluation of disease biomarker candidates: PanelComposer and CombiROC. With PanelComposer analysis the 5 miRNA panel (miR-21-5p, miR-9-5p, let-7d-5p, let-7a-5p and miR-196a-5p) was evaluated as the most successful diagnostic tool with $P < 0.0001$ and area under curve 0.996 and confidence interval (CI) (0.995, 0.997). Analysis with CombiROC was used to discover to most effective combinations of miRNAs with one consisting of 4 miRNAs and the other consisting of 5 miRNAs. The first suggested panel consisted of miR-21-5p, let-7d, miR-155-5p and miR-223-3p (labelled as Combo V) with AUC = 0.971 (error rate = 0.095), when sensitivity (SE) was 0.87 and specificity (SP) was 1. Optimal cutoff for our samples was 0.803 (Figure 15). The second selected combination consisted of 5 miRNAs: miR-21-5p, miR-9-3p, let-7d-5p, miR-155-5p and miR-223-3p (Combo VI). For this combination AUC = 0.977 (error rate = 0.048) with SE = 0.935 and SP = 1 and optimal cut-off value 0.708 (Figure 16).

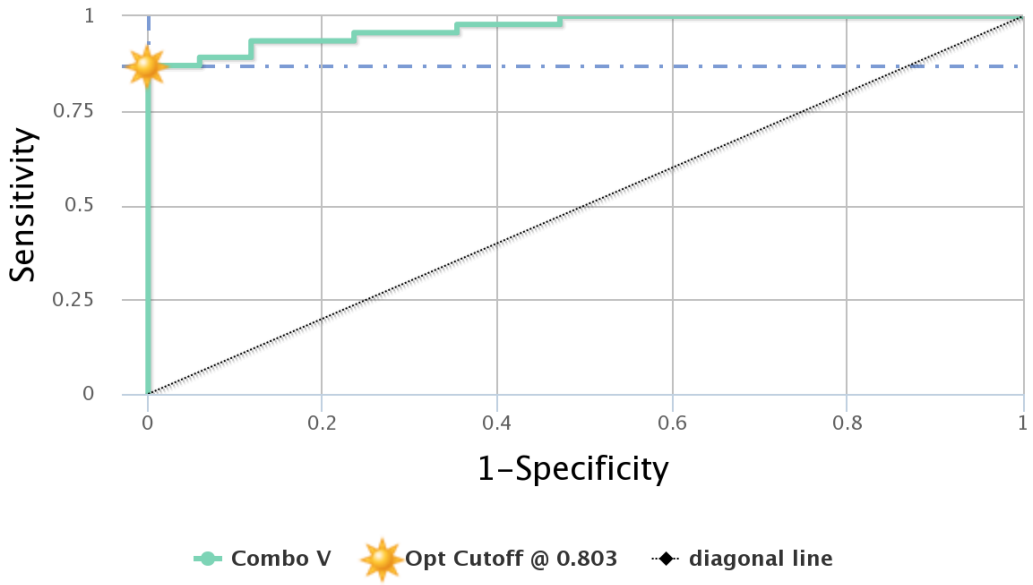

Figure 15. ROC curve for 4 miRNA panel for prediction of SSCC.
Turquoise line represents ROC curve for miR-21-5p, let-7d, miR-155-5p and miR-223-3p panel. Yellow sun pictogram represents Optimal cut-off value. Diagonal line dots represent random guessing.
ROC, receiver operating characteristic.

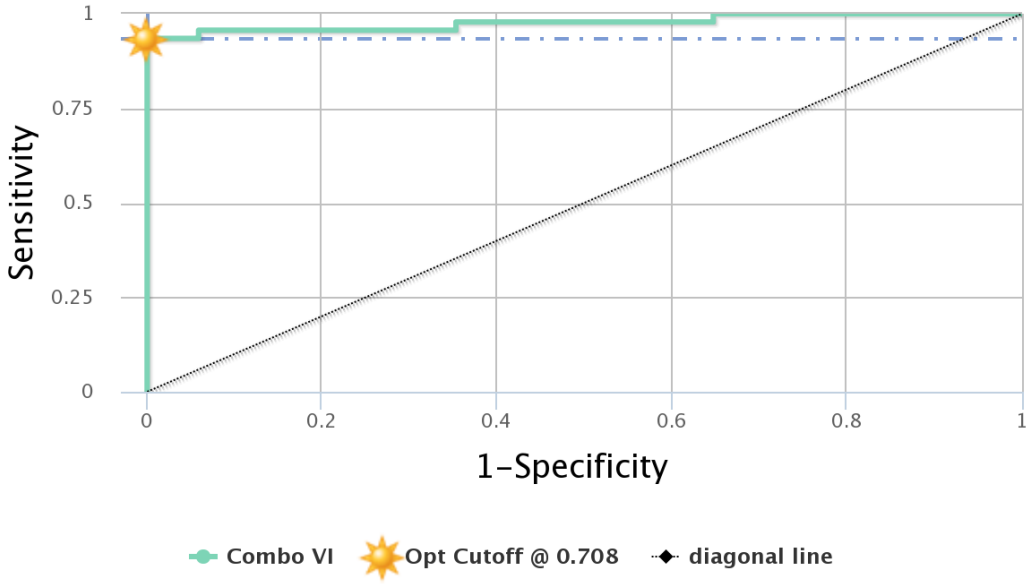

Figure 16. ROC curve for 5 miRNA panel for prediction of SSCC.
Turquoise line represents ROC curve for miR-21-5p, miR-9-3p, let-7d, miR-155-5p and miR-223-3p panel. Yellow sun pictogram represents Optimal cut-off value. Diagonal line dots represent random guessing.
ROC, receiver operating characteristic.

7.1.2 miRNA expression and patients' survival

Follow-up period of the patients ranged from 2 to 111 months with median value of 23 months. For the survival analysis we used Kaplan-Meier analysis to generate survival curves accompanied by Logrank test to obtain P value of prognostic significance of each miRNA expression regarding survival of patients. The patients were divided into two categories for each miRNA based on their expression levels. Only statistically significant outcomes of the analysis or data approaching statistical significance will be presented on following pages.

7.1.2.1 miR-21

The patients were divided into two categories based on quartile values. Samples with miR-21-5p relative expression in the highest quartile formed on group, whereas,

the rest of the samples were categorized as low expression group. The analysis showed that SCC patients with high expression of miR-21 (highest quartile) had impaired survival in comparison to other patients. The results were approaching statistical significance (P=0.0630) (Figure 17).

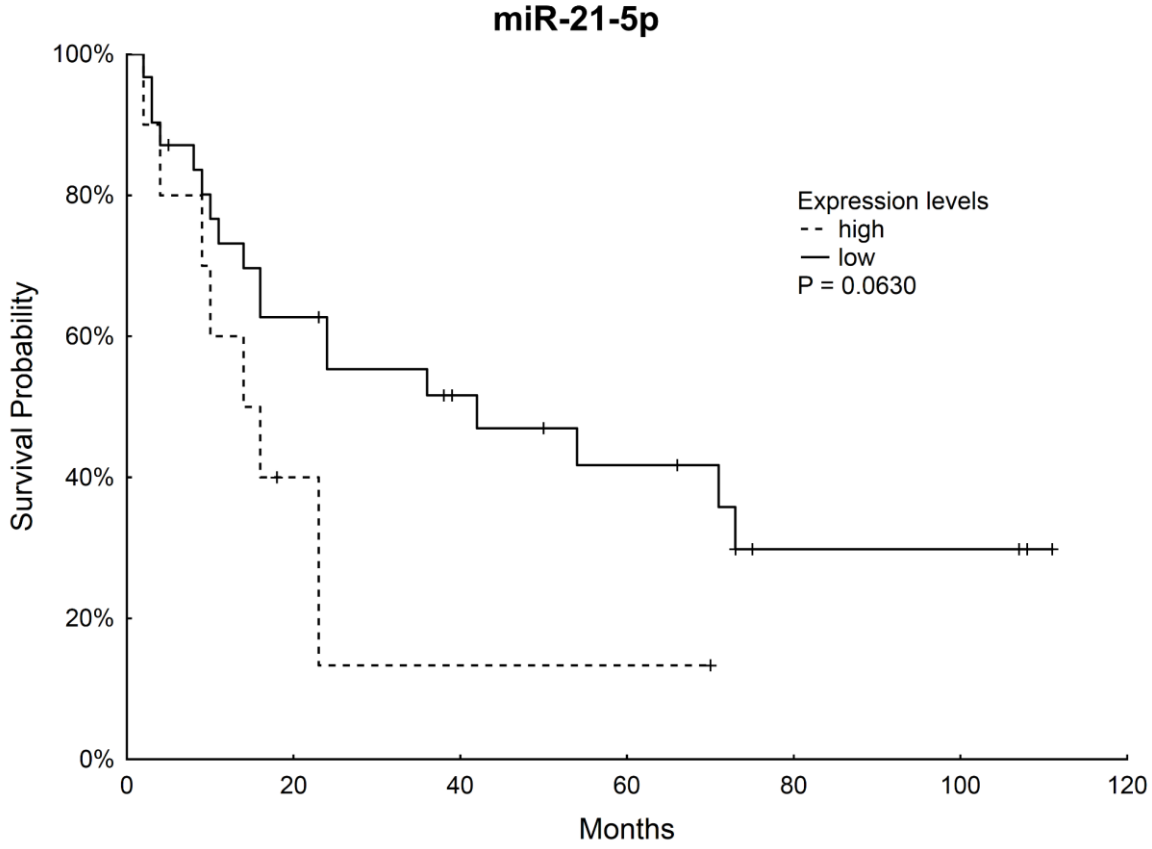

Figure 17. Comparison of survival times of sinonasal SCC patients. Patients with high expression of miR-21 (highest quartile) had lower survival rate than patients with low expression. The difference is approaching statistical significance (Logrank test, P=0.0630). Vertical hatch marks show censored data.

7.1.2.2 miR-9-5p

The results suggested that patients with high expression of miR-9-5p (group of patients with expression levels in the top quartile) had better chance of survival than the patients with lower expression of miR-9-5p (P = 0.0264) (Figure 18).

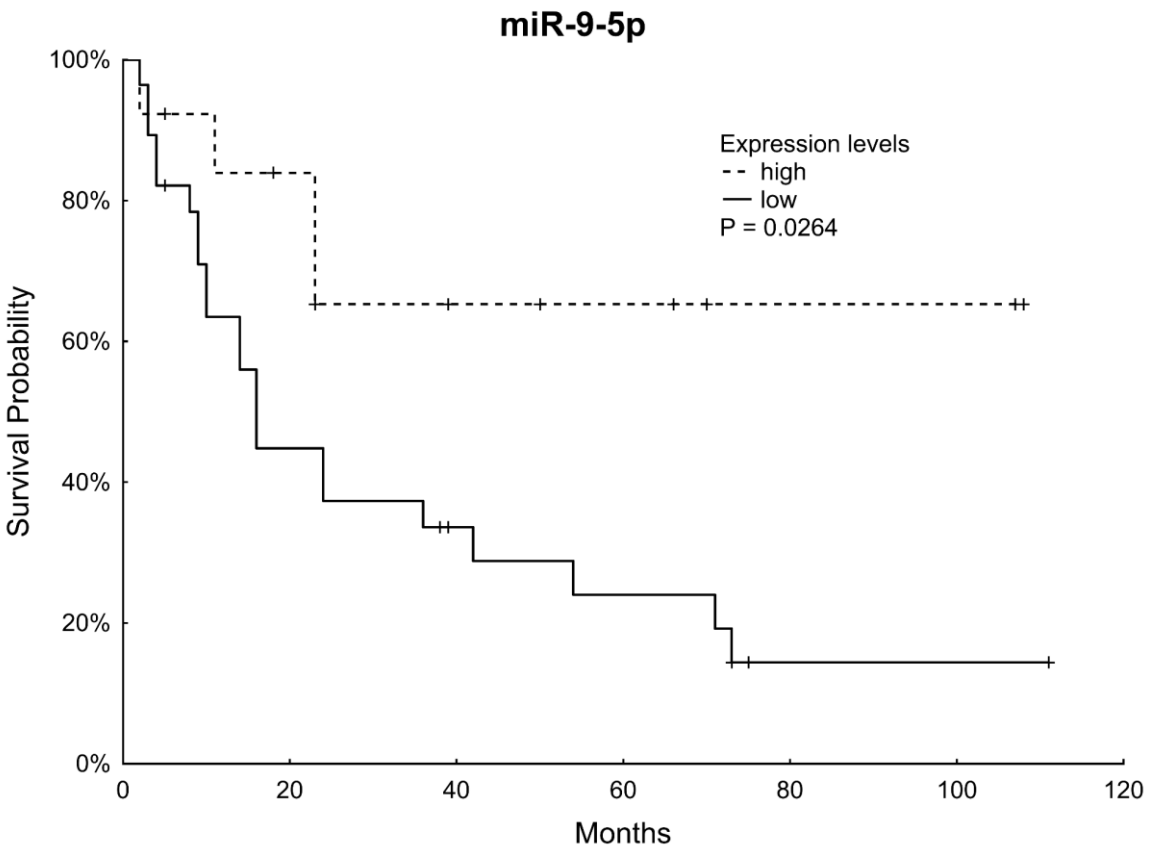

Figure 18. Kaplan-Maier survival plot for miR-9-5p.
Higher expression of miR-9-5p was detected for the patients with longer survival interval with statistical significance (P = 0.0264). Vertical hatch marks show censored data.

7.1.2.3 let-7d-5p

Our data subsequently showed that patients with low expression of let-7d had impaired survival in comparison to patients with higher expression of the same miRNA (based on lower quartile value). These results were statistically significant (P = 0.0417) (Figure 19).

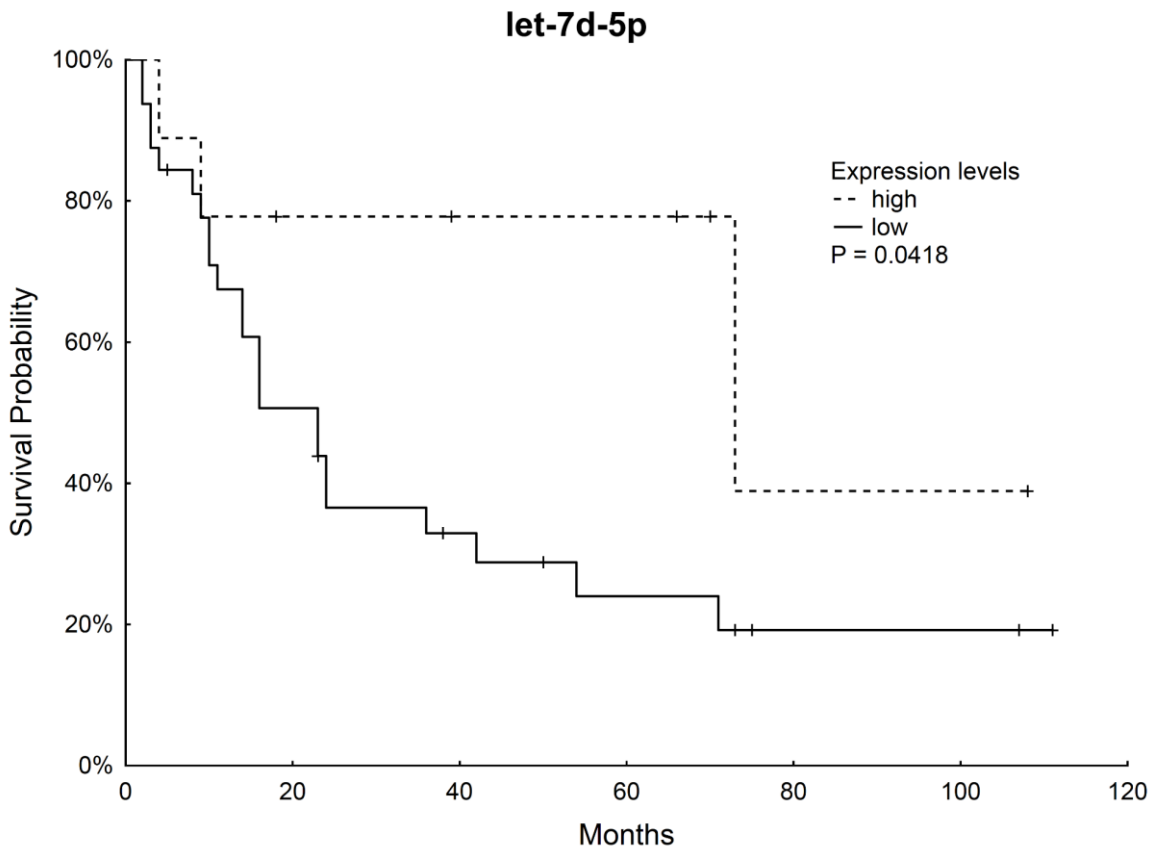

Figure 19. Kaplan-Maier survival plot for let-7d-5p.
Lower expression of let-7d-5p was detected for the patients with shorter survival interval. The results are statistically significant with P value of 0.0417. Vertical hatch marks show censored data.

7.1.2.4 miR-137-3p

On the other hand, patients with higher expression of miR-137-3p had significantly impaired survival interval in comparison to the patients with lower expression (P = 0.0278). The groups were divided based on 3rd quartile value (Figure 20).

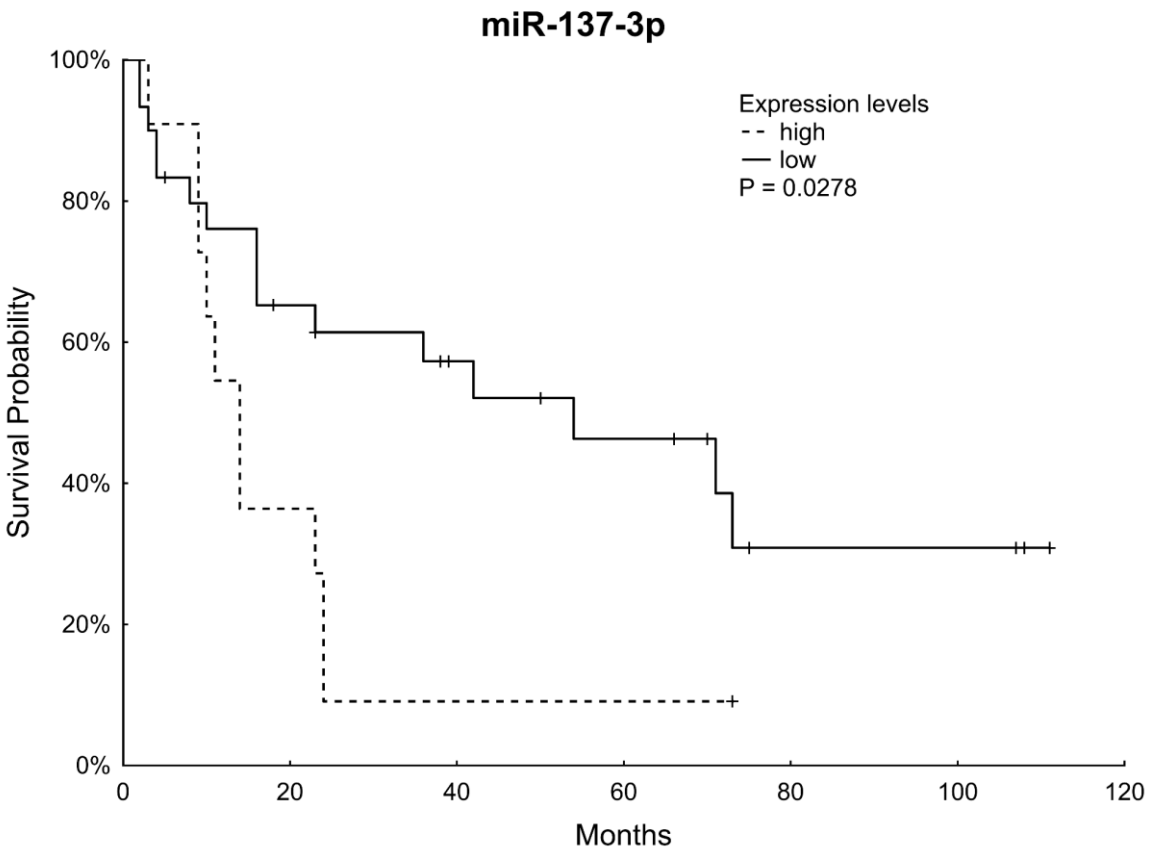

Figure 20. Kaplan-Maier survival plot for miR-137-3p.
Higher expression of miR-137-3p was detected for the patients with shorter survival interval. The results are statistically significant (P = 0.0278). Vertical hatch marks show censored data.

7.1.3 Correlation with clinicopathological characteristics

Overall clinicopathological data of the patients are summarized in Table 2. Age range of the patients at the time of diagnosis was between 27 and 82 years and median age was 65 years. During the follow-up period, 26/41 patients died, of whom 11/39 due to the tumor. Control samples (n = 17) were acquired from 7 male and 10 female patients. The youngest patient was 24 years old and the oldest one was 74 years old while median age was 54 years. Using statistical analysis, we compared relative expression levels of examined miRNAs with clinicopathological characteristics described earlier (Table 8). All statistically significant results and results approaching statistical significance are described in the following text.

Table 8. Correlations of miRNA expression with clinicopathological data in SSCC samples.

miR-		let-7a	let-7d	143-3p	146a-5p	155-5p	196a-5p	200c-3p	21-5p	9-3p	9-5p	137-5p	145-5p	223-3p	484	99a-5p
Clinicopathological data		P value														
Gender	Male	0.74	0.14	0.41	0.91	**0.097**	0.86	**0.072**	0.23	0.28	0.43	0.33	0.22	0.85	0.90	0.43
	Female															
Age (46)	≤ 50	**0.015**	0.57	0.76	0.46	0.87	0.22	0.42	0.72	0.45	0.61	0.74	0.74	0.69	0.30	0.29
	> 50															
Smoking status (39)	Smoker	0.94	0.63	0.50	0.85	0.19	0.54	0.30	0.25	0.12	0.18	0.84	0.30	0.86	0.43	**0.06**
	Non-smoker															
Occupation	High risk	0.40	0.39	0.39	0.22	0.11	**0.0003**	0.50	0.64	**0.031**	0.88	0.33	0.83	0.37	0.42	0.53
	Low risk															
Localization	Nasal cavity	0.57	**0.078**	0.11	**0.007**	**0.011**	0.21	0.34	**0.026**	0.92	0.99	0.78	0.82	0.40	0.40	0.60
	Maxillary sinus															
cT	T1, T2	**0.077**	0.46	**0.08**	0.57	0.88	0.82	0.68	0.28	0.80	0.23	0.46	0.25	0.33	0.63	0.36
	T3, T4															
cN	cN0	0.52	0.68	0.31	**0.025**	**0.009**	0.33	0.87	0.30	0.74	0.74	0.81	0.73	0.55	0.97	0.21
	cN1-3															
cM	M0	0.38	0.18	**0.08**	0.56	0.58	0.57	0.84	0.97	0.20	0.27	0.84	0.13	0.45	0.89	0.26
	M1															
Resection margin	Positive	0.40	0.33	0.29	0.34	0.12	0.55	0.64	0.26	0.52	0.94	0.46	0.25	0.83	0.71	0.92
	Negative															

Grade	G1	0.71	0.22	0.46	0.14	**0.017**	0.94	0.74	0.60	0.42	0.24	0.56	0.15	0.52	0.79	0.77
	G2-3															
Vascular invasion	Yes	0.60	0.36	0.60	0.42	0.71	0.78	0.56	0.73	**0.013**	0.90	0.90	**0.037**	0.65	0.26	0.69
	No															
Perineural invasion	Yes	0.66	0.90	0.32	0.47	0.45	0.40	0.70	0.55	**0.031**	0.74	0.30	0.29	0.46	0.35	**0.0055**
	No															
Local recurrence	Yes	**0.016**	**0.025**	0.13	**0.015**	0.17	0.40	**0.0054**	0.54	0.56	0.11	**0.045**	0.75	0.59	0.54	0.23
	No															
Regional recurrence	Yes	0.19	0.25	0.59	0.78	**0.036**	0.61	0.59	0.38	0.72	**0.045**	0.76	**0.076**	0.81	0.36	0.43
	No															
Distant recurrence	Yes	0.61	0.15	0.93	0.45	0.18	0.75	0.30	0.46	0.32	0.99	**0.09**	0.53	0.83	0.70	0.23
	No															
HPV status	Negative	0.69	0.30	0.68	0.79	0.34	0.42	0.28	0.72	0.23	0.73	0.62	**0.019**	0.89	**0.016**	**0.058**
	Positive															

Statistically significant results and results approaching statistical significance and are marked in bold.

After performing statistical analysis, we concluded that all studied miRNAs apart from miR-223-3p correlated with at least one of the studied parameters. Deregulation mir-200c-3p and miR-155-5p showed a tendency to be related to the gender of the patients (mild upregulation of miR-200c-3p in female patients and downregulation in male patients and vice versa for mR-155-5p) with P = 0.072 and P = 0.097 respectively. Let-7a was significantly more upregulated in patients older than 50 years of age in comparison to younger patients (P = 0.015). Patients with recorder history of smoking or current smokers showed downregulation of miR-99a-5p, whereas nonsmokers had normal levels of this miRNA (P = 0.06).

In samples of patients, where their occupation was assessed as a risk factor upregulation of miR-196a-5p and miR-9-3p was significantly higher in comparison to patients with safe occupations (P=0.0003 and P=0.031). Upregulation of miR-21-5p and let-7d tended to be higher in tumors occurring in maxillary sinus in comparison to tumors localized in nasal cavity (P = 0.026 and P = 0.078). Correspondently, miR-155-5p and miR-146a-5p expression was upregulated in samples originating in maxillary sinus, but mildly downregulated in samples from nasal cavity (P = 0.011 and P = 0.007).

let-7a and miR-143-3p expression tended to be upregulated in lower cT stages of the disease (T1, T2) in comparison to later stages (T3, T4) (P = 0.077 and P= 0.08). miR-146a-5p and miR-155-5p were significantly more upregulated in patients with presence of nodal metastases (cN1-3) in comparison with patients without lymph node metastases (cN0). In patients with recorder presence of long distance metastases (cM0) expression of miR-143-3p tended to be downregulated in comparison to normal expression in patients with no distant metastases (cM0) with P = 0.08. Resection margin had no correlation with miRNA expression.

miR-155-5p was upregulated in patients with high-grade (G2-3) stage tumors. In contrast, it was downregulated in low-grade (G1) tumor samples (P = 0.017). Vascular invasion was present in patients characterized by much higher expression of miR-9-3p (P = 0.013) and lower downregulation of miR-145-5 (P = 0.037). Moreover, perineural invasion was linked to much higher expression of miR-9-3p (P = 0.013) and significant downregulation of miR-99a-5p (P = 0.0055).

There were three types of recurrence events recorded during the follow-up period: local, regional and distant recurrence. Local recurrence was recorded in patients with lower upregulation of let-7a and let-7d (P = 0.016 and 0.025). Furthermore, with significant downregulation of miR-146a-5p (P = 0.015), then significant downregulation of miR-200c-3p (P = 0.0054) and finally significant upregulation of miR-137-3p (P = 0.045). On the other hand, regional recurrence was linked to significantly higher upregulation of miR-155-5p (P = 0.036), significantly lower upregulation of miR-9-5p (P = 0.045) and downregulation of miR-145-5p (P = 0.076). Distant recurrence correlations were limited due to low number of recorded cases. It seemed to be linked to miR-137-3p upregulation (P = 0.09).

HPV infection presence seemed to have influence on expression of miR-145-5p, which was less downregulated in HPV positive patients (P = 0.019), miR-484, which was more downregulated in HPV+ patients and miR-99a-5p, which showed upregulation in HPV positive patients.

7.1.4 Methylation analysis

Methylation status of *miR-145* gene, *miR-143* gene and their host gene (*miR-143/145*) was investigated by methylation specific high-resolution melting analysis to determine, if the deregulation of the miRNAs is caused by DNA methylation regulation. The cycling conditions and optimal master-mix composition was specifically optimized prior to the analysis of the samples. Methylation above 10 % (control containing 10 % of methylated DNA) was considered as methylated sample (Figure 21).

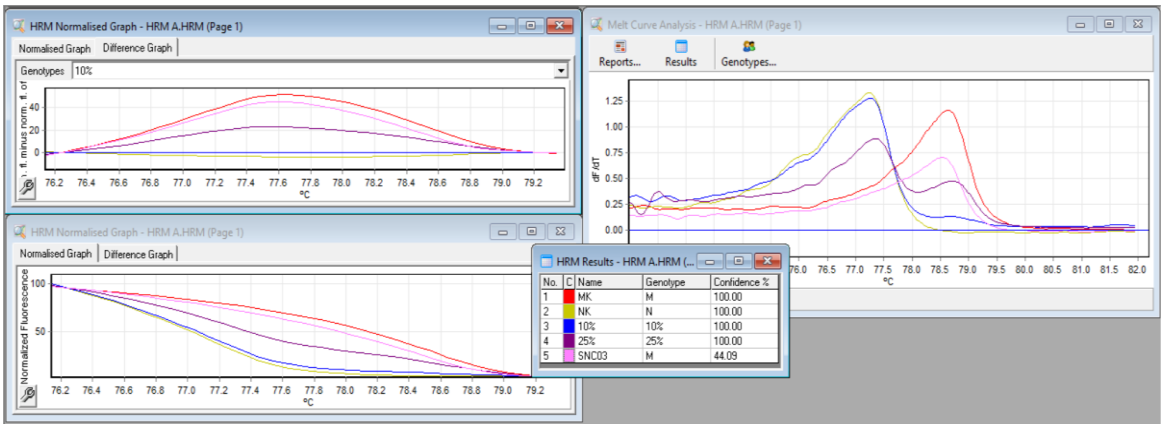

Figure 21. Methylation specific high-resolution melting analysis results. In the figure in the top left corner HRM the difference graph based on 10 % methylation control is displayed. In the bottom left corner Normalized HRM graph is displayed with melt curves in the right side of the figure. Red line is methylated control, yellow line is non-methylated control, blue line is 10 % methylated control purple line is 25 % methylated control and pink line is methylated sample.

The results of the methylation analysis are inconclusive, due to low number of samples with successful amplification and subsequent DNA methylation analysis. The gene for *miR-143* was methylated in 25/27 (92.6 %) of the sinonasal cancer samples. However, it was methylated in 7/7 (100 %) of the control samples. The first part if the *miR-145* gene was methylated in 29/29 (100 %) of the samples and it was also methylated in 6/6 (100 %) of the control samples. The second part of the *miR-145* gene was methylated in 16/28 (57.1 %) of the samples and it was methylated in 5/7 (71.4 %) of the control samples. The host gene miR-145/miR-143 was methylated in 20/27 (74.1 %) of cancer samples and in 5/6 (83.3 %) of control samples. There was no significant change in methylation of the studied genes, when comparing tumor and control samples. Moreover, there was no significant correlation/regression between DNA miRNA gene methylation status and miRNA expression.

7.2 Differentially expressed miRNAs in oropharyngeal carcinoma

7.2.1 microRNA microarray

In the first part of screening phase of the study Agilent miRNA microarray analysis and appropriate bioinformatical analysis were performed. We identified multiple miRNAs to be deregulated between tumor vs. control and metastasis vs. control sample sets and no miRNA to be significantly deregulated in comparison of tumor vs. metastasis samples.

Analysis has identified 71 miRNAs to be deregulated in tumor samples as oppose to control samples. miRNAs were considered deregulated if FC > 2 or < -2 and concurrently adjusted P < 0.05 (Figure 22). The most significantly upregulated miRNAs were miR-3656 (FC = 16.00 and P < 0.0001), miR-4530 (FC = 11.02 and P < 0.0001), miR-4443 (FC = 10.36 and P = 0.0005) and miR-6088 (FC = 10.06 and P < 0.0001). The most significantly downregulated miRNAs included miR-142-5p (FC = -14.79 and P = 0.009 and miR-223-3p with FC = -6,84 and P= 0.034 (Appendix Table 1).

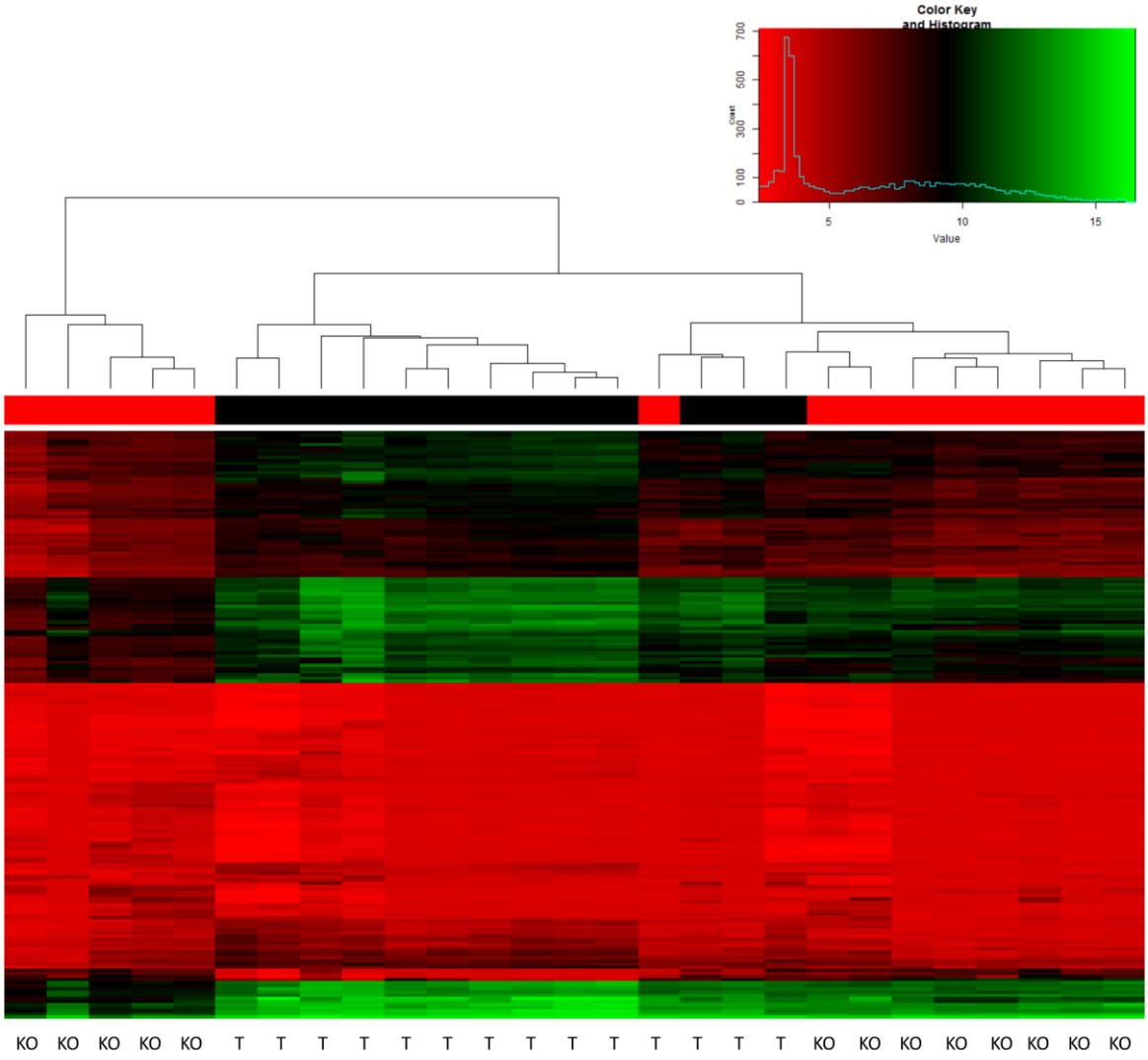

Figure 22. Heat-map of differentially expressed miRNAs.
The map includes color key and histogram of color representation. The clustering analysis shows two groups of samples: tumors in the middle of the heat-map and controls on the right side and left side of the map.
KO, control samples; T, tumor samples.

The second part of the study was to analyze if there are differentially expressed miRNAs when comparing their expression in metastasis samples and control samples. We have found 100 miRNAs to be significantly deregulated in metastasis samples (91 upregulated and 9 downregulated) (Figure 23). The most upregulated miRNAs included miR-3656 with FC = 14.51 and adjusted P < 0.0001, miR-4653-3p (FC = 11.08 and P = 0.0036) and miR-4530 (FC = 10.13 and P = 0.0001). The most

significantly downregulated miRNAs were miR-142-5p with FC = -11.31 and adjusted P = 0.019 and miR-133b (FC = -8.54, P = 0.023) (Appendix Table 2).

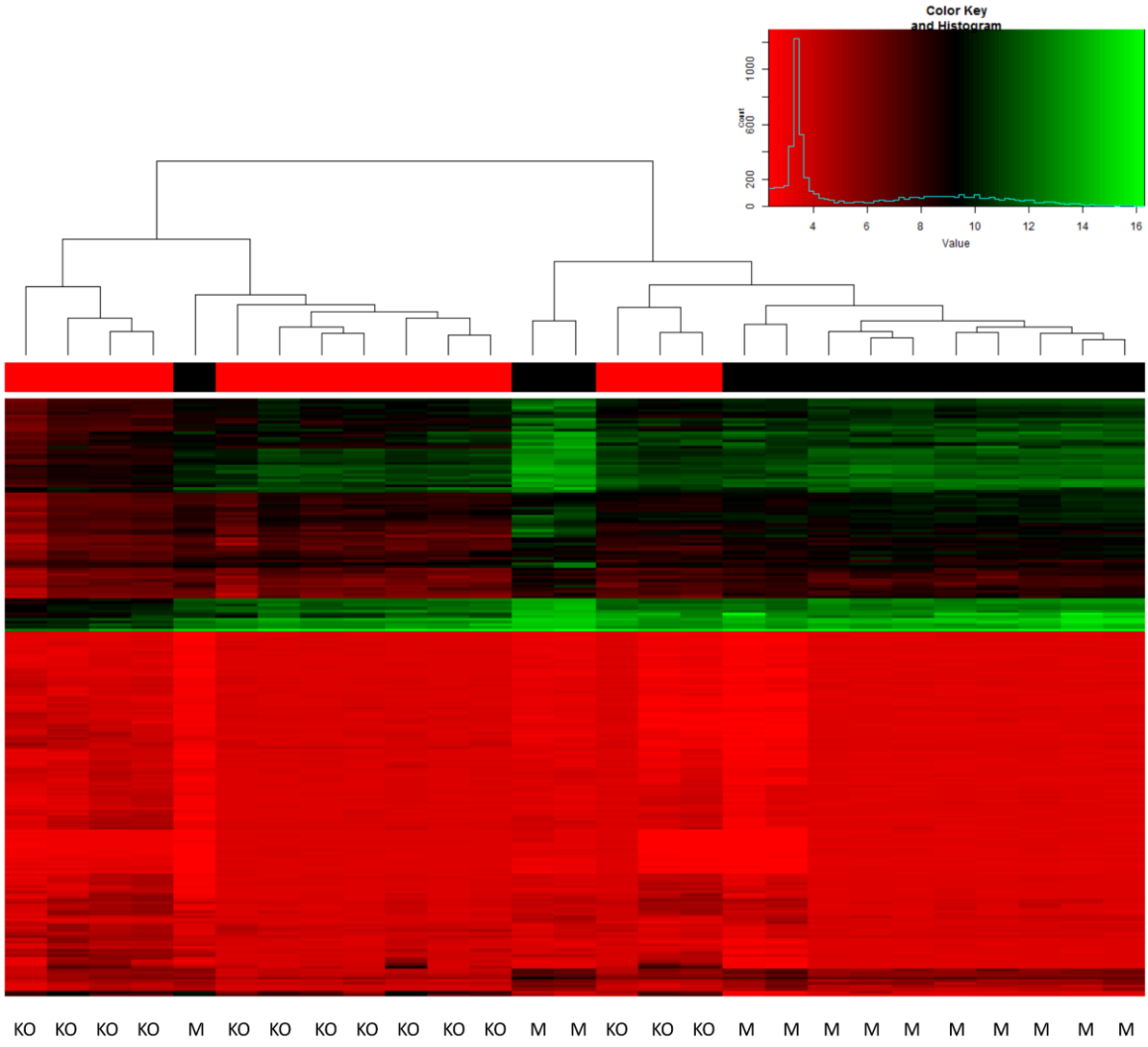

Figure 23. Heat-map of differentially expressed miRNAs.
The figure includes color key and histogram of color representation. The clustering analysis shows two mixed groups of samples. Controls are grouped on the left side of the heat-map as opposed to metastases, which are grouped on the right side of the map with some outliers in the center.
KO, control samples; M, metastasis samples.

Thirdly, analysis of deregulated miRNAs between metastasis samples and tumor samples was performed (tumor samples were considered as controls). There were 7 miRNAs deregulated (5 upregulated and 2 downregulated) (Figure 24). However, the deregulation was not statistically significant measured by adjusted P value.

The trend of upregulation was present for miR-133b (FC = 6.64 and P = 0.625) and for miR-206 (FC = 3.34 and P = 0.276). The trend of downregulation was present for miR-8485 (FC = -2.18, P = 0.995) and miR-150-5 (FC = -2.14, P = 0.995) (Appendix Table 3).

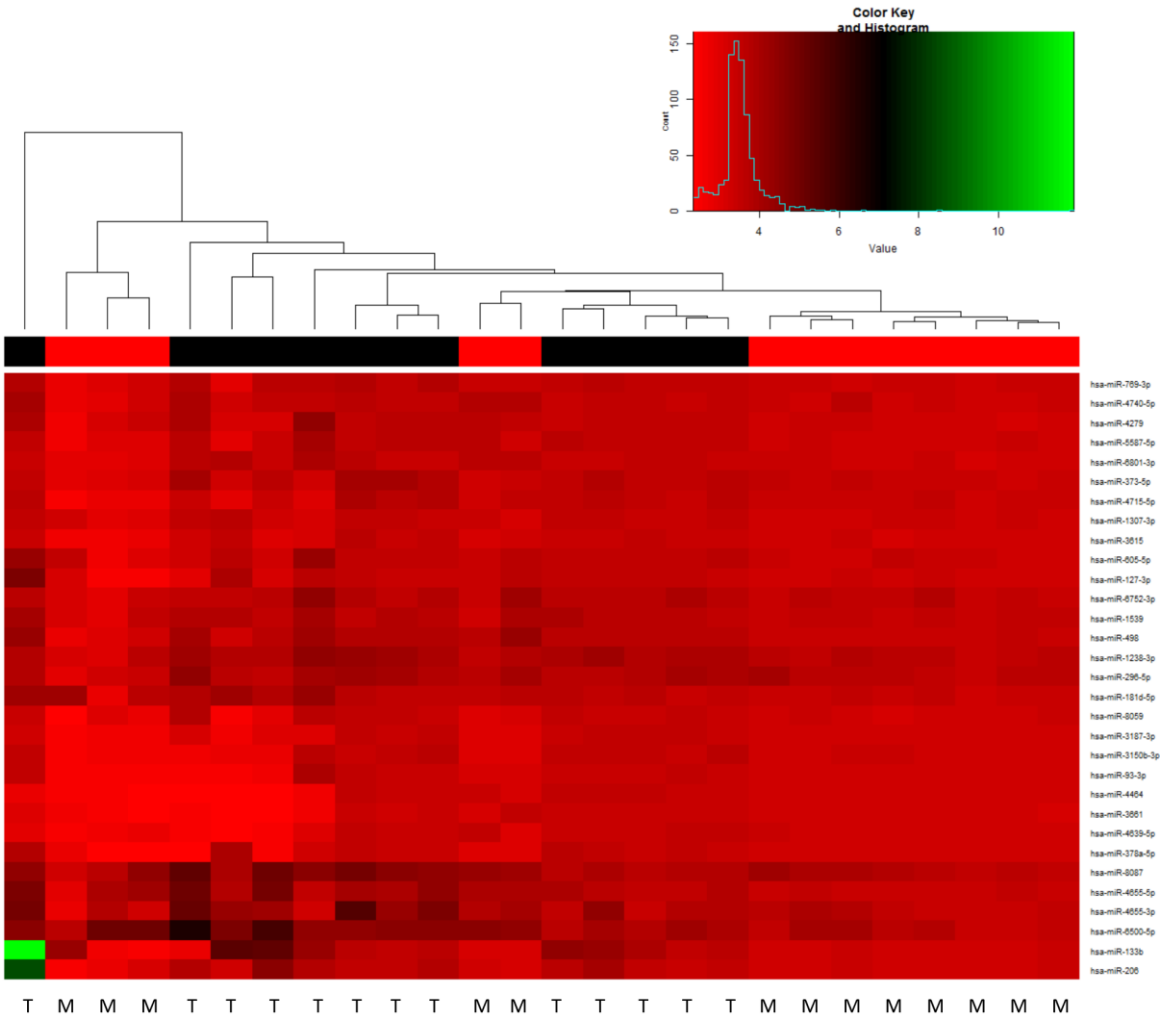

Figure 24. Heat-map of differentially expressed miRNAs.
The map includes color key and histogram of color representation. The clustering analysis shows two mixed groups of samples (tumors in the middle of the heat-map and metastases on the sides of the map). Although, no miRNAs were significantly deregulated, miRNAs with most significantly changed fold change were chosen for heat-map visualization.
T, tumor samples; M, metastases.

From all the results miR-3656 and miR-206 were selected for further validation second phase of the study – validation analysis by real-time PCR. miR-3656 was

selected based on its very significant deregulation in tumor and metastasis samples and comfortable expression levels throughout all the samples for subsequent analysis. miR-206 was selected, because it showed the most significant trend of deregulation between tumor and metastasis samples and it had stable levels of expression in all analyzed samples.

7.2.2 Small RNA Next-generation sequencing

In the second part of screening phase of the study next generation sequencing of sRNAs with focus on miRNAs was performed on MiSeq Illumina sequencer using NEBNext Small RNA Library Prep Kit. Initial quality control and mapping to reference genome (hg38) was performed in sRNA Detection module of Oasis 2.0. Samples with less than 90 % of trimmed reads and less than 40 % of uniquely mapped reads were considered as outliers. As a result, 33 samples (9 tumors, 12 metastases and 11 control samples were used in subsequent analysis and 6 samples discarded as outliers or low-quality samples. Mean value of initial number of reads was 1,793,318 with mean value of 98 % of trimmed reads and 37 % of uniquely mapped reads. The most expressed miRNAs in all samples are pictured in Figure 25.

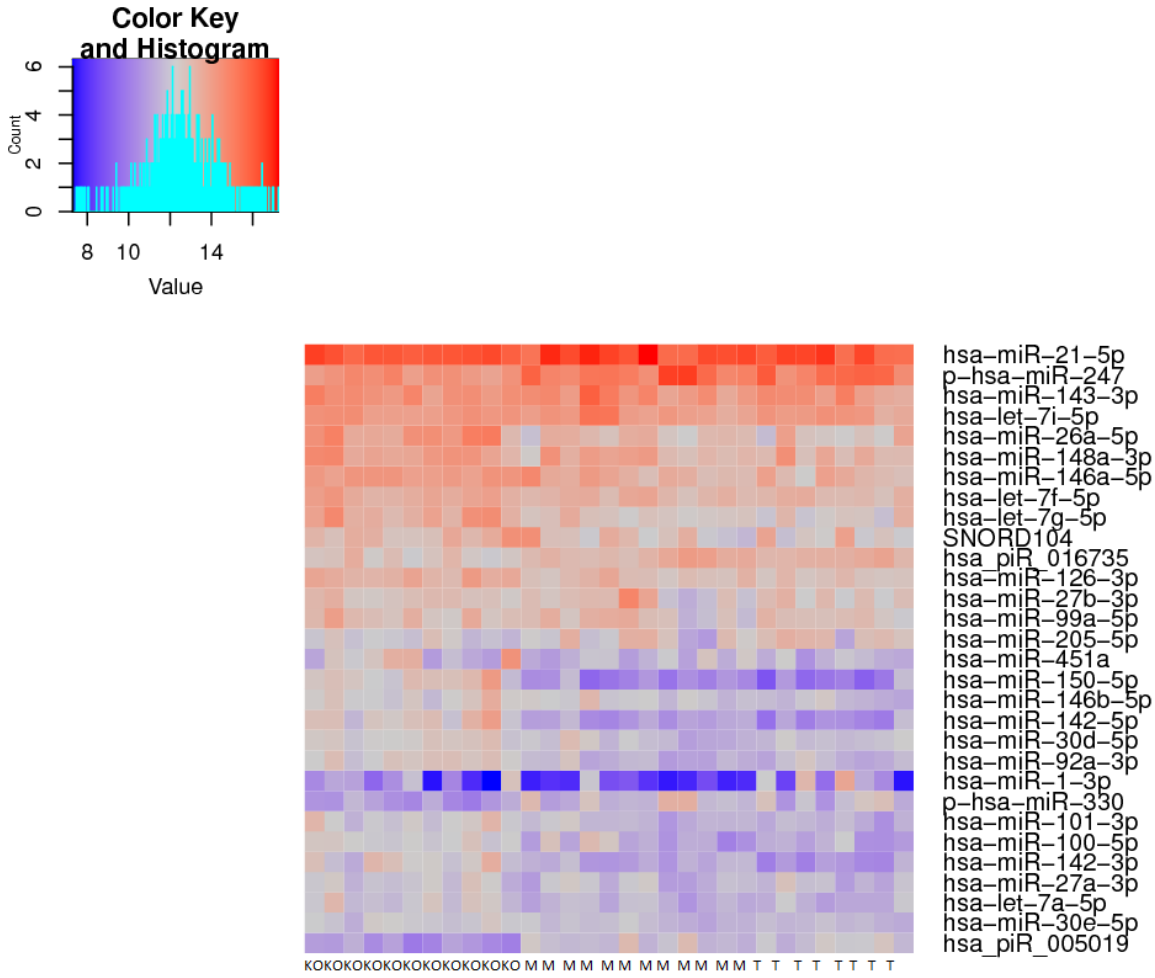

Figure 25. Heat-map of 30 the most expressed sequences in tumors, metastases and control samples.

The heat-map visualizes 30 the most expressed sRNAs in the analyzed NGS counts based on regularized log transformation. The color key and histogram in the top left corner shows distribution of values and colors.

Four different analyses were performed using Oasis 2.0 DE Analysis module (All three sets of samples of tumors corresponding metastases and control were compared with each other and together). Clustering of the three sets of samples is shown in Figure 26. Clustering analysis showed us one distinct group of control samples and one heterogenic group of tumor samples with metastasis samples.

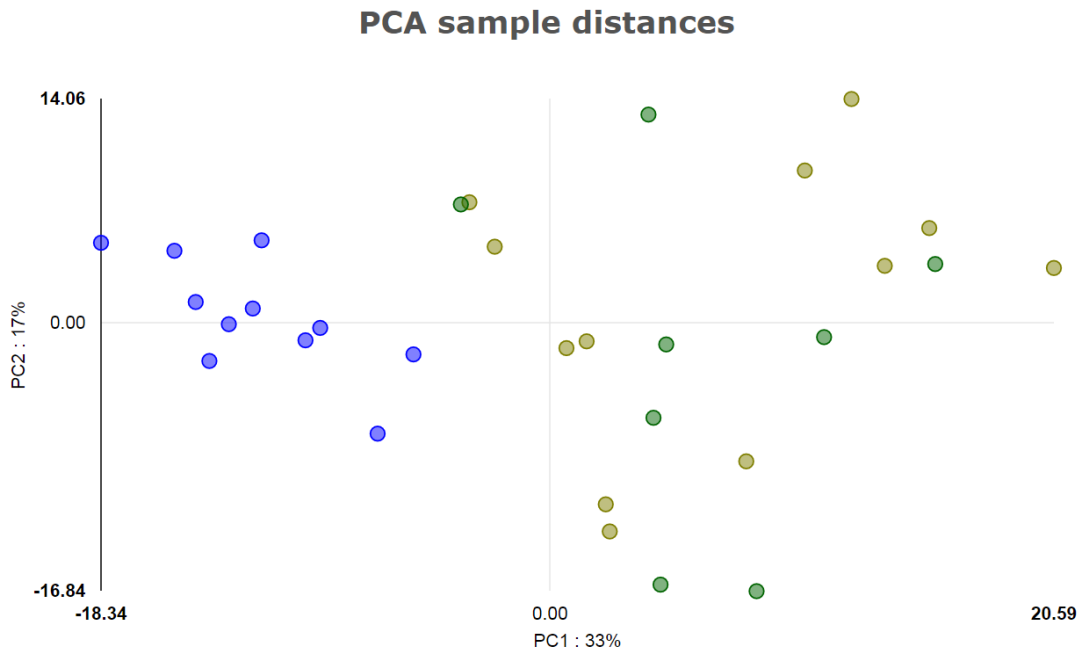

Figure 26. Clustering of individual samples based on sRNA expression profiles obtained by sRNA NGS.
Blue dots represent control sample, green dots represent tumors and khaki dots represent metastases. PCA, Principal Component Analysis, PC, Principal Component.

After differential expression analysis of tumor sample and control samples counts, the analysis provided 673 uniquely mapped reads (with minimum of 5 reads per sample). As a result, we have identified 673 sRNAs to be expressed in the samples. Of the total count of sRNAs 396 RNAs were identified as miRNAs, 15 results were identified as novel miRNAs (miRNAs that have not been annotated in miRbase, 113 were identified as piRNAs, 109 as snoRNAs, 17 as snRNAs and 23 as rRNAs. After selecting miRNA species from the analysis, 156 individual statistically deregulated miRNAs have been found, 52 of them were upregulated and 104 were downregulated (Appendix Table 4). The most upregulated miRNAs were miR-196a-5p (FC = 22.22 and adjusted P < 0.0001), miR-187-3p (FC = 11.26 and P < 0.0001), and miR-9-5p (FC = 8.11 and P < 0.0001). The most significantly downregulated miRNAs were miR-150-5p (FC = -9.87 and adjusted P = P < 0.0001), miR-142-5p (FC = -7.45, P < 0.0001) and miR-342-3p (FC = -6.75, P < 0.0001) (Figure 27).

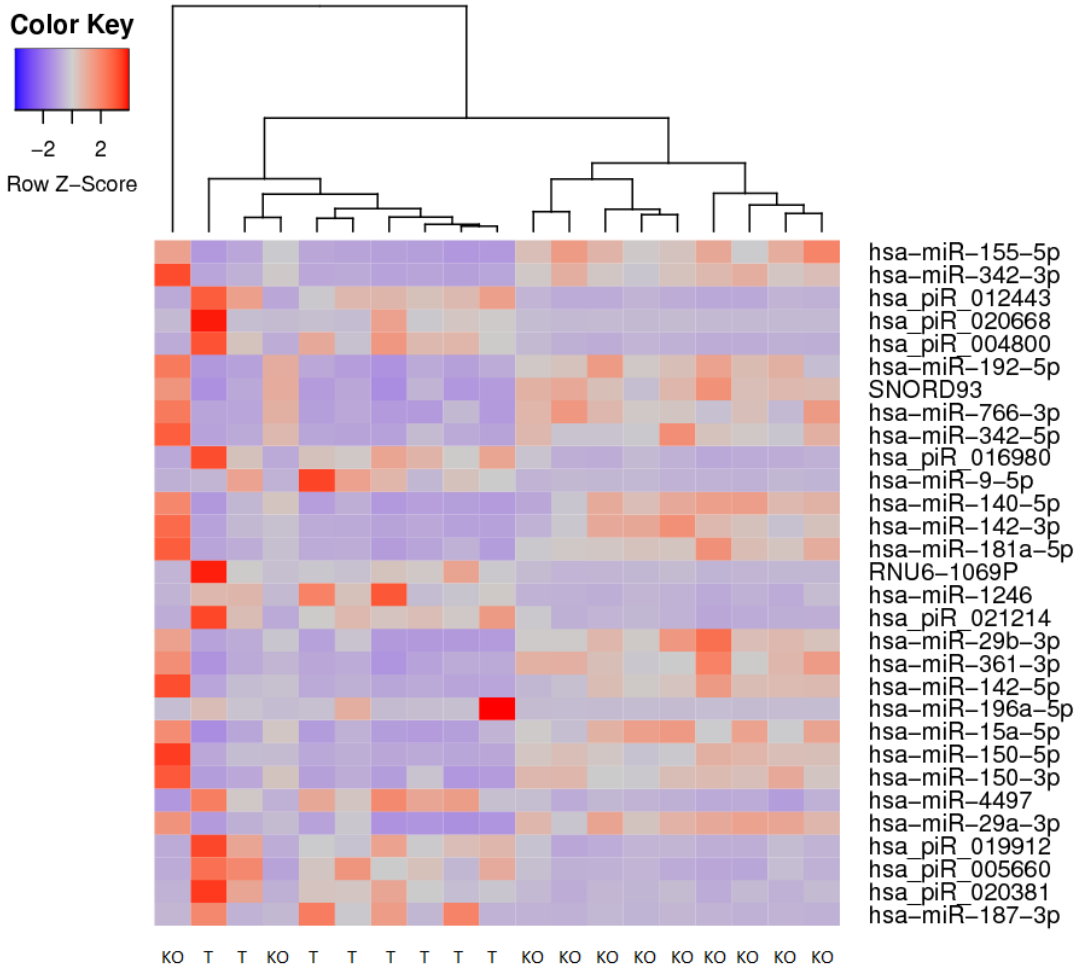

Figure 27. Heat-map of significantly deregulated sequences in tumor samples. The heat-map visualize 30 most significantly deregulated sequences in the analyzed NGS counts based on $\log_2$ fold change expression data (adjusted p value less than 0.1). The color key is in the top left corner of the figure.

Differential expression analysis of metastases and control samples of NGS mapped counts revealed 690 uniquely mapped reads, which means that they were found in the reference sequence and had a minimum of 5 reads per sample. This corresponds with identifying 690 sRNAs among these samples. Of these RNAs, 399 were uniquely mapped miRNAs, 15 were novel miRNAs, 118 were piRNAs, 28 were rRNAs, 110 were snoRNAs and 20 were snRNAs.

Focusing strictly on miRNA species revealed 71 significantly upregulated miRNAs and 75 significantly downregulated miRNAs (Appendix Table 5). The most

upregulated miRNAs in metastasis samples compared to control samples were miR-615-3p with FC = 19.93 and adjusted P value < 0.0001, miR-196-5p (FC = 19.34 and P < 0.0001) and miR-9-5p (FC = 11.23 and P < 0.0001). The most downregulating miRNAs were miR-150-5p (FC = -9.18, P < 0.0001), miR-766-3p (FC = -5.90 and P < 0.0001) and miR-142-5p (FC = -5.64, P < 0.0001) (Figure 28).

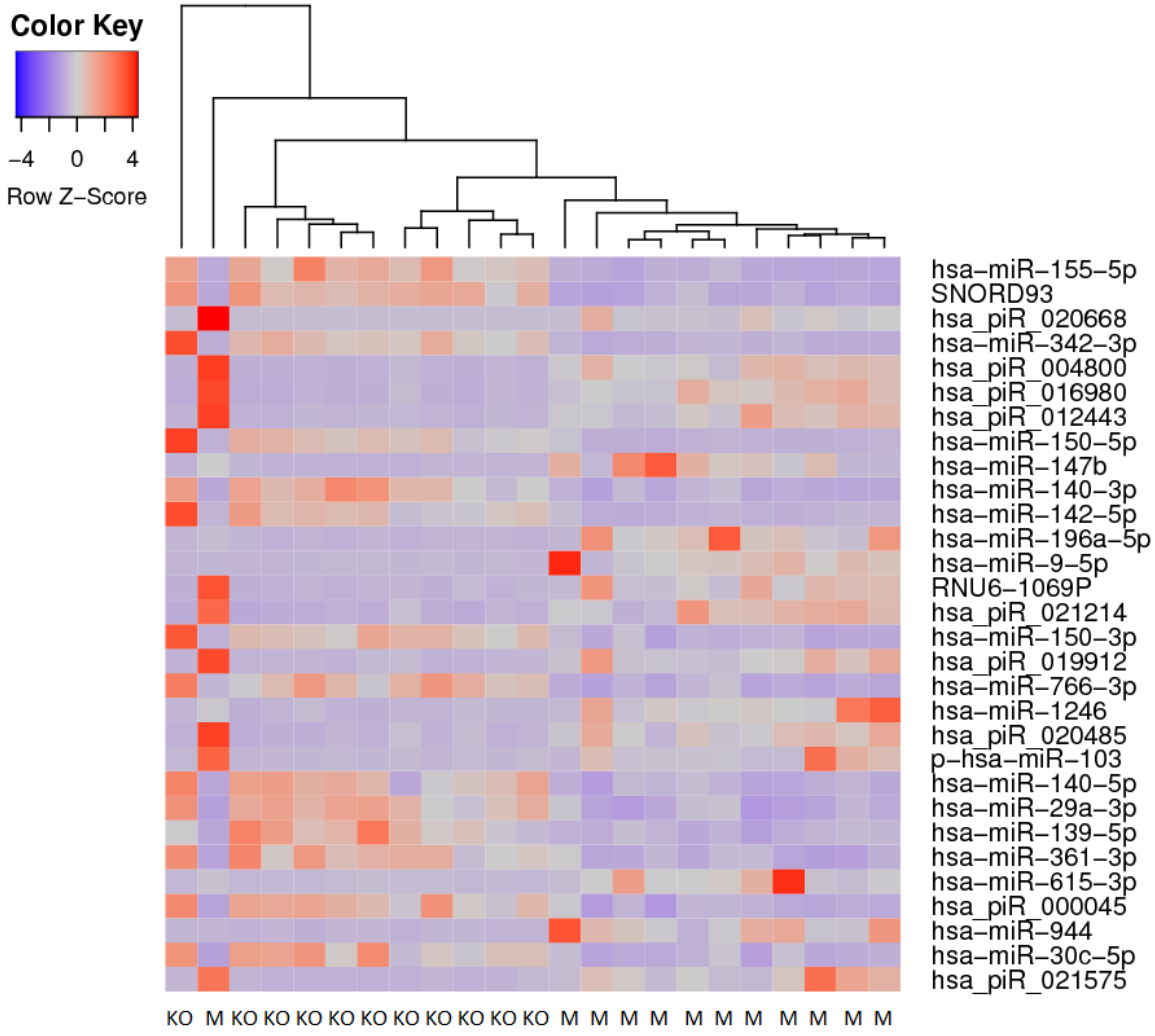

Figure 28. Heat-map of significantly deregulated sequences in tumor samples. The heat-map visualize 30 most significantly deregulated sequences in the analyzed NGS counts of metastasis data based on $\log_2$ fold change expression data (adjusted p value less than 0.1). The color key is in the top left corner of the figure.

Using Oasis 2.0 differential expression analysis of tumor samples versus metastasis samples (tumor samples were marked as control group and metastasis

samples were marked as treatment group for the analysis) we have identified 700 uniquely mapped NGS reads. Consequently, we have identified 700 small RNA species to be captured at minimum of 5 reads per sample. Out of 700 sRNAs, 393 were miRNAs and 16 were novel miRNAs. From the other sRNAs species 125 piRNAs were present, 30 rRNAs, 115 snoRNAs and 21 snRNAs. After other than miRNA small RNA species filtering, we have found 9 miRNAs to be significantly deregulated (Appendix Table 6). Significantly upregulated miRNAs were: miR-514a-3p with FC = 2.89 and adjusted P value = 0.0350 and miR-218-5p with FC = 2.42 and P = 0.0245. The most significantly downregulated miRNAs were: miR-375 (FC = -8.12 and P < 0.0001), miR-1-3p (FC = -4.46 and P = 0.0018) and miR-4532 (FC = -3.88 and P = 0.0015) (Figure 29).

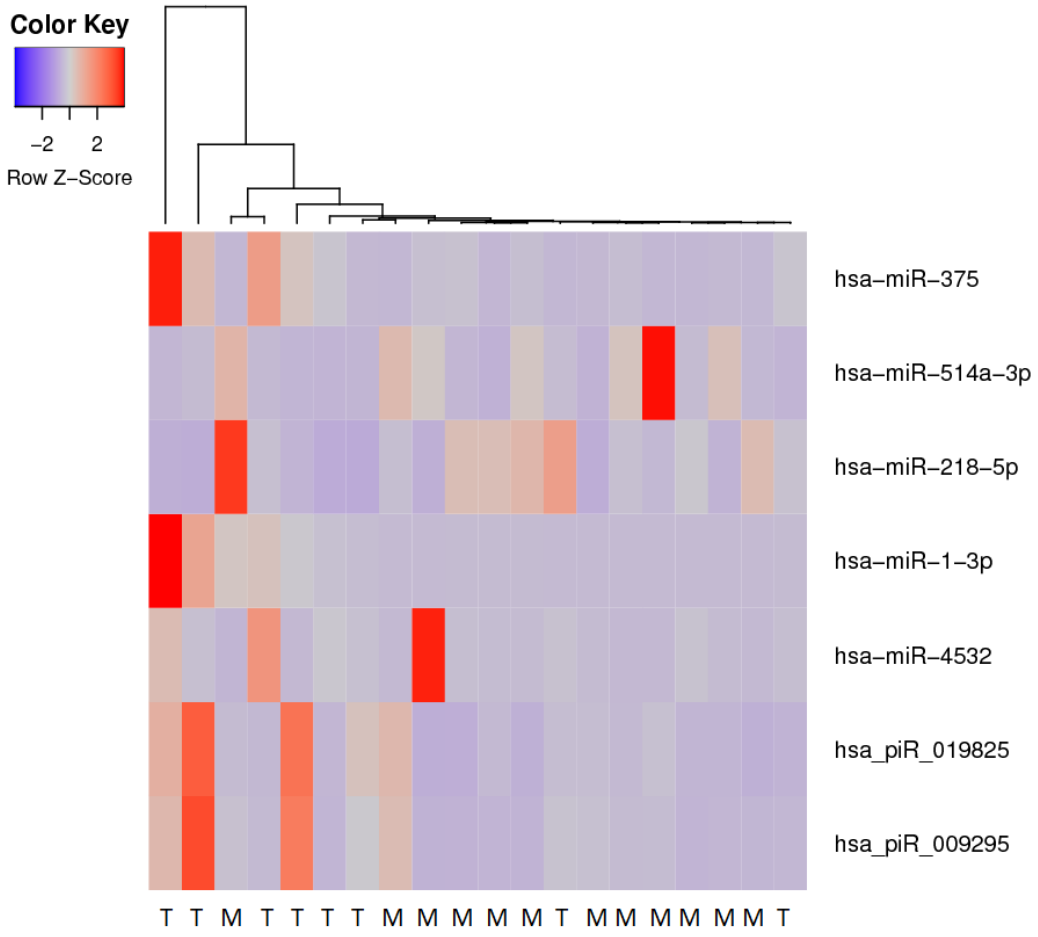

Figure 29. Heat-map of significantly deregulated sequences in metastases versus tumor samples.

The heat-map visualize the most significantly deregulated sequences in the analyzed NGS counts of metastasis data based on $\log_2$ fold change

expression data (adjusted p value less than 0.1). The color key is in the top left corner of the figure.

After evaluation of all small RNAs NGS data two miRNAs were selected for further validation by real-time PCR: miR-375-3p and miR-150-5p. miR-375-3p (previously known as miR-375) was selected based on its significant deregulation in metastasis samples compared to tumor samples and the expression was enough for further analysis. miR-375 was also validated as the best biomarker for tumors and metastases differentiation using Classification Analysis module of Oasis 2.0 with feature importance 1.049. On the other hand, miR-150-5p was selected for its significant downregulation in both tumor and metastasis samples. This miRNA was also among the selected as potentially good biomarker of tumor samples vs. control samples differentiation by Classification Analysis by Oasis 2.0 with feature importance 0.298.

7.2.3 Real-time PCR evaluation analysis

7.2.3.1 Relative expression verification analysis

In the second phase of the study, differentially expressed miRNAs in oropharyngeal cancer samples and corresponding metastases were analyzed using real-time PCR TaqMan Advanced approach. miRNAs for verification analysis were selected based on Agilent miRNA microarray results and small RNA NGS Illumina sequencing. Four miRNAs were selected to be suitable for phase two of the study: miR-3656, miR-206, miR-150-5p and miR-375-3p.

The analysis confirmed that miR-3656 was significantly upregulated in tumor samples (FC = 6.56 and P = 0.0006) and less but significantly upregulated in metastasis samples (FC = 3.04 and P = 0.012) compared to control samples. Moreover, deregulation between the three sets of samples was significant according to ANOVA (P = 0.0012). Correspondently, miR-150-5p was significantly downregulated in tumor samples (FC = -14.75 and P < 0.0001) and significantly

downregulated in metastasis samples compared to control samples (FC = -8.94 and P < 0.0001). Moreover, ANOVA confirmed statistical significance in expression between all three sets of samples (P < 0.0001) and there was difference in expression between metastases and tumors (FC = 1.30 and P = 0.049) (Figure 30).

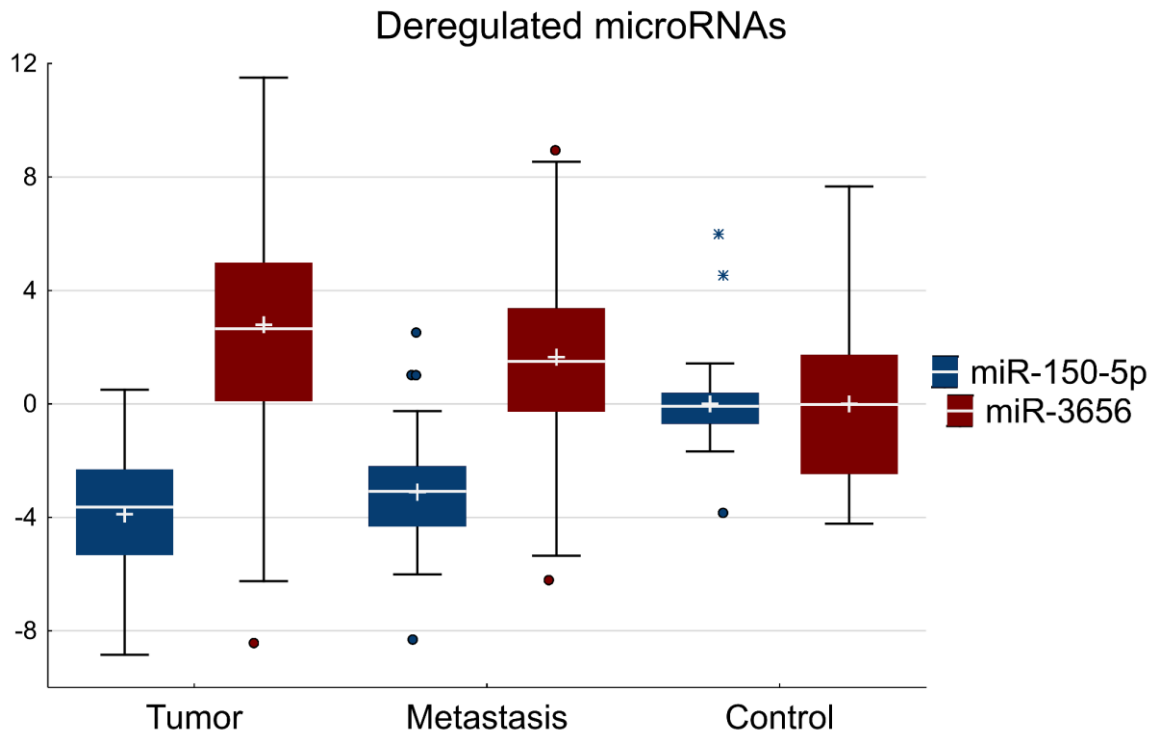

Figure 30. Deregulated miRNAs in the second phase of the study. miR-3656 and miR-150-5p were confirmed to be significantly deregulated in tumor and metastasis samples by real-time PCR analysis with TaqMan™ Advanced Assays. Whiskers show Minimum and maximum values with outliers symbolized as dots and extremes symbolized as asterisks.

On the other hand, miR-206 was significantly downregulated in metastasis samples compared to tumor samples (FC = -39.99 and P < 0.0001). It was significantly upregulated in tumor samples compared to controls (FC = 10.93 and P < 0.0001). In contrast, there was a significant downregulation in metastasis samples compared to control samples (FC = -3.93 and P = 0.0061). Finally, there was a trend of downregulation of miR-375 in metastasis samples compared to tumor samples (FC = -2.45 and P = 0.146). Even though, the deregulation was statistically

significant according to small RNA NGS data, we were unable to confirm the statistically significant results using real-time PCR analysis (Figure 31).

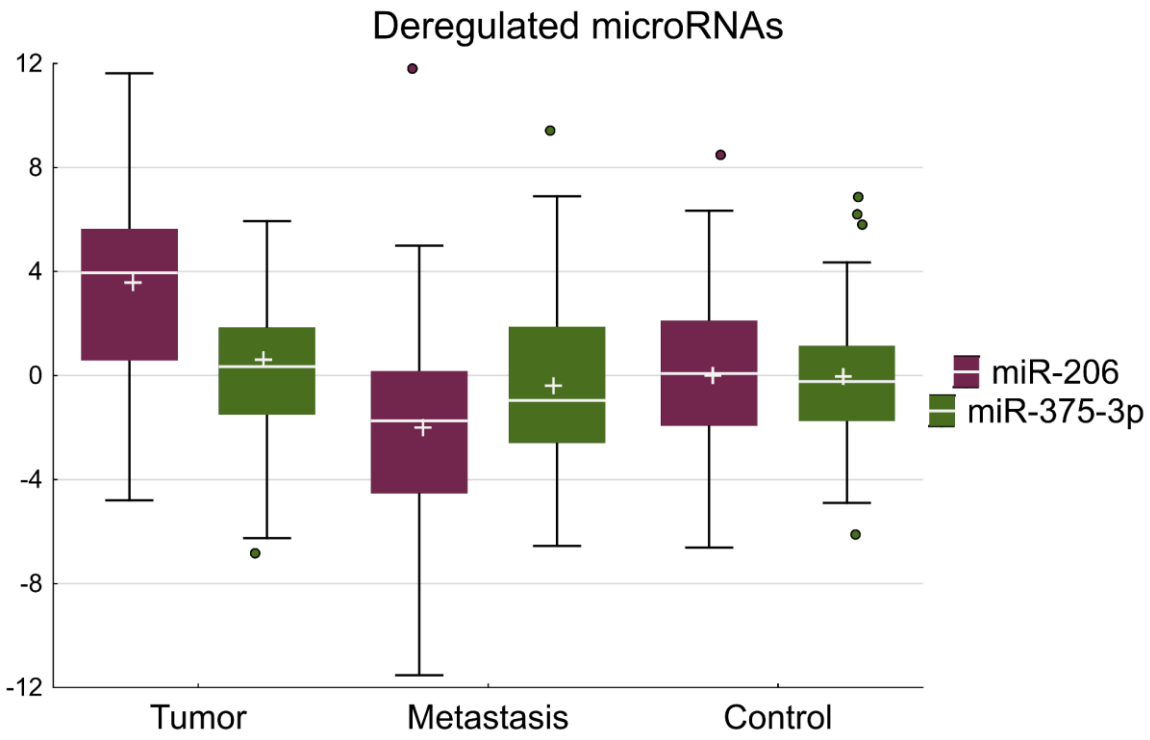

Figure 31. Deregulated miRNAs in the second phase of the study. miR-206 and miR-375-3p were confirmed to be deregulated in metastasis samples compared to tumor samples by real-time PCR analysis with TaqMan™ Advanced Assays. However, only the results for miR-206 were significantly deregulated. Whiskers show Minimum and maximum values with outliers symbolized as dots and extremes symbolized as asterisks.

The four selected miRNAs were further analyzed to determine their diagnostic biomarker potential using PanelComposer and CombiROC web-based tools. Tumor and metastasis samples were considered as positive samples and control samples were considered as negative samples. According to PanelComposer analysis miR-150-5p is the best diagnostic biomarker for oropharyngeal cancer with AUC = 0.934, confidence interval (0.934, 0.935) and $P < 0.0001$. The most efficient panel is: miR-3656, miR-206 and miR-150-5p with AUC = 0.908, CI = (0.908, 0.909) and $P < 0.0001$. The best miRNA panel suggested by CombiROC

tool is miR-3656 combined with miR-150-5p and miR-375-3p with AUC = 0.948 (ER = 0.084), SE = 0.922, SP = 0.09 and optimal cut-off value 0.705 (Figure 32).

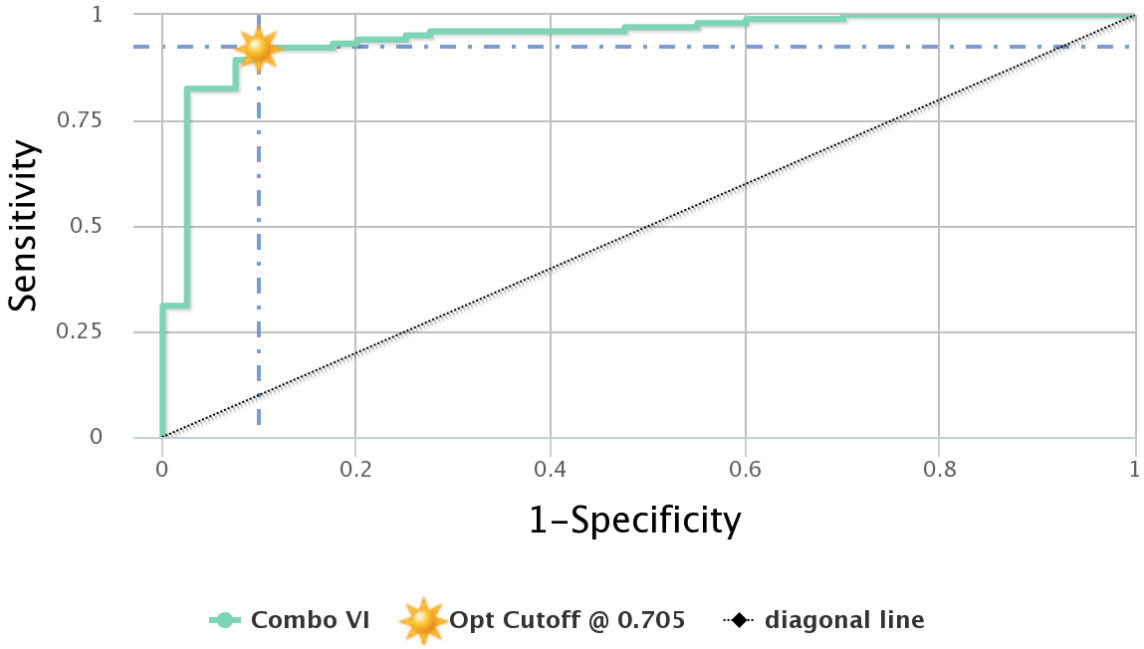

Figure 32. ROC curve for 3 miRNA panel for prediction of OPSCC.
Turquoise line represents ROC curve for miR-3656, miR-150-5p and miR-375-3p panel. Yellow sun pictogram represents Optimal cut-off value. Diagonal line dots represent random guessing.
ROC, receiver operating characteristic

7.2.3.2 miRNA expression and patients' survival

Follow-up period of the patients ranged from 3 to 180 months (until March 2019) and median value was 82 months. For the survival analysis we used Kaplan-Meier analysis to generate survival curves accompanied by Logrank test to obtain P value of prognostic significance of each miRNA expression regarding survival of the patients. The patients were divided into two categories for each miRNA based on their expression levels. Only expression of miR-150-5p was associated with longer survival intervals.

The expression data were divided into two categories independently for tumor samples and metastasis samples based on median values. The tumor samples

with lower expression of miR-150-5p were grouped together (P = 0.0124) (Figure 33) and metastasis samples with lower expression of miR-150-5p were grouped into one category (P = 0.0192) (Figure 34).

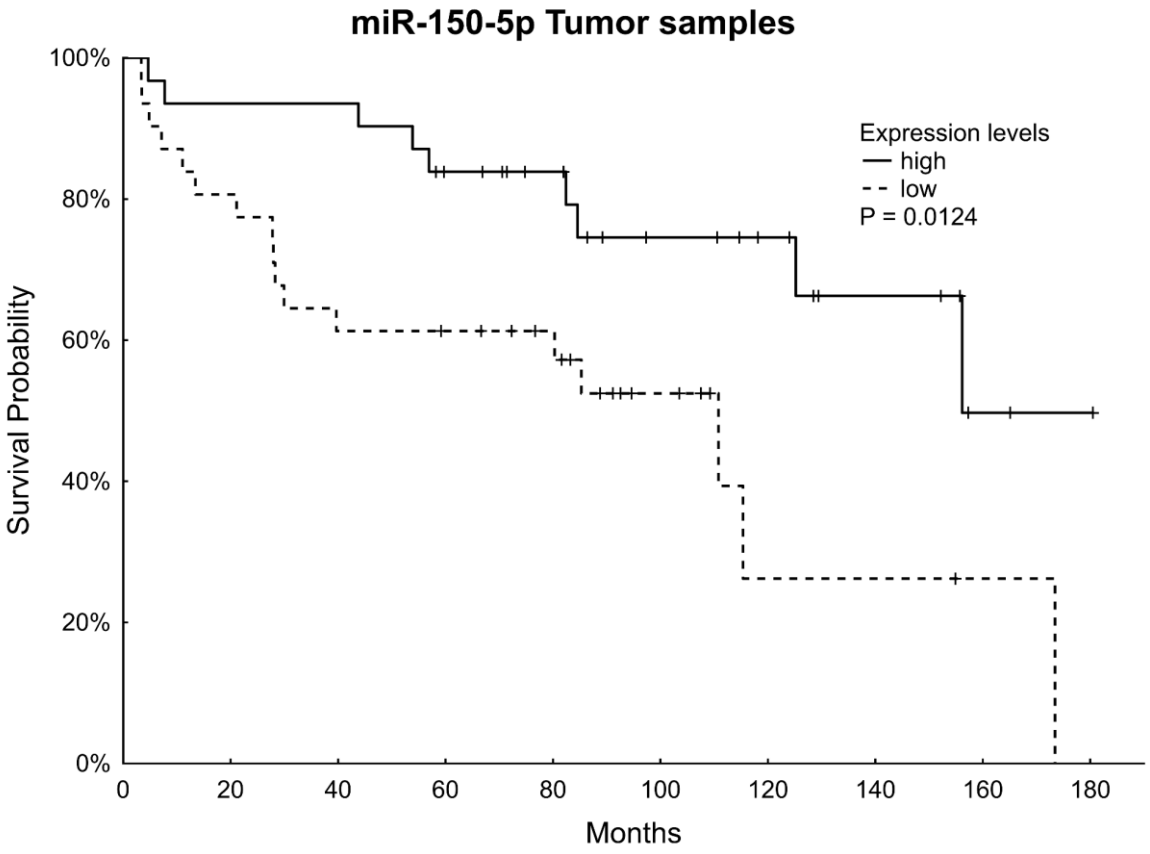

Figure 33. Comparison of survival times of OPSCC patients based on miR-150-5p expression in primary tumors.
Patients with lower expression of miR-150-5p (based on median value) had impaired survival in comparison to higher expression group. The difference is statistically significant with P = 0.0124 (Logrank test). Vertical hatch marks show censored data.

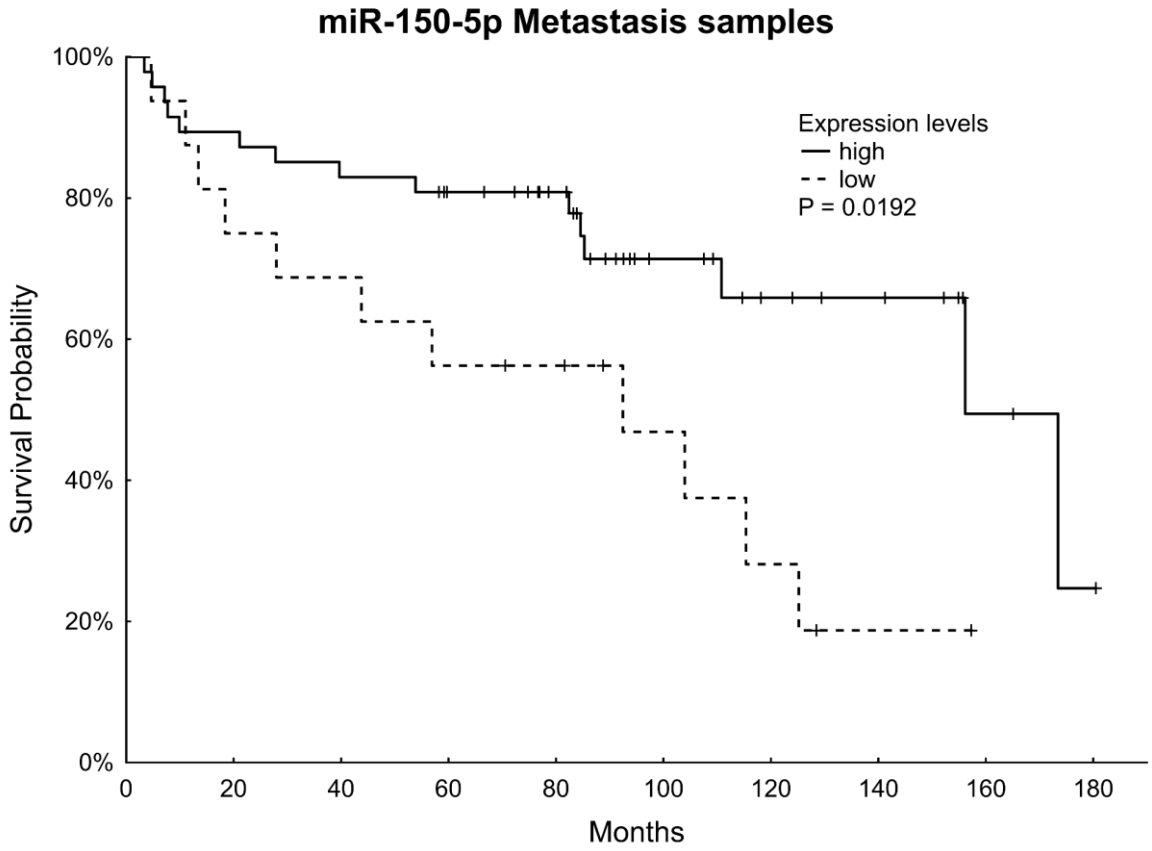

Figure 34. Comparison of survival times of OPSCC patients based on miR-150-5p expression in metastases.
Patients with lower expression of miR-150-5p showed impaired survival in comparison to higher expression group. The difference is statistically significant with P = 0.0192 (Logrank test). Vertical hatch marks show censored data.

7.2.3.3 Correlation with clinicopathological characteristics

Overall clinicopathological data of the oropharyngeal cancer patients are summarized in Table 3. Age range of the patients at the time of diagnosis was between 41 and 80 years and median age was 58 years of age. During the follow-up period 31/83 patients died, of whom 10/31 directly due to the tumor. Control samples were acquired from patients with non-malignant diagnosis treated for chronic tonsillitis. All known clinicopathological data was compared to relative expression of four miRNAs that were part of second phase of the study (Table 9). All statistically

significant results and results approaching statistical significance are described in the following text.

Table 9. Correlations of miRNA expression with clinicopathological data in OPSCC samples.

Clinicopathological Characteristics		miR-3656	miR-206	miR-150-5p	miR-375-3p
Gender	Male	0.11	0.40	0.70	**0.034**
	Female				
Age	≤ 55	0.19	0.27	0.30	0.67
	> 55				
HPV status	Negative	0.87	0.56	**0.071**	0.42
	Positive				
Smoking	Smoker	0.43	0.98	0.32	0.67
	Non-smoker				
Alcohol	Yes	0.25	0.85	0.68	0.90
	No				
Localization	Tonsils	0.74	0.72	0.57	0.31
	Oropharynx				
	Tonsillar fossa				
	Base of tongue				
Typing	Keratinizing	0.18	0.39	**0.010**	0.34
	Non-keratinizing				
pT	T1 – T2	**0.061**	**0.09**	**0.0016**	0.103
	T3 – T4				
pN	N1	0.88	0.80	**0.028**	0.99
	N2 – N3				
Perineural invasion	Yes	0.76	0.28	**0.0061**	0.93
	No				
Extracapsular invasion	Yes	0.71	0.25	0.24	0.24
	No				
Recurrence	Yes	0.60	0.78	**0.053**	0.99
	No				

Statistically significant results and results approaching statistical significance and are marked in bold.

All the miRNAs selected for second phase of the study correlated with at least one of the tracked clinicopathological characteristics. miR-3656 correlated with size and extension of the primary tumor (pT), while the less extensive tumors (T1 – T2) had less prominent downregulation of the miRNA than more extensive tumors (T3 – T4) with P = 0.0016. Concurrently, the more extensive tumors had high

upregulation of miR-206 in comparison to less extensive tumors with results approaching statistical significance (P = 0.09).

On the other hand, miR-150-5p correlated with several recorded parameters. The trend of higher downregulation of miR-150-5p in HPV positive samples was observed with P = 0.071. Keratinizing tumors had lower expression of miR-150-5p than non-keratinizing tumors (P = 0.010). Size and extension of the tumor also correlated with miR-150-5p expression, when lower expression of the miRNA was present T3 – T4 stage tumors (P = 0.0016). Moreover, downregulation of miR-150-5p was less prominent in tumors with higher regional lymph nodes metastases involvement (N2 – N3) (P = 0.028). miR-150-5p expression also correlated with recurrence (three types of recurrence were recorded: local, regional and distant) of the disease and the downregulation was more significant in patients with recorded follow-up recurrence (P = 0.053). Finally, miR-375-3p correlated with gender, when its expression was lower in women than in men (P = 0.034).

8 Discussion

Sinonasal and oropharyngeal cancers as well as other tumors arising from anatomically complex head and neck area are characterized by difficult treatment, poor prognosis and low survival rate [11]. The accumulation of aberrant epigenetics events (such as posttranscriptional deregulation by miRNAs and DNA methylation) can influence head and neck cancer pathology [92]. microRNAs have been recognized as key molecules in cancer development and progression in various types of tumors. They have been established as promising prognostic cancer biomarkers due to their resistance to degradation in many tissue types (including FFPE tissues) and various body fluids (such as plasma, serum, urine, or saliva). The amount of research papers published that are focused on miRNA research grows rapidly each year [93; 94].

Up- or down-regulation of miRNAs occurs usually in the same direction across most of the cancer subtypes. For example, miR-21 is upregulated in almost all human cancer types and its oncogenic effect has been extensively described [95-97].

Number of studies focused on miRNA expression in sinonasal cancer has been limited. Ogawa et al. [71] studied correlation between downregulation of miR-34a and resistance to CDDP treatment. The downregulation of miR-34a in SSCC was further confirmed by Zhao and Wang [72] and the further associated it with poor prognosis of the patients. HPV positive OPSCC is currently the hot topic of oropharyngeal cancer research. Deregulation of various miRNAs (such as miR-31, miR-24, miR-146a, etc.) has been previously described [75].

In the current study, we investigated relative expression of several preselected miRNAs and the relationship between their expression and clinicopathological characteristics of the patients. miRNAs of interest for this study were selected based on literary review focused on other types of squamous cell head and neck cancer (sinonasal cancer study) and high-throughput miRNA expression methods in the phase I of the oropharyngeal cancer study.

8.1 Differentially expressed miRNAs in sinonasal carcinoma

Using real-time assay-based approach for relative expression analysis of miRNAs followed by $2^{-\Delta\Delta Ct}$ analysis has been a gold standard for miRNA expression studies for decades. The main reasons are unmatched specificity, simplicity of the procedures and affordable price of the chemistry [98]. However, the main downside of the method is limitation in number of miRNAs that can be detected during one analysis.

Using relatively new miRNA detection design by TaqMan™ Advanced Assays we were able to detect relative expression of 22 miRNAs in several steps of the SNC study in a span of three years. cDNA from a single tube of obtained from one step synthesis by TaqMan™ Advanced Synthesis Kit with non-specific miRNA reverse-transcription primers was used as an input material for all real-time PCR reactions. With Advanced TaqMan technology, we were able to study both 5p and 3p variants of the same miRNA, because of the sequence specificity of the pre-designed Assays [99]. All pre-designed assays were mapped to the newest version of miRBase (v22).

Assay selection for the SNC study was based on literary review of previously studied miRNAs. However, because miRNA expression studies in SNC are rare, assays were predominantly selected based on previously published research on head and neck squamous cell cancer. As a result, not all selected miRNAs were expressed or deregulated (according to the criteria of P value < 0.05 and Fold change more significant than 2) in our set of sinonasal cancer samples.

Moreover, for some miRNAs only one strand of mature miRNA was expressed. In our experiments, miR-200c-3p did not reach the detection limit of real-time PCR analysis. However, miR-200c-5p strand of the miRNA was abundantly expressed in SNC and control tissue, even though, the upregulation did not reach the statistical significance. These data correlate with results of Kawakubo-Yasukochi et al., who reported miR-200-3p involvement in invasiveness of oral squamous cell carcinoma [100]. On the other hand, in case of miR-9 both mature strands (5p and 3p) were upregulated in the samples. Our findings about the expression of mature forms

of miR-200 and miR-9 support arguments that expression of both strands (guide and passenger) of one miRNA can play different biological roles in cancer pathology [101].

One way of increasing specificity of miRNAs as diagnostic biomarkers of the disease is designing a panel of multiple miRNAs. After ROC analysis we suggest two panels of studied miRNAs for this purpose with AUC larger than 0.95: 4 miRNA panel: miR-21-5p, let-7d, miR-155-5p and miR-223-3p and 5 miRNA panel: miR-21-5p, miR-9-3p, let-7d-5p, miR-155-5p and miR-223-3p. The tissue specificity of the miRNA panel needs to be further confirmed in different tumor types.

8.1.1 miR-9

Our experiment shows that both 3' and 5' miR-9 strands are abundantly expressed and upregulated in SSCC samples in comparison to controls. In concordance with our results Salazar et al. [102] were able to find upregulation of miR-9 in small amounts of saliva of HNSCC patients.

The upregulation was more significant for the 5' strand of miR-9 with fold change of 6.80. 3' form of the same miRNA was significantly upregulated as well with fold change of 3.07. More importantly, we detected significantly longer survival interval in patients with higher expression of miR-9-5p ($P = 0.0264$). Citron et al. [103] identified upregulation of miR-9 in patients with head and neck squamous cell carcinoma and also concluded that this particular miRNA is an important mediator of recurrence formation in HNSCC by regulating EMT process. Similarly, Yu et al. [104] reported that nicotine treated cells had significantly higher expression of miR-9 leading to promotion of metastatic processes by E-cadherin repression. Their conclusions support our findings of correlation between vascular invasion and upregulation of miR-9-3p and, consecutively, of correlation between perineural invasion and the same microRNA strand. Moreover, our results showed relationship between regional recurrence of SSCC recorded in our patients and relative expression of miR-9-5p. We have not found any correlation between miR-9

expression and presence of HPV in our samples. Even though Vojtechova et al. [105] concluded that miR-9-5p is specific for HPV positive tonsillar tumors.

8.1.2 miR-21

miR-21 is the most commonly deregulated miRNA across diseases and tissue types. Survival analysis of our samples showed that SSCC patients with high expression of miR-21 had impaired survival approaching statistical significance in comparison to patients with lower upregulation of miR-21. Relationship between patients' survival time and level of miR-21 upregulation was observed by many other authors studying head and neck cancerous tissue such as Avissar et al. [106] and Ko et al. [107]. Moreover, we observed trends towards higher levels of miR-21 expression in samples from sinonasal cancer patients in advanced stages of the disease. However, the results were not statistically significant, or they had limited statistical power due to low number of samples. The same trend was previously observed by Hu et al. [108] in oral SCC. Mir-21 limitation as a biomarker is low tissue and disease specificity. It has been reported to be involved in many different pathological processes in the human body [109].

8.1.3 miR-143-3p and miR-145-5p and their regulation

Both miRNAs have been reported to be deregulated in various types of head and neck cancer. It was proposed that miR-143 functions in the apoptosis, invasion and migration processes of head and neck cancer [110; 111]. We did not find any significant deregulation of miR-143-3p in our SSCC samples in comparison to control samples, even though they have been abundantly expressed in the studied tissue.

Even though, miR-143 and miR-145 are coded next to each other on the genome, their expression was dissimilar in our samples which we thought might have been attributed to methylation of their coding genes, so we further investigated the methylation status by MS-HRM analysis. miR-145 and miR-143 are expressed

together by transcription of their host gene *CARMIN* and then cleaved by DGCR8/Drosha into the two individual pre-miRs [112]. However, the presence of individual promoters for both miRNAs has also been reported. There was no difference of methylation in the promotor regions of the genes. The hypermethylation of promotor CpG islands was high not only in sinonasal patients' samples, also control samples were hypermethylated. Our results indicate that the expression of miR-143 is not regulated by DNA methylation or that our primers did not span the right regulation region of the gene.

8.1.4 miR-155-5p

MiR-155-5p have been found to be deregulated in HNSCC and its oncogenic role in development of various types of head and neck cancer and the ability to promote metastatic processes has been reported [113]. Although we did not find any statistically significant differences in expression of miR-155-5p between carcinoma samples and control samples, we were able to identify several correlations between miR-155-5p expression and several clinicopathological data suggesting the idea of miR-155-5p relevance as prognostic biomarker of HNSCC.

8.1.5 Let-7d and Let-7a

From our experiments it seems that microRNA let-7a and let-7d are upregulated in SSCC samples in comparison to sinonasal control tissue. Members of miRNA let-7 family are generally considered to be upregulated in aggressive tumors of head and neck [114]. According to our data analysis we concluded that the patients with extremely high expression of let-7d had better chance of survival than the patients with lower upregulation of the same miRNA. It implies that lower levels of let-7 are associated with impaired survival in HNSCC.

8.1.6 Other miRNAs

miR-146a-5p was not significantly deregulated in our samples as was suggested by Yang et al. [115] in chronic rhinitis. However, we have found the miRNA to be locally downregulated in nasal cavity. Our results also suggest its' involvement in lymph node metastases formation, as suggested by Scapoli et al [116].

miR-196a-5p was significantly upregulated in our samples, which corresponds with results of Maruyama et al. [117]. It was also upregulated in patients with occupational exposure to cancerogenic substances, which implies its role in sinonasal inflammation and subsequent tumorigenesis.

MiR-137 has been reported to be downregulated in HNSCC and to have a role in suppression of oncogenes [118]. We have not found miR-137-3p to be deregulated in our set of samples. We were able to associate higher expression of miR-137-3p with impaired survival of the patients with SSCC and we found correlation between local recurrence of the sinonasal tumors and higher expression of miR-137-3p.

Reports of deregulation of miR-99a in head and neck cancer have been published, which we could not confirm in SNC. However, we have found downregulation of miR-99a in patients with recorded history of smoking, which might be connected to possible suggested regulation of miR-99a DNA damage response [119].

8.2 Differentially expressed miRNAs in oropharyngeal carcinoma

Malignancies localized in the oropharyngeal area are typically characterized by small primary tumor occurrence and significant lymph node metastases, which might by diagnosed prior to primary tumor [14]. Therefore, in this study we characterized relative expression of miRNAs not only in primary OPSCC tumor tissue, but also in related metastatic tissue of the patients. Expression of miRNAs was then compared to set of non-malignant control samples from the area of oropharynx. This study design enabled us to characterize differences of miRNA

expression in cells that have metastatic potential and went through metastatic process.

The incidence of oropharyngeal cancer as oppose to other types of head and neck cancers (such as sinonasal cancer) has a rising tendency. Growing frequency of HPV infection in (especially Western) population is considered responsible for this phenomenon. Most of the oropharyngeal cancers are nowadays caused by ongoing high-risk HPV infection of the area. Oropharynx is more prone to HPV related tumors thanks to its immune system component. Although, HPV positive OPSCC tumors tend to respond to treatment better than HPV negative tumors, it is still a serious disease with impact on the life of the patient. Most of the current research regarding cancer of the area is for obvious reasons focused on studying HPV positive tumors [34]. In this study, we included HPV positive and HPV negative samples into the cohort to investigate their miRNA related differences.

In oropharyngeal cancer several epigenetic (focused on miRNAs) studies has been published [74; 75]. For this reason, we selected different approach in the OPSCC study than in the SSCC study, so we did not select studied miRNAs based on literary review. The first phase of the study was preselection of interestingly deregulated miRNAs using high-throughput miRNA expression methods. The second phase of the study was designed to verify the changes in expression of pre-selected miRNAs by real-time PCR. High-throughput methods for miRNome profiling involve microarrays, real-time PCR based arrays and next-generation sequencing [120]. We have selected two of these methods (microarray and NGS) to be used in this study based on the premise that we can use them to investigate all miRNAs expressed in the sample. Agilent miRNA microarray and NebNext Small RNA technology was used due to high sensitivity, up to date content from the most current data on miRBase and availability of the technology at our laboratory. Moreover, these methods target mature miRNAs (as oppose to stem loop sequence) so we can investigate relative expression of both 5' and 3' mature miRNA strands.

NGS sequencing of small RNA is a method also used for novel miRNA identification studies. However, for this purpose ~10 million NGS reads per sample are required. Furthermore, with appropriate bioinformatics tools small RNA NGS

analysis allows differentiation between various isoforms of the same miRNA (isomiRs), which are variants of the same miRNA that differ from canonical mature sequence. isomiRs could be eventually used as more specific biomarkers for various types of cancer [121; 122]. Even though, analysis of our data in Oasis 2 software identified several novel miRNAs and potential isomiRs, but sequencing was not deep enough to draw any reliable conclusions and it was not aim of the current study.

8.2.1 miR-150-5p

miR-150-5p was selected for its significant downregulation in both tumor and metastasis samples. This miRNA was also among the selected as potentially good biomarker of tumor samples vs. control samples differentiation by Oasis 2.0 Classification Analysis. In concordance, comparison of results obtained from microarray data confirms significant downregulation of the miRNA in tumor samples and less prominent downregulation in metastases samples. Real-time PCR analysis was in complete agreement with first phase of the study results and confirmed downregulation of miR-150-5p.

Downregulation of miR-150-5p in head and neck squamous cell carcinoma was detected by Koshizuka et al. [123], who also reported contribution of the downregulation of miR-150 to the aggressiveness of the tumor. In concordance, we have found that patients with very low expression of miR-150-5p had impaired survival in comparison to patients with less significant downregulation of the miRNA. The same results are valid for tumor samples and for metastasis samples of the same patients. This miRNA was also less expressed in HPV positive tumors and in keratinizing tumors. Moreover, more advanced stages of the disease and recurrent tumors parameters correlated with very low expression of the disease. All these results suggest that miR-150-5p could be used as a prognostic biomarker of the oropharyngeal cancer. Correspondently, it was evaluated as the best biomarker by PanelComposer and CombiROC analysis (with miR-3656 and miR-375-3p). Downregulation of miR-150-5p could help with characterization of the

lymph node mestasasis and original subsite of the tumor. Further analyses need to be performed to determine specificity of the miRNA for studied tissue.

8.2.2 miR-206

From the results of miRNA microarray analysis, we can see that many miRNAs are upregulated in the tumor samples and metastasis samples with high statistical significance. Most of the upregulated miRNAs in tumor samples are also upregulated in metastasis samples with less significant fold change value. miR-206 was according to microarray not deregulated in tumors but downregulated in metastases. Moreover, microarray analysis also shows that this miRNA is downregulated in metastasis samples compared to tumor samples. NGS results shows upregulation of miR-206 in tumor samples, downregulation of miR-206 in metastases and downregulation of miR-206 in metastases versus tumors. For these reasons miR-206 was selected for second phase of the study. miR-206 expression was further validated using real-time PCR, which fully confirmed the results obtained from NGS and trends suggested in miRNA microarray. With discrepancy to our results, Liu et al. [124] reported downregulation of miR-206 in head and neck squamous cell carcinoma samples. However, in epithelial ovarian cancer, downregulation of miR-206 was associated with metastasis and poor prognosis [125].

8.2.3 miR-3656

This miRNA was selected for further validation based on its very significant deregulation in tumor and metastasis samples according to microarray analysis. It was the most significantly upregulated miRNA based on fold change value not only in tumors but also in metastases. However, this trend was not observed in NGS analysis were miR-3656 was slightly upregulated only in tumor samples and not in metastasis samples. Eventually, real-time PCR verification of miR-3656 expression confirmed the results of miRNA microarray and showed that miR-3656 is upregulated in OPSCC samples. One of the reasons for disparity between

expression results of miR-3656 might be the fact, that stem loop sequence of miR-3656 was excluded from the newest version of miRBase (version 22). In the newest version of the database only a mature sequence of the miRNA is annotated, because the mature sequence might derive from annotated 28S rRNA sequences. Hence, this update of the database might cause differences in results, because miRNA microarray (based on miRBase v.21) was done prior to NGS analysis (aligned to miRBase 22) and real-time PCR assays was purchased prior to version 22 database release and it is no longer available under the same catalogue number [126]. Due to complicated about the nomenclature, miR-3656 and rarity of the miRNA, there are not many studies that would focus on miR-3656 expression. However, it has been found upregulated in breast cancer [127] and in pancreatic cancer [128].

8.2.4 miR-375-3p

miR-375-3p (previously known as miR-375 in previous version of miRBase) was selected based on its significant deregulation in metastasis samples compared to tumor samples in the same manner as mir-206. The main reason for selection of this miRNA were results obtained from small RNA NGS data, were it was suggested as the most valuable biomarker for distinction of metastases and tumors. According to NGS results miR-375 was significantly upregulated in tumors and significantly downregulated in metastases. In miRNA microarray data there was no deregulation detected in any of the evaluations. On the other hand, real-time PCR showed a trend of miR-375 downregulation on metastases compared to tumors with significant fold change. However, the p value was not significant due to high variability of results within the sample set. As a result, we were not able to confirm the results obtained from NGS by real-time PCR analysis. In literature miR-375 has been reported to be downregulated in head and neck squamous cell carcinoma [129; 130].

8.2.5 Overview

Even though we were not able to confirm some of the results compared to miRNA expression studies in different tissue types, our results may be valuable for OPSCC research. Due to its unique place among head and neck cancers (HPV positivity, lymph node metastasis) expression values of miRNAs can be unique tool for characterization of the tumors.

All methods used for miRNA expression analysis have its advantages and disadvantages. Real-time PCR is the most cost effective and more sensitive method for miRNA studies but has its limitations in quantity of samples that can be analyzed in reaction and especially in low number of target miRNAs. It is clear from our results that both high-throughput methods are mostly reliable. The weakness of miRNA microarray from our point of view is the inability to quantify miRNAs expressed in low quantities and labor-intensive workflow. Small RNA NGS seems to have more advantages than miRNA microarray and nowadays it is a preferred method of miRNA expression studies for many scientists with its high sensitivity, variability and many library preparation kits options on the market. However, small RNA NGS requires prior experience with NGS and bioinformatical analysis of the results is very complex and time-consuming. From our perspective small RNA NGS is the more reliable and elegant method.

9 Conclusions

My doctoral study research activities were focused on investigation of relative microRNA expression (and its possible regulation by DNA methylation) in two unique sets of samples of head and neck squamous cell carcinoma.

Following up on previously established objectives of the study, I conclude that:

1. I have successfully used two high-throughput methods (Agilent miRNA microarray and small RNA NGS with NEBNext Small RNA Library Prep Kit for Illumina) to select significantly deregulated microRNAs in oropharyngeal carcinoma samples for further validation. A low-throughput method (real-time PCR with TaqManTM Advanced Assays) was used to confirm deregulation of previously selected microRNA expression in sinonasal squamous cell carcinoma (based on literary review) and oropharyngeal squamous cell carcinoma (based on high-throughput methods results).

2. I have examined possibility that miR-145-5p and miR-143-3p deregulation is caused by other epigenetic modification – DNA methylation. Methylation status of *miR-145* gene, *miR-143* gene and their host gene (*miR-143/145*) was investigated by methylation specific high-resolution melting analysis. I have found no significant relationship between DNA miRNA gene methylation status and miRNA expression. Even though, the samples were hypermethylated in promotor regions of the genes, the observed hypermethylation was not cause of miRNA deregulation.

3. I have found many correlations between expression of miRNAs and recorded clinicopathological data (such as gender, age, occupation, HPV status, smoker status, alcohol use, localization and typing of the tumor, TNM classification, invasion and recurrence) of the patients using

appropriate statistical software. Especially, deregulation of miRNAs in advanced stage of the disease were considered relevant (mir-9 and miR-150-5p). Moreover, I have found relationship between significant deregulation of several miRNAs (miR-21-5p, miR-9-5p, let-7d-5p, miR-137-3p and miR-150-5p) and impaired survival of the patients.

4. I have concluded that (among others) miR-9 might be the best potential biomarker for sinonasal squamous cell carcinoma. The experiment showed that both 3' and 5' miR-9 strands are abundantly expressed in the tissue, its expression was related to survival of the patients and it correlated with several clinicopathological parameters. miR-150-5p (among others) may be the best potential biomarker for oropharyngeal squamous cell carcinoma. miR-150-5p was significantly in both tumor and metastasis samples, its low expression in tumor and metastasis samples was associated to impaired survival of the patients and it was less expressed in advanced stages of the disease.

10 Literature

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11 Appendices

11.1 Appendix A: Deregulated miRNAs (Agilent microarray)

Appendix Table 1. Differentially expressed miRNAs in oropharyngeal tumor samples compared to control samples.

Name	FC Upregulation	Adjusted P Value	Name	FC Downregulation	Adjusted P Value
hsa-miR-3656	**16.01**	**0.0000**	hsa-miR-150-5p	-17.46	0.0783
hsa-miR-4530	11.02	0.0000	hsa-miR-142-3p	-16.59	0.0608
hsa-miR-4443	10.37	0.0005	hsa-miR-142-5p	-14.79	0.0093
hsa-miR-6088	10.06	0.0000	hsa-miR-29c-3p	-7.40	0.1435
hsa-miR-4653-3p	9.50	0.0072	hsa-miR-223-3p	-6.84	0.0341
hsa-miR-4430	9.36	0.0012	hsa-miR-3653-3p	-5.92	0.0481
hsa-miR-4281	9.34	0.0000	hsa-miR-29b-3p	-5.31	0.1512
hsa-miR-1207-5p	9.22	0.0001	hsa-let-7g-5p	-5.12	0.2814
hsa-miR-642a-3p	8.90	0.0190	hsa-miR-26b-5p	-4.98	0.1827
hsa-miR-2861	8.80	0.0001	hsa-miR-140-3p	-4.91	0.0316
hsa-miR-6125	8.66	0.0000	hsa-miR-342-3p	-4.36	0.2810
hsa-miR-1915-3p	8.65	0.0000	hsa-miR-29a-3p	-4.06	0.4263
hsa-miR-3665	8.58	0.0000	hsa-miR-101-3p	-3.80	0.0625
hsa-miR-6803-5p	8.58	0.0020	hsa-miR-144-3p	-3.70	0.0190
hsa-miR-6821-5p	8.43	0.0000	hsa-miR-203a-3p	-3.62	0.2370
hsa-miR-1202	8.21	0.0001	hsa-miR-19b-3p	-3.60	0.4226
hsa-miR-4741	8.20	0.0007	hsa-miR-126-3p	-3.38	0.3167
hsa-miR-6087	8.18	0.0001	hsa-miR-30b-5p	-3.37	0.2820
hsa-miR-6090	8.05	0.0000	hsa-miR-15a-5p	-3.29	0.2930
hsa-miR-6800-5p	7.92	0.0001	hsa-miR-146a-5p	-3.24	0.3321
hsa-miR-4687-3p	7.89	0.0001	hsa-miR-19a-3p	-3.23	0.2113
hsa-miR-4763-3p	7.81	0.0000	hsa-miR-145-5p	-3.15	0.3393
hsa-miR-197-5p	7.80	0.0001	hsa-miR-140-5p	-2.93	0.0783
hsa-miR-4497	7.76	0.0000	hsa-miR-195-5p	-2.82	0.2745
hsa-miR-3195	7.69	0.0054	hsa-miR-100-5p	-2.80	0.2775
hsa-miR-1268a	7.52	0.0000	hsa-miR-451a	-2.74	0.6041
hsa-miR-1268b	7.50	0.0001	hsa-miR-365a-3p	-2.68	0.1827
hsa-miR-6749-5p	7.35	0.0003	hsa-miR-4446-3p	-2.65	0.2376
hsa-miR-638	7.28	0.0005	hsa-miR-342-5p	-2.61	0.0139
hsa-miR-6068	7.05	0.0004	hsa-miR-3607-3p	-2.61	0.0004
hsa-miR-1225-5p	7.00	0.0009	hsa-miR-497-5p	-2.60	0.2311
hsa-miR-3960	6.93	0.0001	hsa-let-7i-5p	-2.57	0.6065
hsa-miR-5787	6.80	0.0190	hsa-miR-26a-5p	-2.55	0.6609

hsa-miR-762	6.47	0.0005
hsa-miR-7107-5p	6.36	0.0040
hsa-miR-3162-5p	6.28	0.0001
hsa-miR-940	6.21	0.0029
hsa-miR-4466	5.99	0.0000
hsa-miR-4516	5.89	0.0003
hsa-miR-642b-3p	5.83	0.0550
hsa-miR-6089	5.83	0.0001
hsa-miR-5739	5.83	0.0001
hsa-miR-1224-5p	5.69	0.0000
hsa-miR-7110-5p	5.61	0.0232
hsa-miR-1229-5p	5.32	0.0005
hsa-miR-6869-5p	5.13	0.0037
hsa-miR-4733-5p	5.11	0.0000
hsa-miR-205-5p	5.02	0.2879
hsa-miR-1185-1-3p	4.83	0.0008
hsa-miR-4672	4.72	0.0001
hsa-miR-4739	4.61	0.0097
hsa-miR-7150	4.58	0.0200
hsa-miR-6775-5p	4.55	0.0008
hsa-miR-328-5p	4.48	0.0113
hsa-miR-6085	4.43	0.0003
hsa-miR-4507	4.37	0.0185
hsa-miR-6893-5p	4.26	0.1166
hsa-miR-6127	4.24	0.0066
hsa-miR-4270	4.19	0.0115
hsa-miR-937-5p	4.12	0.0000
hsa-miR-6826-5p	4.04	0.0058
hsa-miR-1227-5p	4.03	0.0058
hsa-miR-6891-5p	3.97	0.0031
hsa-miR-1185-2-3p	3.91	0.0219
hsa-miR-3940-5p	3.89	0.0001
hsa-miR-1587	3.85	0.0085
hsa-miR-6132	3.85	0.0179
hsa-miR-6124	3.84	0.0136
hsa-miR-939-5p	3.82	0.0003
hsa-miR-4505	3.81	0.0069
hsa-miR-8069	3.81	0.0000
hsa-miR-4534	3.80	0.0139
hsa-miR-575	3.79	0.0445
hsa-miR-4465	3.76	0.1311
hsa-miR-6786-5p	3.74	0.0096
hsa-miR-7108-5p	3.74	0.0108
hsa-miR-7704	3.71	0.0117

hsa-miR-664a-5p	-2.52	0.1525
hsa-miR-30e-5p	-2.49	0.4144
hsa-miR-107	-2.48	0.4608
hsa-miR-3607-5p	-2.44	0.0040
hsa-miR-374a-5p	-2.43	0.2197
hsa-miR-30c-5p	-2.34	0.3272
hsa-miR-29c-5p	-2.34	0.0004
hsa-miR-106b-5p	-2.32	0.5338
hsa-miR-199a-5p	-2.32	0.4487
hsa-miR-15b-5p	-2.31	0.6191
hsa-miR-17-5p	-2.25	0.5429
hsa-miR-146b-5p	-2.24	0.3569
hsa-miR-148a-3p	-2.23	0.4152
hsa-miR-20a-5p	-2.23	0.6544
hsa-miR-186-5p	-2.22	0.1171
hsa-miR-10b-5p	-2.21	0.2198
hsa-miR-5699-5p	-2.17	0.3378
hsa-miR-103a-3p	-2.14	0.6579
hsa-miR-590-5p	-2.11	0.0009
hsa-miR-361-3p	-2.09	0.1166
hsa-miR-28-5p	-2.07	0.0671
hsa-miR-425-5p	-2.07	0.2863
hsa-miR-199a-3p	-2.06	0.6569
hsa-miR-331-3p	-2.03	0.4144
hsa-miR-4636	-2.03	0.0118
hsa-miR-455-3p	-2.02	0.1025
hsa-let-7f-5p	-2.02	0.7588
hsa-let-7d-5p	-2.02	0.5517

hsa-miR-663a	3.64	0.0697
hsa-miR-6791-5p	3.63	0.0002
hsa-miR-4788	3.59	0.0050
hsa-miR-6724-5p	3.57	0.0066
hsa-miR-6126	3.52	0.0010
hsa-miR-4632-5p	3.48	0.0017
hsa-miR-4327	3.43	0.0272
hsa-miR-4488	3.43	0.0001
hsa-miR-6812-5p	3.42	0.0020
hsa-miR-4721	3.41	0.1108
hsa-miR-3679-5p	3.40	0.0066
hsa-miR-6510-5p	3.37	0.1166
hsa-miR-4462	3.37	0.0014
hsa-miR-4428	3.36	0.0252
hsa-miR-4459	3.19	0.0993
hsa-miR-6769b-5p	3.17	0.2071
hsa-miR-4433a-3p	3.15	0.0192
hsa-miR-6076	3.13	0.0018
hsa-miR-6740-5p	3.09	0.1025
hsa-miR-6165	3.04	0.0005
hsa-miR-4322	3.03	0.0002
hsa-miR-188-5p	3.03	0.1968
hsa-miR-5088-5p	3.02	0.1266
hsa-miR-7847-3p	3.01	0.0294
hsa-miR-6794-5p	3.00	0.0588
hsa-miR-1471	3.00	0.2446
hsa-miR-1287-5p	2.97	0.0838
hsa-miR-320c	2.94	0.0341
hsa-miR-574-3p	2.92	0.4670
hsa-miR-630	2.89	0.2769
hsa-miR-6789-5p	2.87	0.0178
hsa-miR-4463	2.86	0.1577
hsa-miR-4485-5p	2.85	0.2282
hsa-miR-125a-3p	2.84	0.1257
hsa-miR-1275	2.81	0.2311
hsa-miR-150-3p	2.80	0.0190
hsa-miR-4417	2.78	0.1357
hsa-miR-6799-5p	2.78	0.0017
hsa-miR-6785-5p	2.77	0.2976
hsa-miR-6727-5p	2.74	0.0536
hsa-miR-3135b	2.73	0.1630
hsa-miR-671-5p	2.72	0.2062
hsa-miR-4695-5p	2.71	0.0275
hsa-miR-134-5p	2.70	0.0414

hsa-miR-5001-5p	2.70	0.0113
hsa-miR-8063	2.69	0.1242
hsa-miR-4442	2.63	0.0349
hsa-miR-4532	2.61	0.0116
hsa-miR-6849-5p	2.60	0.0289
hsa-miR-3188	2.60	0.1074
hsa-miR-6756-5p	2.53	0.1270
hsa-miR-4745-5p	2.53	0.1212
hsa-miR-371b-5p	2.52	0.0166
hsa-miR-6879-5p	2.52	0.1432
hsa-miR-5100	2.52	0.2339
hsa-miR-5006-5p	2.51	0.2733
hsa-miR-205-3p	2.47	0.0490
hsa-miR-4634	2.47	0.1910
hsa-miR-6728-5p	2.46	0.0438
hsa-miR-371a-5p	2.46	0.0805
hsa-miR-4271	2.45	0.0249
hsa-miR-3141	2.43	0.1021
hsa-miR-1249-5p	2.42	0.2561
hsa-miR-6752-5p	2.41	0.0671
hsa-miR-6875-5p	2.41	0.2746
hsa-miR-3196	2.40	0.0671
hsa-miR-4787-5p	2.40	0.1751
hsa-miR-1273g-3p	2.39	0.2863
hsa-miR-6780b-5p	2.38	0.1758
hsa-miR-4787-3p	2.36	0.0640
hsa-miR-4689	2.35	0.3569
hsa-miR-6850-5p	2.35	0.0805
hsa-miR-5195-3p	2.32	0.1374
hsa-miR-4499	2.31	0.1819
hsa-miR-4746-3p	2.30	0.0671
hsa-miR-8089	2.29	0.1357
hsa-miR-1236-5p	2.28	0.1635
hsa-miR-5196-5p	2.28	0.0603
hsa-miR-6763-5p	2.27	0.0096
hsa-miR-4485-3p	2.26	0.3569
hsa-miR-4800-3p	2.23	0.0000
hsa-miR-483-5p	2.21	0.3272
hsa-miR-6831-5p	2.18	0.3088
hsa-miR-3197	2.17	0.0020
hsa-miR-7845-5p	2.17	0.0651
hsa-miR-3648	2.17	0.2500
hsa-miR-5703	2.16	0.4221
hsa-miR-1343-5p	2.13	0.1291

hsa-miR-99b-3p	2.11	0.0249
hsa-miR-4800-5p	2.10	0.1166
hsa-miR-6075	2.10	0.0281
hsa-miR-4758-5p	2.10	0.5733
hsa-miR-208a-5p	2.10	0.1734
hsa-miR-6867-5p	2.09	0.3272
hsa-miR-4486	2.09	0.1092
hsa-miR-8072	2.07	0.4221
hsa-miR-4257	2.07	0.0805
hsa-miR-7106-5p	2.06	0.1143
hsa-miR-1246	2.06	0.4187
hsa-miR-7159-5p	2.05	0.1074
hsa-miR-6790-5p	2.05	0.0165
hsa-miR-4707-5p	2.04	0.0204
hsa-miR-320a	2.03	0.0640
hsa-miR-8485	2.00	0.6388

Bold text marks miRNA selected for future analysis. FC, fold change.

Appendix Table 2. Differentially expressed miRNAs in oropharyngeal metastasis samples compared to control samples.

Name	FC Upregulation	Adjusted P Value	*Name*	FC Downregulation	Adjusted P Value
hsa-miR-3656	**14.51**	**0.0000**	*hsa-miR-142-5p*	-11.31	0.0187
hsa-miR-4653-3p	11.08	0.0036	*hsa-miR-142-3p*	-9.55	0.1339
hsa-miR-4430	11.05	0.0006	*hsa-miR-133b*	-8.54	0.0231
hsa-miR-4530	10.13	0.0001	*hsa-miR-150-5p*	-8.15	0.1989
hsa-miR-4443	9.37	0.0009	*hsa-miR-223-3p*	-7.20	0.0273
hsa-miR-6125	9.27	0.0001	*hsa-miR-29c-3p*	-5.76	0.1915
hsa-miR-6803-5p	9.26	0.0014	*hsa-miR-203a-3p*	-5.72	0.0733
hsa-miR-3665	8.62	0.0000	*hsa-miR-29b-3p*	-5.22	0.1369
hsa-miR-2861	8.34	0.0001	*hsa-let-7g-5p*	-4.61	0.2650
hsa-miR-6088	8.33	0.0001	*hsa-miR-342-3p*	-4.44	0.2223
hsa-miR-4497	8.15	0.0000	*hsa-miR-26b-5p*	-4.39	0.1989
hsa-miR-197-5p	7.53	0.0002	*hsa-miR-140-3p*	-4.23	0.0543
hsa-miR-4281	7.29	0.0002	*hsa-miR-1-3p*	-4.11	0.0484
hsa-miR-6087	7.28	0.0003	*hsa-miR-3653-3p*	-3.88	0.1382
hsa-miR-4741	7.22	0.0017	*hsa-miR-29a-3p*	-3.54	0.4198
hsa-miR-638	7.13	0.0007	*hsa-miR-30b-5p*	-3.35	0.2362
hsa-miR-1268b	7.01	0.0003	*hsa-miR-19b-3p*	-3.30	0.4019
hsa-miR-6821-5p	6.86	0.0001	*hsa-miR-101-3p*	-3.29	0.0937
hsa-miR-6800-5p	6.76	0.0003	*hsa-miR-19a-3p*	-3.05	0.2008
hsa-miR-642a-3p	6.75	0.0448	*hsa-miR-365a-3p*	-3.00	0.1084

hsa-miR-1915-3p	6.70	0.0002
hsa-miR-1207-5p	6.66	0.0005
hsa-miR-762	6.50	0.0007
hsa-miR-1225-5p	6.35	0.0017
hsa-miR-6132	6.35	0.0008
hsa-miR-6068	6.33	0.0009
hsa-miR-3195	6.32	0.0114
hsa-miR-6090	6.28	0.0002
hsa-miR-3162-5p	6.06	0.0001
hsa-miR-205-5p	5.98	0.1948
hsa-miR-1224-5p	5.92	0.0000
hsa-miR-7107-5p	5.80	0.0061
hsa-miR-574-3p	5.71	0.1432
hsa-miR-4687-3p	5.66	0.0009
hsa-miR-1268a	5.53	0.0001
hsa-miR-4763-3p	5.49	0.0004
hsa-miR-6089	5.30	0.0004
hsa-miR-1202	5.25	0.0028
hsa-miR-5787	5.22	0.0478
hsa-miR-940	5.21	0.0070
hsa-miR-3960	5.07	0.0010
hsa-miR-5739	5.06	0.0005
hsa-miR-6749-5p	4.88	0.0045
hsa-miR-6826-5p	4.72	0.0018
hsa-miR-4466	4.72	0.0001
hsa-miR-4507	4.72	0.0113
hsa-miR-4465	4.70	0.0602
hsa-miR-4721	4.64	0.0296
hsa-miR-1185-1-3p	4.61	0.0013
hsa-miR-7110-5p	4.58	0.0484
hsa-miR-6893-5p	4.54	0.0842
hsa-miR-6127	4.48	0.0043
hsa-miR-6869-5p	4.43	0.0080
hsa-miR-642b-3p	4.37	0.1084
hsa-miR-8485	4.36	0.1802
hsa-miR-4733-5p	4.35	0.0000
hsa-miR-6775-5p	4.29	0.0014
hsa-miR-6769b-5p	4.25	0.0812
hsa-miR-6126	4.20	0.0003
hsa-miR-4516	4.19	0.0036
hsa-miR-4462	4.14	0.0003
hsa-miR-4428	4.14	0.0066
hsa-miR-4505	4.14	0.0036
hsa-miR-4672	4.08	0.0006

hsa-miR-126-3p	-2.99	0.3205
hsa-miR-146a-5p	-2.93	0.3280
hsa-miR-199a-3p	-2.80	0.4149
hsa-miR-148a-3p	-2.79	0.2100
hsa-miR-206	-2.72	0.0527
hsa-miR-103a-3p	-2.65	0.4778
hsa-miR-133a-3p	-2.63	0.0478
hsa-miR-199a-5p	-2.61	0.3147
hsa-miR-144-3p	-2.57	0.1070
hsa-miR-30c-5p	-2.54	0.2197
hsa-miR-186-5p	-2.54	0.0513
hsa-miR-107	-2.53	0.3851
hsa-miR-3607-5p	-2.52	0.0024
hsa-let-7i-5p	-2.46	0.5543
hsa-miR-140-5p	-2.45	0.1378
hsa-miR-100-5p	-2.41	0.3177
hsa-miR-29c-5p	-2.41	0.0003
hsa-miR-15a-5p	-2.39	0.4188
hsa-miR-30e-5p	-2.36	0.3818
hsa-miR-374a-5p	-2.34	0.2034
hsa-miR-331-3p	-2.24	0.2685
hsa-miR-193b-3p	-2.21	0.1935
hsa-miR-3607-3p	-2.16	0.0047
hsa-miR-195-5p	-2.14	0.4002
hsa-let-7d-5p	-2.13	0.4486
hsa-miR-199b-5p	-2.12	0.2429
hsa-miR-4636	-2.11	0.0070
hsa-miR-15b-5p	-2.11	0.6005
hsa-miR-10b-5p	-2.10	0.2136
hsa-miR-145-5p	-2.04	0.5416
hsa-miR-146b-5p	-2.04	0.3697
hsa-miR-17-5p	-2.03	0.5439
hsa-miR-451a	-2.01	0.6795

hsa-miR-937-5p	3.93	0.0001
hsa-miR-6812-5p	3.88	0.0007
hsa-miR-6791-5p	3.85	0.0002
hsa-miR-4485-5p	3.81	0.0881
hsa-miR-3135b	3.76	0.0447
hsa-miR-6085	3.76	0.0013
hsa-miR-1587	3.69	0.0094
hsa-miR-1229-5p	3.69	0.0070
hsa-miR-939-5p	3.65	0.0007
hsa-miR-6785-5p	3.64	0.1339
hsa-miR-7150	3.62	0.0553
hsa-miR-7108-5p	3.61	0.0122
hsa-miR-3196	3.54	0.0042
hsa-miR-4327	3.54	0.0206
hsa-miR-7704	3.50	0.0154
hsa-miR-1227-5p	3.46	0.0135
hsa-miR-4485-3p	3.40	0.1031
hsa-miR-4270	3.40	0.0342
hsa-miR-6875-5p	3.32	0.0884
hsa-miR-3940-5p	3.20	0.0007
hsa-miR-6740-5p	3.18	0.0802
hsa-miR-6724-5p	3.13	0.0146
hsa-miR-6076	3.12	0.0019
hsa-miR-4632-5p	3.08	0.0047
hsa-miR-8069	3.02	0.0003
hsa-miR-6124	2.99	0.0506
hsa-miR-4534	2.98	0.0502
hsa-miR-5100	2.97	0.1219
hsa-miR-4788	2.91	0.0199
hsa-miR-574-5p	2.91	0.1080
hsa-miR-4299	2.91	0.2100
hsa-miR-4488	2.90	0.0008
hsa-miR-8063	2.89	0.0813
hsa-miR-6780b-5p	2.89	0.0734
hsa-miR-6891-5p	2.87	0.0278
hsa-miR-3679-5p	2.85	0.0205
hsa-miR-1185-2-3p	2.83	0.0912
hsa-miR-371b-5p	2.78	0.0066
hsa-miR-6799-5p	2.76	0.0018
hsa-miR-5006-5p	2.74	0.1838
hsa-miR-1275	2.72	0.2064
hsa-miR-1287-5p	2.72	0.1070
hsa-miR-1273g-3p	2.68	0.1846
hsa-miR-188-5p	2.67	0.2211

hsa-miR-4746-3p	2.66	0.0222
hsa-miR-6786-5p	2.62	0.0715
hsa-miR-6165	2.59	0.0028
hsa-miR-125a-3p	2.59	0.1476
hsa-miR-6879-5p	2.56	0.1193
hsa-miR-141-3p	2.55	0.4992
hsa-miR-4745-5p	2.54	0.1031
hsa-miR-1246	2.52	0.2120
hsa-miR-4739	2.51	0.1432
hsa-miR-4695-5p	2.51	0.0448
hsa-miR-5088-5p	2.50	0.1980
hsa-miR-4459	2.50	0.1913
hsa-miR-4515	2.50	0.0734
hsa-miR-5001-5p	2.49	0.0194
hsa-miR-4800-5p	2.48	0.0387
hsa-miR-1273f	2.41	0.1310
hsa-miR-1249-5p	2.39	0.2145
hsa-miR-8089	2.38	0.1031
hsa-miR-4322	2.36	0.0045
hsa-miR-7847-3p	2.31	0.1084
hsa-miR-7106-5p	2.31	0.0502
hsa-miR-5585-3p	2.31	0.2044
hsa-miR-4478	2.31	0.1717
hsa-miR-1471	2.31	0.3533
hsa-miR-4442	2.30	0.0755
hsa-miR-4433a-3p	2.28	0.1080
hsa-miR-6510-5p	2.28	0.2831
hsa-miR-6794-5p	2.28	0.1667
hsa-let-7b-5p	2.26	0.5322
hsa-miR-371a-5p	2.23	0.1139
hsa-miR-4271	2.23	0.0478
hsa-miR-150-3p	2.22	0.0806
hsa-miR-328-5p	2.22	0.2106
hsa-miR-6727-5p	2.22	0.1316
hsa-miR-3141	2.20	0.1339
hsa-miR-575	2.20	0.2507
hsa-miR-1281	2.20	0.1994
hsa-miR-92a-3p	2.17	0.4571
hsa-miR-4417	2.17	0.2482
hsa-miR-663a	2.15	0.2999
hsa-miR-6789-5p	2.14	0.1031
hsa-miR-6075	2.11	0.0252
hsa-miR-4758-5p	2.11	0.5082
hsa-miR-1181	2.09	0.0872

hsa-miR-6763-5p	2.08	0.0199
hsa-miR-4787-5p	2.05	0.2405
hsa-miR-4463	2.04	0.3390
hsa-miR-6756-5p	2.04	0.2322
hsa-miR-320c	2.01	0.1938
hsa-miR-6849-5p	2.01	0.1250
hsa-miR-205-3p	2.00	0.1369

Bold text marks miRNA selected for future analysis. FC, fold change.

Appendix Table 3. Differentially expressed miRNAs in oropharyngeal metastasis samples compared to tumor samples.

Name	FC Deregulation	Adjusted P Value
hsa-miR-8485	2.18	0.9954
hsa-miR-150-5p	2.14	0.9954
hsa-miR-328-5p	-2.02	0.9868
hsa-miR-133a-3p	-2.21	0.8642
hsa-miR-1-3p	-2.53	0.9721
hsa-miR-206	**-3.34**	**0.2758**
hsa-miR-133b	-6.64	0.6251

Bold text marks miRNA selected for future analysis. FC, fold change.

11.2 Appendix B: Deregulated miRNAs (small RNA NGS)

Appendix Table 4. Differentially expressed miRNAs in oropharyngeal tumor samples compared to control samples.

Name	FC Upregulation	Adjusted P Value	*Name*	FC Downregulation	Adjusted P Value
hsa-miR-196a-5p	22.22	0.0000	***hsa-miR-150-5p***	**-9.78**	**0.0000**
hsa-miR-187-3p	11.26	0.0000	*hsa-miR-142-5p*	-7.45	0.0000
hsa-miR-615-3p	9.93	0.0000	*hsa-miR-342-3p*	-6.75	0.0000
hsa-miR-9-5p	8.11	0.0000	*hsa-miR-142-3p*	-6.09	0.0000
hsa-miR-147b	6.68	0.0000	*hsa-miR-1273h-3p*	-5.55	0.0000
hsa-miR-377-5p	6.60	0.0009	*hsa-miR-150-3p*	-5.53	0.0000
hsa-miR-1246	5.80	0.0000	*hsa-miR-342-5p*	-5.51	0.0000
hsa-miR-205-3p	5.35	0.0002	*hsa-miR-138-5p*	-5.37	0.0000
hsa-miR-944	4.75	0.0000	*hsa-miR-7-1-3p*	-5.22	0.0000
hsa-miR-382-5p	4.47	0.0007	*hsa-miR-155-5p*	-5.20	0.0000
hsa-miR-7641	4.25	0.0000	*hsa-miR-766-3p*	-5.08	0.0000
hsa-miR-409-3p	4.06	0.0004	*hsa-miR-140-3p*	-4.69	0.0000
hsa-miR-206	3.92	0.0446	*hsa-miR-26b-5p*	-4.35	0.0000
hsa-miR-141-5p	3.80	0.0001	*hsa-miR-223-5p*	-4.12	0.0000
hsa-miR-4488	3.79	0.0027	*hsa-miR-29b-3p*	-4.09	0.0000
hsa-miR-1307-5p	3.79	0.0006	*hsa-miR-140-5p*	-4.08	0.0000
hsa-miR-6087	3.75	0.0007	*hsa-miR-26a-5p*	-3.93	0.0000
hsa-miR-493-3p	3.70	0.0011	*hsa-miR-26b-3p*	-3.84	0.0000
hsa-miR-141-3p	3.56	0.0001	*hsa-miR-3613-3p*	-3.74	0.0001
hsa-miR-485-5p	3.42	0.0117	*hsa-miR-184*	-3.74	0.0090
hsa-miR-4492	3.37	0.0009	*hsa-miR-532-3p*	-3.64	0.0000
hsa-miR-134-5p	3.30	0.0046	*hsa-miR-29a-3p*	-3.63	0.0000
oan-miR-1386	3.22	0.0000	*hsa-miR-664b-3p*	-3.63	0.0001
hsa-miR-4497	3.18	0.0000	*hsa-miR-361-3p*	-3.57	0.0000
hsa-miR-370-3p	3.18	0.0089	*hsa-miR-671-3p*	-3.51	0.0036
hsa-miR-196b-5p	3.11	0.0002	*hsa-let-7g-5p*	-3.48	0.0000
hsa-miR-127-3p	3.06	0.0052	*hsa-miR-504-5p*	-3.47	0.0001
hsa-miR-4508	3.05	0.0031	*hsa-miR-194-5p*	-3.43	0.0001
hsa-miR-375	2.99	0.0218	*hsa-miR-191-5p*	-3.41	0.0000
hsa-miR-27b-5p	2.84	0.0001	*hsa-miR-28-5p*	-3.40	0.0000
hsa-miR-127-5p	2.80	0.0183	*hsa-miR-145-5p*	-3.39	0.0000
hsa-miR-381-3p	2.79	0.0257	*hsa-miR-181a-5p*	-3.39	0.0000
hsa-miR-224-5p	2.73	0.0008	*hsa-miR-590-3p*	-3.36	0.0005
hsa-miR-1-3p	2.72	0.1154	*hsa-miR-195-5p*	-3.35	0.0001
hsa-miR-210-3p	2.71	0.0000	*hsa-miR-664a-3p*	-3.34	0.0010
hsa-miR-503-5p	2.63	0.0022	*hsa-miR-15a-5p*	-3.27	0.0000
hsa-miR-3687	2.62	0.0029	*hsa-miR-192-5p*	-3.26	0.0000
hsa-miR-34c-5p	2.57	0.0002	*hsa-miR-576-5p*	-3.26	0.0001
hsa-miR-1180-3p	2.55	0.0003	*hsa-miR-1271-5p*	-3.20	0.0027
hsa-miR-33a-5p	2.50	0.0005	*hsa-miR-223-3p*	-3.19	0.0000
hsa-miR-379-5p	2.48	0.0229	*hsa-miR-30c-5p*	-3.14	0.0000
hsa-miR-200a-3p	2.46	0.0010	*hsa-miR-361-5p*	-3.09	0.0000
hsa-miR-1972	2.45	0.0160	*hsa-miR-374a-5p*	-3.08	0.0001

hsa-miR-133a-3p	2.45	0.1910
hsa-miR-1273g-3p	2.40	0.0003
hsa-miR-432-5p	2.39	0.0938
hsa-miR-27a-5p	2.36	0.0006
hsa-miR-133a-5p	2.33	0.2242
hsa-miR-3960	2.29	0.0638
hsa-miR-200a-5p	2.23	0.0072
hsa-miR-378a-3p	2.22	0.0164
hsa-miR-185-3p	2.19	0.0277
hsa-miR-450b-5p	2.18	0.0091
hsa-miR-299-3p	2.17	0.1285
hsa-miR-452-5p	2.15	0.0003
hsa-miR-1291	2.11	0.0021
hsa-miR-424-3p	2.11	0.0938
hsa-miR-21-3p	2.09	0.0043
hsa-miR-675-5p	2.08	0.1311
hsa-miR-3656	2.07	0.0553
hsa-miR-452-3p	2.01	0.0447

hsa-miR-181a-3p	-3.04	0.0000
hsa-miR-139-5p	-3.03	0.0004
hsa-miR-20a-5p	-2.97	0.0000
hsa-miR-144-5p	-2.95	0.0192
hsa-miR-574-3p	-2.94	0.0000
hsa-miR-197-3p	-2.93	0.0000
hsa-miR-455-3p	-2.92	0.0000
hsa-miR-451a	-2.90	0.0073
hsa-miR-331-3p	-2.85	0.0000
hsa-miR-582-5p	-2.84	0.0014
hsa-miR-1260b	-2.84	0.0000
hsa-miR-32-5p	-2.82	0.0005
hsa-miR-30e-3p	-2.74	0.0000
hsa-miR-16-5p	-2.69	0.0000
hsa-miR-100-5p	-2.68	0.0000
hsa-miR-135a-5p	-2.67	0.0092
hsa-miR-339-3p	-2.65	0.0000
hsa-miR-514a-3p	-2.65	0.0632
hsa-miR-338-5p	-2.62	0.0065
hsa-miR-101-3p	-2.62	0.0001
hsa-miR-484	-2.61	0.0021
hsa-miR-128-3p	-2.54	0.0008
hsa-miR-126-5p	-2.53	0.0010
hsa-miR-330-3p	-2.51	0.0007
hsa-miR-625-3p	-2.50	0.0004
hsa-miR-598-3p	-2.48	0.0006
hsa-miR-181b-5p	-2.40	0.0000
hsa-miR-505-3p	-2.38	0.0000
hsa-let-7e-5p	-2.38	0.0015
hsa-miR-744-5p	-2.38	0.0005
hsa-miR-186-5p	-2.37	0.0000
hsa-miR-618	-2.37	0.0417
hsa-miR-92a-3p	-2.36	0.0001
hsa-miR-148b-3p	-2.35	0.0135
hsa-miR-146a-5p	-2.34	0.0001
hsa-miR-548e-3p	-2.34	0.0032
hsa-miR-125a-5p	-2.31	0.0003
hsa-miR-454-3p	-2.30	0.0005
hsa-miR-29c-3p	-2.30	0.0000
hsa-miR-6842-3p	-2.29	0.0013
hsa-miR-589-5p	-2.28	0.0004
hsa-let-7g-3p	-2.27	0.0071
hsa-miR-628-5p	-2.26	0.0204
hsa-miR-125b-5p	-2.25	0.0003
hsa-miR-30b-5p	-2.23	0.0001
hsa-miR-497-5p	-2.22	0.0014
hsa-miR-660-5p	-2.19	0.0001
hsa-miR-126-3p	-2.17	0.0000
hsa-miR-548o-3p	-2.17	0.0167
hsa-miR-146b-5p	-2.17	0.0013
hsa-miR-340-3p	-2.13	0.0908
hsa-miR-339-5p	-2.13	0.0000

hsa-miR-374a-3p	-2.12	0.0094
hsa-miR-19b-3p	-2.09	0.0023
hsa-miR-548f-5p	-2.09	0.0356
hsa-miR-218-5p	-2.06	0.0001
hsa-let-7a-3p	-2.06	0.0340
hsa-miR-28-3p	-2.05	0.0000
hsa-miR-144-3p	-2.05	0.0882
hsa-let-7a-5p	-2.03	0.0041
hsa-miR-32-3p	-2.03	0.0402
hsa-miR-221-5p	-2.02	0.0013
hsa-miR-1249-3p	-2.02	0.0163
hsa-miR-3615	-2.01	0.0164

Bold text marks miRNA selected for future analysis. FC, fold change.

Appendix Table 5. Differentially expressed miRNAs in oropharyngeal metastasis samples compared to control samples.

Name	FC Upregulation	Adjusted P Value	*Name*	FC Downregulation	Adjusted P Value
hsa-miR-615-3p	19.93	0.0000	*hsa-miR-206*	-25.13	0.0000
hsa-miR-196a-5p	19.34	0.0000	*hsa-miR-133a-5p*	-14.09	0.0000
hsa-miR-1269a	12.84	0.0018	***hsa-miR-150-5p***	**-9.18**	**0.0000**
hsa-miR-147b	12.50	0.0000	*hsa-miR-184*	-8.26	0.0000
hsa-miR-9-5p	11.23	0.0000	*hsa-miR-766-3p*	-5.90	0.0000
hsa-miR-7641	7.04	0.0000	*hsa-miR-142-5p*	-5.64	0.0000
hsa-miR-944	6.62	0.0000	*hsa-miR-342-3p*	-5.49	0.0000
hsa-miR-1246	6.56	0.0000	*hsa-miR-135a-5p*	-4.70	0.0002
hsa-miR-141-5p	6.07	0.0000	*hsa-miR-140-3p*	-4.63	0.0000
hsa-miR-493-3p	5.77	0.0004	*hsa-miR-155-5p*	-4.43	0.0000
hsa-miR-214-5p	5.47	0.0000	*hsa-miR-150-3p*	-4.36	0.0000
hsa-miR-205-3p	5.24	0.0000	*hsa-miR-26b-5p*	-4.16	0.0000
hsa-miR-141-3p	4.77	0.0000	*hsa-miR-4521*	-4.06	0.0000
hsa-miR-187-3p	4.71	0.0018	*hsa-miR-139-5p*	-3.87	0.0000
hsa-miR-196b-5p	4.62	0.0000	*hsa-miR-7-1-3p*	-3.75	0.0000
hsa-miR-4508	4.35	0.0002	*hsa-miR-26a-5p*	-3.69	0.0000
hsa-miR-370-3p	4.34	0.0019	*hsa-miR-664b-3p*	-3.67	0.0000
hsa-miR-6087	4.30	0.0002	*hsa-miR-664a-3p*	-3.64	0.0000
hsa-miR-3196	4.10	0.0018	*hsa-miR-128-3p*	-3.42	0.0000
hsa-miR-27b-5p	4.06	0.0000	*hsa-miR-361-3p*	-3.37	0.0000
hsa-miR-493-5p	3.68	0.0041	***hsa-miR-375***	-3.36	0.0086
hsa-miR-27a-5p	3.63	0.0000	*hsa-miR-30c-5p*	-3.36	0.0000
hsa-miR-1972	3.39	0.0007	*hsa-miR-576-5p*	-3.34	0.0001
hsa-miR-127-5p	3.24	0.0085	*hsa-miR-484*	-3.34	0.0000
hsa-miR-382-5p	3.24	0.0074	*hsa-miR-144-5p*	-3.32	0.0033
hsa-miR-210-3p	3.23	0.0000	*hsa-miR-28-5p*	-3.29	0.0000
hsa-miR-3687	3.21	0.0012	*hsa-miR-625-3p*	-3.26	0.0000
hsa-miR-409-3p	3.16	0.0043	*hsa-miR-191-5p*	-3.25	0.0000
hsa-miR-224-5p	3.14	0.0015	*hsa-miR-1271-5p*	-3.25	0.0000

hsa-miR-134-5p	3.12	0.0058	*hsa-miR-342-5p*	-3.20	0.0000
hsa-miR-33a-5p	3.01	0.0000	*hsa-miR-142-3p*	-3.16	0.0000
hsa-miR-96-5p	2.96	0.0002	*hsa-miR-26b-3p*	-3.16	0.0000
hsa-miR-21-3p	2.96	0.0000	*hsa-let-7g-5p*	-3.10	0.0000
hsa-miR-6510-3p	2.92	0.0112	*hsa-miR-29a-3p*	-3.09	0.0000
hsa-miR-1307-5p	2.91	0.0010	*hsa-miR-1-3p*	-3.06	0.0700
hsa-miR-452-5p	2.86	0.0000	*hsa-miR-3615*	-3.01	0.0000
hsa-miR-1291	2.85	0.0000	*hsa-miR-504-5p*	-3.01	0.0001
hsa-miR-452-3p	2.84	0.0029	*hsa-miR-532-3p*	-2.99	0.0001
oan-miR-1386	2.81	0.0000	*hsa-miR-145-5p*	-2.96	0.0000
hsa-miR-200a-5p	2.80	0.0043	*hsa-miR-330-3p*	-2.90	0.0000
hsa-miR-200a-3p	2.79	0.0009	*hsa-miR-181a-5p*	-2.90	0.0000
hsa-miR-4492	2.78	0.0031	*hsa-miR-20a-5p*	-2.89	0.0000
hsa-miR-378g	2.75	0.0294	*hsa-miR-194-5p*	-2.88	0.0000
hsa-miR-4497	2.74	0.0002	*hsa-miR-223-3p*	-2.85	0.0000
hsa-miR-127-3p	2.73	0.0185	*hsa-miR-133a-3p*	-2.82	0.1767
hsa-miR-432-5p	2.72	0.0461	*hsa-miR-140-5p*	-2.82	0.0000
hsa-miR-224-3p	2.71	0.0011	*hsa-miR-29b-3p*	-2.80	0.0000
hsa-miR-23b-5p	2.60	0.0149	*hsa-miR-339-3p*	-2.78	0.0000
hsa-miR-4488	2.55	0.0204	*hsa-miR-486-5p*	-2.78	0.0070
hsa-miR-582-3p	2.48	0.0050	*hsa-miR-181a-3p*	-2.73	0.0000
hsa-miR-3182	2.43	0.0115	*hsa-miR-223-5p*	-2.70	0.0000
hsa-miR-135b-3p	2.41	0.0228	*hsa-miR-361-5p*	-2.69	0.0001
hsa-miR-1180-3p	2.40	0.0031	*hsa-miR-30e-3p*	-2.68	0.0000
hsa-miR-9-3p	2.38	0.0107	*hsa-miR-671-3p*	-2.61	0.0115
hsa-miR-4443	2.35	0.0125	*hsa-miR-138-5p*	-2.60	0.0009
hsa-miR-5585-3p	2.33	0.0035	*hsa-miR-92a-3p*	-2.55	0.0000
hsa-miR-200b-5p	2.31	0.0058	*hsa-miR-340-3p*	-2.54	0.0143
hsa-miR-182-5p	2.30	0.0056	*hsa-miR-29b-2-5p*	-2.50	0.0005
hsa-miR-10a-3p	2.28	0.0169	*hsa-miR-451a*	-2.43	0.0148
hsa-miR-576-3p	2.27	0.0132	*hsa-miR-15b-5p*	-2.41	0.0277
hsa-miR-514a-3p	2.27	0.1099	*hsa-miR-125b-5p*	-2.40	0.0000
hsa-miR-34c-5p	2.27	0.0010	*hsa-miR-148b-3p*	-2.40	0.0026
hsa-miR-200c-3p	2.24	0.0158	*hsa-miR-195-5p*	-2.39	0.0011
hsa-miR-450b-5p	2.17	0.0119	*hsa-miR-125a-5p*	-2.36	0.0000
hsa-miR-1273g-3p	2.16	0.0010	*hsa-miR-1249-3p*	-2.27	0.0059
hsa-miR-183-5p	2.14	0.0209	*hsa-miR-197-3p*	-2.25	0.0002
efu-miR-9226	2.13	0.0170	*hsa-miR-455-3p*	-2.22	0.0025
hsa-miR-758-3p	2.12	0.1265	*hsa-miR-107*	-2.16	0.0240
hsa-miR-25-5p	2.12	0.0224	*hsa-miR-590-3p*	-2.10	0.0250
hsa-miR-3960	2.11	0.1265	*hsa-miR-331-3p*	-2.08	0.0108
hsa-miR-10a-5p	2.06	0.0036	*hsa-miR-129-5p*	-2.06	0.0416
hsa-miR-200b-3p	2.06	0.0101	*hsa-miR-328-3p*	-2.04	0.0019
hsa-miR-151a-3p	2.05	0.0097	*hsa-miR-425-5p*	-2.02	0.0012
hsa-miR-24-1-5p	2.03	0.1231	*hsa-miR-192-5p*	-2.02	0.0008
hsa-miR-149-5p	2.00	0.0159	*hsa-let-7d-5p*	-2.01	0.0000
			hsa-miR-181b-5p	-2.01	0.0001
			hsa-let-7a-5p	-2.00	0.0011

Bold text marks miRNA selected for future analysis. FC, fold change.

Appendix Table 6. Differentially expressed miRNAs in oropharyngeal metastasis samples compared to tumor samples.

Name	FC Deregulation	Adjusted P Value
hsa-miR-514a-3p	2.89	0.0350
hsa-miR-214-5p	2.42	0.1528
hsa-miR-218-5p	2.42	0.0245
hsa-miR-96-5p	2.18	0.1151
hsa-miR-208b-3p	-2.01	0.4584
hsa-miR-95-3p	-2.02	0.4584
hsa-miR-377-5p	-2.12	0.5103
hsa-miR-4521	-2.15	0.2121
hsa-miR-381-3p	-2.26	0.3189
hsa-miR-675-5p	-2.46	0.2296
hsa-miR-133a-5p	-2.86	0.0779
hsa-miR-129-5p	-2.97	0.0616
hsa-miR-133a-3p	-3.17	0.0350
hsa-miR-206	-3.39	0.0194
bta-miR-2889	-3.67	0.0039
hsa-miR-4532	-3.88	0.0015
hsa-miR-486-5p	-4.16	0.0018
hsa-miR-1-3p	-4.46	0.0018
hsa-miR-375	**-8.12**	**0.0000**

Bold text marks miRNA selected for future analysis. FC, fold change.