CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biochemical Sciences

DNA methylation changes in oropharyngeal carcinoma

Diploma thesis

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Hradec Králové 2020 Natália Birknerová

Author ‘s Declaration

Declaration:

I declare that this thesis is my original author's work, which has been composed solely by myself (under the guidance of my consultant). All the literature and other resources from which I drew information are cited in the list of used literature and are quoted in the paper. The work has not been used to get another or the same title.

Hradec Králové, 2020 **Signature of the author**

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ABSTRAKT

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Název diplomové práce: Zmeny v metylácií DNA u orofaryngeáneho karcinómu

Orofaryngeálny karcinóm sa radí medzi karcinómy hlavy a krku, ktoré sú siedmym najčastejším malígnym ochorením v celosvetovom meradle. Prevažná väčšina (viac ako 90%) sú karcinómy skvamóznych buniek. Orofaryngeálny karcinóm zahŕňa nádory jazyka, mandlí, mäkkého podnebia a hltanu. Okrem tradičných rizikových faktorov, ľudský papillomavírus bol identifikovaný ako ďalší rizikový faktor pre vývoj týchto nádorov.

Epigenetické zmeny sú dedičné zmeny v génovej expresii, pri ktorých nedochádza k zmenám sekvencie DNA a môžu prispievať ku karcinogenéze. Zahrňujú DNA metyláciu, modifikáciu histónov a post-transkripčnú génovú reguláciu pomocou mikroRNA.

Cieľom tejto štúdie bolo preskúmať hladiny metylácie vybraných tumor supresorových génov u spinocelulárneho karcinómu orofaryngeálnych buniek (OPSCC) v porovnaní s nenádorovým kontrolnými vzorkami. Hladiny metylácie DNA vybraných tumor supresorových génov boli analyzované metylačne špecifickou multiplexnou amplifikáciou sond závislej od ligácie (MS-MLPA) v nádorových vzorkách, v ich prislúchajúcich metastázach, v nemetastázujúcich nádorových vzorkách a v kontrolných vzorkách (nenádorové mandle). Pomocou 15% medznej hodnoty pre metyláciu sme pozorovali štatisticky významne vyššiu metyláciu v génoch *PAX5*, *CADM1, WT1* ($P < 0.01$) a *RARβ, PAX6* ($P < 0.05$) u pacientov s orofaryngeálnym karcinómom v porovnaní s kontrolnou skupinou.

Na základe výsledkov z MS-MLPA a publikácií zameraných na hypermetyláciu niektorých promótorových oblastí spojených s HPV infekciou pri rakovine krčka maternice, sme zvolili analýzu génu *CADM1* pomocou metylačne špecifickej analýzy topenia s vysokým rozlíšením (MS-HRM). Bolo zistené, že metylácia *CADM1* v OPSCC je zvýšená prítomnosťou HPV infekcie, čo zodpovedá metylácii génu *CADM1* v karcinóme krčka maternice. Hladiny metylácie génu *CADM1* pravdepodobne neboli ovplyvnené metastatickým procesom, pretože naše výsledky ukazujú, že zodpovedajúce metastázy majú rovnaký metylačný status ako ich primárne nádory. Všetky kontrolné vzorky boli nemetylované, čo platí aj pre všetky HPV-negatívne nádorové vzorky.

Zistenia tejto štúdie ukazujú sľubných kandidátov na prognostické biomarkery OPSCC a môžu mať dôsledky pre budúce individualizované terapie založené na epigenetických zmenách.

ABSTRACT

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Title of diploma thesis: DNA methylation changes in oropharyngeal cancer

Oropharyngeal carcinoma (OPC) is a type of head and neck cancer (HNC) that represents the seventh most common malignancy worldwide. The vast majority (more than 90%) of cases are squamous cell carcinomas (SCC). OPC develops in the tissue of the tongue, tonsils, soft palate, and pharynx. In addition to traditional risk factors, human papillomavirus (HPV) has been identified as an additional independent risk factor for the development of these tumors.

Epigenetic alterations refer to heritable changes in gene expression that occur without changes in the underlying DNA sequence and can contribute to carcinogenesis. They include DNA methylation, histone modification and non-coding RNAs effecting gene expression.

This study aimed to investigate methylation levels of selected tumor-suppressor genes in oropharyngeal squamous cell carcinoma (OPSCC) in comparison to normal oropharyngeal tissue. DNA methylation levels of selected tumor-suppressor genes were analyzed using Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) in metastatic tumor samples, corresponding metastases samples, non-metastatic tumor samples and control tissue samples (non-cancerous palatine tonsils). Using a 15% cut-off for methylation we observed statistically significant higher methylation in the *PAX5*, *CADM1, WT1* ($P<0.01$) and *RARβ*, *PAX6* ($P<0.05$) genes of patients with OPC compared with the control group.

Based on results from MS-MLPA and literary review focused on hypermethylation of some promoter regions associated with HPV in cancer of the cervix, we chose analysis of *CADM1* gene using methylation-specific high-resolution melting analysis (MS-HRM). *CADM1* methylation in OPSCC is increased by the presence of HPV infection, which is corresponding to methylation of the *CADM1* gene in the cervix carcinoma. Based on our results it seems that *CADM1* gene methylation levels are probably not influenced by the metastatic process, because methylation levels of corresponding metastases have the same methylation status as their primary tumors. All control samples were unmethylated which also applies to all HPV negative cancer samples.

The findings of this study show promising candidates for prognostic OPSCC biomarkers and may have implications for future individualized therapies based on epigenetic changes.

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Abbreviations

5-mC	5-methylcytosine
APC	Antigen-presenting cells
APG	Antigen-presenting group
BC	Bisulfite conversion
bp	Base pair
CpG	Cytosine-phosphate-Guanine
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase
dsDNA	Double-stranded DNA
ECIS	European Cancer Information System
EtOH	Ethanol
FFPE	Formalin-Fixed, Paraffin-Embedded
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HIV	Human immunodeficiency virus
HMT	Histone methyltransferase
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	High-risk
LINE	Long interspersed element
LR	Low-risk
MBD-seq	Methyl-CpG binding domain sequencing
MiRNA	MicroRNA
MS-HRM	Methylation-specific high-resolution melting analysis
MS-MLPA	Methylation-Specific Multiplex Ligation-dependent Probe Amplification
NSCLC	Non-small cell lung carcinoma
OPC	Oropharyngeal carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1

PD-L1	Programmed death-ligand 1
QMSP	Quantitative methylation-specific PCR
Rb	Retinoblastoma protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT	Radiotherapy
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCC	Squamous cell carcinoma
SINE	Short interspersed element
Tm	Melting temperatures
URR	Upstream regulatory region
WHO	World Health Organization

1 INTRODUCTION

1.1 Head and Neck Cancer

Head and neck cancers constitute the seventh most common malignant tumors worldwide (the fifth most common in men and the 12th most common in women) affecting approximately 888,000 patients and causing almost 350,000 cancer deaths annually (Bray et al. 2018). The vast majority (more than 90%) of cases are histologically designated as squamous cell carcinomas (SCCs). The term head and neck cancer is used to describe all carcinomas arising from the epithelium lining the sinonasal tract, oral cavity, pharynx, and larynx and showing microscopic evidence of squamous differentiation (Figure 1.) (Pai and Westra 2009, National Cancer Institute 2017).

The most important risk factors for head and neck SCC (HNSCC) are smoking and alcohol consumption, which seem to have a synergistic effect. Besides these traditional risk factors, infection with high-risk types of the human papillomavirus (HPV) has been identified as a third major risk factor for HNSCC, mainly in the oropharynx (Leemans, Braakhuis and Brakenhoff 2011), and especially in young, non-smoking patients in developed countries (Young et al. 2015). This discovery led to an increase in research focusing on the biological principles, overlap, and differences between HPV- and non-HPV related HNSCCs (Leemans et al. 2011).

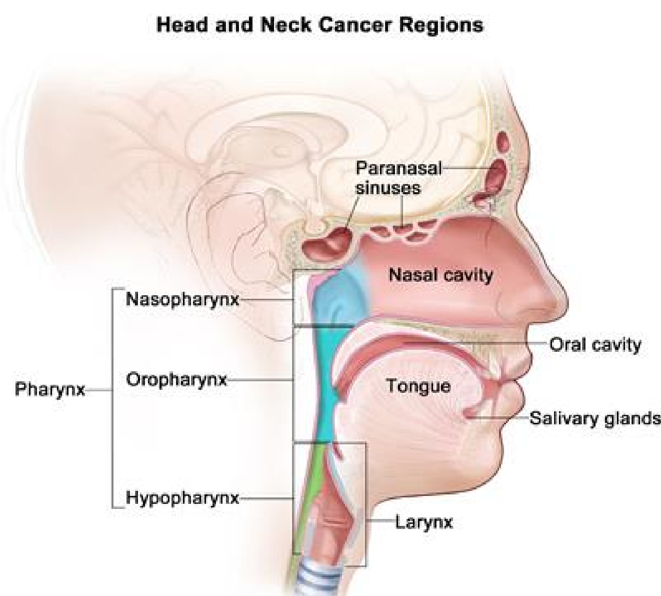


Figure 1. Head and neck cancer regions, adopted from (Winslow 2012)

1.1.1 Oropharyngeal cancer

Anatomically, the oropharynx is a part of the pharynx and it is located posterior to the oral cavity and superior to the upper margin of the epiglottis (Figure 2.). It is bounded posterolaterally by the muscular pharyngeal wall and anteriorly by the upper part of the posterior one-third of the tongue, which contains the lingual tonsils. The lateral walls of the oropharynx are composed of the tonsillar fossa with the palatine tonsils (Tshering Vogel, Zbaeren and Thoeny 2010). The most commonly occurring malignant tumor in the oropharynx is squamous cell carcinoma (Trotta et al. 2011). According to International Classification of Diseases 10th version (2016), oropharyngeal squamous cell carcinoma (OPSCC) refers to all carcinomas of the following codes: C01, C02.4, C05.1, C05.2, C05.8, C09.0, C09.1, C09.8, C09.9, C10.0, C10.2, C10.3, C10.8, and C10.9. The incidence rates of OPSCC are increasing mainly in the base of the tongue region and tonsils that are associated with HPV infection (Boscolo-Rizzo et al. 2013).

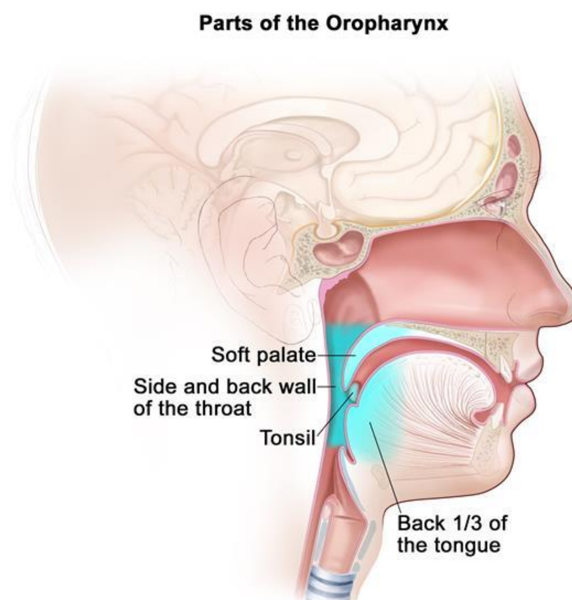


Figure 2. The anatomical location of oropharynx, adopted from (Winslow 2016)

1.1.1.1 Epidemiology

Worldwide, approximately 300,000 new cases of oral cancers are diagnosed annually, of which in 140,000 cases are oropharyngeal cancers. OPSCC occurs more frequently in men than in women with a better survival among women (Saba et al. 2011, de Souza et al. 2012)(Figure

3.). The predominance of men over women in OPSCC could be partly interpreted by men engaging more often in high-risk behaviors such as smoking, heavy alcohol use, oral sex, and having a higher number of sex partners than women. Also, Chaturvedi et al. (2018) have shown, that HPV vaccination has a low effect on population of men due to low HPV vaccine uptake compared to women

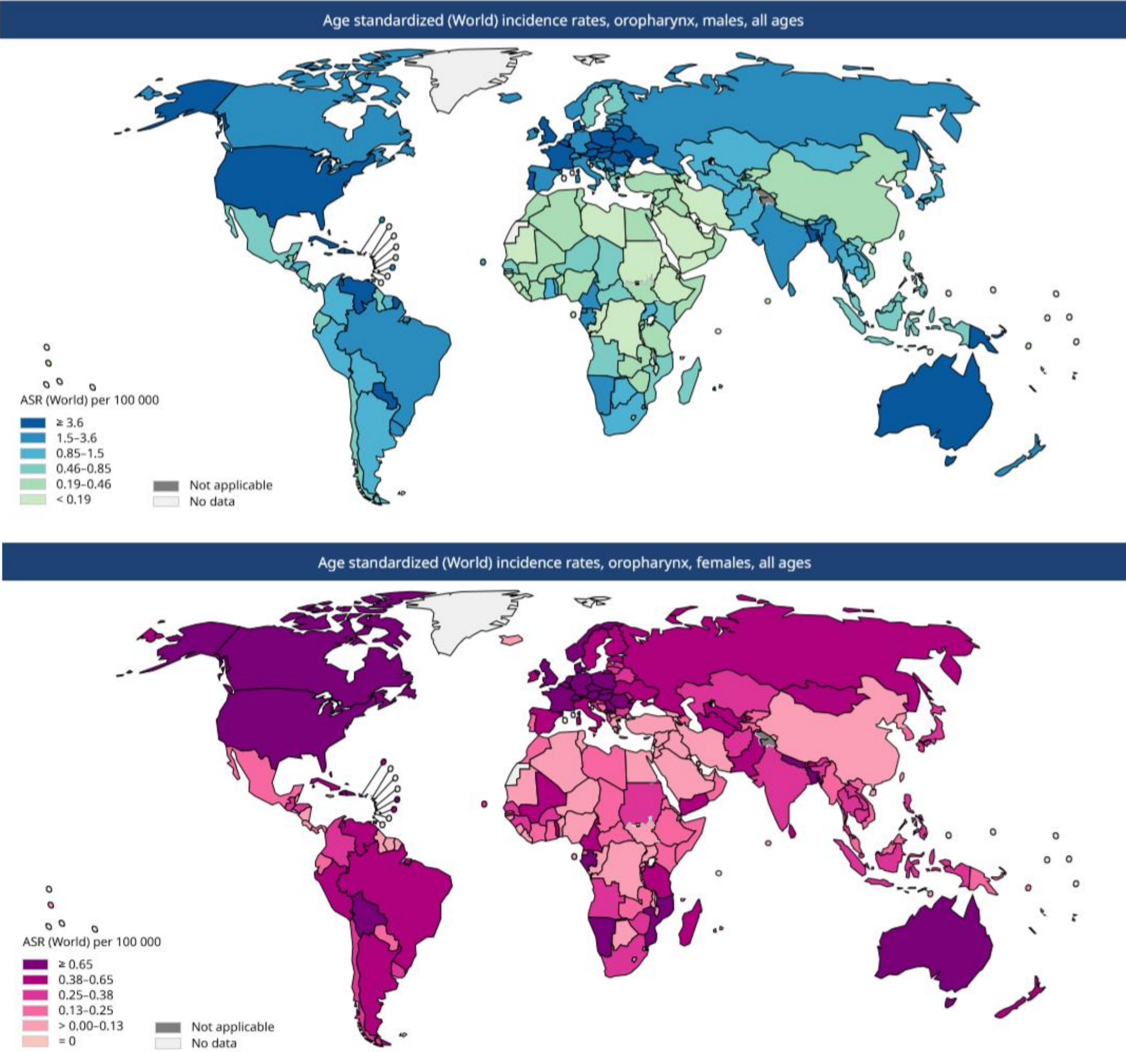


Figure 3. Estimated incidence in both sexes of oropharyngeal cancer worldwide (GLOBOCAN, 2018).

High-income regions such as North America and northern Europe are characterized by higher incidence of HPV-related oropharyngeal cancer, which represent about 70-80 % of cases (Chaturvedi et al. 2011). HPV16 in head and neck cancers has greater predominance than other HPV types. Globally, 84.9% of HPV related head and neck cancers are attributable to

HPV16/18; for HPV6/11/16/18/31/33/45/52/58, the proportion is 89.7% (Castellsagué et al. 2016).

In Europe, the highest incidence of OPSCC is in Denmark, Hungary, France, Romania, and Slovenia. In the Czech Republic, (estimated data from 2018) 562 new cases of OPSCC were recorded, with an increasing incidence of 5.2/100,000 and mortality 1.8/100,000. (Figure 4.) Data from European Cancer Information System (2018) show, that Slovakia is the country with the highest OPC related mortality in Europe (4.1/100,000)

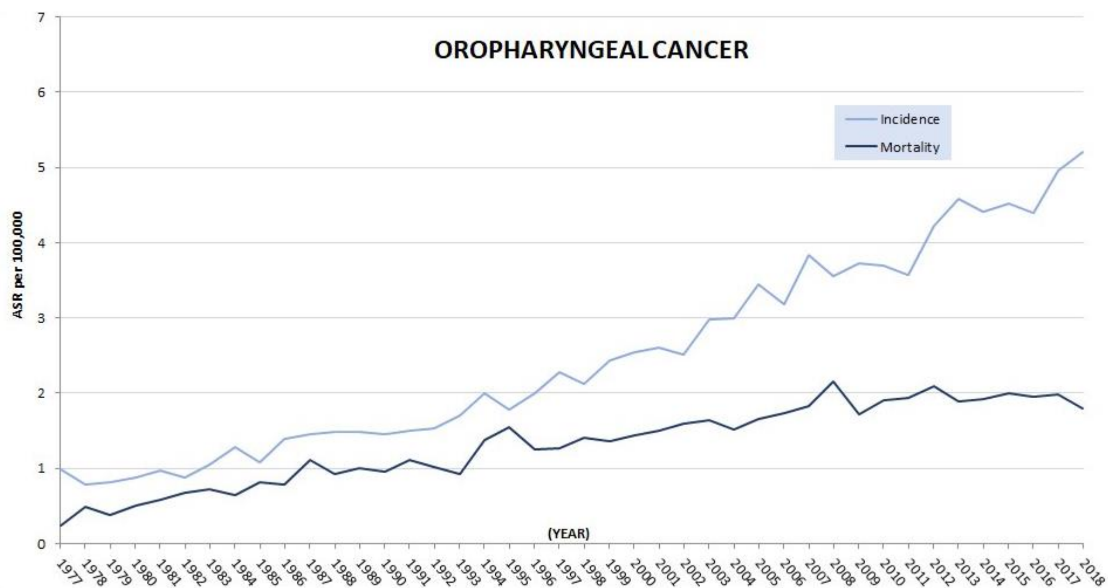


Figure 4. Estimated incidence and mortality of oropharyngeal cancer, Czech Republic (2018)

Knowledge of these cancers has significantly improved over the last decades, but the therapeutic outcome has not changed, with a ~ 50% chance of survival for five years. The more advanced stages of cancer are in time of diagnosis, the lower the response rate to treatment; also patients diagnosed with OPSCC at a high stage are at high risk of developing metastases (lymph nodes and distant) and relapse (Mydlarz, Hennessey and Califano 2010). Oropharyngeal carcinoma differs significantly epidemiologically according to HPV status. Patients with HPV-positive OPSCC tend to be younger (median 50-56 years) consume less tobacco and alcohol, and have higher socioeconomic status and education. In contrast, HPV-negative OPSCC is more common in men in the seventh decade of age, heavy smokers, and with a history of alcohol addiction (Marur et al. 2010). HPV status also significantly affects prognosis; specifically the HPV positive OPSCC is associated with a better prognosis compared to the negative cases of HPV OPSCC (Chaturvedi et al. 2008).

1.1.1.2 Risk factors

Excessive alcohol consumption and smoking are among the traditional risk factors with a synergic effect for HNSCC. In recent decades, there has been a significant decline in the incidence of HNSCC as a result of preventive strategies targeting these risk factors. The greatest decrease was observed in the incidence of carcinomas of the larynx and hypopharynx. The incidence of squamous cell carcinoma of the oropharynx is increasing in contrast to the promising figures, especially in young patients without a history of smoking or excessive alcohol consumption. The absence of these traditional risk factors indicates the occurrence of another risk factor. In recent years, HPV has been identified as another independent risk factor for OPSCC development (van Kempen et al. 2014a).

In addition, sexual behavior is now established as a risk factor for HPV-related OPSCC, with lifetime number of oral sex partners as the factor most strongly associated with OPSCC (Gillison et al. 2015).

Moreover, risk factors such as dietary, immune, and heritable factors may increase the risk of developing OPSCC. Dietary risk factors include vitamin D or iron deficiency which may increase the risk of developing HNSCC. The stability and integrity of the immune system is an important factor in preventing cancer development. Immunocompromised patients, such as those with human immunodeficiency virus (HIV) or organ transplant recipients, are at high risk of developing oral/oropharyngeal carcinoma (Chi, Day and Neville 2015). Other risk factors include poor oral hygiene, smoking marijuana, drinking hot beverages such as maté, and some occupational exposures, such as metal smelting and textile production (Wild et al. 2020).

1.1.1.3 Etiopathogenesis

1.1.1.3.1 From infection to cancer

Understanding of the process of carcinogenesis from HPV infection to cancer in OPSCC is still limited and different than the cervical cancer model. Pai and Westra (2009) reported that reticular epithelium of tonsils and tonsillar crypts is useful in immune protection of mucous membranes, allowing direct passage of immune cells like lymphocytes and antigen-presenting cells (APC) (Figure 5). However, this structure is advantageous for HPV, because the virus can easily migrate through the epithelium and enter the basal cells where it replicates. It can even play a key role in cancer progression by promoting early invasion and metastasis. In the cervix,

HPV infection requires mechanical disruption of the epithelium followed by placement of the virus on the exposed basement membrane (Roberts et al. 2007).

Another characteristic of tonsillar epithelium is the expression of programmed death-ligand 1 (PD-L1) on the epithelial cell membrane observed in benign tonsils. PD-L1 induces immune suppression by binding to the programmed cell death-1 receptor (PD-1) on T cells and converting them from activated to anergic. The PD-L1 / PD-1 pathway may, therefore, be important for the persistence of HPV infection and immune resistance during malignant progression (Lyford-Pike et al. 2013)

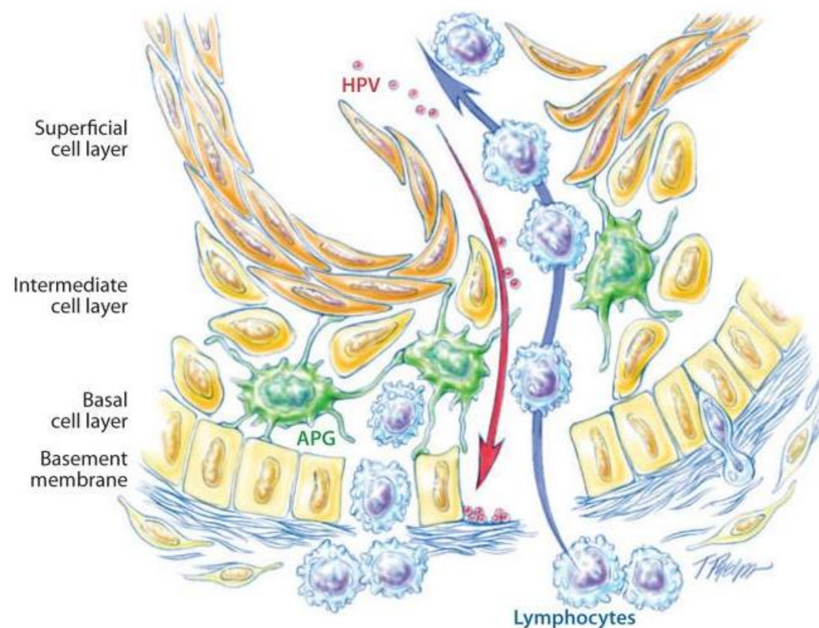


Figure 5. Reticulated epithelium in the tonsillar crypts.

In the crypts of the palatine tonsils, the squamous epithelium is reticulated to allow migration of lymphocytes and cells from the antigen-presenting group (APG.) Loss of structural integrity facilitates entry of HPV into the basal cells (Pai and Westra 2009).

1.1.1.3.2 Human papillomavirus (HPV)

HPVs are small, non-enveloped, circular, double-stranded DNA viruses targeting the basal cells of stratified epithelia of the genital, the skin, and upper respiratory tracts. HPV is classified as a sexually transmitted virus, with the majority of infections transmitted human-to-human via genital-to-genital or oral-to genital contact. Based on their oncogenic potential, mucosal HPV types are divided into two groups: low-risk (LR) types such as HPV 6 and 11, which are mainly

associated with benign genital warts, and high-risk (HR) category, which include serotypes 16, 18, 31, and 33 of HPVs, which are causative agents of cervical, anogenital and oropharyngeal cancers. HPV-16 has shown a much higher association with OPSCC (>90%) relative to other serotypes (zur Hausen 2002).

Their viral genome is organized into three major regions: (a) an upstream regulatory region (URR) that is the origin of replication and includes transcription factor-binding sites and controls gene expression; (b) an early region, encoding for six genes involved in multiple functions including viral replication and cell transformation (E1, E2, E4, E5, E6, E7), and (c) a late region bared information for the expression of L1 and L2 capsid proteins, which later fit together on their own resulting in the creation of a virion (Taberna et al. 2017).

E2 regulates the expression of E6 and E7. Integration of the HPV genome into the DNA of a host cell or E2 binding site with a highly methylated E2 binding site results in loss of E2 expression and thereby deregulation of E6 and E7 oncoprotein expression. As a result, both E6 and E7 are disproportionately expressed. E6 binds and inactivates the p53 protein, resulting in a substantial loss of p53 activity. The p53 plays an important role in responding to DNA damage by arresting cells in G1 or by inducing apoptosis that allows repair of host DNA. Due to the expression of E6, the apoptotic pathway mediated by p53 is inactivated, making these cells susceptible to genomic instability. The E7 protein binds and inactivates the retinoblastoma protein (Rb), causing the cell to enter S phase, resulting in cell cycle disruption, proliferation, and malignant transformation (Marur et al. 2010).

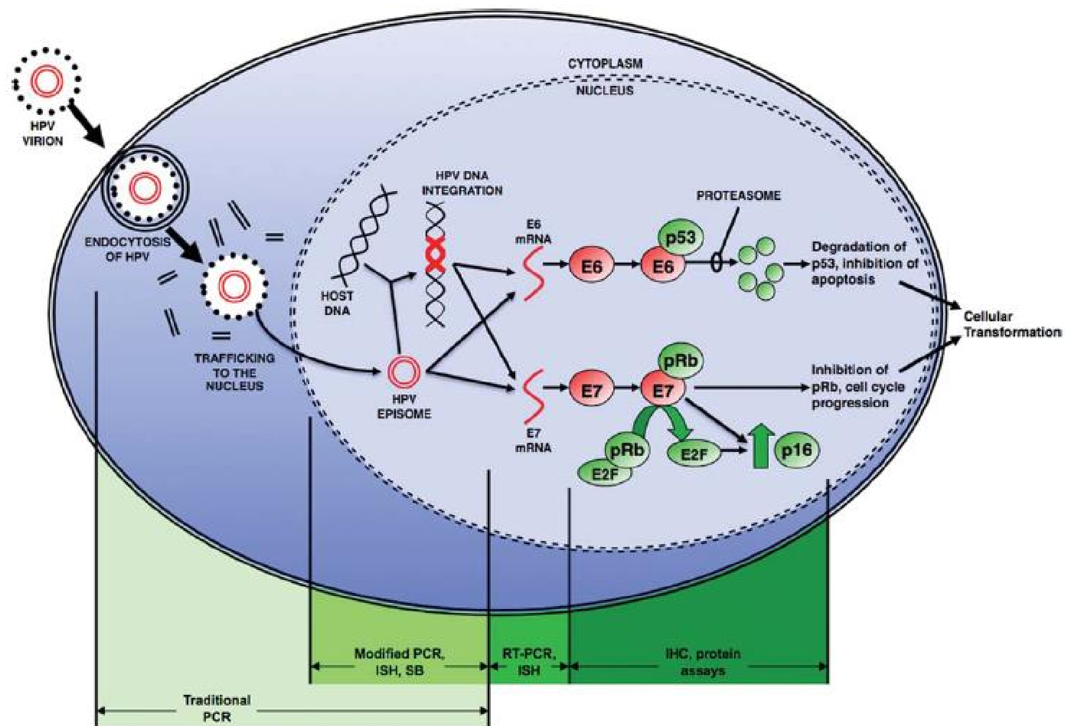


Figure 6. Schematic of HPV infection of a mucosal cell

Upon entering the virion through endocytosis, the virus creates a persistent infection as a viral episome or integrates into the host genome. HPV E6 and E7 oncoproteins are expressed from both forms of viral DNA, resulting in the degradation of p53 and Rb inhibition (Allen et al. 2010).

1.1.1.4 Symptoms, diagnosis and treatment

Oropharyngeal cancer symptoms vary slightly depending on the stage but typically patients present with sore throat, dysphagia, odynophagia, dysarthria, and otalgia. A common symptom of patients with oropharyngeal cancer is neck mass, often cystic. Because the symptoms of oropharyngeal cancer are similar to the symptoms of common upper respiratory tract infections, it often takes many months for patients to reach a specialist. Symptoms of advanced OPC include metastatic involvement of lymph nodes, foetor ex ore, an admixture of blood in saliva, dyspnea, as well as general symptoms of advanced cancer like weight loss and cachexia (MUDr. Michal Rosoľanka 2017).

OPSCC is frequently diagnosed at an advanced symptomatic stage. OPSCC treatment requires a special approach. Cures consist of various combinations of chemotherapy, surgery and radiotherapy (RT). The stage and subsite of the tumor, the functional changes and the morbidity

are main roots for choosing the proper treatment. As we know, the different combination therapies can affect quality of life. Identification of biomarkers could redound to the treatment approaches and/or relapse prediction of OPSCC patients. After radical treatment, risk of locoregional relapse in advanced OPSCC patients varies between 20 and 52% (Nguyen-Tan et al. 2014).

According to Guo et al. (2015), with recent improvements in both surgical and radiotherapy treatments, OPSCC relapse is, for the most part, a time-limited phenomenon, since the majority of recurrences appears within the first 2 years after treatment for both HPV-positive (66.0%) and HPV-negative (89.3%) OPSCC patients. Their study confirmed that relapse of OPCSS usually occurs earlier in HPV-negative than in HPV-positive patients (median time to recurrence 9.9 vs. 19.6 months). In other point of view a significantly worse prognosis have OPSCC patients with radiation-resistant tumors who relapse within 1 year (Spencer et al. 2008).

The propitious outcomes in HPV-positive OPSCC patients are probably caused by stimulation of the immune response directed to HPV-specific antigens, which may play a role in the improved response to therapy (Lyford-Pike et al. 2013) in comparison to HPV-negative OPSCC (O'Rorke et al. 2012). Although HPV positivity is a strong prognostic factor for improving survival and reducing the risk of relapse, in addition, we are missing a lack of suitable prognostic biomarkers to predict clinical outcomes in HPV-negative OPSCC patients with higher risk of relapse despite intensive treatment (Furlan et al. 2017).

The increasingly recognized role of aberrant epigenetic modifications in the OPSCC suggests the ability to test epigenetic markers as potential indicators of disease prognosis and response to treatment. The ability to detect epigenetic changes in premalignant lesions, serum, and saliva can also provide valuable biomarkers for early detection of OPSCC and for monitoring its recurrence (Koffler, Sharma and Hess 2014).

With the increasingly known role of aberrant DNA methylation in HNSCC, various studies have reported that methylation of individual genes has the potential to predict the clinical outcome of OPSCC. Many of them consisted not only of observing aberration in the OPSCC but also in other HNSCCs. Taioli et al. (2009) studied correlation between methylation of *MGMT, CDKN2A* and *RASSF1* with OS and tumor recurrence in OSCC and OPSCC. The results demonstrated that *MGMT* promoter methylation was significantly associated with increased recurrence rates, poor survival, and poor prognosis of OPSCC, consistent with the critical role of *MGMT* in DNA repair.

In recent years, several studies have sought to establish a correlation between promoter methylation and improved survival rates in HPV-positive OPSCC. Gubanova et al. (2012)

demonstrated that down-regulation of serine / threonine-protein kinase *SMG-1* by hypermethylation of the promoter correlated with HPV-positive status and improved OPSCC patient survival, as well as an increased response to radiotherapy in HPV-positive HNSCC cell lines.

1.2 Epigenetic Mechanisms in Cancer

The importance of epigenetics was first introduced by Conrad Waddington in 1942 (Tronick and Hunter 2016). Epigenetics refers to all mitotic hereditary changes in gene expression and associated phenotypic traits that are not encoded in the DNA sequence itself. Epigenetic modifications include DNA methylation, histone modifications, and non-coding RNAs. These changes can be induced by environmental and nutritional factors, and they play a crucial role in carcinogenesis and other chronic disorders. Epigenome dysregulation is associated with many cancer risk factors, including aging, inflammation, tobacco smoking, alcohol consumption, fungal toxins, biological agents, and diet as well as air and water pollution and certain endocrine disrupters (Tiffon 2018). In addition, epigenetic changes, especially DNA methylation present clinical potential in the diagnosis, prognostic assessment, treatment, and screening of cancer (Lopez et al. 2009).

1.2.1 DNA Methylation

The first and most investigated modification of chromatin is the DNA methylation, covalent modification of DNA, which leads to the attachment of the methyl group to the fifth carbon of the cytosine to form 5-methylcytosine (Robertson 2005). Cytosine-phosphate-Guanine (CpG) sites occur with high frequency in genomic regions called CpG islands. There are commonly situated in the 5′ promoter region of genes where their methylation is generally associated with transcriptional repression. In the transcriptionally active genes, these CpG islands are usually unmethylated (Langevin et al. 2015).

A family of enzymes called DNA methyltransferases (DNMTs) controls methylation by catalyzing the transfer of the methyl group from S-adenosylmethionine (SAM) to the cytosine.

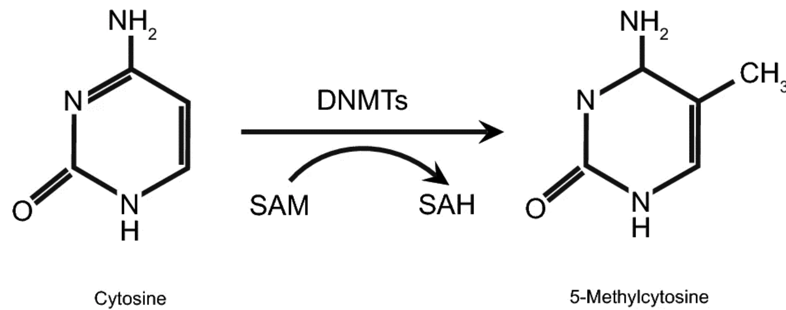


Figure 7. Methylation of cytosine

DNA methyltransferase (DNMT) catalyzes the conversion of cytosine to 5-methylcytosine. The source of the methyl groups is S-adenosylmethionine (SAM), which is converted to S-adenosylhomocysteine (SAH) (Gazdzicka et al. 2020)

Three DNMTs (i.e., DNMT1, DNMT3A, and DNMT3B) are fundamental to the methylation in mammals. DNMT1, called maintenance enzyme, is involved in restoring the parental DNA methylation profile after DNA replication, and demonstrate a preference for hemimethylated DNA, ensuring the methylation status to the future cell generations. DNMT3A and DNMT3B are regarded as *de novo* DNMTs because they create new methylation patterns during embryogenesis and germ-cell development by methylating CpG dinucleotides previously unmethylated on strands (Arantes et al. 2014). DNA methylation plays an indispensable role of gene regulation and in maintaining cell function and cellular integrity. Aberrant methylation may lead to significant changes in gene expression profiles, which may result in the emergence of malignancies. DNA methylation has a decisive role in a number of physiological processes, such as tissue differentiation, organization of chromatin, inactivation of the X-chromosome, genomic imprinting and protection against transposon reactivation. On the other hand, tumor cells show (a) hypermethylation promoter sequences of genes accompanied by inactivation of tumor suppressor genes, (b) global hypomethylation, which leads to genomic instability and cellular transformation. Both hypermethylation and hypomethylation occur frequently in oropharyngeal cancer, leading to the activation of oncogenes, loss of imprinting, genomic instability and subsequently to tumorigenesis (Bhat et al. 2016).

1.2.1.1 Hypomethylation

Global hypomethylation of DNA in repetitive sequences, transposons, or CpG dinucleotides located in introns can affect genome instability. On the other hand, it can stimulate the activation of oncogenes or latent viruses (Castilho, Squarize and Almeida 2017). The hypomethylation of the gene on the promoter has been documented in several OPSCC studies. Lower methylation in retrotransposon elements, such as long interspersed elements (LINEs) or short interspersed elements (SINEs), affects carcinogenesis through genome destabilization. In normal mammalian cells LINE sequences have a high methylation status, while during cancer development they are hypomethylated, which contributes to the activation of sequence transcription, leading to genome instability and carcinogenesis. *LINE-1* has reduced methylation in various cancer cells compared to normal cells indicating, that *LINE-1* is associated with advanced cancer (Kitkumthorn and Mutirangura 2011, Gazdzicka et al. 2020). Furlan et al. (2017) showed, that hypomethylation of *LINE-1* in OPSCC patients have a 3.5-fold higher risk of early relapse compared to cases with high level of methylation (Gazdzicka et al. 2020).

1.2.1.2 Hypermethylation

In normal cells, CpG islands are poorly methylated in transcriptionally active genes, while in cancer cells high level of methylation in promoters of genes is characteristic. This hypermethylation can lead to transcriptional silencing of tumor suppressor genes and consequently promote malignant transformation (Gazdzicka et al. 2020) (Figure 8.).

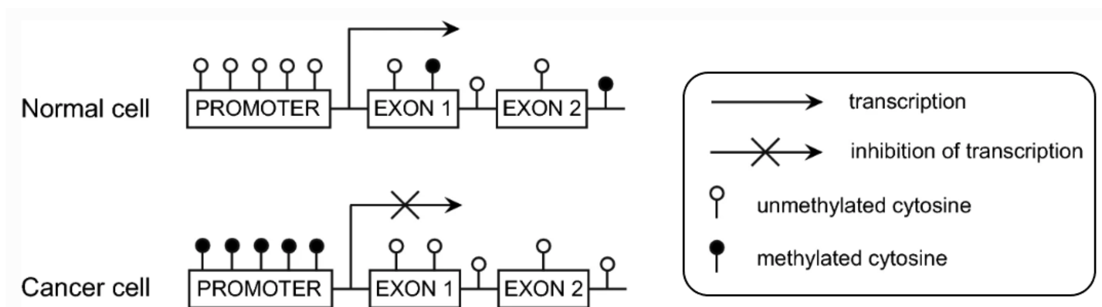


Figure 8. Methylation of promoter region in normal and cancer cells (adopted from (Hatziapostolou and Iliopoulos 2011)).

In several studies have been observed differences in DNA methylation profiles between HPV-positive and HPV- negative OPSCC. In contrast with characteristic genome-wide hypo-methylation in HPV-negative cancers, in HPV- positive cases higher methylation level of pro-moters occurs (Boscolo-Rizzo et al. 2017). Silenced genes by abnormal DNA methylation in-volved several pathways, such as apoptosis, cell cycle, DNA repair, and WNT signaling. The most frequently hypermethylated genes in OPSCC are shown in Table 1.

Table 1. The most frequently hypermethylated genes in OPSCC (Boscolo-Rizzo et al. 2017)

Pathway	Gene	Name	Hypermethylated in	Reference
Apoptosis	DAPK	Death-associated protein kinase 1	HPV-negative/positive	(van Kempen et al. 2014b)
	RASSF1	Ras association domain-containing protein 1	HPV-negative	(Colacino et al. 2013; Taioli et al. 2009; Weiss et al. 2011)
	STAT5	Signal transducer and activator of transcription 5	HPV-negative	(Colacino et al. 2013)
Cell cycle	CCNA1	Cyclin A1	HPV-positive	(Weiss et al. 2011; Colacino et al. 2013)
	CDKN2A	Cyclin-dependent kinase inhibitor-2A	HPV-negative	al. 2011)
			HPV-positive	(Lleras et al. 2013)
	CHFR	Checkpoint with forkhead and ring finger domains	HPV-negative	(van Kempen et al. 2014b)
	TP73	Tumor protein p73	HPV-negative/positive	(van Kempen et al. 2014b)
Cell fate determination	APC	Adenomatous polyposis coli	HPV-negative/positive	(van Kempen et al. 2014b)
DNA repair	MGMT	O6-methylguanine-DNA methyltransferases	HPV-negative	(Colacino et al. 2013; Weiss et al. 2011)
Protein glycosylation	TUSC3	Tumor suppressor candidate 3	HPV-positive	(Colacino et al. 2013)
Inflammation	JAK3	Janus kinase 3	HPV-positive	(Colacino et al. 2013)
Invasion and metastasis	CADM1	Cell Adhesion Molecule 1	HPV-positive	(van Kempen et al. 2014b)
	CDH11	Cadherin 11	HPV-positive	(Colacino et al. 2013)
	CDH13	Cadherin 13	HPV-negative/positive	(van Kempen et al. 2014b)
	IGSF4	Immunoglobulin superfamily member 4	HPV-positive	(Chen et al. 2015)
	SPDEF	SAM pointed domain-containing Ets transcription factor	HPV-negative	(Colacino et al. 2013)
	TIMP3	TIMP metallopeptidase inhibitor 3	HPV-positive	(van Kempen et al. 2014; Weiss et al. 2011)
	SYBL1	Synaptobrevin-like 1	HPV-positive	(Colacino et al. 2013)
Signaling	ESR1	Estrogen receptor 2	HPV-negative/positive	(van Kempen et al. 2014b)
	ESR2	Estrogen receptor 2	HPV-negative	(Colacino et al. 2013)
	GALR1	Galanin receptor type 1/2	HPV-positive	(Lleras et al. 2013)
	GRB7	Growth factor receptor-bound protein 7	HPV-positive	(Colacino et al. 2013)
	RARβ	Retinoic acid receptor β	HPV-negative/positive	(van Kempen et al. 2014b)
Transcription	RUNX1T1	RUNX1 translocation partner 1	HPV-positive	(Colacino et al. 2013)
	TCF21	Transcription factor 21	HPV-positive	(Weiss et al. 2013)
WNT signaling	SFRP1	Soluble frizzled receptor protein 1	Drinkers	(Marsit et al. 2006)
	SFRP4	Soluble frizzled receptor protein 4	HPV-positive	(Marsit et al. 2006)
	WIF1	WNT inhibitory factor 1	NA	(Paluszczak et al. 2015)

1.2.2 Histone modification

Transcriptional activity of DNA and regulating chromatin structure is ensured by histone modifications. Cancer is mostly associated with aberrations in histone modifications. The dynamic structure of chromatin involves numerous pathways that regulate cell metabolism. The fundamental unit of chromatin is the nucleosome, histone octamer, consisting of four globular proteins (two copies of H2A, H2B, H3, and H4). Around the octamer ~ 147 base pair strand of DNA is wrapped in a left-handed supercoil (Gazdzicka et al. 2020).

Histones are essential proteins consisting of a globular C-terminal domain and N-terminal tails. It is the N-terminal tails that protrude from the nucleosome and are subject to combinations of covalent modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination. These modifications determine how tightly chromatin compacts, play a crucial role in modulating gene expression and serve as docking stations for protein recognition modules that acquire specific functional complexes. The most common epigenetic alterations, histone acetylation and methylation are associated with carcinogenesis (Boscolo-Rizzo et al. 2017). Histone acetylation is the result of the cooperation between histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Fratta et al. 2016).

HATs are responsible for histone acetylation, opposite HDACs are responsible for their removal. Although HATs are generally associated with transcriptional activity, both HAT and HDAC activity is required for properly regulated gene expression (Audia and Campbell 2016).

Histone methylation is catalyzed by histone methyltransferases (HMTs) and it occurs on both the arginine and lysine residues on the tails of the histone compounds H3 and H4. As well as acetylation/deacetylation histone methylation is reversible and demethylation is catalyzed by histone demethylases (HDMs) (Boscolo-Rizzo et al. 2017).

1.2.3 MicroRNA

MicroRNAs (miRNAs) represent a large group of non-coding RNAs post-transcriptionally regulating gene expression. The miRNAs are strictly regulated and play an important role in cell proliferation, angiogenesis, apoptosis, and differentiation. MiRNA genes are transcribed by RNA polymerase II into RNA that creates hairpin structures known as primary miRNA (Fang and Li 2019). This structure is processed by the Drosha endonuclease, which cleaves the overhanging ends to form a miRNA precursor, the pre-miRNA. Exportin-5 transports the pre-miRNA from the nucleus to the cytoplasm. In the cytoplasm, the pre-miRNA is recognized by

the enzyme Dicer, which cleaves the hairpin at the other end of the pre-miRNA, producing miRNA. The mature miRNA duplex is a short term subject because only one of the two strands is retained. The miRNA is then bound to the Argonaut protein and forms the RNA-induced silencing complex (RISC). In this complex, miRNAs act as an adapter that specifically recognizes and binds to individual mRNAs (Gregory and Shiekhattar 2005).

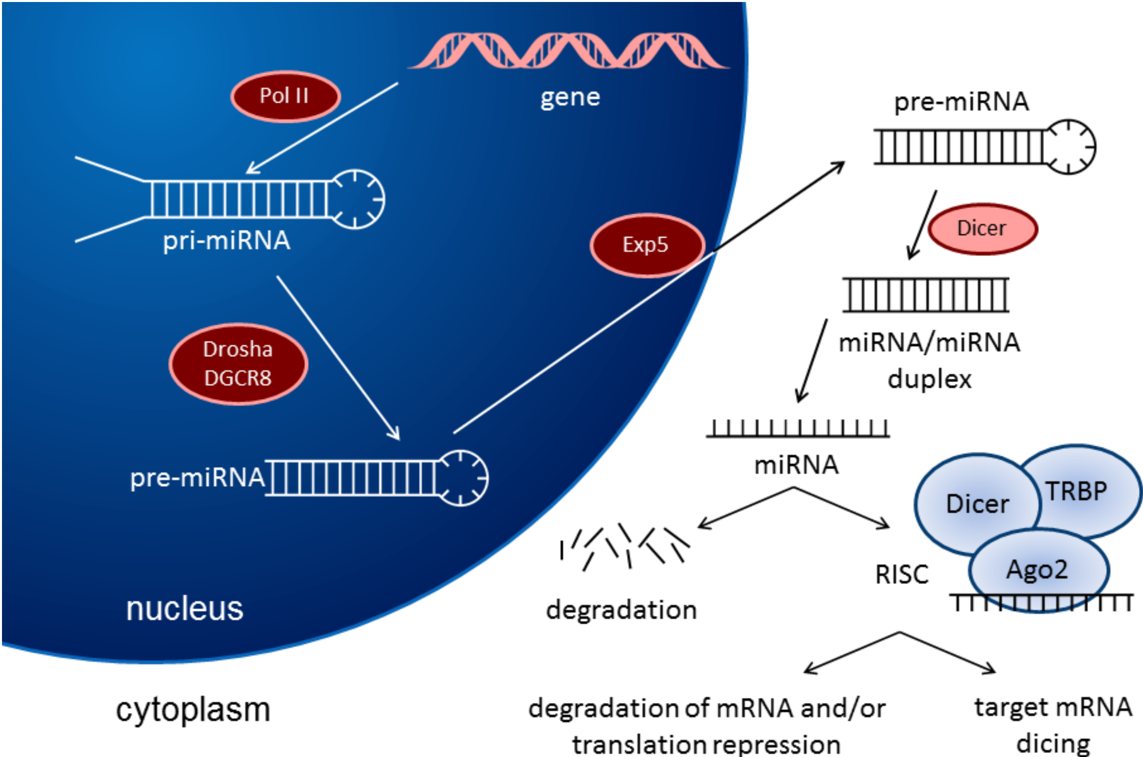


Figure 9. Biogenesis of miRNA, adopted from (Kovaříková 2019)

2 AIMS OF THE STUDY

Until now, numerous studies have tried to identify specific genetic and epigenetic biomarkers, which could contribute to a better understanding of the mechanisms of HPV-positive and HPV-negative OPSCCs to improve diagnostic and therapeutic treatment methods. Analysis of the different epigenetic mechanisms, such as DNA methylation, is essential, as it could help to elucidate the process of OPSCC development, which could subsequently improve treatment options for patients.

Therefore, the specific aims of this study were:

1. Optimization of selected methods used for detection of DNA methylation changes.
2. Investigation of methylation levels of selected tumor suppressor genes in OPSCC by comparison with control tissue.
3. Determine, if methylation of selected genes correlated with clinicopathological characteristics of oropharyngeal cancer.
4. Compare HPV-positive and HPV-negative OPSCCs with methylation levels of examined genes.
5. Determine, whether the methylation levels of genes analyzed in OPSCC may be a useful diagnostic marker and potential therapeutic target for OPSCC.

3 MATERIALS AND METHODS

3.1 Study group

The study group consisted of 94 patients with OPSCC and 44 patients with non-malignant diagnosis (palatine tonsil samples). Total number of 138 formalin-fixed, paraffin-embedded (FFPE) samples (74 metastatic tumors samples and metastases samples associated with their primary tumors, 20 nonmetastatic tumors, and 44 control tissue samples) were analyzed in this study. The control samples of normal tissue were obtained from patients treated for a non-malignant diagnosis such as tonsillitis. The FFPE blocks were retrieved from the archives of the Fingerland Department of Pathology, University Hospital Hradec Králové and Faculty of medicine in Hradec Králové, Czech Republic. All malignant tumors of the oropharynx were diagnosed between the years 1999-2018. According to the current WHO classification (Westra and Lewis 2017), all slides were reviewed by an experienced head and neck pathologist Jan Laco Ethical approval was obtained from the Ethics Committee of the University Hospital in Hradec Králové. Information about gender, age of the patients at the time of diagnosis, tumor size, tumor localization (including oropharynx, the root of the tongue, palatine tonsils, and soft palate) and pathological stage of TNM were recorded for each patient. (Table 2.)

During the follow-up period (unit March 2019), data on recurrence, death, and tumor-related death staging were recorded. Treatment modalities for all patients were radical surgery with radiotherapy and in 22 cases chemotherapy was added. The HPV status was analyzed at the Fingeraland Department of pathology using p16 immunohistochemistry and HPV DNA polymerase chain reaction (PCR) (Laco et al. 2015). Squamous cell carcinomas were classified according to the recently proposed criteria as keratinizing, non-keratinizing, and non-keratinizing with maturation. From these three categories, only keratinizing carcinomas were further graded as well, moderately, or poorly differentiated. Vascular invasion, perineural spread, and status of resection margins were also noted, with the latter being classified as positive (R1) or negative (R0). Due to the presence of lymph node metastases, vascular invasion was present in all patients, not regarding the difference between blood and lymphatic vessel invasion. Furthermore, the extranodal extension of metastasizing tumor cells was evaluated as well (Kovaříková 2019)

Table 2. Clinicopathological data of patients with oropharyngeal carcinoma

Clinicopathological characteristic				N	Quantity	%
Gender		Male		93	68	73%
		Female			25	27%
Age		≤ 55		93	30	32%
		>55			63	68%
Smoking status		Smoker		73*	22	30%
		Non-smoker			26	36%
		Former smoker			25	34%
Alcohol		Yes		73*	21	29%
		No			52	71%
HPV status		Positive		93	78	84%
		Negative			15	16%
Localization		Tonsils		93	65	70%
		Oropharynx			20	22%
		Tonsillar fossa			4	4%
		Base of tongue			4	4%
TNM classification	pT	T1		93	26	28%
		T2			46	50%
		T3			16	17%
		T4	T4a		2	2%
			T4b		3	3%
	pN	N0		93	20	22%
		N1			17	18%
		N2	N2a		27	29%
			N2b		25	27%
			N2c		1	1%
		N3			3	3%
Typing		Keratinizing	Grade 2	93	12	13%
			Grade 3		3	3%
		Non-keratinizing			56	60%
		Non-keratinizing with maturation			22	24%
Invasion		Vascular		93	76	82%
		Perineural		93	11	12%
		Extracapsular		73*	10	14%
Recurrence		Yes		73*	11	15%
		No			62	85%

* Due to the fact that few clinical data are missing, in some cases the partial sums do not add to the total number of involved patients.

3.2 DNA extraction from FFPE tissue

3.2.1 Deparaffinization

Deparaffinization of paraffin-embedded tissue samples was achieved prior to DNA extraction with xylene. 1 ml of xylene was added to 1.5 microcentrifuge tubes with FFPE sections. The samples were vortexed vigorously for 10s then centrifuged at 16,000x g for 3 minutes. The supernatant was carefully discarded by pipetting without disturbing the pellet. The next step was ethanol rehydration. Consequently, 1ml of 96% ethanol (EtOH) was added to the pellet. The samples were mixed by vortexing and centrifuged at 16,000 x g for 3 minutes. The supernatant was removed by pipetting without disturbing the pellet again. The remaining EtOH was allowed to evaporate from the samples.

3.2.2 DNA extraction

DNA isolation was performed with a commercially available extraction kit (QIAamp DNA FFPE Tissue Kit, Qiagen, Germany) according to the manufacturer´s protocol. Isolation of DNA from formalin-fixed tissue samples, embedded in paraffin consists of 6 basic steps. Briefly, after deparaffinization, the next step was lysis of the sample by proteinase K treatment (incubation at 56°C for 1 h or until the sample has been completely lysed). The third step was incubation at 90°C for 1 h in ATL buffer partially reversing formaldehyde modifications of nucleic acids. Longer incubation time or higher incubation temperatures could result in more fragmented DNA. This was followed by the binding of the DNA to the column membrane and column washing to remove contaminating impurities. The last sixth step was the elution of DNA from the membrane. The result was pure and concentrated DNA.

3.2.3 Quantification of genomic DNA

Concentration of DNA in all obtained samples was measured using two methods: spectrophotometric measurement of absorbance at 260 nm wavelength (NanoDrop 1000, Termo Fisher Scientific, USA) and the fluorometric method based on binding of double-stranded DNA (dsDNA)-selective fluorescent dyes (Qubit Fluorometer, Termo Fisher Scientific, USA) according to the manufacturer´s instruction. The quality of extracted DNA was evaluated using an absorbance ratio at 260 nm and 280 nm (A260/A280). Samples with the A260/A280 ratio falling within the range of 1.8–2.0 were considered to be of good quality.

Subsequent Qubit analysis allowed the evaluation of the dsDNA content in isolated samples. The isolated DNA was stored at -20°C until used.

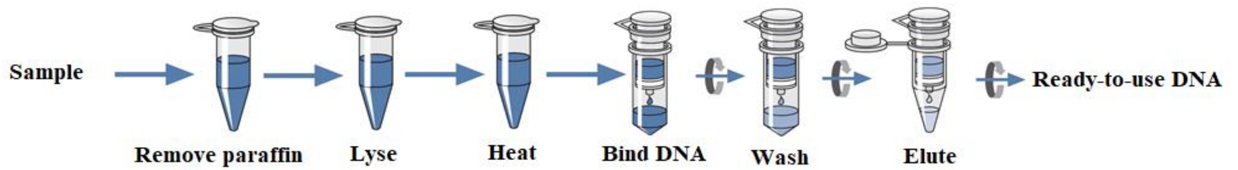


Figure 10. DNA extraction from FFPE tissue

3.3 MS-MLPA

The MLPA technique was first described by Schouten et al. (2002) and an overview is shown in Figure 9. MLPA reactions are easy to perform and require a short hands-on time. MLPA has been used to assess changes in gene copy number (Moelans et al. 2010), gene expression (Eldering et al. 2003), point mutation status (Bunyan et al. 2007) and DNA methylation (Nygren et al. 2005). Due to the short lengths of the target sequences of hemiprobes, MLPA allow us to use more fragmented DNA from FFPE tissue, not only DNA isolated from fresh-frozen tissue. For analysis 50–200 ng of DNA is needed depending on DNA quality. MLPA method is often used in pathology thanks to its ability to create a multiplex copy number and methylation assessment of FFPE samples. A variant of classic MLPA called "methylation-specific MLPA (MS-MLPA)" allows simultaneously semiquantitative detection of the methylation status of genes and their copy number. A key component in MS-MLPA is the methylation-sensitive endonuclease HhaI. Technically, MS-MLPA is similar to MLPA, except that after the ligation step, the samples are divided into two reactions (Moelans et al. 2018) (Figure 9). In one reaction HhaI and ligase is added, in the second reactiononly ligase is added. If the HhaI recognition site is not methylated, HhaI cuts the hybrid DNA with a probe and sample and no PCR product is generated. If the DNA sample is methylated, HhaI digestion is prevented and the fragment will be amplified in subsequent PCR.

For data analysis, MLPA peak patterns of the HhaI-treated and -untreated reactions are compared, providing an estimate of the methylation percentage within a given sample. A disadvantage of using HhaI endonuclease is that the construction of MS-MLPA probes is limited to its recognition sites (GCGC). The major advantage of MS-MLPA compared to other methods for methylation detection such as methylation-specific PCR (MSP), is that it does not require

sodium bisulfite conversion of unmethylated cytosine residues, a step that is often difficult to standardize and leads to degradation of the DNA. Furthermore, with MS-MLPA up to 55 probes can simultaneously analyzed and it can be combined with copy number and point mutation detection in the same reaction (Hömig-Hölzel and Savola 2012).

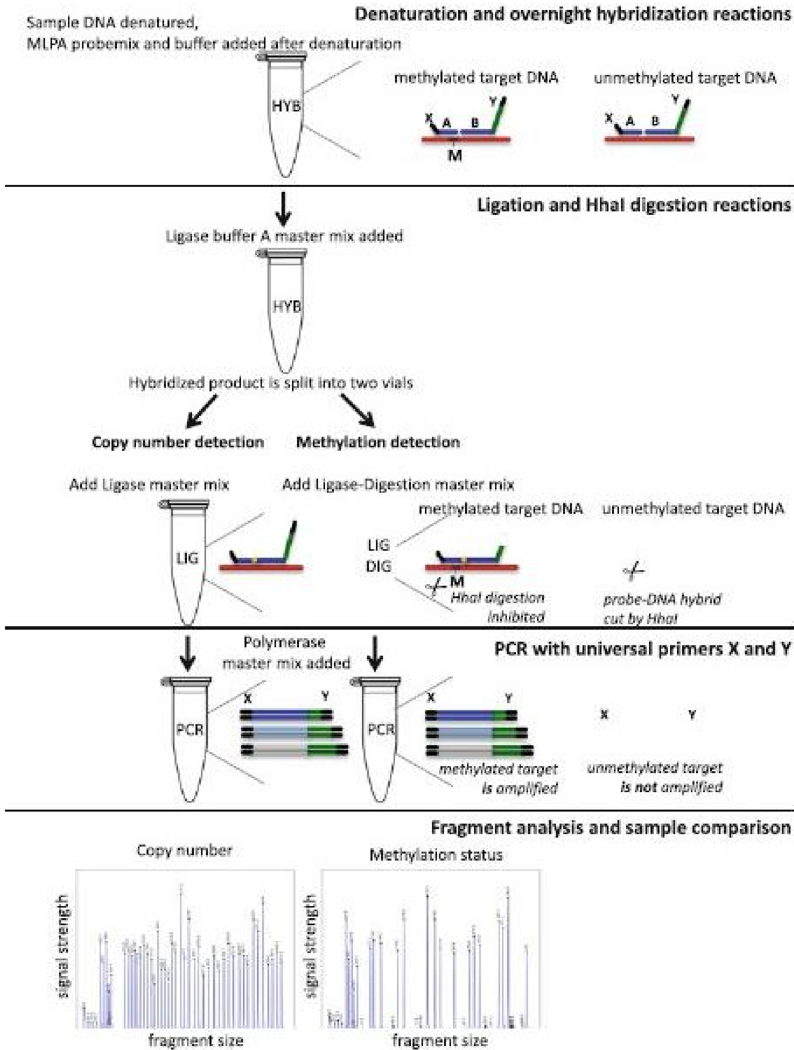


Figure 11. Principle of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), adopted from (Moelans et al. 2018)

For methylation analysis in tumors, metastases, and control tissue MS-MLPA (MRC Holland, Amsterdam, the Netherlands) was performed according to the manufacturer's instructions for a set of 25 tumor suppressor genes (Probe mix ME002-C1; MRC Holland, Amsterdam, The Netherlands). The choice for probe mix ME002-C1 was based on a thorough literature search indicating that genes involved in this particular kit showed promising methylation patterns in head and neck cancer. Moreover, hypermethylation of some promoter regions were associated

with HPV in cervical cancer (Demokan and Dalay 2011). A short overview of the included genes is presented in Table 3.

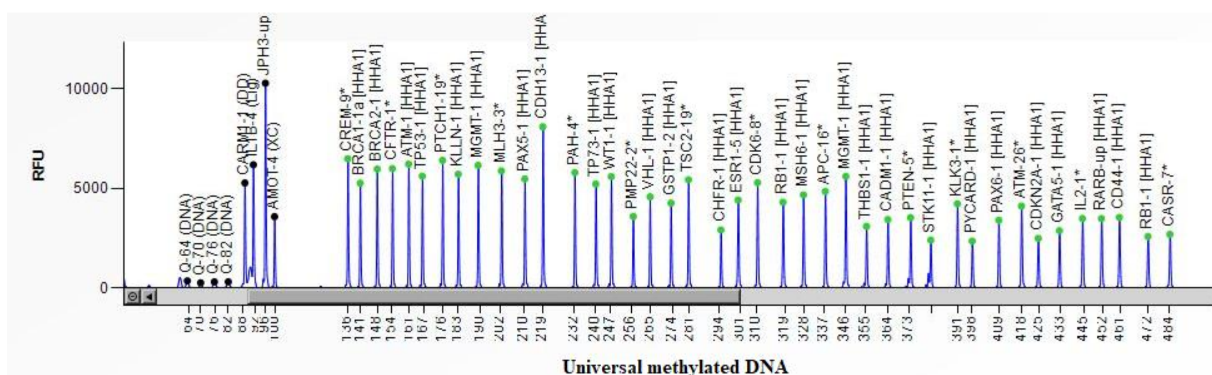
Table 3. Genes included in the MS-MLPA KIT ME002-C1 tumor suppressor

Gene	Name	Probes	Chromosomal location
TP73	tumor protein p73	16004-L23287	01p36.32
MSH6	mutS homolog 6	01250-L00798	02p16.3
VHL	von Hippel-Lindau tumor suppressor	03818-L03850	03p25.3
RARB	retinoic acid receptor beta	04046-L02172	03p24.2
CASR	calcium sensing receptor	02683-L02148	03q21.1
IL2	interleukin 2	00627-L00183	04q27
APC	APC regulator of WNT signaling	01700-L01341	05q22.2
ESR1	estrogen receptor 1	02746-L02173	06q25.1
CDK6	cyclin dependent kinase 6	03184-L02523	07q21.2
CFTR	CF transmembrane conductance	02944-L02376	07q31.2
CDKN2A	cyclin dependent kinase inhibitor 2A	18349-L23290	09p21.3
PAX5	paired box 5	03750-L23113	09p13.2
PTCH1	patched 1	03708-L23221	09q22.32
CREM	cAMP responsive element modulator	00981-L00566	10p11.21
KLLN	killin, p53 regulated DNA replication	13686-L15155	10q23.31
PTEN	phosphatase and tensin homolog	03638-L17142	10q23.31
MGMT(a)	O-6-methylguanine-DNA	05670-L05146	10q26.3
MGMT(b)	O-6-methylguanine-DNA	18346-L23286	10q26.3
PAX6	paired box 6	03749-L03209	11p13
WT1	WT1 – WT1 transcription factor	18347-L23288	11p13
CD44	CD44 molecule (Indian blood group)	04500-L02761	11p13
GSTP1	glutathione S-transferase pi 1	18345-L23787	11q13.2
ATM(a)	ATM serine/threonine kinase	03023-L23862	11q22.3
ATM(b)	ATM serine/threonine kinase	02670-L02137	11q22.3
CADM1	cell adhesion molecule 1	03816-L17141	11q23.3
PAH	phenylalanine hydroxylase	02334-L21324	12q23.2
CHFR	checkpoint with forkhead and ring finger	18344-L23785	12q24.33
BRCA2	BRCA2 DNA repair associated	02285-L01776	13q13.1
RB1(a)	RB transcriptional corepressor 1	02734-L23112	13q14.2
RB1(b)	RB transcriptional corepressor 1	04502-L02199	13q14.2
MLH3	mutL homolog 3	01245-L00793	14q24.3
THBS1	thrombospondin 1	01678-L17140	15q14
TSC2	TSC complex subunit 2	01832-L01397	16p13.3
PYCARD	PYD and CARD domain containing	02252-L01737	16p11.2
CDH13	cadherin 13	02257-L01742	16q23.3
TP53	tumor protein p53	18348-L23289	17p13.1
PMP22	peripheral myelin protein 22	01462-L00927	17p12
BRCA1	BRCA1 DNA repair associated	03296-L01269	17q21.31
STK11	serine/threonine kinase 11	06783-L17143	19p13.3
KLK3	kallikrein related peptidase 3	00713-L23223	19q13.33
GATA5	GATA binding protein 5	03752-L06199	20q13.33

In short, DNA (optimal input range 50-100 ng) samples were diluted up to 5µL with TE buffer (10 mM Tris–HCl with a pH between 8.0 and 9.0). After denaturation (5 min at 98 °C and cooling to 25 °C before opening the thermocycler) of isolated DNA, the probe mix was added, and the samples were incubated overnight at 60°C. For the ligation (digestion) reaction, each sample was divided into two tubes, one of which incubated with Ligase as a standard MLPA reaction and the other with both Ligase and with Hha1 endonuclease (30min., 48°C).

Next, primers and PCR Polymerase were added, and a PCR was performed. In the tube with the Hha1 enzyme, methylated DNA is prevented from being digested by the methylation-sensitive restriction enzyme and therefore the target region is ligated and amplified by PCR. Unmethylated DNA is digested and thus cannot be amplified by PCR. All runs were performed on a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) and the thermal profile of PCR reaction is shown in Table 4.

Universal methylated DNA standard was used as positive (100% methylated) control (ZYMO Research, Freiburg, Germany), and DNA obtained from human blood from a healthy volunteer as a negative (unmethylated) control. Both controls were used in each run of MS-MLPA. Aliquots of PCR reaction products (0.6µl) were mixed with 0.2 µl inner standard GeneScan™ –500 LIZ®(AppliedBiosystems, Foster City, CA, USA) and 9.0 µl formamide, subsequently were denatured and separated by electrophoresis on an ABI 3500 capillary sequencer (Applied Biosystems, Foster City, CA). Methylation levels of selected tumor suppressor genes were performed using Genemapper v4.1 software (Applied Biosystems) and CoffalyserNET analysis (MRC Holland) software (Figure 12.)



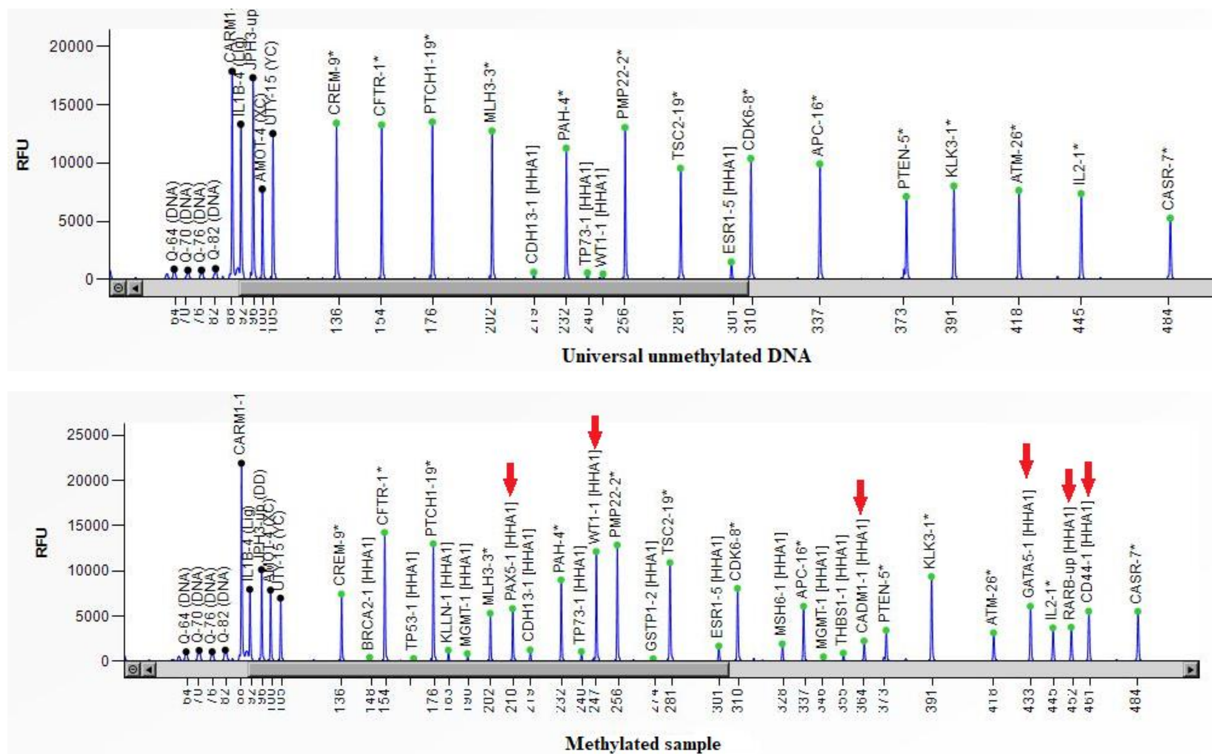


Figure 12. Detection of aberrant methylation using MS-MLPA ME-002 kit. Red arrow showing methylation of several genes including *PAX5, WT1, CADM1, GATA5*, *RARβ* and *CD44*.

Methylation dosage ratio was obtained by the following calculation: $D_m = (P_x / P_{ctrl})_{Dig} / (P_x / P_{ctrl})_{Undig}$, where D_m is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of the peak areas of all control probes, Dig stands for HhaI digested sample, and Undig stands for undigested sample. Based on previous validation experiments a given promoter was considered to show methylation if the methylation dosage ratio was ≥0.15 (corresponding to 15% of methylated DNA).

Table 4. Thermal profile of PCR reaction

PCR thermal profile			
Step		Temperature (˚C)	Time
Initial denaturation		95	5 minutes
35 PCR cycles	Denaturation	95	30 seconds
	Annealing	60	30 seconds
	Extension	72	60 seconds
Final Extension		72	20 minutes
Hold		15	Indefinitely

3.4 Methods based on bisulfite conversion

Bisulfite conversion (BC) is the "gold standard" for DNA methylation analysis and enable identification and quantification of DNA methylation at single-nucleotide resolution. For methods based on DNA modification with bisulfite conversion, the first step is a chemical modification of the analyzed DNA. Already in 1970, it was discovered that sodium bisulfite treatment to single-stranded DNA leads to converting cytosine to uracil. All cytosines are converted to uracil, only methylated cytosines (5-methylcytosine) are protected and remain as cytosines (Hayatsu, Wataya and Kazushige 1970). In the process of converting unmethylated cytosine to uracil, the first step is denaturation of DNA by heating in an alkaline environment. The chemical modification of DNA involves sulfonation and hydrolytic deamination to generate the intermediate (uracil sulphonate), which must be converted to uracil by alkaline desulfonation (Figure 13.)

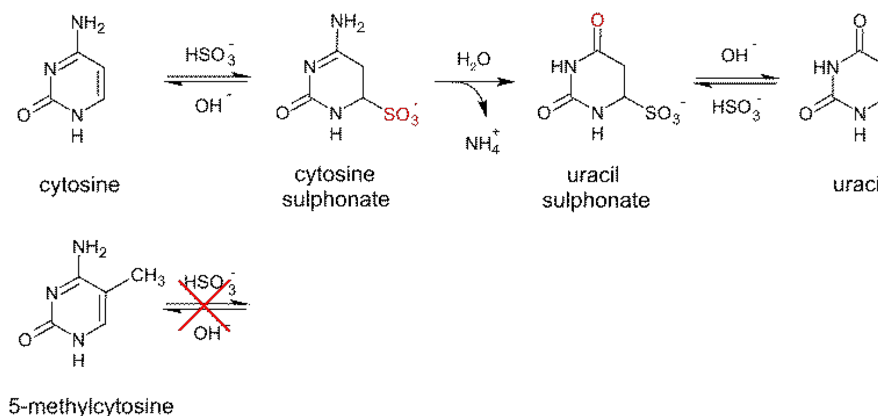


Figure 13. Bisulfite conversion. (Jujubix 2011)

After treatment with sodium bisulfite, unmethylated cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unaffected.

After PCR amplification, uracil is amplified as thymine, while 5-mC residues remain as cytosines, allowing methylated CpGs to be distinguished from unmethylated CpGs (Patterson et al. 2011).

This chemical treatment was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, USA) according to the manufacturer's instruction. Briefly, 500ng of isolated DNA was added 130 µl CT Conversion Reagent mix. After thorough mixing the bisulfite treatment was performed using a thermal cycler Veriti™ Thermal Cycler (Thermo

Fisher Scientific, USA) with the following conditions: denaturation for 10 minutes at 98 °C and incubation for 2.5h at 64 °C, and indefinite hold at 20°C. Binding buffer was added to the spin column before transferring the modified DNA samples to the columns. Subsequently, samples were purified and desulfonated. Desulfonation was followed by further ethanol purification and elution from the column membrane. The samples containing bisulfite-treated DNA were stored in the freezer (-20°C). Bisulfite modification of DNA is relatively inexpensive, rapid, and simple, but the DNA quality and quantity are an important factor in the bisulfite conversion reaction. It is generally recommended to use more than 1 μg of high-quality DNA extracted from cultured cells or fresh frozen tissue samples to obtain consistent results. Therefore, using DNA isolated from the FFPE tissue sections may generate unfavorable results due to the limited quantity of initial DNA and possible DNA degradation caused by bisulfite treatment (Tan and Dobrovic 2001).

3.4.1 Methylation Sensitive High Resolution Melting

Based on results from MS-MLPA and studies focused on hypermethylation of some promoter regions associated with HPV in cancer of cervix we selected CpG with most distinct changes in methylation between tumors and control samples for further analysis. To confirm the hypermethylation of the selected region in the *CADM1* gene, we analyzed 210 FFPE samples using MS-HRM analysis. MS-HRM is a sensitive and specific method for the detection of DNA methylation based on different melting temperatures (Tm) of methylated and unmethylated DNA. Tm is defined as the temperature at which the double-stranded DNA dissociates into single-stranded DNA, characterized by a change in the fluorescent signal due to the release of the intercalating dye, for example SYBR Green, EvaGreen or SYTO9. The Tm is dependent on the DNA base sequence because the CG base pairs are linked by three hydrogen bonds and the AT pairs by only two. BS conversion leads to converting unmethylated cytosine to uracil and, following PCR, uracil becomes thymine. So a different melting profile allows us to distinguish between methylated and unmethylated DNA. The PCR amplification and HRM analysis were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules CA, USA) in white Hard-Shell® 96-Well PCR Plates according to the protocol in Table 5.

Table 5. Protocol for MS-HRM analysis of CADM1 amplicon

<table>
<tr><th colspan="4">PCR setup</th></tr>
<tr><th colspan="2">Component</th><th colspan="2">Volume</th></tr>
<tr><td colspan="2">RNase-free water</td><td colspan="2">2,5 µL</td></tr>
<tr><td colspan="2">2X EpiTect HRM PCR Master Mix</td><td colspan="2">5 µL</td></tr>
<tr><td colspan="2">Forward primer (10 µM)</td><td colspan="2">0.75 µL</td></tr>
<tr><td colspan="2">Reverse primer (10 µM)</td><td colspan="2">0.75 µL</td></tr>
<tr><td colspan="2">Template DNA</td><td colspan="2">1 µL</td></tr>
<tr><th colspan="4">PCR thermal profile</th></tr>
<tr><th colspan="2">Step</th><th>Temperature (˚C)</th><th>Time</th></tr>
<tr><td colspan="2">Initial denaturation</td><td>95</td><td>5 minutes</td></tr>
<tr><td rowspan="3">40 PCR cycles</td><td>Denature</td><td>95</td><td>10 seconds</td></tr>
<tr><td>Anneal</td><td>55</td><td>30 seconds</td></tr>
<tr><td>Extend</td><td>72</td><td>10 seconds</td></tr>
<tr><td colspan="2">HRM</td><td>65-80°C; Δ 0.1</td><td>2 seconds</td></tr>
<tr><td colspan="2">Hold</td><td>40</td><td>1 minutes</td></tr>
</table>

The data were analyzed using the CFX maestro™ for qPCR interpretation and Precision Melt Analysis™ Software for HRM parameter determination (Bio-Rad Laboratories). The selection of primers was based on (Fisser et al. 2015). Primers CADM1-CpG-F (5’-GAGGATTTTTTAAGGGAGAT-3’) and *CADM1*- CpG-R (5’-TCAAAAAAAAATATTCTCCC-3’) amplify a fragment from - 297 to - 148 relative to the *CADM1* transcriptional start site. (Fisser et al. 2015).

In each run were used bisulfite-converted universal methylated DNA, universal un-methylated DNA, and 10% standard which served as a cut-off value for methylation status.

3.5 Statistical analysis

The statistical analysis was carried out using STATISTICA (data analysis software system) version 12 (StatSoft, Inc., Tulsa, OK, USA). All statistical tests were two-sided and statistical significance was claimed for $P<0.01$ or <0.05. Association between clinicopathological factors and DNA methylation status was analyzed using Fisher's exact test and chi-square test. For analysis of the overall survival rate the Kaplan-Maier method and the Logrank test were used.

4 RESULTS

4.1 MS-MLPA

Promoter methylation using a 15% cut-off

DNA methylation levels of selected tumor suppressor genes were analyzed using the Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) probe set ME002-C1 (MRC-Holland). Study group consisted of 102 FFPE OPSCC samples (32 metastatic oropharyngeal tumor sample, 32 correlated metastases, 19 non-metastatic tumor samples, and 19 control samples). All control samples of non- cancerous palatine tonsils were obtained from patients with chronic tonsillitis. CpG universal methylated and unmethylated control was used in each run.

Using a 15% cut-off value for methylation we observed significantly higher methylation in the *PAX5, CADM1, WT1* (P<0.01) and *RARβ, PAX6* (P<0.05) genes oropharyngeal carcinoma patients samples compared to a control group. Moreover, in the *PAX5*, *PAX6,* and *CADM1* genes, there was no methylation in the control samples. We observed a high methylation rate (about 50%) for *MSH6*, *CDH13,* and *GATA5* genes in both tumor and control samples. In contrast, we found out that the *TP73*, *GSTP1*, *CHFR, BRCA2, VHL, CDKN2A, ATM, RB1, PYCARD, BRCA1,* and *STK11* genes did not exhibit methylation in any of the patient samples.

The *CADM1* gene was significantly differentially methylated in HPV-positive and HPV-negative OPSCC (P<0.01) and *RARβ*, *PAX5*, *PAX6,* and *WT1* genes were frequently methylated independent of the HPV status of the tumor samples.

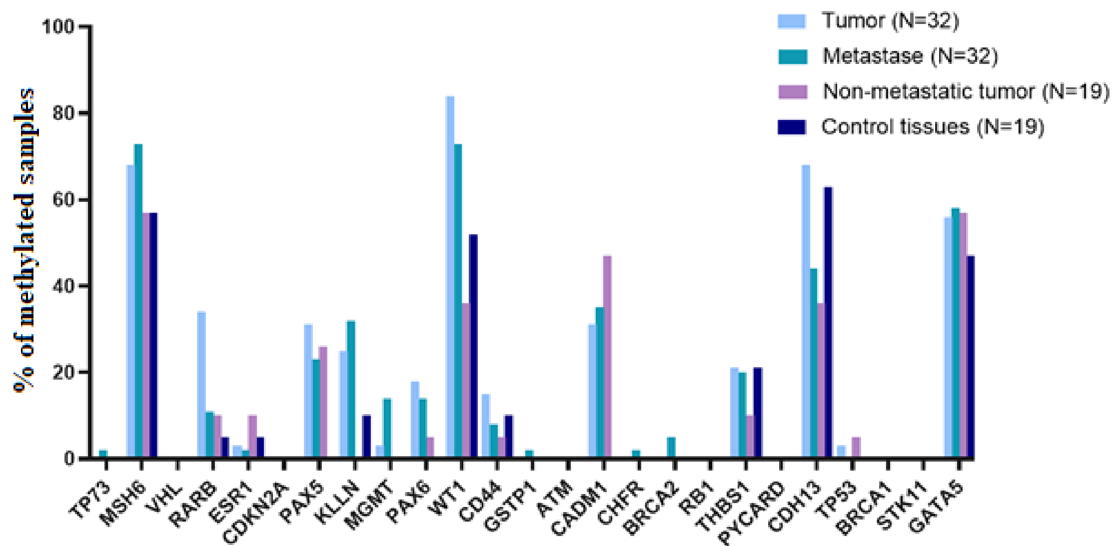


Figure 14. Promoter hypermethylation (>15%methylation) of 25 studied tumor-suppressor genes.

4.1.1 Correlation with clinicopathological features

Median age of oropharyngeal carcinoma patients at the time of diagnosis was 58 years (range between 45 – 80). The analyzed carcinoma group consisted of 37 males and 14 females. The follow-up period ranged from 3 to 180 months (median 82 months). Recurrence was found in 4/32 (12.5%). During the follow-up period 12/32 (37.5%) patients died, of whom 3/32 (9.4%) due to the tumor. HPV positivity was found in 41/51 (80.4%) of tumor samples. Clinicopathological characteristics were compared with methylation results of OPSCC tumor samples. (Table 6.)

Table 6. Clinicopathological data versus % of methylated samples

Clinicopathological characteristic				N	Quantity	%	% of methylated samples				
							RARB	PAX5	PAX6	WT1	CADM1
Gender	Male			51	37	73%	29,70	27,00	13,50	72,97	40,50
	Female				14	27%	14,30	35,70	14,30	50,00	28,60
Age	≤ 55			51	15	29%	20,00	33,30	26,67	73,30	40,00
	>55				36	71%	27,80	27,80	8,33	63,90	36,10
Smoking status	Smoker			32*	10	31%	40,00	40,00	80,00	80,00	30,00
	Non-smoker				12	38%	33,30	41,70	91,67	91,70	33,33
	Former smoker				10	31%	30,00	10,00	80,00	80,00	30,00
Alcohol	Yes			32*	10	31%	60,00**	40,00	13,63	90,00	40,00
	No				22	69%	22,70	27,27	30,00	81,81	27,27
HPV status	Positive			51	41	80%	24,39	31,70	41,63	68,29	46,34
	Negative				10	20%	30,00	20,00	10,00	60,00	0,00 ***
Localization	Tonsils			51	36	70%	19,44	25,00	5,55**	52,78***	33,33
	Oropharynx				9	18%	33,33	66,67	44,44	100,00	55,56
	Tonsillar fossa				3	6%	33,33	0,00	0,00	100,00	0,00
	Base of tongue				3	6%	66,67	0,00	33,33	100,00	66,67
TNM classification	pT	T1		51	15	29%	13,33	0,00***	0,00***	60,00	33,33
		T2			25	49%	32,00	36,00	12,00	64,00	32,00
		T3			9	18%	33,33	55,56	33,33	77,78	55,56
		T4	T4a		1	2%	0,00	0,00	0,00	100,00	0,00
			T4b		1	2%	0,00	100,00	100,00	100,00	100,00
	pN	N0		51	19	37%	10,50	26,32	5,56	36,84**	47,37
		N1			9	18%	44,44	33,33	44,44	88,89	55,56
		N2	N2a		13	25%	20,00	0,00	0,00	60,00	0,00
			N2b		9	18%	11,11	33,33	11,11	88,89	22,22
		N3			1	2%	100,00	100,00	0,00	100,00	0,00
Typing	Keratinizing	Grade 2		51	9	18%	22,22	22,22	0,00	62,50	22,22
		Grade 3			3	6%	66,67	33,33	22,22	100,00	0,00
	Non-keratinizing				29	56%	20,69	31,03	16,00	62,07	44,83
	Non-keratinizing with maturation				10	20%	30,00	30,00	10,00	80,00	40,00
Invasion	Vascular			51	35	69%	31,43	31,43	17,14	80,00 ***	34,29
	Perineural			51	3	6%	33,33	66,67	0,00	66,67	0,00
	Extracapsular			32*	2	6%	0,00	50,00	20,00	100,00 ***	0,00
Recurrence	Yes			32*	4	13%	33,33	33,33	0,00	100,00	0,00
	No				28	87%	34,48	31,03	20,69	82,76	34,48

* Due to the fact that few clinical data are missing, in some cases the partial sums do not add to the total number of involved patients. **(P<0,05), ***(P<0,01)

The *RARβ* gene showed significantly higher methylation in patients who consumed alcohol. (P<0.05). Significantly lower methylation in the *PAX6* gene was associated with tumors localization in tonsils (P<0.05) and T1 size of the tumor (tumor was 2 cm or smaller) (P<0.01). On the other hand, the *WT1* gene showed significantly higher methylation in patients with a tumor of tonsils (P<0.01), cancer cells in lymph nodes (N1-N3) (P<0.05), and in patients with vascular invasion (P<0.01). No correlation was found between DNA methylation and gender, age, smoking status, typing, and recurrence.

Kaplan-Meier survival curve showed a correlation between methylation of the *PAX5* gene in tumors and poor patient survival (P<0.05). Patients with higher methylation levels of *PAX5* gene had impaired survival than those with the unmethylated *PAX5* gene (Figure. 15).

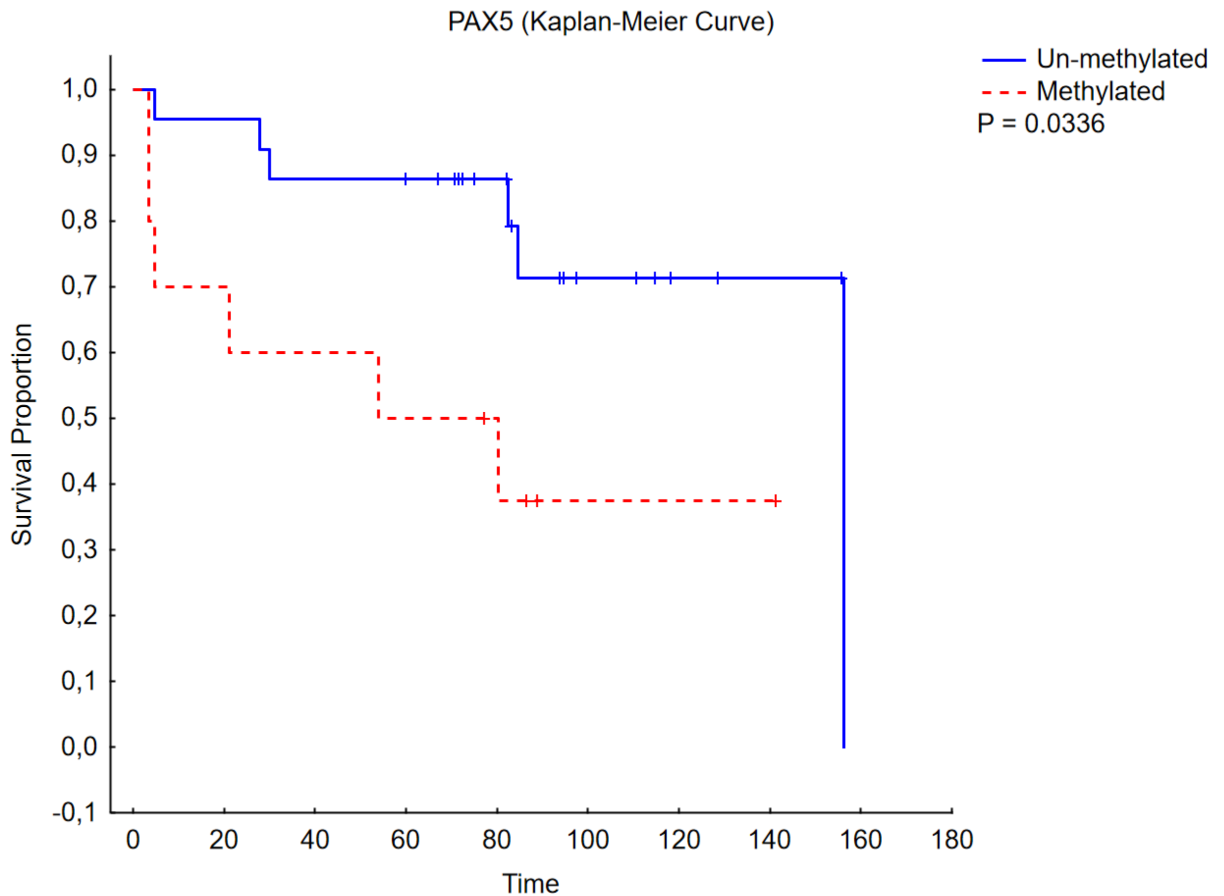


Figure 15. Kaplan-Meier survival analysis curve

4.2 MS-HRM

Of the 210 initially enrolled samples of 133 patients (74 metastatic tumors, 74 corresponding metastases, 22 non-metastatic tumor samples, and 39 control samples), 81 samples were excluded from the analyses due to the insufficient amplification/amount of obtained tissue or poor-quality of isolated DNA.

CADM1 methylation status in bisulfite converted DNA was detected using methylation-specific high-resolution melting analysis (MS-HRM) using Bio-Rad CFX96 Touch™ instrument within a range of 129 samples (35 metastatic tumors and 35 corresponding metastases), 20 non-metastatic tumor samples, and 38 control tissue samples (non-cancerous palatine tonsils). We considered the sample to be methylated when the methylation detected was above the cut-off limit value of 10%.

Methylation was detected in 36.4% (20/55) of the tumor samples (tumor and non-metastatic tumor). In all cases methylation status was the same in both samples of one patient (tumors and associated metastasis). All 38 control samples were unmethylated which also applies to all 11 HPV negative cancer samples. Methylation was detected in 45.5% (20/44) of HPV positive cancer samples (20/44).

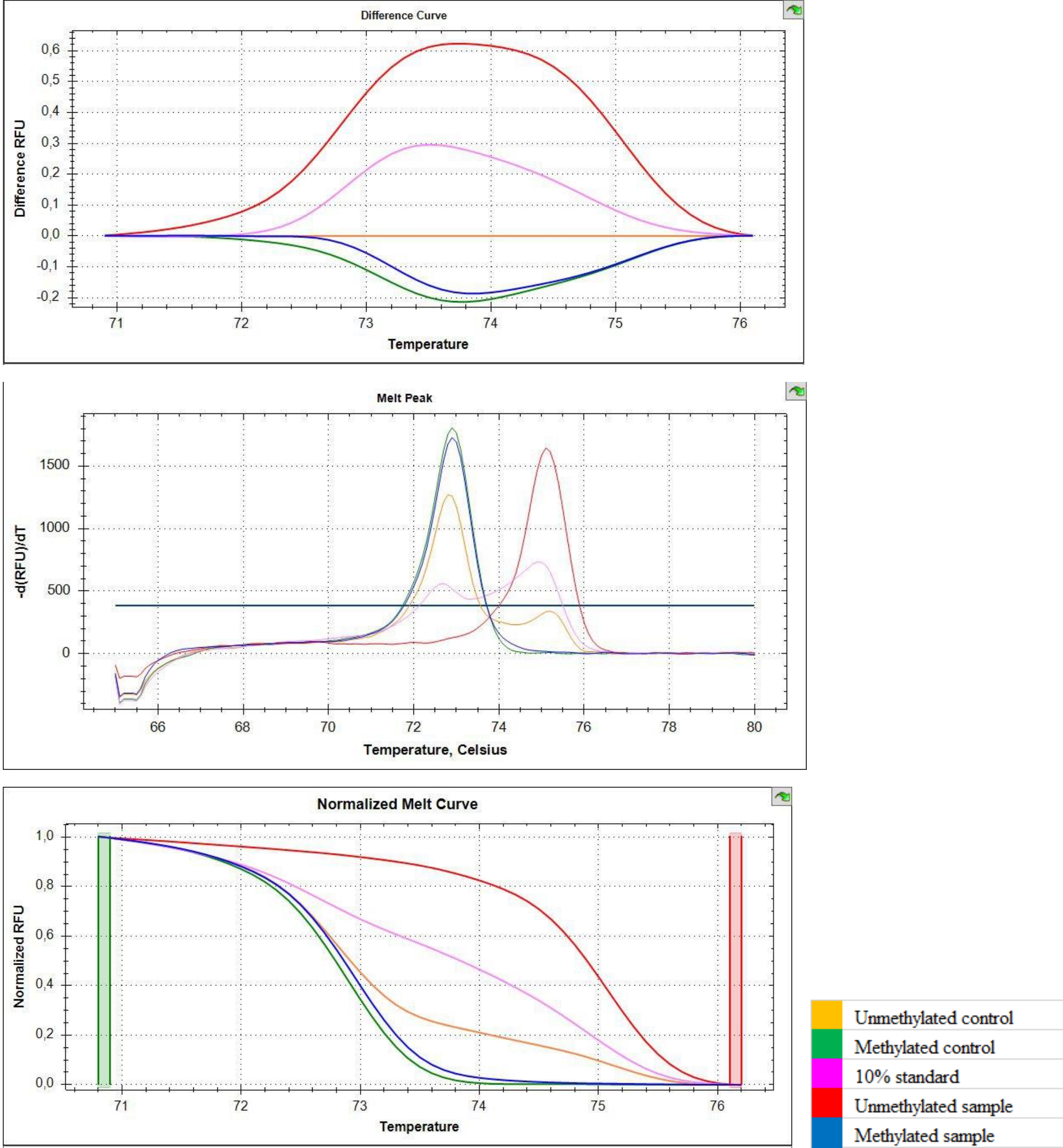


Figure 16. Representative plots from MS-HRM of *CADM1* gene.

In each run were used BC universal methylated DNA, universal unmethylated DNA and 10% standard, which served as a cut-off value for methylation status.

5 DISCUSSION

Oropharyngeal carcinoma, a type of head and neck cancer, is the seventh most common cancer worldwide. The squamous cell carcinomas are the most common histological type. Despite the significant efforts committed during the last decades to its early detection, prevention, and treatment, oropharyngeal cancer prognosis remains very poor with the rising incidence in developing countries and younger population. Because the symptoms of oropharyngeal cancer are similar to those of common upper respiratory tract infections, small percentage of OPSCC is diagnosed at an early clinical stage. Advanced stages of the disease respond poorly to current cancer therapies, with a high incidence of local and regional relapses and lymphoid tissue metastases. Therefore, early detection and better prediction of the disease using for example epigenetic biomarkers is crucial. Over the last decade, epigenetic changes, mainly aberrant DNA methylation, have been shown to play an important role in the OPSCC. The aim of this study was to determine, whether the methylation levels of selected tumor-suppressor genes analyzed in OPSCC, may be useful diagnostic markers and potential therapeutic targets for OPSCC. The most cases of OPSCC are associated with human papillomavirus. The HPV-positive OPSCC is considered as a prognostic advantage compared to HPV-negative cases. We examined promoter methylation status in 25 tumor suppressor genes using Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) probe set ME002-C1 (MRC-Holland). Using a 15% cut-off for methylation we observed significantly higher methylation in the *PAX5*, *CADM1*, *WT1*, ($P<0.01$), and *RARβ, PAX6* ($P<0.05$) genes of patients with oropharyngeal carcinoma compared with a control group. Our results also showed that the frequency of promoter methylation of genes *MSH6*, *CDH13,* and *GATA5* was higher than 50%, but did not show any significant difference between tumor and control samples. In contrast, we found out that the *VHL, TP73, GSTP1, CHFR, BRCA2, CDKN2A, ATM, RB1, PYCARD, BRCA1* and *STK11* genes did not exhibit methylation in any of the patient samples tested, suggesting that methylation of these tumor suppressor genes may not play an important role in carcinogenesis of the oropharyngeal cancer.

5.1 *PAX5* gene

The paired box 5 (*PAX5*) gene can be found at chromosome 9p13. Encoding a nuclear transcription factor that's involved in the control of organ development and tissue differentiation. *PAX5* was originally identified as a B-cell-specific activator protein that has an essential

role in early stages of B-cell differentiation, neural development, and spermatogenesis (Gu et al. 2017).

PAX5 is widely expressed in normal adult and embryonic tissues, as well as in various neoplasms including HNSCC (Guerrero-Preston et al. 2014), gastric cancer (Deng et al. 2014), hepatocellular carcinoma (Liu et al. 2011), breast cancer, and lung cancer (Palmisano et al. 2003). *PAX5* is also mutated in human acute B-cell leukemia (Mullighan et al. 2007).

In our study, methylation of the *PAX5* gene was found in 15/51 (29.4%) of oropharyngeal carcinoma samples and did not exhibit methylation in any of the control tissue samples ($P<0.01$). Using the Kaplan-Meier survival curve it was determined that patients with higher methylation levels of the *PAX5* gene had impaired survival than those with the unmethylated *PAX5* gene ($P<0.05$). The results of our study imply that expression of *PAX5* may be an indicator of potential malignant development in oropharyngeal cancer. Guerrero-Preston et al. (2014) compared *PAX5* methylation in HPV-positive and HPV- negative HNSCCs. Their data showed, that higher level of methylation was in HPV-negative tumors (83%) than in HPV positive tumors (25%). They also demonstrated that tumors from patients with a history of tobacco exposure (57%) had a similar frequency of *PAX* methylation as the patients with no smoking history (67%). Our data confirmed that the frequency of *PAX5* methylation in the patients' tumors was 40% in smokers and 41.7% in non-smokers. Conversely, lower level of methylation was in HPV-negative tumors (20%) a than in HPV-positive tumors (31.7%)

Guerrero-Preston et al. (2014) reported that *PAX5* gene methylation could be an excellent marker for HNSCC detection using methylated DNA-binding domain-based sequencing (MBD-seq) and fluorescence-based quantitative methylation-specific PCR (QMSP). They found out that the marker had high sensitivity (80%) and high specificity (94%) analyzing 76 tumors and 19 normal tissues. In another study, they used droplet digital PCR to show that *PAX5* gene methylation can be used as a molecular marker for surgical margin analysis and as a prognostic marker of HNSCCs (Hayashi et al. 2015).

5.2 *CADM1* gene

Cell adhesion molecules 1 (*CADM1*) gene also known as *TSLC1, NECL-2, IGSF4, SynCAM1*, is a novel tumor suppressor gene that is localized at chromosome 11q23.2. CADM1 protein is a transmembrane glycoprotein with 442 amino acids and belongs to an immunoglobulin cell adhesion superfamily (Murakami et al. 1998). CADM1 protein has structural homology to the neural cell adhesion molecules and cell signaling transduction and it is implicated in

calcium ion independent cell-cell adhesion (Shingai et al. 2003). *CADM1* was first observed in patients with non-small cell lung carcinoma (*NSCLC*) (Murakami et al. 1998). According to Ando et al. (2008), this gene has been shown to suppress tumor growth through antiproliferative and proapoptotic activity and loss of CADM1 expression leads to tumor formation and metastasis.

Hypermethylation of *CADM1* is one of the principal causes of gene silencing (Mao et al. 2004) in many types of cancer including esophageal cancer, cutaneous melanoma, hepatocellular carcinoma, ovarian carcinoma, breast cancer, pancreatic ductal adenocarcinoma, lung cancer, laryngeal squamous cell carcinoma, colorectal cancer, prostate cancer, neuroblastoma, nasopharyngeal carcinoma and in 83% of cervical carcinoma cases (Steenbergen et al. 2004, Chen et al. 2019). However, little is known about the role of *CADM1* hypermethylation in oropharyngeal cancer.

Using MS-MLPA, our results showed significantly higher methylation in the *CADM1* gene (P<0.01) in 19/51 (37.3%) cases of patients with oropharyngeal carcinoma compared with control group 0/19 (0%). Hypermethylation of *CADM1* gene correlated with HPV status, all samples with HPV-negative status 11/11 (100%) were unmethylated. No other correlation was found between DNA methylation and clinicopathological characteristics.

According to Steenbergen et al. (2004), hypermethylation of *CADM1* caused by HPV16 and HPV18 was observed in cervical cancer. It was determined, that *CADM1* inhibits proliferation and invasion of squamous carcinoma cells, which are the fundamental processes to disease progression (Vallath et al. 2016).

Since more than 90% of oropharyngeal cancer cases are histologically designated squamous cell carcinoma, and the most common cause is HPV infection, we used MS-HRM to confirm methylation levels of *CADM1* gene in oropharyngeal cancer samples. Using primers based on Fisser et al. (2015), we were able to detect methylation of the *CADM1* promoter region in 20 of 55 (36.4%) oropharyngeal cancer samples in our study. Because our results show that corresponding metastases have the same methylation status as their primary tumors, methylation of *CADM1* gene is probably not influenced by the metastatic process. Analysis by MS-HRM confirmed, that all control samples were 38/38 (100%) unmethylated, which implies that methylation of the *CADM1* promoter may play an important role in carcinogenesis of oropharyngeal cancer. These data correlate with the research results of van Kempen et al. (2014b), where HPV-positive OPSCC showed a significantly higher cumulative methylation index compared to HPV-negative OPSCC (P<0.01). Also, patients with HPV-positive OPSCC

generally respond more favorably to chemotherapy and radiation than patients with negative HPV status.

Methylation status of the *CADM1* promoter region in HPV-positive cancer cell lines was examined by Woo et al. (2015). *CADM1* promoter methylation was highly increased in HPV-positive cells but was very low in HPV-negative cells. Subsequently, hypermethylated cervical cells of *CADM1* were treated with inhibitor of DNA methylation, 5-aza-2'-deoxycytidine, to determine if the reason for its silencing was *CADM1* methylation. The 5-aza-2'-deoxycytidine treatment induced significant upregulation of gene expression in all highly methylated cell lines. Data from Woo et al. (2015) study indicate, that hypermethylation of the *CADM1* promoter region might be an active mechanism of silencing of *CADM1* gene expression. HPV infection may play a crucial role in the loss of *CADM1* gene expression via aberrant DNA methylation.

Our results suggest, that *CADM1* hypermethylation and identification of HPV status can serve as a biomarker for better prognosis and may play an important role in the choice of treatment.

5.3 *WT1* gene

The *WT1* gene was first identified in kidney tumors on human chromosome 11p13. *WT1* comprises ~5 kb and contains 10 exons; its mRNA spans ~2.9 kb, coding for the renal tumor protein (Wilms tumor protein), which has 449 amino acids (Breslow et al. 1993). Breslow et al. (1993) found that WT1 protein is a transcriptional regulation factor. It can activate or inhibit the expression of target genes, producing different biological effects. *WT1* plays a role in regulating cell proliferation, growth, differentiation, and apoptosis and can be both a tumor suppressor and a carcinogenic inducer (Wu et al. 2018, Scharnhorst, van der Eb and Jochemsen 2001). Moreover, *WT1* has been found hypermethylated in many tumors including glioblastoma (Rankeillor et al. 2014), prostate cancer, and ovarian cancer (Jiang et al. 2014).

We found significantly higher methylation of the *WT1* gene (P=0,014) in 34/51 (67%) cases of tumor samples and 10/19 (53%) in control samples. The *WT1* gene showed significantly higher methylation in patients with tumors of tonsils 19/36 (P<0.01), cancer cells in lymph nodes (N1-N3) (P<0.05), and in patients with vascular invasion 28/35 (P<0.01).

Ribeiro et al. (2016) using MS-MLPA showed methylation of *WT1* in 69/93 OPSCCs. Conversely, methylation levels of control samples were <20%, which did not correlate with our results. This may be due to using a different MS-MLPA kit, which means we analyzed different

specific CpG sites. According Ribeiro et al. (2016) *WT1* gene promoter methylation was associated with T1 and T2. Our data showed a higher methylation level in stage T1 (60%) and T2 (64%) of the tumor.

Oji et al. (2003) suggested that WT1 could serve as a prognostic factor in tumors, as they found that a high WT1 expression significantly correlated with a poor tumor differentiation and, consequently, an advanced tumor stage. They observed overexpression of the WT1 gene in 42 of 56 (75%) cases of examined HNSCC. Whereas in our group of patients there were only a few cases of advanced stages of the tumor; it is not possible to evaluate.

5.4 ***RARβ* gene**

Human retinoic receptor beta (*RARβ*) gene, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators, is located on chromosome 3p24.2. This receptor is localized in the cytoplasm and in subnuclear compartments. It binds retinoic acid, a biologically active form of vitamin A that mediates cell signaling in embryonic morphogenesis, cell growth, and differentiation. It is assumed, that this protein limits growth of many cell types by regulating gene expression (NCBI 2020). The gene was first identified in hepatocellular carcinoma, and the deregulation of this gene has also been detected in uterine cervical carcinoma (Rotondo et al. 2015).

Lotan (1996) confirmed, that retinoid acid suppresses carcinogenesis and inhibits the growth of human HNSCC, so a loss of retinoids and their receptors has been associated with malignant progression in HNSCC (McGregor et al. 2002)

In our study, 15/51 (29.4%) oropharyngeal carcinoma samples were methylated at the observed CpG site of the *RARβ* promotor, while all control non-malignant samples were unmethylated ($P<0.05$). The methylation level of *RARβ* in metastases was 4/32 (12.5%) and correlated with the primary tumor. Maruya et al. (2004) identified methylation of *RARβ* in 15/32 (46.9%) samples of primary oral malignant diseases. With the exception of one pair of primary and metastatic cell lines, the methylation pattern in the cell lines was similar. Conversely, histologically normal mucosa showed a 50% level of methylation.

In Youssef et al. (2004) study, promoter methylation status of *RARβ* gene in HNSCCs was detected in 18/27 (66.7%) tumor samples and in 6/22 (23.3%) normal tissue.

In our study, the *RARβ* gene was significantly methylated in patients who consume alcohol ($P<0.02$). There was no other statistically significant correlation between methylation status and clinicopathological characteristics of oropharyngeal cancer patients.

5.5 *PAX6* gene

Paired-box 6 (*PAX6*), located on chromosome 11p13, encodes a transcription factor that is involved in various developmental processes of the eye and central nervous system (Simpson and Price 2002). *PAX6* is an important transcription factor during embryogenesis and a stem cell factor (Osumi et al. 2008) and may play an important role in tumorigenesis. It was found, in human cancer, the expression and biological role of *PAX6* varies depending on types of cancer. *PAX6* is downregulated in tumors compared to normal tissues and acts as a tumor suppressor gene in glioblastoma (Mayes et al. 2006) and prostate cancer (Shyr et al. 2010), whereas it is overexpressed and acts as an oncogene that facilitates cell growth and suppresses terminal differentiation in pancreatic cancer (Mascarenhas et al. 2009).

To date, no data has been found to address the aberrant methylation of the *PAX6* gene in oropharyngeal carcinoma. Our data showed significantly higher methylation in the *PAX6* gene ($P<0.05$) in samples of patients with oropharyngeal carcinoma compared with control group. We also examined the correlation between methylation status and clinicopathological data. Significantly lower methylation of the *PAX6* gene was associated with tumor localization in tonsils ($P<0.05$). The significantly lower methylation levels of *PAX6* was also in the T1 size of the tumor (tumor is 2 cm or smaller) ($P<0.05$). In T3-T4 a higher level of methylation was found, but due to the small observed group, we cannot draw a conclusion. Since there was no methylation in the control samples, hypermethylation could serve as a potential biomarker, but further studies are needed to understand the role of the *PAX6* gene in the pathogenesis of OPSCC.

6 CONCLUSIONS

Oropharyngeal squamous cell carcinoma is frequently occurring malignancy with heterogeneous clinical behavior. Based on the lack of specific early diagnostic and prognostic biomarkers, the aim of my master´s thesis was to examine the methylation levels of select tumor suppressor genes in oropharyngeal cancer.

Firstly, the selected methods, MS-MLPA and MS-HRM, which are useful for methylation analysis of low quality DNA isolated from paraffin blocks, were successfully optimized.

Our data showed statistically significant changes in the frequency of methylation of observed CpG sites in the *PAX5, CADM1, WT1, RARβ* and *PAX6* genes in oropharyngeal tumor samples by comparison with control tissue.

In the *PAX5, PAX6,* and *CADM1* genes, there was no case of methylation in the control samples. The *CADM1* gene was significantly differentially methylated in HPV-positive and HPV-negative OPSCC ($P<0.01$) and *RARβ*, *PAX5*, *PAX6,* and *WT1* genes frequently methylated in all tumors regardless their HPV status.

The frequency of methylation of selected tumor-suppressor genes even significantly correlated with clinicopathological characteristics such as tumor localization, stage, tumor size, HPV status, alcohol consumption and, vascular invasion. Significance of the association between clinicopathological data and promoter methylation of selected genes may be in the future used in the screening of the oropharyngeal cancer, early detection, prognosis, approach to therapy, and individualization of treatment.

7 LITERATURE

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8 APPENDICES

8.1 TNM classification for oropharyngeal cancer

TNM CATEGORIES			DEFINITION
T	TX		Primary tumor cannot be assessed
	T0		No evidence of primary tumor
	Tis		Carcinoma in situ
	T1		Tumor 2 cm or less in greatest dimension
	T2		Tumor more than 2 cm but not more than 4 cm in greatest dimension
	T3		Tumor more than 4 cm in greatest dimension or extension to lingual surface of epiglottis
	T4	T4a	Tumor invades any of the following: larynx, deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), medial pterygoid, hard palate, or mandible
		T4b	Tumor invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or encases carotid artery
N	NX		Regional lymph nodes cannot be assessed
	NO		No regional lymph node metastasis
	N1		Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
	N2	N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
		N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
		N2b	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
	N3		Metastasis in a lymph node more than 6 cm in greatest dimension
M	M0		No distant metastasis
	M1		Distant metastasis