Charles University
Faculty of Medicine in Hradec Králové

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

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Abstract of the dissertation

Doctoral study programme: Clinical Biochemistry

Hradec Králové

2019

Dissertation thesis was written during residential doctoral study (Ph.D.) study programme Clinical Biochemistry at the Department of Clinical Biochemistry and Diagnostics, Faculty of Medicine in Hradec Králové, Charles University and University Hospital Hradec Králové.

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Thesis defense will take place on 17th of September at 2:00 pm in the auditorium of Department of Clinical Biochemistry and Diagnostics (room B 401), University Hospital Hradec Králové.

This work has been supported by the program MH CZ - DRO (UHHK, 00179906), SVV grant 260 398/2017, by the program PROGRES Q40/11, by European Regional Development Fund-Project BBMRI-CZ.: Biobank network – a versatile platform for the research of the etiopathogenesis of diseases No. EF16 013/0001674.

The dissertation is available for inspection at the Study Department of the Dean´s Office, Faculty of Medicine in Hradec Králové, Charles University, Šimkova 870, 500 03 Hradec Králové (phone 495 816 134).

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in doctoral study programme Clinical Biochemistry

1 Table of Contents

1 Summary CZE 4
2 Summary ENG 5
3 Background 6
3.1 Head and Neck Cancer 6
3.1.1 Sinonasal cancer 6
3.1.2 Oropharyngeal cancer 7
3.2 Epigenetic modifications 8
3.2.1 microRNAs 8
3.2.1.1 microRNA biogenesis and nomenclature 9
3.2.1.2 microRNAs and head and neck cancer 9
3.2.1.3 DNA methylation and microRNAs 10
4 Objectives 11
5 Materials and Methods 12
5.1 Sinonasal cancer patients and samples 12
5.2 Oropharyngeal cancer patients 12
5.3 Relative expression of microRNAs 12
5.3.1 Total RNA isolation and quality control 12
5.3.2 miRNA microarray 13
5.3.3 Small RNA Next generation sequencing 13
5.3.4 Quantitative real-time PCR 13
5.4 DNA methylation analysis 13
5.4.1 Statistical analysis 14
6 Results 15
6.1 Differentially expressed miRNAs in sinonasal carcinoma 15
6.1.1 miRNAs, patients' survival, clinicopathological data 16
6.1.2 Methylation analysis 17
6.2 Differentially expressed miRNAs in oropharyngeal carcinoma 17
6.2.1 microRNA microarray 17
6.2.2 Small RNA Next-generation sequencing 18
6.2.3 Relative expression verification analysis 18
6.2.4 miRNAs, patients' survival, clinicopathological data 20
7 Discussion 21
7.1 Differentially expressed miRNAs in sinonasal carcinoma 21
7.2 Differentially expressed miRNAs in oropharyngeal carcinoma 22
8 Conclusions 24
9 Literature 25
10 Overview of own publications 28

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 Background

3.1 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. Head and neck cancers are predominantly squamous cell carcinomas (HNSCC), which make up to 90 % of head and neck cancers worldwide [1].

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 [2]. 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 [3].

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 [4]. 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]. 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 from 2016) [8].

3.1.1 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 [9; 10]. 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. 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 [11].

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) [12]. Even though aggressive combined treatment is usually used, survival median is less than 18 months and 5-year survival rate is only 20 – 30 % [13]. Early symptoms of the disease are rhinorrhea, epistaxis, epiphora,

and nasal obstruction. Advanced lesion symptoms include blurred vision, diplopia or proptosis [14]. The treatment is usually based on radical surgical resection with postoperative radiotherapy [15].

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 [16]. 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 [17]. Patients with HPV+ tumors have much better prognosis than patients with HPV- tumors [18-20]. Professional exposure to specific cancerogenous substances (such as wood-dust, leather dust and formaldehyde) is another established risk factor for SNC development [21]. Hard wood-dust inhalation causes chronic inflammation in sinonasal area, which leads to *TP53* mutagenesis and subsequently to carcinogenesis in the area [22].

3.1.2 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. 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 [23; 24]. Almost all cases of oropharyngeal cancer are squamous cell carcinomas (OPSCC), other subtypes such as adenocarcinomas are rare.

Most of the tumors develop from premalignant lesions such as leukoplakia, erythroplakia and oral submucous fibrosis that can be often asymptomatic [25]. 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 [25; 26]. 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 [27].

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 [28]. 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 [27]. 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 [29].

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 [30-32]. 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 [27].

3.2 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).

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.

3.2.1 microRNAs

microRNAs are short (~23 nucleotides) non-coding RNA molecules participating in regulation of gene expression. The first evidence of their existence was published by Lee et al. in 1993 [33] 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 [34; 35]. 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 [36].

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 [37].

3.2.1.1 microRNA biogenesis and nomenclature

The canonical pathway of miRNA biogenesis 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 [38; 39].

Furthermore, about 1 % of conserved miRNAs are produced by different non-canonical pathways, which have been recently described. Their structural and functional resemblance to canonical miRNAs was discovered thanks to development of deep sequencing techniques [40; 41]. 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. Non-canonical miRNAs have been linked to various pathological processes such as psoriasis, ischemic disease, diabetes mellitus or epithelial malignancies [41].

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) [42; 43].

3.2.1.2 microRNAs 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) [44]. 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 [45].

3.2.1.3 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 [46]. miRNAs acting as tumor-suppressor genes might be silenced by hypermethylation of CpG islands in their own promoter regions [47]. 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 [48].

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), 148a,b (targeting DNMT1 and DNMT3), miR-185 (targeting DNMT3B and DNMT1), miR-152 (targeting DNMT1) and miR-301 targeting DNMT1 [46].

4 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.

5 Materials and Methods

5.1 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.

5.2 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 and small RNA sequencing 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.

5.3 Relative expression of microRNAs

5.3.1 Total RNA isolation and quality control

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. 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.

Concentration and purity of isolated RNA was first assessed by NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 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.

5.3.2 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). All steps were performed according to manufacturer's protocol with 100 ng of total RNA in the reaction. Expression data were bioinformatically analyzed and divided into three groups (tumors × metastases × controls).

5.3.3 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. 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. 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 [49].

5.3.4 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.

5.4 DNA methylation analysis

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. 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).

Primers for Methylation specific-High resolution melting analysis were designed in MethPrimer [50] to the promotor region of *miR-143* and *miR-145* genes and their mutual miR-143-3p and miR-145-5p host gene (*CARMIN*). 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).

5.4.1 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. For best diagnostic marker selection two web-based interactive tools were used: PanelComposer and CombiROC. CombiROC.

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.

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.

6 Results

6.1 Differentially expressed miRNAs in sinonasal carcinoma

We detected significant upregulation of 6 miRNAs: let-7a-5p, let-7d-5p, miR-196a-5p, miR-21-5p, miR-9-3-p, miR-9-5p (Figure 1) and significant down-regulation of 1 miRNA: miR-145-5p (Figure 2). 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.

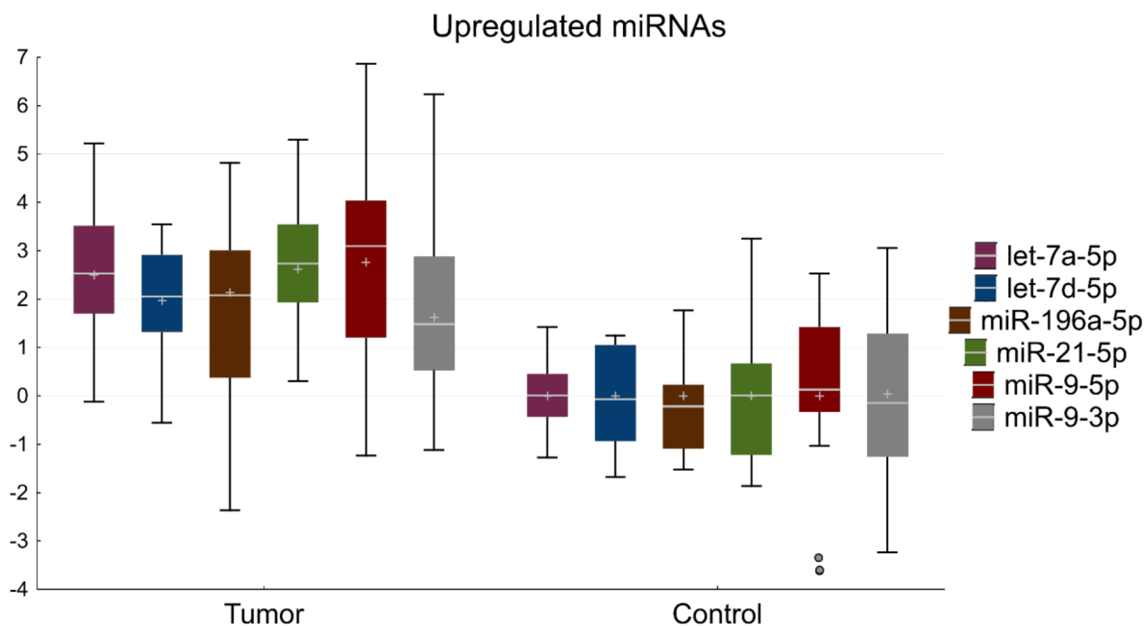

Figure 1. Box plot of significantly upregulated miRNAs in SSCC samples. Horizontal line in the box marks median value and plus sign marks mean value. The box bounds Interquartile Range. Whiskers show Minimum and maximum values with outliers symbolized as dots.

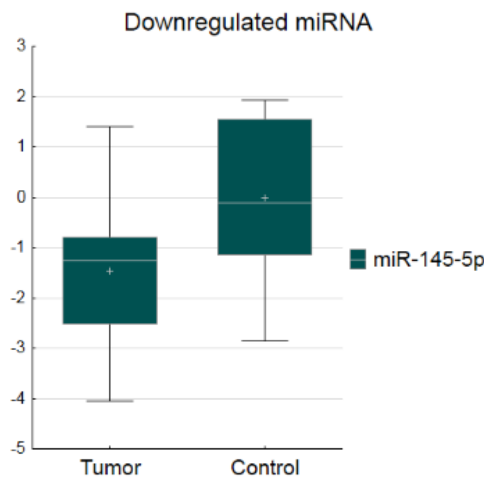

Figure 2. Box plot of significantly downregulated miRNA in SSCC samples. Horizontal line in the box marks median value and plus sign marks mean value. The box bounds Interquartile Range. Whiskers show Minimum and maximum values with outliers symbolized as dots.

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.

6.1.1 miRNAs, patients' survival, clinicopathological data

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. 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). 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). 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). 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. After performing statistical analysis, we concluded that all studied miRNAs apart from miR-223-3p correlated with at least one of the studied parameters.

6.1.2 Methylation analysis

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 of 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.

6.2 Differentially expressed miRNAs in oropharyngeal carcinoma

6.2.1 microRNA microarray

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. 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.

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). 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).

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). 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).

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.

6.2.2 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.

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 analysis showed us one distinct group of control samples and one heterogenic group of tumor samples with metastasis samples.

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.

6.2.3 Relative expression verification analysis

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 = - 4.75 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 3).

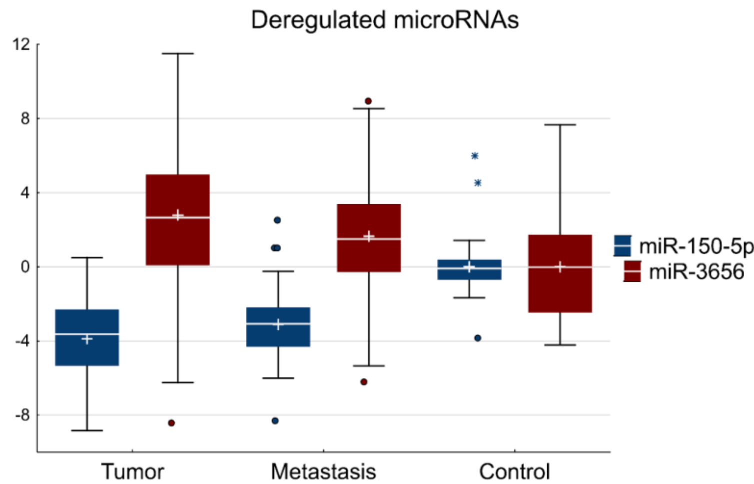

Figure 3. Deregulated miRNAs in the second phase of the study.
miR-3656 and miR-150-5p were confirmed to be significantly deregulated in tumor and metastasis samples. Whiskers show Minimum and maximum values with outliers symbolized as dots and extremes symbolized as asterisks.

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 4).

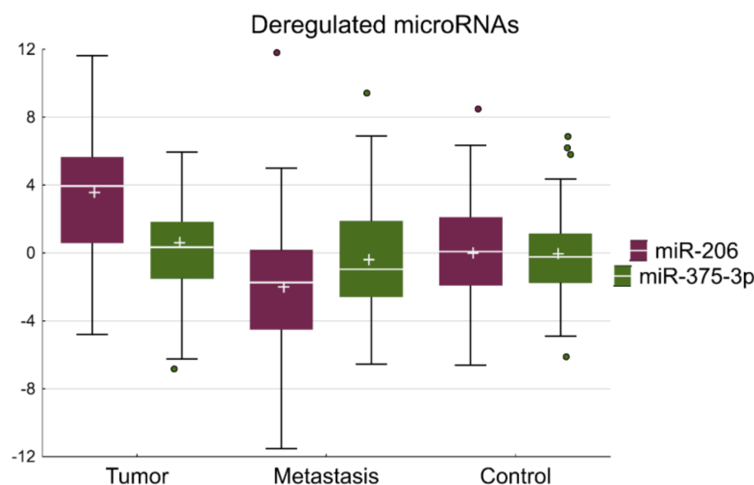

Figure 4. Deregulated miRNAs in the second phase of the study.
miR-206 and miR-375-3p were confirmed to be deregulated in metastasis samples compared to tumor samples. Whiskers show Minimum and maximum values with outliers symbolized as dots and extremes symbolized as asterisks.

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.

6.2.4 miRNAs, patients' survival, clinicopathological data

Patients with lower expression of miR-150-5p in primary tumors and metastases (based on median value) had impaired survival in comparison to higher expression group. The difference is statistically significant with P = 0.0124 and P = 0.0192 respectively.

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).

7 Discussion

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 [51; 52].

Number of studies focused on miRNA expression in sinonasal cancer has been limited. Ogawa et al. [53] 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 [54] 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 [55].

In my doctoral study, I 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.

7.1 Differentially expressed miRNAs in sinonasal carcinoma

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 [56]. 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 [57]. 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 [58].

Citron et al. [59] 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. [60] 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.

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.

7.2 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 [29]. 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.

In oropharyngeal cancer several epigenetic (focused on miRNAs) studies has been published [55; 61]. 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 [62]. 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.

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. [63], 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 metastases and original subsite of the tumor. Further analyses need to be performed to determine specificity of the miRNA for studied tissue.

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.

8 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.

9 Literature

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10 Overview of own publications

Original scientific papers published in an impacted journal

Kovarikova, H., I. Bubancova, J. Laco, K. Rozkosova, H. Vosmikova, M. Vosmik, P. Dundr, K. Nemejcova, J. Michalek, V. Palicka and M. Chmelarova „Deregulation of selected microRNAs in sinonasal squamous cell carcinoma: searching for potential prognostic biomarkers". *Accepted for publication, Folia Biologica.* (IF = 1.044)

Ren H., X. Hou, P. W. Eiken,J. Zhang, K. E. Pierson, A. A. Nair, J. I. Davila, H. Kovarikova., J. S. Jang, S. H. Johnson, J. R. Molina, R. S. Marks, P. Yang, J. E. Yi, A. S. Mansfield, J. Jen (2019). „Identification and Development of a Lung Adenocarcinoma PDX Model With STRN-ALK Fusion." Clin Lung Cancer. 2019 Mar;20(2):e142-e147. (IF = 4.204)

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Kovaříková, H., M. Chmelařová and V. Palička (2016). "Role mikroRNA u nádorů hlavy a krku se zaměřením na sinonazální karcinom." Čas. Lék. čes. 2016(155): 99-104.

Scientific lectures

Kovaříková H., I. Bubancová, J. Laco, K. Sieglová, H. Vošmiková, P. Dundr, K. Němejcová, J. Michálek, M. Vošmik, V. Palička, M. Chmelařová (2016). „Deregulation of selected microRNAs in sinonasal carcinoma and their value as prognostic biomarkers" 12. fakultní konference studentů DSP, Hradec Králové.

Kovaříková H., I. Bubancová, J. Laco, P. Dundr, K. Němejcová, J. Michálek, M. Vošmik, V. Palička, M. Chmelařová (2017). "In search for potential biomarkers: Deregulation of selected microRNAs in squamous cell sinonasal carcinoma." 13. fakultní konference studentů DSP, Hradec Králové (1. místo).

Kovaříková H., I. Bubancová, J. Laco, P. Dundr, K. Němejcová, J. Michálek, M. Vošmik, V. Palička, M. Chmelařová (2017). "In search for potential biomarkers: Deregulation of selected microRNAs in squamous cell sinonasal carcinoma." 14. mezinárodní lékařská postgraduální konference, Hradec Králové.

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Kovaříková H., Ryška A., Laco J., Palička V. „BBMRI Hradec Králové." XXXVIII. Imunoanalytické dny s mezinárodní účastí 2017, Plzeň.

Poster presentations

Chmelarova, M., I. Baranova, H. Kovarikova, A. Mrkvicova, M. Rezacova, J. Laco, I. Sedlakova and V. Palicka. Role of DNA methylation in resistance to platinum-based chemotherapy in ovarian cancer cells. 4th International Congress on Epigenetics & Chromatin, London, September 3 – 5, 2018.

Baranová, I., H. Kovaříková, J. Laco, O. Dvořák, I. Sedláková, V. Palička and M. Chmelařová. Změny v metylaci protokadherinů u high-grade serózního ovariálního karcinomu. XLII. Brno Oncology days and XXXII. Conference for medical professionals, Brno.

Kovaříková, H., I. Baranová, J. Laco, M. Vošmik, P. Dundr, K. Němejcová, J. Michálek, V. Palička and M. Chmelařová. Upregulace miR-9 u dlaždicobuněčného sinonasálního karcinomu: potenciální prognostické biomarker. XLII. Brno Oncology days and XXXII. Conference for medical professionals, Brno.

Laco, J., H. Kovaříková, M. Chmelařová, H. Vošmiková, K. Sieglová, I. Bubancová, P. Dundr, K. Němejcová, J. Michálek, M. Vošmik and A. Ryška. MicroRNA expression in SMARCB1/INI1-deficient sinonasal carcinoma: A clinicopathological, immunohistochemical and molecular genetic study. 29th European Congress of Pathology, Amsterdam.

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