MicroRNA Profile in Site-specific Head and Neck Squamous Cell Cancer

DAVID KALFERT[1], MARTIN PESTA[2,4], VLASTIMIL KULDA[3], ONDREJ TOPOLCAN[4], ALES RYSKA[5], PETR CELAKOVSKY[1], JAN LACO[5] and MARIE LUDVIKOVA[2]

[1]*Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Hradec Kralove, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic;*
[2]*Institute of Biology, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic;*
[3]*Department of Biochemistry, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic;*
[4]*Central Immunoanalytical Laboratory, Department of Nuclear Medicine, Faculty Hospital in Pilsen, Pilsen, Czech Republic;*
[5]*The Fingerland Department of Pathology, University Hospital Hradec Kralove, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic*

Abstract. *Background/Aim: MicroRNAs (miRs) are non-coding RNA molecules regulating diverse cellular processes essential in carcinogenesis. Little is known regarding miRs in head and neck squamous cell cancer (HNSCC). The aim of the present study was to investigate miRs in relation to the clinicopathological features of site-specific HNSCC. Materials and Methods: The study comprised of 51 patients with HNSCC (23 oropharyngeal, 24 laryngeal and 4 hypopharyngeal carcinomas). Total RNA was extracted from tumor tissue and normal squamous epithelium using the miRNeasy FFPE Kit. A quantitative estimation of let-7a, miR-21, miR-200c, miR-34a, miR-375 was performed by a real-time polymerase chain reaction (PCR) method using the TagMan® MicroRNA assay. Additionally, p16 expression was detected by immunohistochemistry. Results: Significant differences of let-7a, miR-200c, miR-34a levels between oropharyngeal and laryngeal cancers were found ($p<0.05$). Compared to non-neoplastic tissues, miR-21, miR-200c, miR-34a were up-regulated and miR-375 was down-regulated in tumors of all sites. MiR-34a tumor levels significantly correlated with oropharyngeal origin ($p=0.0284$) and p16 positivity ($p=0.0218$). Conclusion: The microRNA profile seems to play a potential role in the pathobiology of oropharyngeal and laryngeal HNSCC. Up-regulation of miR34a in p16-positive oropharyngeal cancer has not been so far described and additional studies are warranted.*

Correspondence to: Marie Ludvikova, Institute of Biology, Faculty of Medicine in Pilsen, Charles University in Prague, alej Svobody 1655/76, 304 60 Pilsen, Czech Republic. Tel: +420 378024270, e-mail: ludvikova.m@email.cz and marie.ludvikova@lfp.cuni.cz

Key Words: MicroRNA, let-7a, miR-21, miR-200c, miR-34a, miR-375, head and neck squamous cell carcinoma, oropharyngeal, laryngeal, hypopharyngeal cancer.

The head and neck squamous cell cancer (HNSCC) represents a broad scale of tumors from the oral cavity to larynx (1). HNSCC of different anatomical sites seems to be associated with different etiopathogenesis, molecular characteristics and clinical outcomes, despite the same histological type. The majority of previous studies on HNSCC were performed irrespective of tumor location and published results were not site-specific. Detailed knowledge of the molecular basis of these tumors is, thus, required and new HNSCC biomarkers are warranted.

Nowadays, there is an increasing interest in the role of microRNAs (also known as miRNAs or miRs) in physiological and pathological cell processes, which bring new insights to cancer pathobiology. MicroRNAs are newly-recognized, non-coding, regulatory RNA molecules, 18-25 nucleotides in length. Their biogenesis is a multi-step process under the control of several enzymes and enzymatic complexes (Figure 1). Briefly, this process starts in the nucleus, where the primary-micro RNA (pri-micro RNA) with long nucleotide sequence is produced. During the next step, the hairpin-shaped pri-micro RNA enters a complex consisting of the enzyme Drosha and an essential cofactor Pasha to be processed into pre-microRNA and then transported to the cytoplasm. Enzymes Dicer and helicase are responsible for shortening of double stranded RNA and subsequently unwinding of this duplex into two mature microRNAs. They are incorporated into the RNA-induced silencing complex (RISC), which regulates the final effect of microRNA. For detailed description of micro RNA biogenesis, we recommend earlier reviews (2-4). Expression of

microRNA is tissue-specific and each alteration of tissue microRNA profile is associated with distinct disease-status. MicroRNAs participate in post-transcriptional regulation of gene expression to control development and maintain diverse cellular processes, including proliferation, apoptosis, senescence, differentiation, motility and morphogenesis. Each microRNA can regulate a considerable number of genes downstream by targeting many messenger RNA (mRNA) transcripts. One mRNA may be influenced by more types of microRNA. The final effect of this interaction is mRNA cleavage or translational repression (depending on the degree of complementarity with the target mRNA) with simultaneous quick degradation of microRNA. Thus, microRNA can control more than one-third of the protein-coding genes in the human genome inclusive of genes coding for transcription factors and RNA regulating proteins (5). Transregulation represents subsequent functional effects of microRNAs that may alter the levels of other RNAs (5).

MicroRNAs are involved in many diseases, both non-neoplastic and cancers. Their link to cancer is not surprising taking into consideration that microRNAs are involved in many processes essential in carcinogenesis and cancer progression (*e.g.* proliferation, migration, apoptosis). MicroRNA dysregulation is attributed to germ-line or somatic mutations of microRNA genes or epigenetic changes (6, 7). Two principal effects of microRNAs in human cancer have been described: oncogenic and tumor-suppressor. The former is linked to negative regulation of tumor suppressor genes, while the latter is mediated by translational repression of oncogenes (8, 9). However, a dual effect of some microRNAs, both oncogenic and tumor suppressing, has also been described (10).

More than 2,500 mature microRNAs have been identified in humans (see miRBase Sequence Database, Release 21, available online at www.miRbase.org) with different roles in cancerogenesis (11). In our study, we focused on 5 selected microRNAs (in Table I) with potentially crucial roles in the pathobiology of site-specific HNSCC: (i) Let-7a belongs to the large let-7 family. The let-7a precursor was the first microRNA identified from the study of developmental timing in *Caenorhabditis elegans*. The ubiquitously expressed let-7/miR-98 was one of the first mammalian microRNAs to be described. The let-7 family is often present in multiple copies in the genome and isoforms with slightly different sequences are indicated by a letter (12). Along with the general function to promote the differentiation of cells, its tumor suppressor role by targeting multiple oncogenes, namely *RAS*, in various human cancers, including head and neck cancer, was recently described (13). Moreover, its repressive role in cancer stem cells with stem-like properties ablation was also recently described (14). (ii) MicroRNA-21 is one of the first microRNAs identified in a number of cancers also having an important role as an oncogene (15). MiR-21 has been proven to be up-regulated in a wide variety of malignant tumors, namely in human glioblastomas, breast, colon, lung, pancreas, prostate cancer and in head and neck squamous cell carcinoma as well (16, 17). MiR-21 over-expression was significantly associated with worse outcome promoting cell proliferation (16, 18). Recently, miR-21 was introduced as a promising diagnostic and prognostic biomarker and therapeutic target as well (17, 19-21). (iii) MicroRNA-200c is one of the five members of microRNA-200 family that regulates the epithelial-mesenchymal transition (EMT) by targeting EMT-related gene expression. MiR-200c exhibits tumor suppressive properties. Its down-regulation promotes EMT-facilitating tumor cell invasion and progression of cancer, while up-regulation induces mesenchymal-epithelial transition (22, 23). (iv) MicroRNA-34a, like one of the three members of miR-34 family, is characterized by its tumor suppressor action that induces apoptosis, cell-cycle arrest, senescence and EMT. The p53 family has been described as one of the principal inducers of miR-34a gene expression, although alternative regulatory pathways also exist (24, 25). MiR-34a dysregulation plays an important role in many human cancers; this small molecule is, usually, down-regulated and associated with poor outcome (26). Common causes of miR-34a low expression are deletion, CpG promoter methylation of the *MIR-34A* gene (RNA gene) on chromosome 1 or the alteration of p53. This shows the important therapeutic potential of miR-34a as an anticancer drug (27). However, little is known regarding the role and expression status of miR-34a in head and neck squamous cell carcinomas. (v) MicroRNA-375 is typically expressed in the pancreas and pituitary gland. It has a great impact on development and endocrine function of the pancreas. MiR-375 has been implicated in a number of different cancers being down-regulated in cancer cells when compared to adjacent non-neoplastic tissues. Since there exist several molecular targets and different pathways of miR-375 regulation, it is necessary to investigate the expression pattern in specific cancers (28). Generally, miR-375 acts as tumor suppressor in human cancer, although an oncogenic function in breast cancer has been described (29).

Therefore, the aim of the present study was to investigate the profile of the above-mentioned microRNAs in HNSCC of different anatomical sites in relation to etiological factors and other clinicopathological features of these tumors.

Patients and Methods

Patients. The study patient population comprised of 51 patients with HNSCC treated from July 2009 to December 2012 at the Department of Otorhinolaryngology and Head and Neck Surgery, Faculty Hospital in Hradec Kralove, Czech Republic. The HNSCC groups included three sub-groups represented by histologically-verified squamous carcinomas of the oropharynx (n=23 patients), hypopharynx (n=4 patients) and larynx (n=24 patients). Patients included in the study were both smokers and non-smokers. Grading of tumors was obtained from pathological reports. Staging of tumors

Table I. *Selected microRNAs evaluated in the present study.*

MicroRNA (miR)	Principal effect of miR in cancerogenesis	Principal function mRNA of miR/target
let-7a	TS	Promotes differentiation of cells, represses CSC
miR-21	ONC	Promotes cell proliferation
miR-200c	TS	Regulates EMT/MET
miR-34a	TS	Induces apoptosis, cell cycle arrest, senescence
miR-375	TS/dual in distinct cancers	Regulates development of pancreas and glucose-induced insulin secretion

TS, Tumor suppressive; ONC, oncogenic; CSC, cancer stem cells; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

was evaluated according to pTNM Union for International Cancer Control (UICC) pathological staging criteria (30). All participants were informed on the research study and written inform consent was obtained from each patient. This work was approved by the Ethics Committee of the University Hospital in Hradec Kralove. Patients with other malignancies, inflammatory diseases or infections were excluded. Treatment decision-making was based on clinical status of patients and on grading and staging of tumors. All patients underwent surgery with resection of the tumor. The clinicopathological profile of the study populations is shown in Table II.

Tissue samples. Formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with HNSCC were prepared by standard laboratory technique and stored at room temperature until use. Samples, collected during a 4-year period (from 2009 to 2012), were processed at the Fingerland Institute of Pathology at the University Hospital in Hradec Kralove. Four-μm-thick paraffin sections were stained with hematoxylin and eosin and microscopically evaluated to ascertain regions suitable for macrodissection. Minimally, two FFPE tissue samples from each patient were histologically analyzed by a pathologist (M.L.) in order to find sites with cancer cells and sites of adjacent non-cancerous epithelial tissue. All selected areas destined for microRNA analysis were manually highlighted on hematoxylin and eosin stained slides. Thereafter, FFPE tissue samples were cut into 50 μm sections and parts of these sections, corresponding with previously highlighted areas, were separated. A total tissue area of approximately one cm^2 was used for RNA extraction. The estimation was performed in 51 paired (tumor and control) tissue samples of HNSCC.

RNA isolation. RNA was extracted from 50 μm FFPE sections following macrodissection of HNSCC tumor tissue and adjacent non-cancerous tissue using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany).

Real-time quantitative PCR (qRT-PCR). A quantitative estimation of let-7a, miR-21, miR-200c, miR-34a, miR-375 and U6snRNA was performed by a qRT-PCR method using TaqMan® MicroRNA Assays (supplier, address). A two-step protocol requires reverse transcription with a miRNA-specific primer followed by a real-time PCR with TaqMan® probes (give more details and/or reference).

Table II. *The clinicopathological profile of the study population.*

Feature		Anatomic site					
	N	Oropharynx (n=23/45.1%)		Hypopharynx (n=4/7.8%)		Larynx (n=24/47.1%)	
		N	%	N	%	N	%
Sex							
Male	41	16	69.6	3	75.0	22	91.7
Female	10	7	30.4	1	25.0	2	8.3
Age							
≤60	28	15	65.2	3	75.0	10	41.7
>60	23	8	34.8	1	25.0	14	58.3
Mean	60.33		58.26		58.50		62.63
Median	60		57		60		61.5
Min-Max	45-85		45-70		53-61		46-85
Abuses							
Non-smoker	13	12	52.2	0	0	1	4.2
Smoker	38	11	47.8	4	100.0	23	95.8
TNM staging[a]							
I-II	14	4	17.4	0	0	10	41.7
III-IV	37	19	82.6	4	100.0	14	58.3
Histological grading							
G1-2	30	14	60.9	2	50.0	14	58.3
G3	21	9	39.1	2	50.0	10	41.7
Nodal status							
N0	24	6	26.1	0	0	18	75.0
N1-3	27	17	73.9	4	100.0	6	25.0
P16 status							
$P16^+$	17	17	73.9	0	0	0	0
$P16^-$	34	6	26.1	4	100.0	24	100.0
p-values				$p<0.0001$[b]			
Local recurrence/persistence							
No	43	18	78.3	3	75.0	20	83.3
Yes	8	3	21.7	1	25.0	4	16.7

LQ, Low quartile; UQ, upper quartile; SD, standard deviation. [a]UICC, 7th edition, [b]Chi-square test

The assays target only mature microRNAs, not their precursors. We used RNU6B (U6snRNA) as a normalizer. Each sample was assessed twice in parallel. The Ct values were corrected using calibrators for the elimination of differences between runs of the Stratagene Mx3000P Real-Time PCR apparatus (Agilent Technologies, Santa Clara, CA, USA). For the statistical analysis, we used the ddCt approach (2-Δ Δ CT algorithm; Applied Biosystems, Foster City, CA, USA).

Determination of p16 status. In our Hospital (Faculty Hospital in Hradec Kralove, Czech Republic), p16INK4a (hereafter denoted as p16) expression is routinely evaluated by immunohistochemistry within the field of activity of the histological examination of every HNSCC. Therefore, p16 status data are available as they were retrieved from hospital records. Immunohistochemistry was performed with the CINtec® Histology Kit (Roche mtm laboratories AG, Heidelberg, Germany). The p16 immunostaining was scored as follows: negative (0-50% tumor cells-nuclei and/or cytoplasmstained); positive (51-100% tumor cells-nuclei and/or cytoplasm-stained).

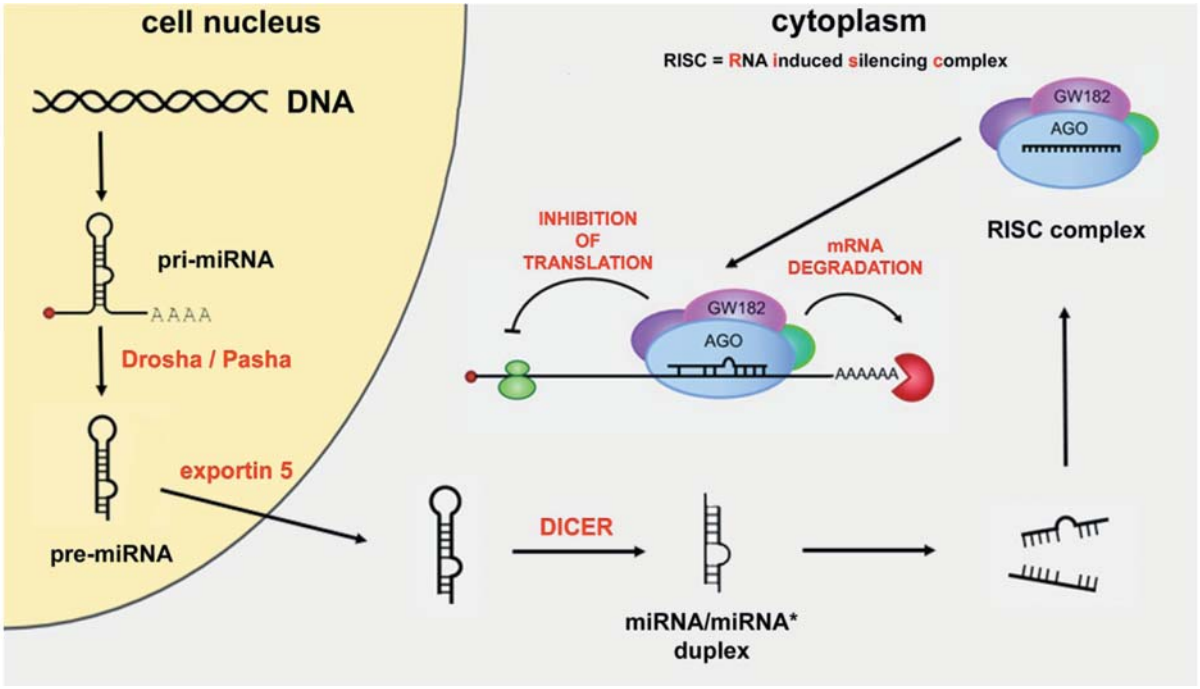

Figure 1. *Biogenesis of microRNA.*

Statistical analysis. Data analysis was performed using the SAS 8.02 software (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by the Wilcoxon non-parametric two sample test. Values of p lower than 0.05 were considered to be statistically significant in all analyses.

Results

The clinicopathological data of the HNSCC patients in the studied cohort are summarized in Table II. The expression levels of let-7a, miR-21, miR-200c, miR-34a and miR-375 were compared between site-specific sub-groups (oropharyngeal, hypopharyngeal and laryngeal) of HNSCC and between neoplastic and non-neoplastic tissues of the same patient. Comparison of the expression ratio of studied microRNAs with the clinicopathological and etiological characteristics of tumors was also performed.

Comparison of expression status of microRNAs between neoplastic and non-neoplastic tissues and between site-specific tumor subgroups. Comparing the expression values of the studied microRNAs between tumorous (irrespective to tumor site) and normal tissues of the same patient, we found statistically significant differences in miR-21 (p=0.0001) and miR-200 (p=0.0335), as well as in miR-375 (p=0.0020) expression values. The first two mentioned microRNAs were up-regulated, while the last one was down-regulated in the tumor tissue. All measured values of the expression of the studied microRNAs in tumorous and healthy tissues are summarized in Table III. MiR-34a (p=0.0155) was significantly up-regulated in oropharyngeal carcinomas when only comparing neoplastic and normal epithelial cells.

Statistically significant differences were recorded in the expression of let-7a (p=0.0242) and miR-200c (p=0.0378) between oropharyngeal and laryngeal cancers, in the expression value of miR-34a between oropharyngeal and laryngeal (Table IV), oropharyngeal and hypopharyngeal, as well as laryngeal and hypopharyngeal carcinomas (p=0.0178; p=0.0355; p=0.0317, respectively). In all cases, the highest expression levels of miR-34a were found in oropharyngeal cancers.

Expression tumor status of microRNAs in relation to clinico-pathological features of HNSCC patients. No correlation was observed between microRNA tumor expression and traditional clinicopathological factors, like sex and age. Furthermore, taking all HNSCC tumors together (irrespective to tumor site), statistically significant differences were found in tumor expression levels of let-7a and miR-200c between low stage (I-II) and high stage (III-IV) cancers (p=0.0204; p=0.0492, respectively), whereas higher tumor microRNA expression was associated with high HNSCC stage in both biomarkers. We also recorded significantly higher expression of miR-200c (p=0.0092) in less differentiated HNSCC (G3) and miR-375 (p=0.0231) in lymph node-positive (N1-3)

Table III. *Significant differences of microRNAs expression: tumor versus control non-neoplastic tissue.*

Group	N	miR-21				mir-200c				miR-375			
		25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value
HNSCC	51	6.62	20.1	52.5	0.0001	4.78	7.56	13.09	0.0335	0.02	0.06	0.29	0.0020
Control tissue	51	2.10	5.99	20.7		2.13	4.62	9.60		0.09	0.53	1.76	

Med, median.

laryngeal carcinomas. There were no significant differences in the miR-21, miR-34a, let-7a and miR-375 or miR-200c expression between low-grade and high-grade tumors, cancer lymph node status (N0 *versus* N1-3) and non-smoker and smoker patient groups. However, a significantly higher miR-21 (p=0.0005) and lower miR-375 (p=0.0010) expression in tumor than in healthy tissues was found in smokers but not in non-smokers.

HNSCC microRNAs expression in relation to p16 status. Comparing the p16 expression in site-specific head and neck tumors, p16 positivity has a statistically significant relationship to oropharyngeal cancer (p=0.0001) but not to hypopharyngeal and laryngeal tumors. Moreover, oropharyngeal carcinomas had a significant relationship between p16 expression and non-smoking (p=0.0428) and response to therapy (p=0.0018). Taking all HNSCC cases together, a statistically significant p16 tumor positivity was proven in patients with lymph node metastases (p=0.0173), in non-smokers (p=0.0001) and in tumors without recurrence/ persistence (p=0.0294). With respect to tumor site, the aforementioned significant relationship was confirmed in oropharyngeal tumors only (p=0.0018).

Down-regulation of miR-375 in HNSCC neoplastic cells was significantly associated with p16 tumor negativity (p=0.0038). Comparing all the site-specific p16 negative cancer sub-groups, the highest statistically significant miR-375 expression was found in oropharyngeal tumors (p=0.0441).

MiR-34a was significantly up-regulated in oropharyngeal tumors (p=0.0155) and in p16 positive carcinomas (p=0.0267). MiR-21, let-7a, miR-375 and miR-200c levels did not significantly correlate with p16 expression.

Discussion

HNSCCs represent about 6% of all cancer cases worldwide, with the majority being oropharyngeal and laryngeal squamous carcinomas (31). Smoking, alcohol abuse and human papillomavirus (HPV) infection have been acknowledged by the International Agency for Research against Cancer (IARC) as basic etiological factors of HNSCC (32). Generally, HNSCC is considered to be an aggressive neoplasm with unfavorable prognosis, despite the improvement of therapy in the last decades, and is often studied together as a single disease. However, behavior diversity of these tumors in different head and neck locations probably reflects miscellaneous pathways of cancerogenesis and specific intrinsic tumor properties, which are known from clinical practice (33). The locoregional lymph node metastasis is an important prognostic factor for these cancers. Moreover, many node-negative (N0) classified HNSCCs patients harbor occult neck node metastases (34). HNSCC remains difficult to be clinically managed. Therefore, a better understanding of the molecular pathobiology of head and neck squamous cell cancer is required. MicroRNAs represent new molecules regulating gene expression at the post-transcriptional level by the translational repression and/or degradation of target mRNA (35). MicroRNAs have distinct expression profiles in multiple pathophysiological conditions, including cancer. A number of studies on microRNA expression profiles of breast, prostate, colorectal, brain and other cancers have been so far published (18, 21, 35-37). However, little is known regarding the role of microRNAs in head and neck squamous carcinomas mainly with respect to their site of origin. Therefore, further studies of microRNA expression profile in site-specific HNSCC with potential clinical efficacy for diagnosis, prognosis and treatment seem to be fully substantiated (38). Certain studies have focused on microRNA expression profile of head and neck cancer (35, 39). The results seem to be inconclusive as various microRNAs were studied under different methods in different samples/cell lines and in different clinicopathological conditions. Although a vast number of de-regulated microRNAs have been reported, only a limited number of microRNAs share a common type of de-regulation pointing out to their important role in head and neck cancerogenesis and their potential prognostic function (35). In the study of Avissar *et al.* (2009), comparing neoplastic and normal tissues, it was shown that miR-21, miR-221, miR-18 were up-regulated in HNSCC tumors, whereas miR-375 was down-regulated (40). Moreover, the expression ratio of miR-221/miR-375 was introduced to be a promising diagnostic marker in distinguishing tumor from normal tissue. The same study revealed significant discrepancies in microRNA expression between cell lines and tumor tissues warning

Table IV. *Significant differences of microRNAs expression: oropharyngeal tumors versus laryngeal tumors.*

Group	N	let-7a				mir-200c				miR-34a			
		25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value
Oropharynx	23	1.96	3.40	5.37	0.0242	5.98	9.09	15.48	0.0378	0.64	1.01	1.64	0.0178
Larynx	24	0.84	1.72	3.05		4.35	6.75	8.75		0.19	0.43	0.90	

Med, median.

about limited utility of cell lines as a model system for the identification of clinically relevant microRNA biomarkers (40). MicroRNAs tested in the present study have been previously described to play crucial role(s) in carcinogenesis either in HNSCC or other cancer types.

In the present study, by comparing tumors to healthy tissues, we found significant differences in miR-21, miR-200c and miR-375 expression. Up-regulation of miR-21, a repeatedly confirmed finding, was first revealed in HNSCC (39-42). Down-regulation of miR-375 (being under-expressed by 9-fold compared to normal tissue in the present study) has also been reported in previous studies (40). Little is known about the deregulation of miR-200c in head and neck cancer, although its tumor-suppressive role in renal cell carcinoma, prostate, bladder, breast, pancreatic and gastric cancers has been described (35). Up-regulation of miR-200c was proven to inhibit the cancer stem cell-like properties and support MET by targeting the *ZEB1/ZEB2*, *BMI-1* and *SOX2* genes (23, 35). However, the role of miR200c has not been yet reported in the regulation of tumorigenicity and clinical behavior of HNSCC. MiR200c was significantly up-regulated and a considerable correlation of its over-expression with less differentiated cancers, but not with high-stage cancers, was also revealed in our recent study. However, this over-expression, with respect to its tumor-suppressive function, could be expected to be related to low-grade cancer. The small number of cases studied and incomplete elucidation of miR-200c interaction are shortcomings for better interpretation of these results.

Concerning the evaluation of microRNA expression profiles in different site-specific sub-groups of HNSCC and comparing them with each other, we found that let-7a, miR-200c and miR-34a revealed significant differences between oropharyngeal and laryngeal tumors. Surprisingly, miR-34a was significantly more up-regulated in oropharyngeal carcinomas. We are aware of only one study by Hui *et al.* that focused on microRNA profiling in subgroups of HNSCC (43). However, the authors did not find any differences in microRNAs expression of the oropharynx, the larynx and the hypopharynx. Besides miR-21 and miR-375, the other evaluated microRNAs were different from those explored in our study (43).

Taking all HNSCC cases together, we found significant differences between let-7a, as well as miR-200c expression, and stage of tumors. In laryngeal carcinomas, only miR-200c expression was higher and significantly associated with high stage of cancer, whereas miR-375 was associated with laryngeal lymph node-positive carcinoma. Generally used prognostic parameters, like disease free interval (DFI) and/or overall survival (OS), could not be evaluated in relation to microRNA expression levels due to relatively short follow-up in our study. Nevertheless, the monitoring of let-7a, miR-200c and miR-375 seems to have some potential prognostic significance. The prognostic role of selected microRNAs has been evaluated in HNSCC by several authors but the results seem to be heterogeneous and inconclusive for clinical practice (44).

Cancer pathobiology and the molecular differences between the anatomical locations and tumors of different etiology (viral, smoking or alcohol-induced) are important for developing new molecular targeted strategies. MicroRNA patterns are tissue-specific and may be influenced by the diversity of environmental factors.

Smoking-related cancers are the common object of microRNA studies, namely lung cancer. There exist different pathways of microRNA dysregulation: modification of microRNA gene expression followed by mutation or epigenetic regulation of distinct genes or by alteration of tumor suppressor or oncogenic microRNA function (45). Smoke-induced cancers show usually a bimodal microRNA expression profile with an initial down-regulation and a final up-regulation in advanced stages of cancer. In our study, we revealed significantly higher miR-21 (p=0.0005) and lower miR-375 (p=0.0010) expression in the tumor of smokers than in healthy tissues.

Alcohol represents one of the risk factors of HNSCC pathobiology. Alcohol has been described to contribute to dysregulation of microRNAs by several mechanisms, like the irritation of inflammation, interference with the absorption of folate and/or its degradation into carcinogenic acetaldehyde. Expression of miR-375 has been shown to increase with alcohol consumption in HNSCC (46). Our research group did not reveal any association between the studied microRNAs and alcohol use. However, it is necessary to take into consideration that clinical data in relation to drinking can be subject of variability.

The role of HPV/p16 in the regulation of microRNA expression in head and neck cancers is largely unknown (47). P16 positivity of HNSCC is considered to be a surrogate

marker of HPV etiology. However, other causes of p16 expression are also suggested. Clinical features and prognosis of HNSCC are greatly influenced by the HPV/p16 status, which is favorable in p16-positive tumors (48). The function of p16 seems to be more complex and differs between tumors of various locations. Therefore, the study of p16 role in tumors, in relation to its prognostic and therapeutic consequences, should be site-specific (49, 50). Oncogene-induced senescence represents a barrier against tumorigenesis. P16 is known as a key regulator of cellular senescence in cooperation with senescence-associated micro-RNAs. Namely, accumulation of miR-34a has been described during senescence in a range of cell types (25, 49, 51). The tumor-suppressive function of miR-34a has been reported in multiple cancers. Tumor suppressive effects consist of deregulation of oncogene expression and are mediated by changes in a number of target mRNAs, including *MYC*, cyclin B1 (*CCNB1*), *BCL-2*, *E2F3* and surivivin (*BIRC5*) (11, 25, 52, 53). In the majority of cancers, miR-34 is down-regulated and associated with tumor progression (11, 26). Ogawa *et al*. described miR-34 down-regulation to be associated with cis-diamminedichloroplatinum (CDDP) resistance of sinonasal squamous cell carcinoma (54). Contrary to the majority of cancers, miR-34 was over-expressed in our study, namely in p16-positive oropharyngeal carcinomas. This fact could be responsible for more favourable prognosis of these tumors described earlier (48). Unfortunately, the number of cases in our pilot study was limited. Therefore the studies of larger cohort of p16-positive oropharyngeal carcinomas are required to confirm miR-34a overexpression. The elucidation of its underlying pathobiological mechanisms and possible association miR-34a up-regulation with oncogene-induced senescence and clinical consequences are warranted.

Published articles on head and neck microRNA de-regulations are still scarce but expanding. However, since different conclusions have been formulated in reported studies, the use of microRNA profile of HNSCC in routine clinical practice cannot be currently asserted (42, 47, 55). Our study was performed using FFPE tissue samples. Although FFPE samples do not seem to be useful for mRNA, as well as DNA analysis due to their degradation during the fixation process and deterioration over time, the isolation of small-sized microRNA from these samples seems to be fully substantiated as micro-RNAs are known to be more stable (56). Moreover, the study of microRNAs in HNSCC should be more complex by taking many exogenous and endogenous aspects, including tumor location, into consideration. Nevertheless, based on our pilot study and the literary knowledge, we are convinced that microRNA profiling of HNSCC is behind the diagnostic, prognostic and therapeutic potential of these tumors. Therefore, microRNAs seem to potentially extend the family of tumor biomarkers in the future.

Conclusion

This study revealed that certain microRNA profiles are de-regulated in head and neck cancer (up-regulation of miR-21, miR-200c and miR-34a; down-regulation of miR-375) playing a potential role in the pathobiology of HNSCC. Significant differences of microRNA expression (let-7a, miR34a) between oropharyngeal and laryngeal cancers support the hypothesis of site-specific cancerogenesis in HNSCC. Up-regulation of miR34a expression in p16-positive oropharyngeal carcinomas has not been so far described. The role of miR-34a in carcinogenesis is more complex and remains to be elucidated. Additional microRNA expression studies in site-specific HNSCC in relation to prognosis, smoking and HPV infection/p16 status, respectively, are warranted.

Acknowledgments

This study was supported by a research project of Charles University in Prague, Czech Republic (SVV No.260050), by the Charles University Research Fund (projects PRVOUK No. P36 and P37) and by a research project of the Ministry of Health, Czech Republic, No. 00669806 (Faculty Hospital Plzen).

Conflicts of Interest

The Authors declare that there exist no conflicts of interest regarding this work.

References

1 Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr.

2 Carthew RW and Sontheimer EJ: Origins and Mechanisms of miRNAs and siRNAs. Cell 136: 642-655, 2009.

3 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*: 281-297, 2004.

4 Ludvikova M, Pesta M, Holubec L, Jr. and Kalfert D: New aspects of tumor pathobiology. Cesk Patol *45*: 94-99, 2009.

5 Liu X, Chen Z, Yu J, Xia J and Zhou X: MicroRNA profiling and head and neck cancer. Comp Funct Genomics: 837514, 2009.

6 Lujambio A and Esteller M: CpG island hypermethylation of tumor suppressor microRNAs in human cancer. Cell Cycle *6*: 1455-1459, 2007.

7 Suzuki H, Maruyama R, Yamamoto E and Kai M: Epigenetic alteration and microRNA dysregulation in cancer. Front Genet *4*: 258, 2013.

8 Metias SM, Lianidou E and Yousef GM: MicroRNAs in clinical oncology: at the crossroads between promises and problems. J Clin Pathol *62*: 771-776, 2009.

9 Hagan JP and Croce CM: MicroRNAs in carcinogenesis. Cytogenet Genome Res *118*: 252-259, 2007.

10 Fabbri M, Ivan M, Cimmino A, Negrini M and Calin GA: Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther *7*: 1009-1019, 2007.
11 Kumar B, Yadav A, Lang J, Teknos TN and Kumar P: Dysregulation of microRNA-34a expression in head and neck squamous cell carcinoma promotes tumor growth and tumor angiogenesis. PLoS One *7*: e37601, 2012.
12 Roush S and Slack FJ: The let-7 family of microRNAs. Trends Cell Biol *18*: 505-516, 2008.
13 Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D and Slack FJ: RAS is regulated by the let-7 microRNA family. Cell *120*: 635-647, 2005.
14 Yu CC, Chen YW, Chiou GY, Tsai LL, Huang PI, Chang CY, Tseng LM, Chiou SH, Yen SH, Chou MY, Chu PY and Lo WL: MicroRNA let-7a represses chemoresistance and tumourigenicity in head and neck cancer via stem-like properties ablation. Oral Oncol *47*: 202-210, 2011.
15 Krichevsky AM and Gabriely G: miR-21: a small multi-faceted RNA. J Cell Mol Med *13*: 39-53, 2009.
16 Fu X, Han Y, Wu Y, Zhu X, Lu X, Mao F, Wang X, He X and Zhao Y: Prognostic role of microRNA-21 in various carcinomas: a systematic review and meta-analysis. Eur J Clin Invest *41*: 1245-1253, 2011.
17 Ren J, Zhu D, Liu M, Sun Y and Tian L: Downregulation of miR-21 modulates Ras expression to promote apoptosis and suppress invasion of Laryngeal squamous cell carcinoma. Eur J Cancer *46*: 3409-3416, 2010.
18 Kulda V, Pesta M, Topolcan O, Liska V, Treska V, Sutnar A, Rupert K, Ludvikova M, Babuska V, Holubec L, Jr. and Cerny R: Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases. Cancer Genet Cytogenet *200*: 154-160, 2010.
19 Pan X, Wang ZX and Wang R: MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol Ther *10*: 1224-1232, 2010.
20 Liu J, Zhu H, Yang X, Ge Y, Zhang C, Qin Q, Lu J, Zhan L, Cheng H and Sun X: MicroRNA-21 is a novel promising target in cancer radiation therapy. Tumour Biol *35*: 3975-3979, 2014.
21 Chen J and Wang X: MicroRNA-21 in breast cancer: diagnostic and prognostic potential. Clin Transl Oncol *16*: 225-233, 2014.
22 Mongroo PS and Rustgi AK: The role of the miR-200 family in epithelial-mesenchymal transition. Cancer Biol Ther *10*: 219-222, 2010.
23 Lo WL, Yu CC, Chiou GY, Chen YW, Huang PI, Chien CS, Tseng LM, Chu PY, Lu KH, Chang KW, Kao SY and Chiou SH: MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells. J Pathol *223*: 482-495, 2011.
24 Agostini M and Knight RA: miR-34: from bench to bedside. Oncotarget *5*: 872-881, 2014.
25 Christoffersen NR, Shalgi R, Frankel LB, Leucci E, Lees M, Klausen M, Pilpel Y, Nielsen FC, Oren M and Lund AH: p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC. Cell Death Differ *17*: 236-245, 2010.
26 Chen F and Hu SJ: Effect of microRNA-34a in cell cycle, differentiation, and apoptosis: a review. J Biochem Mol Toxicol *26*: 79-86, 2012.
27 Li XJ, Ren ZJ and Tang JH: MicroRNA-34a: a potential therapeutic target in human cancer. Cell Death Dis *5*: e1327, 2014.
28 Kinoshita T, Hanazawa T, Nohata N, Okamoto Y and Seki N: The functional significance of microRNA-375 in human squamous cell carcinoma: aberrant expression and effects on cancer pathways. J Hum Genet *57*: 556-563, 2012.
29 Yan JW, Lin JS and He XX: The emerging role of miR-375 in cancer. Int J Cancer *135*: 1011-1018, 2014.
30 Sobin LH, Gospodarowicz MK and Wittekind C: TNM Classification of Malignant Tumours, 7th Edition: Wiley-Blackwell, 2009.
31 Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. CA Cancer J Clin *60*: 277-300, 2010.
32 Ramqvist T and Dalianis T: An epidemic of oropharyngeal squamous cell carcinoma (OSCC) due to human papillomavirus (HPV) infection and aspects of treatment and prevention. Anticancer Res *31*: 1515-1519, 2011.
33 Silva SD, Nonogaki S, Soares FA and Kowalski LP: p16 (INK4a) has clinicopathological and prognostic impact on oropharynx and larynx squamous cell carcinoma. Braz J Med Biol Res *45*: 1327-1333, 2012.
34 Gourin CG, Conger BT, Porubsky ES, Sheils WC, Bilodeau PA and Coleman TA: The effect of occult nodal metastases on survival and regional control in patients with head and neck squamous cell carcinoma. Laryngoscope *118*: 1191-1194, 2008.
35 Chen LH, Tsai KL, Chen YW, Yu CC, Chang KW, Chiou SH, Ku HH, Chu PY, Tseng LM, Huang PI and Lo WL: MicroRNA as a Novel Modulator in Head and Neck Squamous Carcinoma. J Oncol *2010*: 135632, 2010.
36 Heneghan HM, Miller N and Kerin MJ: MiRNAs as biomarkers and therapeutic targets in cancer. Curr Opin Pharmacol *10*: 543-550, 2010.
37 Pesta M, Klecka J, Kulda V, Topolcan O, Hora M, Eret V, Ludvikova M, Babjuk M, Novak K, Stolz J and Holubec L: Importance of miR-20a expression in prostate cancer tissue. Anticancer Res *30*: 3579-3583, 2010.
38 Barber BR, Biron VL, Klimowicz AC, Puttagunta L, Cote DW and Seikaly H: Molecular predictors of locoregional and distant metastases in oropharyngeal squamous cell carcinoma. J Otolaryngol Head Neck Surg *42*: 53, 2013.
39 Janiszewska J, Szaumkessel M and Szyfter K: microRNAs are important players in head and neck carcinoma: a review. Crit Rev Oncol Hematol *88*: 716-728, 2013.
40 Avissar M, Christensen BC, Kelsey KT and Marsit CJ: MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. Clin Cancer Res *15*: 2850-2855, 2009.
41 Wiklund ED, Gao S, Hulf T, Sibbritt T, Nair S, Costea DE, Villadsen SB, Bakholdt V, Bramsen JB, Sorensen JA, Krogdahl A, Clark SJ and Kjems J: MicroRNA alterations and associated aberrant DNA methylation patterns across multiple sample types in oral squamous cell carcinoma. PLoS One *6*: e27840, 2011.
42 Chang SS, Jiang WW, Smith I, Poeta LM, Begum S, Glazer C, Shan S, Westra W, Sidransky D and Califano JA: MicroRNA alterations in head and neck squamous cell carcinoma. Int J Cancer *123*: 2791-2797, 2008.
43 Hui AB, Lenarduzzi M, Krushel T, Waldron L, Pintilie M, Shi W, Perez-Ordonez B, Jurisica I, O'Sullivan B, Waldron J, Gullane P, Cummings B and Liu FF: Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clin Cancer Res *16*: 1129-1139, 2010.

44 Childs G, Fazzari M, Kung G, Kawachi N, Brandwein-Gensler M, McLemore M, Chen Q, Burk RD, Smith RV, Prystowsky MB, Belbin TJ and Schlecht NF: Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol *174*: 736-745, 2009.
45 Momi N, Kaur S, Rachagani S, Ganti AK and Batra SK: Smoking and microRNA dysregulation: a cancerous combination. Trends Mol Med *20*: 36-47, 2014.
46 Avissar M, McClean MD, Kelsey KT and Marsit CJ: MicroRNA expression in head and neck cancer associates with alcohol consumption and survival. Carcinogenesis *30*: 2059-2063, 2009.
47 Lajer CB, Nielsen FC, Friis-Hansen L, Norrild B, Borup R, Garnaes E, Rossing M, Specht L, Therkildsen MH, Nauntofte B, Dabelsteen S and von Buchwald C: Different miRNA signatures of oral and pharyngeal squamous cell carcinomas: a prospective translational study. Br J Cancer *104*: 830-840, 2011.
48 Lajer CB and von Buchwald C: The role of human papillomavirus in head and neck cancer. Apmis *118*: 510-519, 2010.
49 Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Lleonart ME, Castellvi J and Ramon y Cajal S: p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene *30*: 2087-2097, 2011.
50 Leemans CR, Braakhuis BJ and Brakenhoff RH: The molecular biology of head and neck cancer. Nat Rev Cancer *11*: 9-22, 2011.
51 Overhoff MG, Garbe JC, Koh J, Stampfer MR, Beach DH and Bishop CL: Cellular senescence mediated by p16INK4A-coupled miRNA pathways. Nucleic Acids Res *42*: 1606-1618, 2014.
52 Shen Z, Zhan G, Ye D, Ren Y, Cheng L, Wu Z and Guo J: MicroRNA-34a affects the occurrence of laryngeal squamous cell carcinoma by targeting the antiapoptotic gene survivin. Med Oncol *29*: 2473-2480, 2012.
53 Ribeiro J and Sousa H: MicroRNAs as biomarkers of cervical cancer development: a literature review on miR-125b and miR-34a. Mol Biol Rep *41*: 1525-1531, 2014.
54 Ogawa T, Saiki Y, Shiga K, Chen N, Fukushige S, Sunamura M, Nagase H, Hashimoto S, Matsuura K, Saijo S, Kobayashi T and Horii A: miR-34a is downregulated in cis-diamminedichloroplatinum treated sinonasal squamous cell carcinoma patients with poor prognosis. Cancer Sci 103: 1737-1743, 2012.
55 John K, Wu J, Lee BW and Farah CS: MicroRNAs in head and neck cancer. Int J Dent 2013: 650218, 2013.
56 Peiro-Chova L, Pena-Chilet M, Lopez-Guerrero JA, Garcia-Gimenez JL, Alonso-Yuste E, Burgues O, Lluch A, Ferrer-Lozano J and Ribas G: High stability of microRNAs in tissue samples of compromised quality. Virchows Arch 463: 765-774, 2013.

Received December 15, 2014
Revised January 15, 2015
Accepted January 21, 2015