Association of pre-miR-146a rs2910164 Polymorphism with the Risk of Head and Neck Cancer

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Abstract. *Background: Over- or underexpression of miR-146a has been reported in several different human tumor types, and a polymorphism in its precursor form (pre-miR-146a rs2910164 G/C) has been recently studied in connection with cancer susceptibility. The aim of the present study was to investigate the possible influence of the pre-miR-146a rs2910164 polymorphism on the risk of squamous cell carcinoma of the head and neck (HNSCC). Patients and Methods: The study included 468 patients with HNSCC and 468 cancer-free, age-, gender-, and smoking-matched controls. The miR-146a genotypes were determined using polymerase chain reaction with confronting two-pair primers, and their distribution was compared by multivariate binary logistic regression analysis. Results: Occurrence of heterozygous (odds ratio, OR=1.46, 95% confidence interval, CI=1.10-1.95, p=0.009) and C/C homozygous (OR=2.37, 95% CI=1.01-5.60, p=0.048) individuals was significantly more frequent among patients with HNSCC than in the control group. Conclusion: The pre-miR/146a C allele may contribute to an increased susceptibility to HNSCC.*

Squamous cell cancers of the oral cavity, pharynx and larynx are often referred to as head and neck squamous cell carcinomas (HNSCCs), indicating their common etiological, pathophysiological and epidemiological characteristics. HNSCCs are among the most frequent malignant tumors worldwide: according to the most recent available estimates they are responsible for about 630,000 new cases and more than 350,000 deaths annually (1). Besides some less prevalent risk factors, these tumors are strongly correlated with smoking and alcohol consumption (2), and recently, the role of human papillomavirus infection in head and neck carcinogenesis is also being intensively investigated (3). The geographical patterns and time trends in the occurrence of HNSCCs, however, cannot be completely explained by these risk factors. Moreover, only a minority of smokers or alcoholics develop HNSCC, suggesting the participation of other, still not fully-identified, risk factors, such as genetic susceptibility, in the genesis of HNSCC.

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs that participate in the post-transcriptional regulation of gene expression (4). Their primary function is to reduce the expression of their target genes, either by causing the degradation of the messenger RNA, or by other translational repressive mechanisms (5). They bind to the 3' untranslated region of the mRNA, and in cases of a perfect or closely-perfect match, lead to its cleavage, while a non-perfect match inhibits the translational machinery (6). In parallel with the development of our knowledge on miRNAs, the number of known miRNA target genes has been continuously increasing. Currently, it is believed that the majority of the mammalian genes are under miRNA regulation (7).

miRNAs not only modulate several physiological cellular functions (proliferation, differentiation, and senescence) (8) but may also be involved in the development of different pathological processes, such as malignant transformation (9). Several miRNAs have been shown to exhibit a dysregulated expression in different types of cancer (10, 11). miRNAs can behave as oncogenes (showing an increased expression in tumor cells) or tumor suppressor genes (decreased expression in cancer cells) (9). They can affect both the risk of cancer formation and the prognosis of the disease.

The miR-146 family consists of two miRNAs, miR-146a and miR-146b, located on chromosomes 5 and 10, respectively (12). While these two miRNAs differ only in two nucleotides, their biological functions are completely unrelated to each other (12). miR-146a was discovered during the search for

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miRNAs participating in innate immune responses (13). Besides its role in innate and adaptive immune responses, it has been shown to be involved in the regulation of cell differentiation (*e.g.* hematopoietic cells) and in cancer formation (14). Involvement of miR-146a in carcinogenesis is demonstrated by its altered expression in several types of tumor, such as thyroid (15), prostate (16), pancreatic (17), gastric (18), oral (19) and other types of cancer (20, 21). An allelic polymorphism, the rs2910164 G/C single-nucleotide polymorphism, altering the amount of mature miR-146a has been described in the gene encoding for pre-miR-146a, the precursor molecule of miR-146a (15, 22), but only relatively few studies have investigated its effect on the risk of cancer. Some studies suggested a possible relationship between the pre-miR-146a polymorphism and the risk of different tumor types (15, 23-27) while others have not found such a connection (28-30). The only study examining the effect of this polymorphism on the risk of HNSCC did not find such an association when it was analyzed alone, but in combination with other miRNA polymorphisms, the pre-miR-146a C allele seemed to increase the risk of HNSCC (31).

In order to further explore the role of miR-146a in head and neck carcinogenesis, we studied the effect of the rs2910164 polymorphism on the risk of HNSCC in a Hungarian population.

Patients and Methods

Study participants. Four hundred and sixty-eight patients with histologically-verified HNSCC were involved in the study. The patients were recruited from the Department of Otorhinolaryngology and Head and Neck Surgery, Faculty of Medicine, University of Pécs, and from the Department of Oncoradiology, Markusovszky County Hospital, Szombathely, Hungary. The control group (of the same size as the case group) consisted of cancer-free patients from outpatient departments of the two clinics and healthy individuals taking part in screening tests. The control participants were individually-matched to the cases by age (±5 years), gender, and smoking habits (see below). The protocol was approved by the appropriate Ethical Committees, and all the participants entered the study on a voluntary basis. Participants were fully informed of the nature of the study, and written informed consent was collected from them. Data on risk factors were collected from hospital records and personal interviews. Participants who smoked >100 cigarettes in their lifetime were considered as smokers. The number of cigarettes smoked per day (<20 or ≥20) and years of smoking (<30 or ≥30) were used to characterize smoking habits for the control matching. The classification for the level of education was based on a country-specific, simplified version of the current UNESCO guidelines (32) using five educational categories. Alcohol consumption habits were characterized by the average daily number of drinks: abstinent: 0, moderate: >0 and ≤2, intermediate: >2 and ≤5, heavy: >5.

Pre-miR-146a genotyping. The DNA used for genotyping was isolated from peritumoral healthy parts of paraffin-embedded blocks or from peripheral blood. For de-paraffinization, 10-μm-thick sections were made and soaked in xylene twice (for 15 min each time). Sections were rehydrated in a descending ethanol series (100-90-70-50%, for 5 min each), followed by rinsing with distilled water.

In the case of the control participants, 5 ml venous blood were drawn and subsequently treated with a hypotonic solution (repeated centrifugation with double amount of 0.84% ammonium chloride) to remove red blood cells. In order to check the comparability of genotyping results from different source tissues, 100 randomly selected patients were double-genotyped (using paraffin-embedded blocks and blood), with a concordance rate of 100%.

The DNA isolation was performed by the standard phenol-chloroform method (33).

The miR-146a genotyping was carried out by polymerase chain reaction (PCR) with confronting two-pair primers (34, 35). The PCR mix contained 1.5 mM $MgCl_2$, 200 pM dNTP, 1 pM each primer, 0.5 U Taq DNA polimerase (Go Taq; Promega GmbH, Mannheim, Germany), 1× buffer (Promega), 0.5 μg DNA template in 15 μl total volume. After an initial denaturation for 10 min at 95˚C 35 PCR cycles were performed (60 s at 95˚C, 60 s at 63˚C, 60 s at 72˚C), followed by a final extension for 10 min at 72˚C. Sequence of the primers was the following: $forward_1$: AAGCAGCTGCATTGGATT, $reverse_1$: CAGCTGAAGAACTGAATTTCAC, $forward_2$: GTTGT GTCAGTGTCAGACCTC, and $reverse_2$: CAAGCTCTTCAGCAG ACTGA. The amplification resulted in a common 261-bp fragment, while a 128-bp fragment indicated the presence of the C allele, and the G allele was characterized by a 182-bp fragment. The amplification products were visualized in ethidium bromide-stained 2% agarose gel.

Statistical analysis. The Chi-square test was used to compare differences in alcohol consumption habits, level of education, and presence of oral diseases between cases and controls. The average age of patients and controls was compared by using the Student's *t*-test. Hardy-Weinberg equilibrium was tested with the goodness-of-fit chi-square test. Multivariate binary logistic regression analysis was used to describe the association between the studied risk factors and HNSCC, where *p*-values and adjusted (for alcohol consumption, education, and presence of periodontitis or other chronic oral diseases) odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. The statistical analysis was performed by the IBM SPSS Statistics version 19 software package (IBM, Armonk, NY, USA).

Results

The average age of patients with HNSCC was 65.3 (range 41-82) years, which did not present a statistically significant difference from that of the controls (64.8, range 42-80 years), indicating a successful matching. The vast majority of the patients were smokers (35.9% light/intermediate, 51.9% heavy), as illustrated in Table I.

Alcohol consumption habits of patients and controls were as follows: abstinents 14.5% and 30.3%, moderate drinkers 28.6% and 31.0%, intermediate drinkers 35.0% and 25.8%, heavy drinkers 21.8% and 13.9%. Periodontitis, broken teeth or other chronic oral disorders were found in 48.9% of the cases and in 34.8% of the controls. The chi-square test indicated a statistically significant difference in the alcohol

Table I. *Smoking habits of the patients included in this study.*

	Gender		Total
	Male	Female	
Smoking frequency			
Non-smoker	42 (11.6%)	15 (14.2%)	57 (12.2%)
<20/day	129 (35.6%)	39 (36.8%)	168 (35.9%)
≥20/day	191 (52.8%)	52 (49.1%)	243 (51.9%)
Smoking duration (smokers-only)			
<30 years	124 (38.8%)	48 (52.7%)	172 (41.8%)
≥30 years	196 (61.3%)	43 (47.3%)	239 (58.2%)

Table II. *Distribution of genotypes and external risk factors among patients and controls.*

	Controls	Patients
Pre-miR-146a		
GG	323 (69.0%)	284 (60.7%)
GC	136 (29.1%)	168 (35.9%)
CC	9 (1.9%)	16 (3.4%)
GC + CC	145 (31.0%)	184 (39.3%)
Oral disorder		
No	305 (65.2%)	239 (51.1%)
Yes	163 (34.8%)	229 (48.9%)
Alcohol (drinks/day)		
Abstinent	142 (30.3%)	68 (14.5%)
>0 and ≤2	145 (31.0%)	134 (28.6%)
>2 and ≤5	116 (24.8%)	164 (35.0%)
>5	65 (13.9%)	102 (21.8%)
Education		
Below primary	45 (9.6%)	50 (10.7%)
Primary	116 (24.8%)	121 (25.9%)
Lower secondary	135 (28.8%)	133 (28.4%)
Upper secondary	124 (26.5%)	127 (27.1%)
Tertiary	48 (10.3%)	37 (7.9%)

consumption habits and occurrence of oral disorders ($p<0.001$ for both) but not in the educational status ($p=0.21$) between patients and controls. Distribution of pre-miR-146a genotypes among cases and controls was the following: GG 60.7% *vs*. 69.0%, GC 35.9% *vs*. 29.1%, and CC 3.4% *vs*. 1.9%. The allelic distributions fitted to the Hardy-Weinberg equilibrium, for both cases and controls. Detailed information on genotypic frequencies, occurrence of oral diseases, alcohol consumption habits and educational status of the participants is shown in Table II.

The logistic regression analysis revealed an association between the pre-miR-146a allelic polymorphism and HNSCC (Table III). Compared to the GG homozygotes, both heterozygous (OR=1.46, 95% CI=1.10-1.95, $p=0.009$) and CC homozygous (OR=2.37, 95% CI=1.01-5.60, $p=0.048$) individuals occurred with a statistically significantly higher frequency among patients with HNSCC than in the control group. The association was also statistically significant when heterozygotes and CC homozygotes were grouped together and compared to GG homozygotes (OR=1.52, 95% CI=1.15-2.01, $p=0.004$).

Table III. *Association between squamous cell carcinoma of the head and neck and the studied risk factors.*

	OR (95% CI)	*p*-Value
Oral disorder	1.87 (1.43-2.45)	<0.001
Alcohol (drinks/day)		
>0 and ≤2	1.87 (1.28-2.73)	0.001
>2 and ≤5	2.97 (2.03-4.34)	<0.001
>5	3.35 (2.18-5.17)	<0.001
Pre-miR-146a		
GC	1.46 (1.10-1.95)	0.009
CC	2.37 (1.01-5.60)	0.048
GC + CC	1.52 (1.15-2.01)	0.004

Table IV. *Association between pre-miR-146a genotype and squamous cell carcinoma of the nead and neck (CC+GC vs. GG), stratified according to gender and smoking habits.*

	OR (95% CI) (CC+GC *vs*. GG)	*p*-Value
Gender		
Males	1.44 (1.04-1.98)	0.026
Females	1.84 (1.01-3.34)	0.045
Smoking frequency		
Non-smoker	0.97 (0.41-2.27)	0.944
<20/day	1.48 (0.91-2.42)	0.117
≥20/day	1.68 (1.14-2.47)	0.009
Smoking duration (smokers-only)		
<30 years	1.29 (0.81-2.06)	0.281
≥30 years	1.88 (1.26-2.81)	0.002

While it was not the main purpose of our study to examine the role of alcohol consumption in carcinogenesis, a significant connection was found between alcohol intake and HNSCC, in a dose-dependent manner. The strongest effect was seen among heavy drinkers, resulting in an OR of 3.35 (95% CI=2.18-5.17, $p<0.001$). Presence of oral diseases also proved to be a risk factor (OR=1.87, 95% CI=1.43-2.45, $p<0.001$). Educational status, however, did not exert any statistically significant effect on the risk of HNSCC in our study.

In order to further evaluate the role of the pre-miR-146a polymorphism, a stratified analysis was also performed (Table

IV), which found that gender acted as an effect modifier in the relationship between the studied risk factors and HNSCC. Associations between HNSCC and oral disorders (men: OR=1.75, women: OR=2.52), alcohol consumption (heavy drinkers: OR=4.26 *vs*. OR=5.60) and carrying the pre-miR-146a C allele (OR=1.44 *vs*. OR=1.84) were stronger among female than male participants. The stratified analysis revealed an interaction between the pre-miR146-a polymorphism and smoking: the risk-increasing effect of the C allele was only statistically significant in those who smoked ≥30 years or ≥20 cigarettes per day (Table IV).

Discussion

In the present study, we analyzed the effect of pre-miR-146a rs2910164 allelic polymorphism on the risk of HNSCC. Our results demonstrated an association between the presence of the C allele and the occurrence of HNSCC. The effect of the genotype was almost twice as strong in CC homozygotes as in heterozygotes. The association was seen in smokers only, and it was somewhat stronger among women than among men.

To the best of our best knowledge, the only existing study examining the connection between the pre-miR-146a rs2910164 polymorphism and the risk of HNSCC has been published by Liu *et al*. (31). This study did not find any statistically significant association between the pre-miR-146a G/C polymorphism and the occurrence of HNSCC in a non-Hispanic US white population. However, somewhat controversially, combined high-risk genotypes of four miRNAs, including pre-miR-146a C allele, were found to increase the risk of HNSCC in the same study.

The difference between the results of Liu *et al*. and ours may be partially caused by the different proportion of non-smokers in the studied populations. The unusually high proportion (27.7%) of never smokers among patients with HNSCC in the study of Liu *et al*. may reflect the effects of the recent reduction of smoking prevalence in the US, while our study, with 12.2% never smokers, fits the majority of studies on HNSCC epidemiology better. Since smoking is by far the strongest risk factor known in the genesis of HNSCC, it is of particular importance to take its effect into consideration when studying genetic susceptibility. This is why we decided to use triple-matching (by age, gender, and smoking habits) instead of simply adjusting for smoking, since matching is the most powerful method to eliminate confounding or effect modification, while resulting in good statistical power. In the stratified analysis, smoking proved to be an effect modifier, as it was demonstrated by the considerable differences between the layer-specific ORs (Table IV). A risk-increasing effect could only be attributed to the pre-miR-146a C allele in frequent smokers and in those who had smoked for longer periods of time. This type of interaction between lifestyle and genetic factors is often seen in molecular epidemiological studies, and has been also demonstrated for HNSCC (36-38).

To date, only one study has analyzed the association between the pre-miR-146a polymorphism and the prognosis of oral squamous cell carcinoma (19). The C allele was found to be associated with an increased expression of miR-146a in the tumors and with a worse prognosis (higher frequency of advanced nodal metastasis). Since oral cancer comprises the most important group of HNSCCs besides pharyngeal tumors, our analysis is in full accordance with these results, indicating the implication of miR-146a in head and neck carcinogenesis.

The molecular basis of the possible cancer risk-modifying effect of the pre-miR-146a G/C polymorphism is not completely understood, but the involvement of miR-146a has been already demonstrated in important cancer related regulatory processes. miR-146a suppresses the expression of interleukin-1 receptor-associated kinase-1 [involved in the (NF-κB) pathway], epidermal growth factor receptor (EGFR), SMAD4 [participating in the (TGF-β) signaling pathway], and there is growing evidence for the involvement of NF-κB, TGF-β, and EGFR pathways in human carcinogenesis (39-42). The pre-miR-146a polymorphism affects the maturation of miR-146a by altering its processing due to the G:U to C:U change in the stem region, causing a base mismatch. This leads to a reduced amount of mature miR-146a, as has been demonstrated in cells transfected with pre-miR-146a G or C expression vectors (15). The hypothetical tumor-suppressor role of miR-146a is supported by the fact that several studies have found its underexpression in human tumors (16-18, 20). The fact that its up-regulation is rarely found (21, 43) can be considered as a possible feedback reaction to the disrupted regulatory mechanisms in tumor cells.

In conclusion, our study suggests that the pre-miR-146a C allele increases the risk of HNSCC. However, further epidemiological studies are needed to confirm our findings and, along with functional studies on miRNAs, to elucidate the role of the rs2910164 polymorphism in human carcinogenesis.

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