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Genetic Variation in MicroRNA Genes and Risk of Oral Premalignant Lesions

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Abstract

MicroRNAs (miRNAs) have been reported to play a key role in oncogenesis and, recently, studies have examined the role miRNAs might play in the risk of premalignant lesions. To our knowledge, no study has investigated the association between miRNA polymorphisms and risk of oral premalignant lesions (OPL). We genotyped 31 single nucleotide polymorphisms (SNPs) among 21 miRNA-related genes in a case-control study including 136 OPL patients and 136 matched controls. Patients with at least one variant allele of *mir26a-1*:rs7372209 had a significantly increased risk of OPL (OR, 2.09; 95% CI, 1.23–3.56). Likewise, patients with at least one variant allele of *DICER*:rs3742330 had a significantly increased risk of OPL (OR, 2.09; 95% CI, 1.03–4.24). To assess the cumulative effects, we performed a combined unfavorable genotype analysis that included all SNPs showing at least a borderline statistical significance. A significant trend of increased risk of OPL with increasing number of unfavorable genotypes was observed (*P* for trend <0.0001). This study presents the first epidemiologic evidence supporting that individual as well as combined genotypes of miRNA-related variants may be used to predict the risk of OPL, and may be useful for identifying patients with OPL at high risk for progression to oral cancer.

Keywords

microRNA; Oral premalignant lesions; Cancer risk; Genetic variation; Case-control study

INTRODUCTION

MicroRNAs (miRNA) are a group of endogenous, small RNA molecules of about 21–23 nucleotides [1]. Most miRNA genes reside within intergenic or intronic regions of other genes and start off as a 500–3000 base, primary miRNA transcript (pri-miRNA) generated by RNA polymerase II [2]. The pri-miRNA is processed in the nucleus by the microprocessor machinery containing the RNase Drosha and the double-strand RNA binding protein DGCR8 [3]. Hairpin molecules are formed and cleaved by Drosha to form a 60–70-nucleotide precursor miRNA (pre-miRNA) [4]. The pre-miRNA is translocated to the cytoplasm through the assistance of Ran-GTPase and Exportin-5 (XP05), where it is further

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processed by a protein complex that includes DICER, TRBP, AGO1 and AGO2, leading to the production of mature miRNAs [2].

It has been predicted that miRNAs regulate the expression of almost one third of the human genome and are likely to play a key role in human development, cellular differentiation, adaptation to the environment, and host cell interactions with pathogens and oncogenesis [1]. miRNAs may influence tumorigenesis by acting as either oncogenes or tumor suppressor genes [1]. For example, *miR-21* is relatively over expressed in glioblastoma multiforme, cervical cancer, breast cancer, and several other solid tumor types and its increased expression appears to contribute to decreased apoptosis in malignant cells [5–7]. On the other hand, let-7 represses the expression of the RAS oncogenes, thus inhibiting lung tumorigenesis [8]. More recently, studies have suggested that miRNAs might play a role in the development of premalignant lesions [9,10].

Oral leukoplakia, oral submucous fibrosis and erythroplakia are the three major forms of oral premalignant lesions (OPLs) [11]. The development of these lesions, as the result of carcinogenic exposures, may cause simultaneous genetic defects to the upper aerodigestive tract epithelium at different stages of carcinogenesis, and individuals with these lesions are at a high risk for developing oral cancer [12–14]. According to our data, during a follow-up of seven years, 31.4% of OPL patients developed cancers in the upper aerodigestive tract and the overall cancer incidence is 5.7% per year [15]. The overall malignant transformation rate of dysplastic lesions depends on the length of follow-up and varies from 11 to 36% [14]. Tobacco chewing, tobacco smoking and alcohol drinking have been identified as major environmental risk factors for OPLs[16]. Currently, the genetic components of OPL risk are still largely unknown.

Previous studies have shown that common genetic polymorphisms in protein-coding genes play a role in oral tumorigenesis [17,18]. Genetic variation in miRNA genes and their biogenesis pathway has the potential to affect the regulation of multiple cellular pathways instrumental in cancer development and susceptibility [19–23]. However, to our knowledge, no study has investigated the association between polymorphisms in the miRNA biogenesis pathway genes or miRNA genes and risk of OPL, which may be useful for identifying patients with OPL at high risk for progression to oral cancer. Therefore, in this case-control study, we tested whether common sequence variants in miRNA genes and the miRNA biogenesis pathway affect OPL susceptibility.

MATERIALS AND METHODS

Study Population and Epidemiologic Data

A total of 136 OPL patients were identified at The University of Texas M. D. Anderson Cancer Center from 1997 to 2005. The inclusion criteria for cases were the presence of histologically confirmed OPL (leukoplakia and/or erythroplakia). All OPL patients were participants of a chemoprevention trial and only those ages ≥ 18 years were enrolled. Enrollment into the clinical trial also excluded patients with acute intercurrent illnesses or infections and patients who had retinoid or carotenoid therapy within three months prior to study entry. Patients with prior history of cancer (except non-melanoma skin cancer) that had been treated within the preceding two years were also excluded. Furthermore, there were no oral tumors present at the time of the sampling. A self-administered questionnaire was used to collect epidemiological data, including demographical information and tobacco use history. Before the subjects were randomized into the chemoprevention trail, blood samples were obtained in heparnized tubes for molecular analyses.

A total of 136 healthy controls who had no history of cancer were recruited from the Kelsey-Seybold clinics, the largest multispecialty managed-care organization consisting of more than 300 physicians and 23 clinics in the Houston metropolitan area. The potential controls were identified by reviewing short survey forms distributed to patients coming to the clinic for annual health check-ups. Controls had no prior history of cancer (except non-melanoma skin cancer) and were frequency matched to the OPL patients by gender, ethnicity, and age (±5 years). Epidemiologic questionnaire data were obtained through in-person interview for the controls. After the interview, participants were asked to donate a blood sample for molecular analysis. Controls were recruited during 1999–2005.

For both cases and controls, written, informed consent was collected from each participant and approval for conducting human subjects research was obtained from the M. D. Anderson and the Kelsey-Seybold Institutional Review Boards. An individual who had never smoked or had smoked less than 100 cigarettes in his or her lifetime was defined as a never smoker. A former smoker was a person who had quit smoking at least one year prior to diagnosis (cases) or who had quit smoking at least one year prior to the interview (controls). A current smoker was someone who was currently smoking or who had stopped less than one year prior to being diagnosed (cases) or interviewed (controls). Current and former smokers were defined as ever smokers.

Selection of Genes and Polymorphisms

We searched the International HapMap Project, dbSNP and miRBase registry databases to identify potentially functional SNPs and haplotype tagging SNPs with a reported minor allele frequency (MAF) of > 0.01 in the Caucasian population. In the case of multiple candidate SNPs within the same haplotype block (defined by $r^2 > 0.8$), only one SNP was included. We identified more than 200 SNPs located within the pri-miRNA transcript but not in pre-miRNAs. Therefore, we only chose SNPs from eight pri-miRNAs whose mature counterparts have been extensively implicated in cancer etiology or clinical outcome. In total, 31 SNPs were selected: 18 SNPs in eight genes in the miRNA biogenesis pathway, five SNPs in five pre-miRNAs and eight SNPs in eight pri-miRNAs (Table 1).

Genotyping

Genomic DNA was extracted from each participant's blood sample using the QIAamp DNA extraction kit (Qiagen, Valencia, CA) following manufacture's protocol. Samples were frozen at −80°C until genotyping experiments. All SNPs were genotyped blinded to the case-control status of each sample using the SNPlex assay according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 3730 DNA Analyzer. Genotypes were called by GeneMapper software (Applied Biosystems) using a template file provided with each custom SNPlex assay. Internal quality controls and negative controls were used to ensure genotyping accuracy, and 5% of all samples were randomly selected and genotyped in duplicate with 100% concordance.

Statistical Analysis

Statistical analyses were done using Intercooled STATA software (STATA Corporation, College Station, TX). χ^2 analysis was used to assess the differences between cases and controls with regard to categorical variables such as gender and smoking status. Student's *t* test was used to test for continuous variables, including age. The Hardy-Weinberg equilibrium was tested using a goodness-of-fit χ^2 analysis. The OPL risks were estimated as odds ratios (ORs) and 95% confidence intervals (95% CIs) using unconditional multivariate logistic regression adjusted for age, gender, ethnicity and smoking status, where appropriate. The unfavorable genotype analysis included those SNPs showing at least a borderline statistical significance ($P < 0.10$) in the main analysis. The unfavorable genotypes were

collapsed together and categorized according to the tertiles (low, medium and high risk) of the number of unfavorable genotypes in controls. Using the low-risk group as the reference group, we calculated the ORs and 95% CIs for the medium-risk and hi-risk groups using unconditional multivariate logistic regression adjusted for age, gender, ethnicity, and smoking status. All *P* values were two sided and a $P \leq 0.05$ was considered the threshold of statistical significance.

RESULTS

Characteristics of the study population

A total of 136 patients with OPL and 136 frequency matched controls by gender, ethnicity, and age (±5 years) were included in this study. As shown in Table 2, cases and controls were perfectly matched on sex ($P = 1.00$) and ethnicity ($P = 1.00$), and no significant difference was observed for cases (57.65± 12.91 years) and controls (58.76± 12.48 years) on age ($P = 0.47$). However, cases were significantly more likely to be current smokers (29.41%) than controls (8.09%, $P < 0.001$).

Risk Associated with Individual SNPs

Among the 31 SNPs, one SNP (rs17276588 in *let7f-2*) showed departure from Hardy-Weinberg equilibrium at significance level 0.05 and was removed from further analysis. Overall, three SNPs were found to be associated with OPL risk (Table 3). For the pri-mRNA SNP rs7372209 in *mir26a-1*, patients with at least one variant allele had a significantly increased risk of OPL (OR, 2.09; 95% CI, 1.23–3.56). Likewise, patients with at least one variant allele of SNP rs3742330 in *DICER* had a significantly increased risk of OPL (OR, 2.09; 95% CI, 1.03–4.24). In contrast, individuals with *GEMIN3* nonsynonymous rs197412 variant genotypes exhibited a significantly reduced OPL risk (OR, 0.58; 95% CI, 0.33–0.99) compared to individuals with wild type genotype. Two additional SNPs, rs784567 in *TRBP* and rs3744741 in *GEMIN4*, were found to be borderline significantly associated with OPL risk ($P < 0.10$).

Cumulative Effect of Selected SNPs on OPL Risk

We performed an unfavorable genotype analysis for five SNPs that had significant and borderline significant associations with OPL risk ($P < 0.10$), including rs197412 (TT), rs3742330 (AG + GG), rs3744741 (CC), rs7372209 (CT + TT), rs784567 (TT). Compared to the low-risk group with ≤1 unfavorable genotypes, the OR for the medium risk group with 2 or 3 unfavorable genotypes was 3.52 (95% CI, 1.81–6.84) and the OR increased to over 21 (95% CI, 5.08–89.79) for the high risk group with ≥4 unfavorable genotypes. We observed a significantly increased risk of OPL with increasing number of unfavorable genotypes (*P* for trend < 0.0001, Table 4).

DISCUSSION

Given the high risk of oral cancer among OPL patients, it is critical that subgroups at high risk of premalignancy development be identified for early oral cancer prevention. In this study, we assessed the effects of 31 SNPs in genes of the miRNA biogenesis pathway, pre-miRNAs and pri-miRNAs on OPL risk. We found that a pri-miRNA SNP in *mir26a-1*, a SNP in the 3′UTR of *DICER*, and a nonsynonymous SNP in *GEMIN3* were all significantly associated with an altered risk of OPL. We also identified two additional miRNA-related SNPs showing borderline significant associations and showed that the combined unfavorable genotypes of these five selected SNPs had a dramatic affect on OPL risk. To our knowledge this is the first study to provide evidence that common SNPs in miRNAs and their processing pathway might play an important role in the prediction of OPL.

The gene for *miR26a-1* is located on chromosome 3p21, a region frequently deleted in several epithelial cancers and down-regulated in lung cancer [24–26]. In the current study, the variant allele of rs7372209 in *mir26a-1* was associated with a 2.15-fold significantly increased risk of OPL ($P = 0.005$). Abnormal expression of this miRNA has been found in premalignant colon cancer cells [27] and mutations in miRNA could alter its expression. It is possible, then, that the variant allele of rs7372209 is altering the expression of *mir26a-1*, either through down regulation or inactivation, and accounting for the increased risk of OPL. Interestingly, our group previously reported the opposite effect of this rs7372209 SNP on risk of bladder cancer risk [20], highlighting the significance of this miRNA in the cancer development process and the need for further investigation in other cancers and premalignant conditions.

In the current study, the variant allele of rs3742330 located in *DICER* was associated with a 2.09-fold increase in risk of OPL ($P = 0.04$). *DICER* plays an important role in the cleavage of pre-miRNAs into their mature form and has previously been implicated in the oncogenic process of several cancers. Lower levels of *DICER* mRNA expression have been shown to be associated with decreased non-small cell lung [28] and ovarian cancer survival [29]. Likewise, through inhibiting expression of *DICER*, Kumar et. al. observed increased growth in mouse and human cancer cell lines and more aggressive behavior of the tumor cells when injected into in nude mice [30]. However, Chiosea et. al. [9] observed an opposite effect with up-regulation of *DICER* in non-invasive precursor lesions of lung adenocarcinoma. This evidence suggests that *DICER* may have different roles in the tumorigenesis process depending on the stage of cancer development. Interestingly, the significant SNP identified in our study is located within the 3′-UTR of *DICER*. This region is important for RNA stability, and rs3742330 could cause changes in the stability of *DICER* transcripts. Future studies examining the functionality of *DICER* SNPs are warranted to determine if this is the case and to better understand how this SNP modulates risk of OPL.

We observed that individuals with *GEMIN3* nonsynonymous rs197412 variant genotypes exhibited a significantly reduced OPL risk (OR, 0.58; 95% CI, 0.34–0.99) compared to individuals with wild type genotype. Mourelatos et al. [31] observed the presence of the GEMIN3 protein in a 15S ribonucleoprotein complex containing eIF2C, a member of the AGO protein family important in miRNA processing. Several other miRNAs have also been identified as part of this 15S ribonucleoprotein complex, suggesting that GEMIN proteins may be involved in the processing of miRNA precursors [31–33]. Thus, variants such as rs197412 could alter global miRNA homeostasis and have a major effect on cellular signaling pathways. However, studies assessing the functional effect of *GEMIN3* rs197412 are needed.

Borderline significant associations with OPL risk also identified two SNPs in *TRBP* and *GEMIN4* (Table 3). Although the results were only borderline significant, these SNPs are of interest because the proteins encoded by these genes are involved in the synthesis and processing of miRNA transcripts that may have a much larger downstream effect. TRBP is an integral component of a DICER complex and has been found to result in significantly impaired miRNA biogenesis [20,34]. We observed a 1.67-fold increase in OPL risk ($P = 0.092$) for the rare homozygous genotype of the *TRBP* SNP, rs784567. GEMIN4 is a central component of a macromolecule complex that interacts with the survivor or motor neuron protein and is essential in pre-mRNA splicing and ribonucleoprotein assembly [35]. In our study, we observed a borderline significant 40% decreased risk of OPL for the variant genotype of the *GEMIN4* SNP, rs3744741 ($P = 0.097$).

To gain more power and insight, we used a polygenic approach in which we collapsed all the unfavorable genotypes with at least a borderline significant association to assess their

combined effect on OPL risk. Through this strategy, we identified a significant trend between increasing OPL risk and the increasing number of unfavorable genotypes, and reinforced the belief that the development of OPL is a polygenic process and a combined analysis of multiple factors may have a greater ability to characterize populations at highest risk.

Overall, this study presents the first epidemiologic evidence supporting an association between genetic polymorphisms in miRNA-related genes and OPL susceptibility. In this current study, we were unable to determine if these OPL risk alleles were also markers for oral cancer development. Further studies with follow-up of OPL patients will be required to address this question. However, the potential ability to identify patients at the beginning of the carcinogenic processes manifested by OPL is a major strength of the study. There is a possibility that the associations observed in this study were attained by chance, given the loss of significance after multiple comparison adjustments (data not shown). Nevertheless, our results suggest that individual as well as combined genotypes of miRNA-related variants may be used to predict the risk of OPL, and may prove useful for identifying patients with OPL at high risk for progression to oral cancer.

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Table 1

MicroRNA-related genes and polymorphisms evaluated in this study

Gene name	Polymorphism	Position	Nucleotide change	Amino acid change
miRNA biogenesis pathway				
DGCR8	rs1640299	3′ UTR	T>G	
	rs3757	3′ UTR	G>A	
	rs417309	3′ UTR	G>A	
RAN	rs14035	3′ UTR	C>T	
DICER	rs13078	3′ UTR	T>A	
	rs3742330	3′ UTR	A>G	
TRBP	rs784567	Promoter	C>T	
AGO1	rs595961	Intron 10	A>G	
	rs636832	Intron 8	G>A	
AGO2	rs4961280	Promoter	C>A	
GEMIN4	rs2740348	nsSNP	G>C	Glu>Gln
	rs3744741	nsSNP	C>T	Arg>Gln
	rs4968104	nsSNP	T>A	Val>Glu
	rs7813	nsSNP	C>T	Arg>Cys
	rs910924	Promoter	C>T	
GEMIN3	rs197388	Promoter	T>A	
	rs197412	nsSNP	T>C	Ile>Thr
	rs197414	nsSNP	C>A	Arg>Ser
Pre-miRNAs				
miR-196a-2	rs11614913	Pre-miRNA	C>T	
miR-423	rs6505162	Pre-miRNA	A>C	
miR-492	rs2289030	Pre-miRNA	C>G	
miR-604	rs2368392	Pre-miRNA	C>T	
miR-608	rs4919510	Pre-miRNA	C>G	
Pri-miRNAs				
let7f-2	rs17276588	5′ region	G>A	
miR-26a-1	rs7372209	5′ region	C>T	
miR-30a	rs1358379	5′ region	A>G	
miR-30c-1	rs16827546	5′ region	C>T	
miR-100	rs1834306	5′ region	C>T	
miR-124-1	rs531564	5′ region	C>G	
miR-219-1	rs213210	3′ region	T>C	
miR-373	rs12983273	5′ region	C>T	

Abbreviation: nsSNP, nonsynonymous SNP, UTR, untranslated region.

Table 2

Distribution of selected host characteristics by case-control status

Variables	Cases, (n = 136)[2]	Controls, (n = 136)[2]	*P*-value[3]
Age, mean(SD)	57.65(12.91)	58.76(12.48)	0.47
Sex, n (%)[1]			
Male	81(59.56)	81(59.56)	
Female	55(40.44)	55(40.44)	1.00
Smoking status, n (%)[1]			
Never	42(30.88)	79(58.09)	
Former	54(39.71)	46(33.82)	
Current & Recent Quitters	40(29.41)	11(8.09)	<0.0001
Ethnicity, n (%)[1]			
White	122 (89.71)	122 (89.71)	
Hispanic	9 (6.62)	9 (6.62)	
Black	5 (3.68)	5 (3.68)	1.00

[1]Percentages might not sum to 100% because of rounding.

[2]Values might not sum to 136 because of missing data

[3]*P*-value for student's t-test (continuous variables) or χ^2 test (categorical variables).

Table 3

Selected Genetic Polymorphisms in miRNA Related Pathway and OPL Risk

Gene	SNP	Genotype	Cases	Controls	OR (95% CI)[1]	*P*-value
DICER	rs3742330	AA	99	120	Ref	
		AG + GG	29	16	2.09 (1.03–4.24)	0.040
TRBP	rs784567	CC + CT	94	106	Ref	
		TT	38	28	1.67 (0.92–3.04)	0.092
GEMIN4	rs3744741	CC	102	94	Ref	
		CT + TT	33	42	0.60 (0.33–1.10)	0.097
GEMIN3	rs197412	TT	54	43	Ref	
		TC + CC	80	93	0.58 (0.33–0.99)	0.048
miR-26a-1	rs7372209	CC	57	75	Ref	
		CT + TT	76	57	2.09 (1.23–3.56)	0.007

[1]Adjusted for age, gender, ethnicity and smoking status

Table 4

Cumulative Effect Analysis by the Number of Unfavorable Genotypes[1] from miRNA SNPs and OPL Risk

Unfavorable genotypes[1]	Cases	Controls	OR (95% CI)[2]	*P*-value
Low risk (0~1)	24	53	Ref	
Medium risk (2~3)	84	76	3.52 (1.81–6.84)	0.0002
High risk (4~5)	14	3	21.35 (5.08–89.79)	<0.0001
P for trend				<0.0001

[1]Unfavorable genotypes: rs197412(TT), rs3742330(AG + GG), rs3744741(CC), rs7372209(CT + TT), rs784567(TT).

[2]OR adjusted for age, gender, ethnicity, and smoking status