Impacts of MicroRNA Gene Polymorphisms on the Susceptibility of Environmental Factors Leading to Carcinogenesis in Oral Cancer

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Abstract

Background: MicroRNAs (miRNAs) have been regarded as a critical factor in targeting oncogenes or tumor suppressor genes in tumorigenesis. The genetic predisposition of miRNAs-signaling pathways related to the development of oral squamous cell carcinoma (OSCC) remains unresolved. This study examined the associations of polymorphisms with four miRNAs with the susceptibility and clinicopathological characteristics of OSCC.

Methodology/Principal Findings: A total of 895 male subjects, including 425 controls and 470 male oral cancer patients, were selected. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real-time PCR were used to analyze miRNA146a, miRNA196, miRNA499 and miRNA149 genetic polymorphisms between the control group and the case group. This study determined that a significant association of miRNA499 with CC genotype, as compared to the subjects with TT genotype, had a higher risk (AOR = 4.52, 95% CI = 1.24–16.48) of OSCC. Moreover, an impact of those four miRNAs gene polymorphism on the susceptibility of betel nut and tobacco consumption leading to oral cancer was also revealed. We found a protective effect between clinical stage development (AOR = 0.58, 95% CI = 0.36–0.94) and the tumor size growth (AOR = 0.47, 95% CI = 0.28–0.79) in younger patients (age <60).

Conclusions: Our results suggest that genetic polymorphism of miRNA499 is associated with oral carcinogenesis, and the interaction of the miRNAs genetic polymorphism and environmental carcinogens is also related to an increased risk of oral cancer in Taiwanese.

Introduction

MicroRNAs (miRNAs) are small fragment RNAs, which contain approximately 20–22 nucleotides, can target specific miRNAs and negatively regulate their translational efficiency and stability [1]. Previous findings have shown that one miRNA could influence expression of several target genes. According to regulating different target RNAs, miRNAs can participate in cellular processes including proliferation, differentiation, and survival [2]. Altered miRNA expression has been observed in several diseases, especially in cancer [3].

In Taiwan, oral cancer is the fourth most common cancer among men. A rapid increase of incidence of oral cancer occurred in the past few years and crude mortality rate of oral cancer was 10.1 per 100,000 in 2007 and ranked as the sixth cause of cancer death [4]. In the past few years, incidence of oral cancer was particularly high in South Asia. This phenomenon appears especially in populations accustomed to chewing areca (betel nut [5]. Epidemiological studies have also suggested that the susceptibility of oral cancer is mediated by both environmental carcinogens (including alcohol intake, tobacco consumption, and betel nut chewing) and genetic factors [6]. Increasingly more evidence shows that miRNAs are associated with head and neck/oral cancer [7], and several miRNAs have been shown to be unregulated in head and neck cancer [8]. This relationship has also been determined in laboratory research [9].

Single nucleotide polymorphism (SNP) is a variation in the DNA sequence that occurs when nucleotides (A, T, C or G) change in at least 1% of a certain population. Some epidemiologic evidence shows that miRNA genetic variations are associated with progression to oral cancer [10,11]. While miRNAs have received considerable attention in recent years, SNPs in miRNA and premiRNA sequences have been discovered to be connected to their candidate genes [12]. In dbSNP database, over 400 miRNAs SNPs have been documented. To obtain adequate power for evaluating the potential association, we investigated miRNA146a
(rs2910164), miRNA149 (rs2292832), miRNA196 (rs11614913), and miRNA1499 (rs3746444), those with minor allele frequencies ≥5% and also located at the pre-miRNA regions in the Chinese populations [13,14]. In another aspect, these four miRNA SNPs have been reported as important for tumorigenesis due to their targeting on several important genes [13]. Therefore, these four miRNA SNPs were selected in this study. We also considered a unique phenomenon in South Asia, where most oral cancer individuals have a betel nut chewing habit. We combined clinical status and laboratory outcomes to determine the relationship between these SNPs and the susceptibility of oral cancer.

**Results**

The statistical analysis of demographic characteristics is shown in Table 1. There were significant differences in the distributions of betel-quid chewing \( (p < 0.001) \), alcohol consumption \( (p < 0.001) \), and tobacco use \( (p < 0.001) \) between healthy control subjects and OSCC patients. Because these differences between the two groups may be confounders, we adjusted these characteristics in further statistical analysis.

The distribution of miRNA SNP genotypes are described in Table 2. In our healthy controls, miRNA polymorphisms (rs2910164, rs11614913, and rs3746444) conformed to the Hardy-Weinberg equilibrium, except for rs2292832 \( (p < 0.001) \). We used logistic regression model to estimate the adjusted odds ratios \( (AORs) \). After adjusting age, smoking status, alcohol intake and betel nut chewing habits, we found that miRNA1499 CC genotypes exhibited significantly \( (p < 0.05) \) higher risks of 4.52 \((95\% CI = 1.24–16.48)\) of having OSCC compared to the corresponding wild-type (WT) homozygotes. However, there was no significantly higher oral cancer risk for individuals with the miRNA146a, miRNA149 and miRNA196 polymorphic gene compared to those with the WT gene.

Considering that risk factors such as betel nut chewing may modify the genetic susceptibility to oral cancer, and the interactive effects between environmental risk factors and genetic polymorphisms of miRNAs are shown in Table 3, Table S1 and Table S2. Subjects with at least one C allele of miRNA1499 and a betel nut-chewing habit had respective higher risks of 17.33 \((95\% CI = 5.06–14.89)\), 10.98 \((95\% CI = 6.21–19.39)\) of having oral cancer (Table S1). Similarly, tobacco consumption significantly elevated the oral cancer risk in subjects polymorphic for miRNA146a, miRNA149 and miRNA196 compared to individuals with the WT gene but without smoking (Table 3 and Table S2). We further evaluated the gene-environment statistical interaction between the miRNA polymorphisms, smoking and betel quid on oral cancer (Table 3, Table S1 and Table S2). Statistical significance was found for the interaction between all miRNA polymorphisms, smoking and betel quid on oral cancer development \( (p < 0.05) \). The above results suggest that miRNAs gene polymorphisms have a strong impact on oral cancer susceptibility in betel nut and/or smoking consumers.

To explore the impacts of polymorphic genotypes of miRNAs on the clinical status of OSCC, we further classified OSCC patients into two subgroups: one subgroup with at least one polymorphic allele, and the other subgroup with homozygous WT
alleles. Data of the statistical analysis showed that those with polymorphic miRNA499 gene had a protective effect of the tumor size growth (AOR = 0.46, 95% CI = 0.29–0.72) as compared to the patients with wild type (Table 4). However, no significant association between miRNA416a, miRNA149 and miRNA196 gene polymorphisms and the clinicopathologic covariates were observed (data not shown). Moreover, compared to the WT genotype (T/T), patients with at least one polymorphic C allele of miRNA499 showed a protective effect between clinical stage development (AOR = 0.58, 95% CI = 0.36–0.94) and the tumor size growth (AOR = 0.47, 95% CI = 0.28–0.79), in younger patients (age < 60), as shown in Table S3. However, no significant association between miRNA499 gene polymorphisms and the clinicopathologic covariates were observed in elderly patients (age ≥ 60) (Table S4).

Discussion

In this study, we provided novel information of SNPs of miRNA499 on the association of the oral cancer susceptibility. After combining the major risk factor of oral cancer, these genetic affects can also increase the susceptibility of oral cancer. Considering previous findings, miRNA499 has been determined to exhibit high expression in heart and skeletal muscle tissues [16]. Particularly in the heart, previous studies have also found that high expression of miRNA499 increases the risk of cardiomyocyte hypertrophy and cardiomyopathy. Furthermore, some evidence suggests that miRNA499 can regulate mitochondrial dynamics.

| Variable | Control | Case | OR     | AOR<sup>a</sup>   |
|----------|---------|------|--------|-------------------|
|          | N = 425 (%) | N = 470 (%) | (95% CI) | (95% CI)         |
| TT and non-chewing | 283 (66.59) | 75 (15.96) | Reference | Reference         |
| CT or CC or consumer | 125 (29.41) | 288 (61.28) | 8.69 (6.25–12.09)* | 5.95 (4.20–8.44)* |
| CT or CC with betel nut chewing | 17 (4.00) | 107 (22.77) | 23.75 (13.41–42.06)* | 17.33 (9.63–31.17)* |

Test for interaction χ² = 31.28 (1 d.f.), p < 0.001

| Variable | Control | Case | OR     | AOR<sup>b</sup> |
|----------|---------|------|--------|------------------|
|          | N = 425 (%) | N = 470 (%) | (95% CI) | (95% CI)         |
| TT and non-smoker | 185 (43.53) | 40 (8.51) | Reference | Reference        |
| CT or CC or smoker | 209 (49.18) | 320 (68.09) | 7.08 (4.83–10.39)* | 3.03 (1.95–4.70)* |
| CT or CC with smoking | 31 (7.29) | 110 (23.40) | 16.41 (9.71–27.74)* | 6.31 (3.42–11.65)* |

Test for interaction χ² = 5.30 (1 d.f.), p = 0.02

<sup>a</sup>AOR adjusted, age, smoking status and alcohol intake.
<sup>b</sup>AOR adjusted, age, alcohol intake and betel nut chewing.

<sup>*</sup>p < 0.05.

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| Gene | TT | CT/CC | OR   | AOR<sup>+</sup> |
|------|----|-------|------|------------------|
|      | N = 339 | N = 131 | (95% CI) | (95% CI)         |
| Clinical Stage | | | | |
| Stage I+II | 143 (42.18) | 69 (25.67) | Reference | Reference        |
| Stage III+IV | 196 (57.82) | 62 (47.33) | 0.66(0.44–0.98)* | 0.66 (0.44–1.00) |
| Tumor Size | | | | |
| T1+T2 | 195 (57.23) | 98 (74.81) | Reference | Reference        |
| T3+T4 | 145 (42.77) | 33 (25.19) | 0.45(0.29–0.71)* | 0.46 (0.29–0.72)* |
| Lymph node metastasis | | | | |
| Negative | 215 (63.42) | 88 (67.18) | Reference | Reference        |
| Positive | 124 (36.58) | 43 (32.82) | 0.85 (0.55–1.30) | 0.86 (0.56–1.32) |
| Cell differentiation | | | | |
| Well differentiated | 46 (13.57) | 20 (15.27) | Reference | Reference        |
| Moderately or poorly differentiated | 293 (86.43) | 110 (84.73) | 0.87 (0.49–1.54) | 0.87 (0.49–1.54) |

<sup>*</sup>AOR adjusted, age, smoking status, alcohol intake and betel nut chewing.

<sup>p</sup>< 0.05.

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through by targeting specific proteins such as p53\cite{17}. Previous
studies have also shown that miRNA499 is highly associated with
heart diseases by regulate cellular differentiation and proliferation
\cite{18,19}. These findings show that miRNA499 is possibly
associated with heart diseases and also carcinogenesis.

Based on the clinical status, previous studies have found that
miRNA-486, miRNA-30d, miRNA-1, and miRNA-499 exhibited
high expression in serum and were also associated with survival in
non-small-cell lung cancer\cite{20}. Other studies have found that
the genetic variant in pre-miRNA may contribute to the process of
carcinogenesis in breast cancer\cite{14,21}, gastric cancer\cite{22},
cervical cancer\cite{23,24}, and head and neck cancer\cite{15}. In this
study, we first demonstrated that polymorphism of miRNA499 is
associated with oral cancer.

Previous studies have found that miRNA146a may contribute to
the tumor progression, metastasis, prognosis, and survival in
gastric cancer and oral cancer\cite{25,26}. Another finding also
showed that miRNA146a polymorphism can regulate miRNA-146a
expression\cite{27}. Several laboratory studies have also
discovered that miRNA146a could inhibit cell differentiation and
survival in the hematopoietic system\cite{28}. Moreover, connected to
cancer, miRNA146a could suppress cell invasion in pancreatic
cancer\cite{29}. In detail, some evidence connects miRNA146a with
transcription factors such as NF-kB\cite{30}. These findings provide a
new vision that miRNA146a could affect cancer progression by
regulating cell differentiation but may not affect apoptosis.
Although we found no association between miRNA146a and
cellular clinical statuses in our study, based on these findings, future
studies could consider joint prognosis status or even survival rate.

Recent studies have identified that miRNA196 may interact
with several transcription factors and involve in cancer development
and progression\cite{31,32}. Some findings suggest that
overexpression of miRNA196 leads to more favorable prognosis and
survival in leukemia\cite{33}. In addition, miRNA196 is
associated with inflammation in specific cancers\cite{34}. Further-
more, Christensen et al., reports a polymorphism in the mature
sequence of miRNA196a2 in a case-control study (n = 1,039) of
head and neck squamous cell carcinoma (HNSSC)\cite{35}. When
the authors stratified on tumor site they did not observe a
significant association between oral cancer and miRNA196a2,
though the effect estimate was protective, similar to the results
presented in this study. Although the reason for those discrepan-
cies is not well-known, the different results from the report and
the present study may relate to the racial/ethnic difference.

Considering that the causes of carcinomas are complex, we
analyzed several risk factors, such as smoking status and betel nut
chewing habits, in this study. We expect that these gene
polymorphisms could influence the susceptibility of oral cancer.
Based on the results, we observed that these polymorphisms
significantly increased the odds ratio in each group, possibly
because the functions of these miRNAs regulate the differentia-
tions and proliferations in tumor progression. We also found that
genetic polymorphism of miRNA499 is associated with distal
metastasis of OSCC. A previous study for colorectal cancer
has found that overexpression of miRNA499 may facilitate the
migration and invasion of cancer cells in vitro, as well as the
metastasis to lung and liver in vivo\cite{36}. Additionally, this study
also identified forkhead box O4 (FOXO4) and programmed cell
death 4 (PDCD4) as direct and functional targets of miRNA499
\cite{36}. Moreover, Reis et al., also reports that PDCD4 as a
suppressor of migration and invasion and may be a clinically
relevant biomarker with prognostic value in OSCC\cite{37}. These
above-mentioned findings may provide a preliminary explanation
with our finding for the association between miRNA499 and distal
metastasis of OSCC. Detailed relevant mechanisms may warrant
further studies.

One of the limitations of our study is that information on
alcohol, betel nut, and tobacco use is dichotomized into “ever-
user” versus “never-user.” As the result, more detailed analysis
based on amount, length, and past history of betel nut, alcohol,
and tobacco consumption were not able to be performed. Data
collection relied on self-reports, for which some individuals may be
reluctant to report their habitual use of such substances. Hence,
there may be residual confounding effect from betel nut, alcohol,
and tobacco use misclassification. Furthermore, the functional role
of miRNA in growth or metastasis of oral cancer is worth for
further investigation, which will be included in our future work. Clones
containing various genotypes of miRNA499 SNPs will be
constructed to elucidate the possible functions of miRNA499
(proliferation, cell cycle regulation, migration and invasion) in oral
cancer cell lines, as well as the underlying mechanisms.
Furthermore, this study revealed the nonconformity of miRNA
149 (rs2292832) genotypes to Hardy-Weinberg equilibrium in the
control group. A previous study with 107000 genotypes generated
from 443 SNPs has found that genotype distributions for 36 out of
313 assays (11.5%) were deviated from HWE\cite{38}. Upon
searching for the possible reasons, assays for SNPs proved
nonspecific or genotyping errors have been identified. However,
they also found the deviation from HWE for the remaining 10
SNPs was unexplainable. Although the reason for the nonconfor-
mity of miRNA 149 genotypes to HWE in our control group is not
explored yet, results from the abovementioned study may provide
some direction for our future study.

In conclusion, we discovered a significant association between
miRNA499 gene polymorphisms and the susceptibility of oral
cancer. However, there are no connections between the SNPs and
clinical status. After considering betel nut chewing, which is the
most influential factor of oral cancer, we found that those four
miRNAs which carrying the mutation genotype may increase the
susceptibility of oral cancer. These gene mutations may affect
miRNA target efficiency and stability and increase the incidence of
oral cancer, but the detailed mechanisms from gene levels to
protein levels that ultimately affect tumor development should be
classified, perhaps charting a new direction for target therapy.

Materials and Methods

Subjects and Specimen Collection

We recruited 470 male patients at Chung Shan Medical
University Hospital in Taichung and Changhua Christian
Hospital and Show Chwan Memorial Hospital in Changhua,
Taiwan as a case group between 2007 and 2011. Meanwhile,
controls were enrolled from the physical examination during those
days, which are also the facilities that cases were
collected from. At the end of recruitment, a total of 425 male
participants that had neither self reported history of cancer of any
sites were included. In addition, subjects with oral precancerous
disease such as oral submucous fibrosis, leukoplakia, erythroplakia,
 verrucous hyperplasia, etc. were excluded from control group. The
participation rate was approximately 91% for cases and 76% for
controls. Since all cases and controls were consecutively collected
without any selection, and based on information provided on
questionnaires, no significant genetic relationship or family history
were found.

As for cases and controls, exposure information, including betel
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vs. not current heavy drinker), were all obtained from questionnaires. While medical information of cases, including TNM clinical staging, primary tumor size, lymph node involvement and histologic grade, were obtained from medical records. Oral cancer patients were staged clinically at the time of diagnosis according to the TNM staging system of the American Joint Committee on Cancer (AJCC) [39]. Tumor differentiation examined by pathologist according to AJCC classification. This study has been reviewed and approved by Institutional Review Board and informed written consent was obtained from each individual.

DNA Extraction
The whole blood samples, collected from healthy controls and oral cancer patients, were placed into tubes containing EDTA, after centrifuged and stored at −80°C. Venous blood from each subject was drawn into vacutainer tubes containing EDTA and stored at 4°C. Genomic DNA was extracted by QiAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacture’s instructions. DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD260. Final preparation was stored at −20°C and used as templates for polymerase chain reaction.

Polymerase Chain Reaction-restriction Fragment Length Polymorphism (PCR-RFLP)
The miRNAs gene rs2910164, rs11614913, and rs3746444 polymorphisms were determined by PCR-RFLP assay [15]. The primers sequences and the restriction enzyme for analysis of those miRNAs gene polymorphisms were described in Table S3. PCR was performed in total 10 µl volume containing 100 ng DNA template, 1.0 µl of 10× PCR buffer (Invitrogen, Carsibad, CA, USA), 0.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs (Promega, Madison, WI, USA) and 200 nM of each primer (MBBio Inc. Taipe, Taiwan). The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C for miRNA146a, 1 min at 63°C for miRNA196a2, and 1 min at 67°C for miRNA499, and 2 min at 72°C, with a final step at 72°C for 20 min to allow a complete extension of all PCR fragments. The results were shown in Figure 1. For each assay, appropriate controls (nontemplate and known genotype) were included in each typing run to monitor reagent contamination. To validate results from PCR-RFLP and for quality control, around 10% of assays were repeated from different batches and several cases of each genotype were confirmed by the DNA sequence analysis.

Real-time PCR
The allelic discrimination of the miRNA149 rs2292832 gene polymorphisms was assessed with the ABI StepOne™ Real-Time PCR System (Applied Biosystems) and analyzed using SDS v3.0 software (Applied Biosystems), using the TaqMan assay. The final volume for each reaction was 5 µl, containing 2.5 µL TaqMan Genotyping Master Mix, 0.125 µL TaqMan probes mix, and 10 ng genomic DNA. The real-time PCR reaction included an initial denaturation step at 95°C for 10 min, followed by 40 cycles, each consisting of 95°C for 15 sec and 60°C for 1 min.

Statistical Analysis
The distributions of demographic characteristics and genotype frequencies between cases and controls as well as clinicopathological features in different genotypes were analyzed by Fisher’s exact test, since the small sample size was present in some categories of variables. The odds ratios (ORs) with their 95% confidence intervals (CIs) of the association between genotype frequencies and oral cancer were estimated by multiple logistic regression models, after controlling for covariates. Nonparametric method was used due to not normal distribution of some estimated variables. We fitted a logistic regression model with main effects (miRNAs genetic polymorphism, smoking status and betel quid chewing status), as well as an interaction term between them (miRNAs genetic polymorphism*demographic characteristic), comparing the model against a model with only the main effects. Interaction effect was defined as the difference of their deviance, and further assessed using the likelihood ratio test to calculate χ² and p values [40]. A p value of less than 0.05 was considered significant. The data were analyzed on R statistical software.

Supporting Information
Table S1 Association of miRNA genotype and betel nut chewing status.
(DOC)
Table S2 Association of miRNA genotype and smoking status.
(DOC)
Table S3  Relationship of clinical status and miRNA499 genotypes in oral cancer patients (≤60 only, N = 332).

(DOC)

Table S4  Relationship of clinical status and miRNA499 genotypes in oral cancer patients (>60 only, N = 138).

(DOC)

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Table S5  Primer sequences and PCR conditions for amplification of miRNA SNPs.

(DOC)

Author Contributions

Conceived and designed the experiments: SFY. Performed the experiments: YHC CWL. Analyzed the data: MKC SFY MHC. Contributed reagents/materials/analysis tools: MKC SFY SLT. Wrote the paper: YHC SFY.