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Original article

miRNA genetic variants: As potential diagnostic biomarkers for oral cancer

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ABSTRACT

MicroRNAs (miRNAs) comprise a novel class of small, non-coding endogenous RNAs that have a role in the plethora of regulatory activities by directing their target mRNAs for degradation or translational repression. Single nucleotide polymorphisms (SNPs) in miRNA genes can lead to alteration in mRNA expression, resulting in serious consequences. Detection of miRNA-polymorphisms can potentially improve diagnosis, treatment, prognosis in patients and has extreme implications in the fields of pharmacogenomics and personalization of medicine. The aim of this study is to investigate the association between miR-499 A/G and miR-149 C/T polymorphisms with susceptibility to development of Oral Squamous Cell Carcinoma (OSCC). 200 histopathologically diagnosed and confirmed samples from OSCC patients and 200 control samples from the general population were recruited for the study. All OSCC patients were graded based on their differentiation, and genetic analysis was performed by PCR-RFLP and sequencing. qRT-PCR was carried out to compare the expression of miR-499 and miR-149 in different grades of various stages of OSCC patients concerning to the controls. Further Immunohistochemistry (IHC) was performed to study the target gene of miR-499. The study shows a probable association of miR-499 A/G and miR-149 C/T with susceptibility of OSCC. Random sequencing analysis and Immunohistochemistry contribute to the result that miR-499 A/G increases the susceptibility of OSCC by targeting SOX-6. PCR- Restriction Fragment Length Polymorphism (RFLP) and multivariable logistic analysis revealed that there is a significant association between miR-149 CT + TT and CT and susceptibility of OSCC. Our study suggests that miR-499 A/G and miR-149 C/T polymorphisms may play crucial roles in susceptibility and development of OSCC in Indian population.

1. Background

Oral Squamous Cell Carcinomais defined as the cancer of lip, tongue, and mouth which is becoming a serious and growing problem across the globe. OSCCis rated as one of the top three cancers occurring in India [1]. Severe use of alcohol, tobacco like cigarettes, smokeless tobacco, betel nut chewing, and human papilloma virus (HPV) are the most likely causes of OSCC. Most of the time, OSCC is diagnosed in the latter stages which lead to increase in the cost of treatment and decrease in treatment outcomes.

In the previous century, physicians and surgeons believed that diagnosis and treatment of the tumor with surgery and radiation therapy were the only options; this resulted in poor prognosis and caused more cancer-related deaths. In the present biological era, the prognosis of this deadly disease has improved to some extent because of the enhancement of technologies [2], but diagnosing the tumor at its initial stages relapse, and metastasis are the major challenge to improve the scope of patient survival [3]. These targets can be achieved by giving more emphasis on the molecular aspects of Oral Squamous Cell Carcinoma and biomarkers that act at the molecular levels. Among these biomarkers, miRNAs are the ones that have gained attention as the crucial regulators of neoplastic transformation.

miRNAs are small non-coding RNA molecules that are 18–25 nucleotides in length. Besides the fact that they are noncoding in nature still, they influence the expression of many protein-coding genes [4]. At the molecular extent, they are related to mRNA degradation and translation, thus affecting several biological processes such as cell proliferation, differentiation, migration, apoptosis, and signal transduction [5–7]. Thereby, these can be implicated in tumor development through the modification of cellular levels of specific oncogenes or

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tumor suppressor genes [8,9]. Besides the key biological roles of miRNAs, their expression levels have been shown to be linked with the clinicopathological variables as well as to have diagnostic and prognostic importance [10].

Now it is well established that tobacco, betel quid, and alcohol users are more prone to develop OSCC, But not all the patients who are tobacco or alcohol users develop OSCC. Now, this fact of variability can be explained by the term Genetic disposition. A variation in pre-miRNA sequence can affect the transcriptional step of pri-miRNA, mature miRNA processing, and alteration of their target genes, which in turn will influence the susceptibility of OSCC [11–13].

Genetic variation or polymorphisms in the miRNA pathway are concomitant to the prognosis and progression of OSCC and drug responses [14,15]. For this reason, the detection of miRNA polymorphism will probably improve the diagnosis, treatment, and prognosis of OSCC in patients as well as make it a powerful mechanism to study the its-biology [16]. In this study, we have investigated the effect of miR-149 (T > C) and miR-499 (A > G) concerning the susceptibility of OSCC. Many of the studies already conducted on a vivid range of people, and these studies have proved the association of these miRNA gene polymorphisms with various cancers. Studies have also shown clear association of miRNA gene with lung cancer because lung cancer and OSCC share the same etiology (i.e., tobacco). By considering this clue, we have investigated their association with OSCC in North Indian population. Moreover, to the best of our knowledge, no study has been conducted so far to assess whether polymorphism in both *miR-149* and *miR-499* genes are associated with OSCC susceptibility in North Indian population.

2. Material and methods

2.1. Identification of study subjects and case & control sample collection

A total of 200 OSCC patients from Lucknow Cancer Institute were contacted for this study. This study had been reviewed and approved by the ethics committee of CSIR-CDRI, Lucknow. The information gathered from the patients or their attendants were obtained only after getting written consent from them.

A] Inclusion criteria for cases:

1. All stages of clinically and histopathologically diagnosed OSCC cases
2. Patients more than 18 years of age

Inclusion criteria for controls:

1. Patients with no history of cancer or any chronic illness
2. Patients more than 18 years of age

B] Exclusion criteria for both cases and controls

1. Patients suffering from known systemic diseases like cardiovascular diseases, diabetes mellitus, osteoporosis, Alzheimer's disease and renal diseases.
2. Patients suffering from infectious diseases like HIV/AIDS, blood cancer, lung cancer, breast cancer and other malignancies.
3. Patients suffering from known systemic nutritional deficiencies
4. Pregnant or lactating females
5. Patients who refuse to give consent

Each subject was requested to fill a questionnaire by providing his or her demographic information and the usage status of tobacco and alcohol. Information on tumor subtype and stage of disease were obtained from medical records of the patients.

Blood samples (1–2 ml) were collected from both the cases and controls by venepuncture and then the blood samples were transferred to vacutainer tubes containing EDTA as anticoagulant with which the blood was immediately mixed to prevent clotting. The vacutainers were storedat 4 °C until further processing. Total 70 tissue specimens of cancerous and 20 peripheral control tissues of oral cancer region were gathered. After collecting a part of the tissues, these were immediately submerged in formal saline, and another part in RNAlater solution [Thermo Scientific] and kept on −20 °C till processing.

2.2. Cell culture and maintenance

Human tongue squamous cell carcinoma cell line CAL 27 was procured from American Type Culture Collection (ATCC, USA). Human embryonic kidney HEK-293 cell line was purchased from cell culture repository of National Centre for Cell Sciences (NCCS, Pune, India). Both the cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were maintained in 5% CO 2 incubator at 37 0C under humidified condition.

2.3. DNA isolation from cancer and control samples

DNA was isolated from the blood of Oral Squamous Cell Carcinoma (OSCC) cases and the control samples using blood mini kit (Qiagen, Germany) according to manufacturer's protocol. DNA was quantified by UV–vis spectrophotometer (Quawell Q5000, San Jose, CA USA). Furthermore, DNA quality was assessed by agarose gel electrophoresis. DNA samples were stored at −20 °C.

2.4. RNA isolation from tissue and cDNA preparation

40–50 mg tissue was taken in 500 µl of all protect reagent for stabilizing the tissue content. Before RNA isolation, the tissues were equilibrated at room temperature, and RNA was isolated manually using Trizol (Thermo Fisher Scientific). RNA samples were stored at −80 °C. About 1 µg of RNA was used in cDNA preparation. cDNA was prepared by Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's protocol. Prepared cDNA was quantified using UV–vis spectrophotometer and kept at −20 °C for further use.

2.5. Histopathology of tissue samples

50 mg OSCC tissue samples were stored in formal saline for performing histopathological grading of cases. Sections were paraffin embedded and cut by a microtome. Next, sections were mounted on slides for hematoxylin and eosin staining. After staining, the sections were graded according to their differentiation.

2.6. PCR standardization and amplification

Gradient PCR was carried out for the standardization of DNA amplification conditions and optimization of annealing temperature for *miR-499* and *miR-149*. Further, PCR reactions were performed for cases and control samples using specific primers PCR of *miR-149*; C > T using the following primer sequence; forward primer **5′-CATCTCATGTCCAGGACCACAACCTGT-3′** andreverse primer **5′-AAACACCCGTAAGATATGGGAGCTCCC-3′** were carried out to yield 470 bp product. Similarly, PCR was performed for *miR-499*, A > G using the following primer sequence; forward primer **5′-CCTTGTCTCTATTAGCTGAGCCCCCA-3′** and reverse primer **5′-GGGAAAGGCACAAAGGGGGCA-3′** were carried outto generate499 bp product. The PCR conditions included an initial denaturation step at94 °C for 5 min, followed by for 30 cycles at 94 °C for 30s, by annealing at 60° C for 30s, extension at 72 °C for 30 s and the final extension at 72 °C for 2 mins in case of the *miR-499* gene. However, for *miR-149* annealing was performed at 58 °C. Gene products were then visualized on 1.2% agarose gel.