Analyzing the Expression of MicroRNA-375 and its Target Gene p53 in Oral Squamous Cell Carcinoma and its Implication in Oral Carcinogenesis

Sangeetha Narasimhan¹*, Malathi Narasimhan², Shishir Ram Shetty¹, Sharada T Rajan², Sausan Al Kawas¹ and Vijaya Nirmala Subramani²

¹Department of Craniofacial Health Sciences, College of Dental Medicine, University of Sharjah, Sharjah, United Arab Emirates.
²Sri Ramachandra Dental College, Department of Oral Pathology and Microbiology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India.
*Corresponding Author E-mail: snarasimhan@sharjah.ac.ae

https://dx.doi.org/10.13005/bpj/2781

(Received: 06 September 2023; accepted: 07 October 2023)

Oral mucosal cancers are the 11th most common human malignancies worldwide with a five-year survival rate of ≤ 50%. The lacunae of reliable diagnostic and prognostic markers pose an enormous challenge to the timely identification and prediction of disease progression in oral cancer. MicroRNAs (miRNAs) are emerging molecular markers associated with cancer initiation, progression, and therapy. The present study evaluated the microRNA-375 (miR-375) expression and its target p53 gene in Oral squamous cell carcinoma (OSCC) to validate its utility as a diagnostic marker of the disease. This case-control study targeted histopathologically diagnosed cases of OSCC. miR-375 was quantified from 22 cases of OSCC and corresponding control tissues using qRT-PCR. Mutant p53 expression in cases and controls was determined by subjecting the tissues to immunohistochemical analysis. Significant downregulation of miR-375 was noted in OSCC tissues (68.1%) compared to the control tissues with a mean fold change of 83.9 (p<0.05). Significant downregulation of miR-375 was noted in Paan and tobacco chewing patients (77.8%). Men exhibited considerable downregulation compared to women (p<0.05). The miR-375 expression levels did not correlate with the patient’s age, location, size, nodal status, and histopathological grade of the tumor. About 63.6% of OSCC tissues showed mutant p53 positivity. Mutant p53 expression was noted in 73.3% of miR-375 downregulated tumors. Smokers exhibited higher expression of mutant p53 contrary to non-smokers (p<0.00). P53 immunopositivity showed a correlation with tumor size, histopathological grade, and nodal metastasis. The findings of the study indicate that miR-375 downregulation may have a crucial effect on oral carcinogenesis by targeting p53. miR-375 should be further evaluated as a potential marker for oral cancer diagnosis.

Keywords: Immunohistochemistry; Oral cancer; MicroRNA; RT-PCR; p53; Tumor suppressor gene.
tool. Results of the research studies conducted during the past several years with similar intentions are yet to be clinically translated4.

MicroRNAs are endogenous RNAs that possess enormous gene modulatory potential. Based on their sequence and complementarity, microRNAs combine with specific mRNAs and regulate protein synthesis either by target mRNA degradation or translation repression5, 6. In this manner, the human microRNAs can control about 60% of the human genome. Every cellular process such as cell differentiation, proliferation, mobility, and apoptosis are subject to microRNA-dependent regulation7. Expression of microRNAs is tightly controlled by various transcription factors. MicroRNA expression is heavily dysregulated in human cancers and compelling evidence has shown that they affect all the hallmarks of cancer8. The variations in the expression of many microRNAs have assisted as diagnostic and prognostic predictors in specific tumors and might become potential and promising therapeutic targets in cancer treatment in the future9.

MicroRNA 375 is a tumor-suppressive microRNA located in 2q3510. It is an islet-specific miRNA that regulates insulin secretion and glucose homeostasis. Anti-tumor effects of miR-375 are potentiated by modulating its oncogenic targets JAK2, IGF1R, AEG-1 and YAP1 genes that moderate many processes like apoptosis, invasion, and metastasis. Many studies reveal that miR-375 is widely present in other human tissues and is significantly downregulated in many malignancies such as melanoma, glioma, Gastric, Laryngeal, Esophageal, and hepatocellular carcinoma11,12.

The p53 is a tumor suppressor gene with pro-apoptotic function13. The p53 transcription factor is considered the “guardian of the genome” as it senses the DNA damage and mediates cell cycle arrest or promotes apoptosis14. Mutations in p53 are associated with genomic instability and increased susceptibility to cancer15. In normal cells, the half-life of p53 protein is very short lasting up to 5-20 minutes making it difficult to be detected in normal tissues16. However, a mutated p53 protein is not easily digested and has a prolonged half-life. Therefore, it accumulates inside the cancer cells and can be immunohistochemically detected in premalignant and malignant tissues17, 18. p53 gene regulates, and in turn, is also regulated by several microRNAs19. p53 has been reported as a gene target for miR-37520,21. There are no previous studies in the literature that studied the association between miR375 and p53 expression in oral cancer. This study analyzed the expression of miR-375 and its gene target p53 in OSCC tissues and correlated their expression to the clinical and pathological parameters of the study samples to understand their role in oral carcinogenesis.

**MATERIALS AND METHODS**

The study was conducted in the Oral Pathology department of the University Dental Hospital. The Institutional Review Board approved the study((SRIHER)- IEC-N1/12/MAR/27) and all the patients signed informed consent before sample collection. The study population comprised histopathologically diagnosed cases of squamous cell carcinoma using incisional biopsy samples. Only the patients with primary tumors were included in the study. Recurrent tumors and cases with previous treatments such as radiotherapy and chemotherapy were excluded from the study. After exclusions about 22 cases were included in the study. Resected tumors and the corresponding adjacent normal mucosa tissues were obtained from OSCC patients. Normal mucosal tissue was obtained from the contralateral side of the lesion proper. Fresh tissue samples were preserved in RNAlater (sigma Aldrich) and stored at -40°C until PCR analysis. Tissue for the histopathological study was stored in formalin at room temperature and routinely processed.

**Quantitative Real-time (qRT-PCR) assay**

The tumor and normal tissues were homogenized using mortar and pestle and extraction of total DNA was performed by the trizol method (Invitrogen, USA). Stem loop primers (table 1) and primerscript RT reagent kit ((Takara Bio Inc) were used for synthesizing the Complementary DNA from the extracted RNA. Reverse transcriptase reactions included 7µl of RNA and 3µl of master mix containing RT buffer, stem loop primers and RT enzyme. 10µL aliquots were prepared and incubated for 15 min at 37°C, and 15 seconds at 85°C, followed by maintenance at 4°C. The primeScript SYBR Green PCR kit (Qiagen NV) was used to perform the qPCR analysis. One microliter of cDNA was used as a template in 10µL
reactions. The master mix was prepared separately for the microRNAs and the control gene with the forward and reverse primers (Table 1), SYBR green dye, ROX dye and water. Gene expression was quantified using triplicates in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The 10µL aliquots PCR plates were denatured for 95°C for 30 seconds, annealed at 95°C for 30 seconds, 60°C for 35 seconds (40 cycles), 95°C for 15 seconds and extended for one minute at 60°C.

**Analysis of relative gene expression**

The relative gene expression in the tumor and normal tissues was calculated using the $2^{-\Delta\Delta C_q}$ method (Livak method) by normalizing it to the U6 housekeeping gene. The first step in the analysis involved the determination of the threshold cycle (Ct) value of individual samples. Using the threshold cycle value of each microRNA and control gene the $\Delta C_t$ value was calculated using the formula $\Delta C_t= C_t \text{miR375} - C_t \text{U6}$. The $\Delta \Delta C_t$ value was calculated from the $\Delta C_t$ values using the formula ($\Delta \Delta C_t= \Delta C_t \text{Cancer} - \Delta C_t \text{Normal}$). Finally, the fold value ($2^{-\Delta \Delta C_t}$ value) was calculated. A Fold value more than 1, is considered as an upregulation of the gene and a fold value of less than 1 signifies a downregulation of the gene.

**Immunohistochemistry (IHC)**

The tumor and control tissues were routinely processed, and 4-um paraffin sections were taken on charged slides. The sections were dewaxed using xylene, dehydrated in graded alcohols, and subsequently rinsed thoroughly using distilled water. Endogenous peroxidase activity was blocked by applying peroxide block to the tissue sections for 5 minutes at 37°C. The pressure cooker method was used for antigen retrieval. Further p53 primary antibody (Mouse monoclonal, clone DO7, class-IgG1 -BioGenex Life sciences Pvt Ltd) was applied to the tissue sections for 1 hour. Further, the sections were thoroughly rinsed with citrate buffer and incubated with universal secondary antibody for 30 minutes (Super Sensitive™ Polymer HRP Kit Manual- BioGenex life sciences Pvt Ltd). Finally, the sections were thoroughly rinsed with distilled water and stained with the chromogen diaminobenzidine (DAB), counterstained with Harris hematoxylin, air dried, cleared and mounted with dibutyl phthalate xylene (DPX). Breast cancer tissues were used as a positive control for mutant p53. Internal negative controls were used to validate the staining.

All the slides were scored by two certified pathologists. Mutant p53 positivity was confirmed by the presence of brown staining of the nucleus of the cells. The sections were considered negative when <5% of the cells were stained for p53. Further positive sections were graded as mild positive (5-25%), moderate positive (25-50%) and intense positive (> 50%) based on the number of cells that were immune-positive for mutant p53.

**Data collection/statistical analysis**

The clinical parameters of the study samples were obtained from the medical records session. The study sample comprised 16 men and 6 women. The mean age of the patients was 54.5. Eleven cases were excised from buccal mucosa, 5 from the tongue, 3 from alveolar mucosa, 2 from the lip, and one from the floor of the mouth. The revised International TMN Staging System was used to assess the tumor stage. 15/22 cases had nodal metastasis of the disease. However, none of the patients had distant organ metastasis confirmed by a PET scan. The date of the surgery was considered the beginning of the disease. Follow-up data was obtained for 5 years. Recurrence was noted in 2 of the 22 cases. The statistical analysis of the study results was performed using version 18 of SPSS software. Descriptive statistics were presented as numbers and percentages. Mean and SD were calculated for all the data. Statistical differences between clinicopathological parameters and the miRNA375 levels were evaluated using non-parametric tests. The difference in microRNA expression between the lesion and control tissues was calculated student T-test. A chi-square test was used for comparison between two attributes. Inter-observer reliability was calculated using Cohens Kappa. A p-value less than 0.05 was considered statistically significant.

**RESULTS**

The study analyzed the MicroRNA375 expression in OSCC and its paired normal tissues by using qRT-PCR. The tissues were also analyzed for the expression of p53 using immunohistochemistry. The microRNA and p53 expression were also compared to the clinical and pathological attributes of the tumor.
Expression of miR-375 in oral cancer and normal tissues

A significant difference was noted in the expression level of miR-375 between the tumor and adjacent normal tissues. Among 22 OSCC cases, 15 (68.1%) tumor samples demonstrated downregulation of miR-375 ($p < 0.05$) (Figure 1). A mean fold change of 83.96 was noted in the tumor tissues compared to the controls.

MicroRNA-375 expression correlation to clinico-pathological parameters

Table 2 displays miR-375 expression based on the clinicopathological parameters. All the cases below 40 years, 50% of cases between 40-60 years and 77.8% of cases above 60 years of age demonstrated miR-375 downregulation. Men showed significant downregulation of miR-375 compared to women ($p < 0.05$). Cases with pan/gutka chewing habits (77.8%) showed significant ($p < 0.05$) miR-375 downregulation compared to cases without habits. Smokers and non-smokers did not exhibit any significant difference in miR-375 expression ($p > 0.05$). 80% of the cases with N2 nodal involvement exhibited downregulation of miR-375. However, this finding was not statistically significant. The miR-375 expression levels did not reveal any statistically significant relationship with patient age, tumor size, location, nodal metastasis, histopathological grade, and survival of the patient. The Kaplan–Meier analysis could not yield a meaningful interpretation.

p53 Expression in OSCC

The positive control breast cancer tissue stained intensely with p53 antibody (Figure 2), and the internal negative controls did not exhibit any p53 positivity. The interobserver variation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-375 stem loop RT primer</td>
<td>5'-GTCGTATCCAGTGGAGGGTCCGAGGTATTCGCACTGG</td>
</tr>
<tr>
<td></td>
<td>ATACGACTACCGCG-3'</td>
</tr>
<tr>
<td>RNU6 stem loop RT primer</td>
<td>5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG</td>
</tr>
<tr>
<td></td>
<td>ATACGACTATGGAAC-3'</td>
</tr>
<tr>
<td>miR-375 Forward Primer</td>
<td>5'-GCCCCTTTTGTTCCGCTTG-3'</td>
</tr>
<tr>
<td>RNU6 Forward Primer</td>
<td>5'-GTGCTCGCTTCCGAGCAC-3'</td>
</tr>
<tr>
<td>Universal Reverse Primer</td>
<td>5'-GTGCAGGGTCCGAGGT-3'</td>
</tr>
</tbody>
</table>

Table 1. Primer sequence for RT- PCR

MiR-375 expression in OSCC

Fig. 1. Downregulation of miR-375 in OSCC
or the measurement of agreement between the observers was statistically analyzed and was highly significant \(p < 0.00\) The kappa score was calculated to analyze the overall significance of p53 expression between the normal and OSCC sections and was statistically significant \(p < 0.00\)

In our study mutant p53 expression was observed in the nuclei of the dysplastic epithelial cells of OSCC. Staining ranged from focal positivity in the tumor islands to intense positivity in sheets of dysplastic squamous cells in the underlying connective tissue (Figure 3-5). Among 22 tumor cases, 14 tissues (63.6%) exhibited immunopositivity for mutant p53 (4 - mild positive; 5- Moderate positive & 5- intense positive).

### Table 2. Clinco-pathological Correlation of OSCC patients to miR-375 and p53 expression

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>miR375</th>
<th>p53 expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Downregulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weak</td>
<td>Moderate</td>
</tr>
<tr>
<td>Age in years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; = 40</td>
<td>3</td>
<td>3</td>
<td>0.191</td>
</tr>
<tr>
<td>41 - 60</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>&gt; 60</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>13</td>
<td>*0.032</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>11</td>
<td>9</td>
<td>0.471</td>
</tr>
<tr>
<td>Tongue</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lip</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Alveolus</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Habits- Pan chewing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>18</td>
<td>14</td>
<td>*0.040</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>9</td>
<td>8</td>
<td>0.083</td>
</tr>
<tr>
<td>Non-Smokers</td>
<td>13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>11</td>
<td>6</td>
<td>0.323</td>
</tr>
<tr>
<td>T2</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>7</td>
<td>5</td>
<td>0.717</td>
</tr>
<tr>
<td>N1</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WDOSCC</td>
<td>12</td>
<td>7</td>
<td>0.33</td>
</tr>
<tr>
<td>MDOSCC</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PDOSSC</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05 Statistically Significant, p > 0.05 Non Significant
patients without nodal metastasis showed weak to moderate immunostaining \((p < 0.05)\). There was no significant association between the mutant p53 expression with age, gender, or the clinical location of the tumor \((P>0.05)\).

The relative expression of mutant p53 was compared with miR-375 expression in OSCC cases and about 73.3\% (11/15) of miR-375 down regulated cases showed mutant p53 expression in OSCC demonstrating a very significant association within the genes.

**DISCUSSION**

MicroRNAs are molecular regulators of physiological cellular processes. MicroRNA dysregulation has been noted in various diseases including cancer\(^8\). In current study analyzed miR-375 expression in 22 OSCC tissues and the corresponding normal tissues. The results reveal that MicroRNA-375 expression is considerably downregulated in OSCC compared to non-tumor tissue which is supported by the previous studies\(^23-29\). In the current study miR-375 was 83.9 \% -fold downregulated compared to the adjacent paired normal tissues. MicroRNA studies have shown a 10 to 22-fold downregulation of miR-375 in OSCC and a 32-fold downregulation in HNSCCs\(^24,26,87,30\). This very high level of downregulation of miR-375 in our study is attributed to a single case of poorly differentiated squamous cell carcinoma which showed a very high Ct value in the PCR analysis.

Identified as a tumor suppressor gene, miR375 suppresses the malignant properties of cancer cells. miR-375 loss has been noted in various human malignancies including gastric,
Pharyngeal, esophageal, hepatic and breast cancers. Lian et al demonstrated that miR-375 targets tyrosine kinase Janus kinase 2 (JAK2) and inhibits cell proliferation in gastric cancer. Further miR-375 could suppress rapid cancer cell growth by inhibiting aerobic glycolysis via the PI3K-Akt signaling pathway. On the other hand, miR-375 upregulation results in cell cycle arrest at the G0/G1 phase in oral cancer cell lines thereby inhibiting tumor growth. MicroRNA-375 downregulation is also a contributing factor for the progression of potentially malignant disorders to oral cancer. Our study results show that miR-375 downregulation in oral cancer showed a positive association with paan chewing habits. Our findings along with the literature support provide evidence that miR-375 loss in oral cancer could contribute to oral carcinogenesis.

Men demonstrated significant downregulation of miR-375 versus women in our study. This distribution could be attributed to a higher number of men involved in the study compared to the women. miR-375 expression did not correlate to the oral cancer disease progression in our study. By contrast, Siow MY et al revealed that miR-375 downregulation is associated with tumor size and disease progression in oral cancer. Zhang B et al reported reduction in miR-375 levels correlates with increased lymph node metastasis and reduced survival rate in OSCC patients.

The p53 gene is one of the most frequently mutated genes in human cancers. About 63.3% of the tumor tissues demonstrated mutant p53 immunopositivity in this study which is consistent with the results of Patil NN et al (61%), Dave KV et al (62%) and Ghanghoria S et al (63%) in OSCC cases. Further previous immunohistochemical studies have documented significant variability in the expression of mutant p53 in oral cancer, spanning from 36% to 80%. Detection of p53 in oral cancer confirms the mutation of p53 in oral cancer tissues. However, the presence of p53 beyond the basal layer of epithelium denotes an early sign of initiation of oral carcinogenesis, thereby demonstrating the fact that the genomic mutations take place well in advance of the observable morphological alterations in the affected tissue. Thus, the deactivation of the p53 protein or alteration in its coding gene might have a significant impact on the development of oral cancer.

Mutant P53 expression was noted in all the smokers involved in this study. Santos FD et al also recorded an association between p53 expression and smoking. A wide spectrum of p53 mutations were diagnosed in cancers reported among both active and former smokers. Exposure to cigarette carcinogens cause a variety of gene mutations that are closely related to tumorigenesis. Langdon JD’s findings indicate that p53 mutations were frequently detected in tumors from individuals who had a history of heavy smoking. This suggests that genetic damage to p53 could be a contributing factor in this patient group. Among 9 smokers included in the study 8 cases (88.9%) showed miR-375 downregulation. However, this finding was not statistically significant. It can be hypothesized that nicotine associated carcinogens might downregulate miR-375. Further, miR-375 dysregulation might initiate carcinogenesis by inactivating the p53 pathway. Further molecular studies are required to substantiate the above statement.

The current study results also demonstrated a greater intensity of expression of mutant p53 with increasing tumor size, tumor grade and nodal metastasis. This finding indicates the role of p53 in tumor progression. Mutated p53 gene forfeits its ability to inhibit cancer and behaves like an oncogene and promotes tumor growth by stimulating cell division. The findings of the present study show that expression of mutant p53 was noted in 66.7% of miR-375 down regulated cases of OSCC demonstrating a very significant association within the genes. Liu Y et al study results revealed that microRNA-375 directly binds to the 3' -UTR regions of p53 and mediates down-regulation of the gene. Studies have shown that miR-375 overexpression leads to inactivation of the p53 pathway and reduces the p53 protein levels in gastric cancer cells. Further, it aids in the evasion of apoptosis after damage to the DNA. Song L et al demonstrated that miR-375 regulates radio resistance of cervical cancer cells through the p53 pathway. Based on our findings, and literature evidence miR-375 downregulation may induce mutation in the p53 gene which might further result in the initiation of oral carcinogenesis.
CONCLUSION

In summary, MicroRNA-375 is a tumor-suppressive microRNA and its significant down-regulation in oral cancer highlights its association with oral carcinogenesis. miR-375 favors oral carcinogenesis by targeting the p53 gene, which is the frequently mutated gene in oral cancer. Further studies are required to analyze the molecular pathways involved in the interaction between these genes. Loss of miR-375 in oral cancer holds diagnostic implications and should be evaluated further as early diagnostic markers for OSCC. Targeting miR-375 could provide a promising strategy for oral cancer intervention in the future.

ACKNOWLEDGMENTS

We extend our sincere thanks and gratitude to Prof. Ganesh Venkatraman and Prof Rayala Suresh Kumar for providing laboratory access to conduct our PCR experiments.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest regarding the publication of this paper.

Funding Sources

No funding resources to be declared.

REFERENCES

16. Midgley CA, Lane DP. p53 protein stability
in tumour cells is not determined by mutation but is dependent on Mdm2 binding. Oncogene. 1997 Sep; 15(10):1179-89. doi: 10.1038/sj.onc.1201459.


