# EXPRESSION AND CORRELATION OF MICRORNA-34C AND CD68 IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA VERSUS NORMAL ORAL MUCOSA

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## Abstract:

**Background**: Oral cancer is one of the most prevalent cancers worldwide, with squamous cell carcinomas being the most common type, accounting for 90% of all oral cancers with high recurrence in many cases. Thus, the development of new methods to predict the prognosis of OSCC is essential. **Aim:** To investigate and correlate the expression profile of miR-34c using RT-qPCR and quantify CD68 antibody usingimmunohistochemistry in normal oral mucosal tissues versus different grades of OSCC.**Methodology:** Cases of oral squamous cell carcinomas were retrieved from archival blocks between 2016 to 2021, from the Department of Oral and Maxillofacial Pathology, Faculty of dentistry.**Results:** All grades of OSCC revealed significant lower mean expression of miR-34c in comparison to normal mucosal tissue samples at p-value (0.000) with the lowest expression noticed in well differentiated OSCC at p-value (0.000).CD68+ Macrophages in each group of OSCC were found to be significantly higher compared to the normal oral mucosa with the highest expression seen at (Well-Moderate group) at p-value of (0.000).

**Keywords:** OSCC;miRNA-34c; RT-Qpcr; Mir-34 family,TAMs,CD68

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## Introduction:

In the oral cavity, OSCC is the most prevalent malignant tumor. It represents the majority of head and neck cancers with more than half million patients being affected each year worldwide. **(Haddad & Shin, 2008)** Moreover, OSCC has a very poor prognosis because of its invasive nature, and despite advances in treatment and diagnostics, the five-year survival rate is below 50% and has not been improved in the last three decades. **(Ge et al., 2015).**

Therefore, early detection and diagnosis of OSCC results in improved survival rates. On the other hand, delayed detection may result in a high OSCC mortality rate. Furthermore, high recurrence rate of OSCC has been found in many cases. Thus, the development of new methods to predict the prognosis of OSCC is essential**(Gao et al., 2018).**

MicroRNAs (miRNAs) have revolutionized molecular biology over the past few years and have become important players in the development of cancer. They have been found in different tumor types, demonstrating that various sets of miRNAs are typically dysregulated in different cancers. Moreover they have also shown to effectively differentiate between various cancer types, grades, and tumour stages, making them a potential diagnostic and prognostic marker to find changes in normal cellular function in tumor tissue. **(Condrat et al., 2020 and Wang et al., 2018**).

Thus, a deeper knowledge of these dynamic molecules holds the key to the process of oral cancer initiation and progression, which could also aid in the clinical management of oral cancer. **(Rishabh et al., 2021.)**The tumor suppressor miR-34c controls chemotherapy resistance, stem cell renewal, migration, proliferation, apoptosis, and stem cell renewal **(Bommer et al, 2007; Corney et al, 2007; Migliore et al, 2008; Catuogno et al, 2012; Hagman et al., 2013**).It has been shown to be downregulated in several malignancies, including neuroblastoma **(Cole et al, 2008)**, breast **(Yu et al, 2012)**, lung cancer **(Liang, 2008; Liu et al, 2009)**, colorectal cancer **(Toyota et al, 2008)** prostate cancer as mentioned by **(Hagman et al., 2013)** and in laryngeal SCC **(Hu & Liu, 2015).**

Regarding the dysregulation of MicroRNA 34c in HNSCC, the expression of MicroRNA 34c were frequently decreased in OSCC cell lines. (**Kozaki et al., 2008),** In laryngeal SCC **(Hu & Liu, 2015)** and nasopharyngeal carcinoma (**Li et al., 2015).** On the other hand, A study by **Metheetrairut et al., 2019** showed that expression levels of miR-34-family were significantly up-regulated in head and neck squamous cell carcinoma with wild-type *TP53* genes (*n* = 23); while such up-regulation was notfound in tumors with mutant *TP53* (*n* = 19).

Tumor microenvironment is defined as a complex environment that promotes cancer growth, neoplastic cell proliferation, and invasion of nearby tissues and It is regarded as an emerging field in cancer therapy. It is made up of stromal cells, including fibroblasts, endothelial cells, pericytes, and immune cells, as well as cancer cells. **(Hanahan & Coussens, 2012; Quail & Joyce, 2013)**.One of the major inflammatory components in the tumor tissue is tumor associated macrophages (TAMs).

Macrophages present in different tissues are polarized according to changes in their environment, forming different macrophage sub-types, such as M1 macrophages and M2 macrophages**(Martinez & Gordon, 2014).**M1 macrophages are generally considered to be tumor-killing macrophages, primarily anti-tumor and immune-promoting**.**(Yunna et al., 2020)**.**On the other hand, tumor cells release chemokines and polarizing cytokines, which cause M2 prototype macrophages to be recruited. As a result, tumor cells are able to evade immune system attack and are protected from being destroyed and subsequently they are allowed to proliferate. **(Heusinkveld & van der Burg, 2011).** Hence,they promote tumor growth and metastasis, and are associated with poor prognosis of tumors.

Interestingly, recent studies have also reported that M1 macrophages can have the effect of promoting cancer cell metastasis and proliferation(**Chen et al., 2018; Quaranta et al., 2018).**In their recent research **Lv et al., 2022**demonstrated that M1 macrophages potentiate proliferation, migration, and invasion got in vitro and in vivo xenograft formation of OSCC cells. This contradiction of M1 macrophages role might be connected to cell-type selectivity, numbers of M1 macrophages, and heterogeneity among tumors originating from distinct tissue

Finally, this research is conducted to estimate a possible risk and prognosis by correlation between the change of expression level of MicroRNA- 34c as well as Tumor associated macrophages (TAMs) density in the different histological grades of OSCC versus normal oral mucosal tissues.

## Material and Methods:

### 1. Materials

### Study population

A total of 40 cases were enrolled in this study, the cases were divided into four groups as follow: 10 cases of normal oral mucosa,10 cases of well differentiated OSCC, 10 cases of moderately differentiated OSCC and 10 cases of poorly differentiated OSCC. Cases of oral squamous cell carcinomas were retrieved from archival blocks between 2016 to 2021 from the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry - Cairo University that are diagnosed according to histopathologic criteria set by World Health Organization.

The control group of normal oral mucosal tissues, were obtained from patients undergoing esthetic gingivoplasty, (after thorough oral prophylaxis and reduction of gingival inflammation) from the Department of Periodontology, Faculty of Dentistry Cairo university.

### 2. Methods

### a. RNA extraction and RT-PCR protocol:

RT-qPCR of mature miR-34c and B-actin, as endogenous control, was performed for all tumor samples and normal oral mucosal tissues.

- **RNA purification from FFPE samples:**

  Total RNA including miRNA was extracted from tissues using (miRNeasy® FFPE kit Catalogue no. 217504, QIAGEN)following the manufacturer's protocol. Briefly, FFPE tissue scrolls were deparaffinized using deparaffinization solution followed by Proteinase K digestion and nucleic acid isolation; DNA was removed from the RNA sample by a DNase digestion step. Next, the lysate is mixed with buffer RBC.Ethanol

is added to provide appropriate binding condition for RNA and the sample was collected with repeated RNeasy MinElute spin. Total RNA including miRNA binds to membrane and contaminants are efficiently washed away. Total RNA including miRNA is then eluted in RNase-free water.

- **Rt-qpcr for MiRNA-34c expression:**

MiRNAs in the total RNA sample were converted into Complementary DNAs (cDNAs) with (miScript II RTkit Catalogue no. 217504, QIAGEN). RT-PCR was performed using (iTaq™ Universal SYBR® Green supermix) detection protocol as outlined by the manufacturer. DNA was then amplified using the following cycling conditions: One initial PCR activation step at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 53°C for 30 sec, and elongation at 72°C for 30 sec. Model used for Real-time PCR device-Manufacturer (Corbett Research RG-6000 Real Time PCR Thermocycler Qiagen Roto-Gene Q). Primer sequences used are demonstrated in table 1 and table 2.

- **Primers sequences:**

**Table 1: Sequence ofmiR-34c-5p Primers**

| | |
|---|---|
| Forward Sequence: | GGCAGTGTAGTTAGCTG |
| Reverse Sequence: | GAACATGTCTGCGTATCTC |

**Table 2:Sequence of β-actin Primers**

| | |
|---|---|
| Forward Sequence | CACCATTGGCAATGAGCGGTTC |
| Reverse Sequence: | AGGTCTTTGCGGATGTCCACGT |

**b. Data Analysis**

Evaluation of relative expression of miR-34c was analyzed first by obtaining an amplification curve in which Ct is inversely proportional to initial sample quantity. Secondly normalizing the data to an internal control dCt = (Ct of mir34c-Ct B-actine). Thirdly, calculating 2^(-ΔCT) values that is the measure of the miRNA expression level in each sample analyzed.

Finally, the Expression of miR-34c as mean fold change was calculated {mathematically: ratio of mean $2^{\wedge(-\Delta CT)}$ values} between each group of OSCC and the control group (Reference sample) as described in (**Schmittgen & Livak, 2008).** This method is described for obtaining fold change when analyzing different patient samples (independent samples).

**mmunohistochemistry protocol**

**For immunohistochemical analysis**

- For immunohistochemical staining of OSCC different grades with CD 68: only two places allowed us to obtain positive slides: Cairo University, Faculty of dentistry, Oral and Maxillofacial Pathology Department and Cairo university faculty of medicine (general pathology department). The archival blocks utilized were in the period from 2016 to 2021.

- Immunohistochemical analysis was done using anti- CD68 (KP-1), Primary antibody purchased from Ventana Medical Systems (Ventana). In brief, slides were left in Peroxidase-Blocking Reagent for 5 min, incubated with primary antibodies for 20–30 min, horseradish peroxidase (HRP) polymer reagent for 20 min, and diaminobenzidine (DAB) chromogen/substrate working solution for 10 min. Finally, counterstaining with hematoxylin was done.

The stained sections were assessed by two methods:

**1. Ordinary light microscopy:**

All immunohistochemistry (IHC)-stained slides along with the corresponding H and E slides were evaluated by two qualified observers to minimize the subjective

bias. In addition, detection and localization ofthe positive immunohistochemical reaction of antiCD68 were also performed.

**2. Computer image analysis:**

The stained sections were examined by the image analyzer computer system using the software Leica Qwin 500 (Germany).The image analyzer was calibrated automatically to convert the measurement units (pixels) into actual micrometer units. The area percent of CD68 positive cells were measured in a measuring frame of 61934 μm², where three fields were measured for each slide using a magnification (x400). Areas of the most intense staining were selected then the computer system converted the picture into a green binary color that could be measured

**Statistical methods:**

Data were statistically described in terms of mean ± standard deviation (± SD). Data were tested for the normal assumption using Kolmogorov Smirnov test. Comparison between the study groups was done using One Way Analysis of Variance (ANOVA) test with posthoc multiple 2-group comparisons. Two-sided *p* values less than 0.05 was considered statistically significant. IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows was used for all statistical analyses.

## Results:

**Patient characteristics**

The clinicopathological characteristics of the 30 oral cancer patients included in this study are summarized in (Table 3). The age of the patients ranged from 25 to 70 yrs. With a mean of 52.7yrs. There were a higher proportion of males than females (66.6%). Tongue was the most common site of tumor occurrence (40%). Most patients were positive for regional lymph node involvement (66.6%). Tumors were distributed evenly between well, moderate and poorly differentiated histology (33.3 %).

**Table 3:Clinicopathological characteristics of OSCC**

| Characteristics | Number of Patients (%) |
|---|---|

| | |
|---|---|
| **Gender** | |
| Male | 20 (66.6%) |
| Female | 10 (33.3%) |
| **Age** | |
| ≤60 | 22 (73.3%) |
| >60 | 8 (25.8%) |
| **Site** | |
| Floor of Mouth | 3 (10 %) |
| Tongue | 12 (40 %) |
| Palate | 4 (13.3%) |
| Buccal Mucosa | 1(3.3%) |
| Lower Lip | 2 (6.6%) |
| Lower Alveolus | 8 (26.6%) |
| **Lymph node Involvement** | |
| Positive | 20 (66.6%) |
| Negative | 10 (33 %) |
| **Tumor size** | |
| ≤ 3 | 17 (56.6%) |
| > 3 | 13 (43.3%) |
| **Histological Grading** | |
| Well | 10 (33.3%) |
| Moderate | 10 (33.3%) |
| Poor | 10 (33.3%) |

**microscopic Examination**

Using ordinary light microscope, H&E-stained sections were examined to confirm the diagnosisbefore sections from each block were subjected to RT-QPCR analysis for quantification of miR-34c and immunostaining of CD68.Normal oral mucosal epithelium, covered by Keratinized stratified squamous epithelium, showing all four layers of oral epithelium (basal layer, prickle layer, granular layer and keratinized layer).Well differentiated squamous cell carcinoma, showing malignant epithelial cellsinvading through the connective tissue with prominent central keratinization with formation of 'keratin pearls**.** Moderately differentiated squamous cell carcinomas showing a higher degree of cellular atypia and a lesser degree of keratinization, with nuclear and cellular pleomorphism, and hyperchromatic nuclei, can be noted.Poorly differentiated squamous cell carcinomas showing considerable atypia, often with bizarre pleomorphic cells, and dysplastic malignant cell invade in

the form of non-cohesive pattern with fine cords, small islands and single cells infiltrating through the connective tissues.**(Figure 1).**

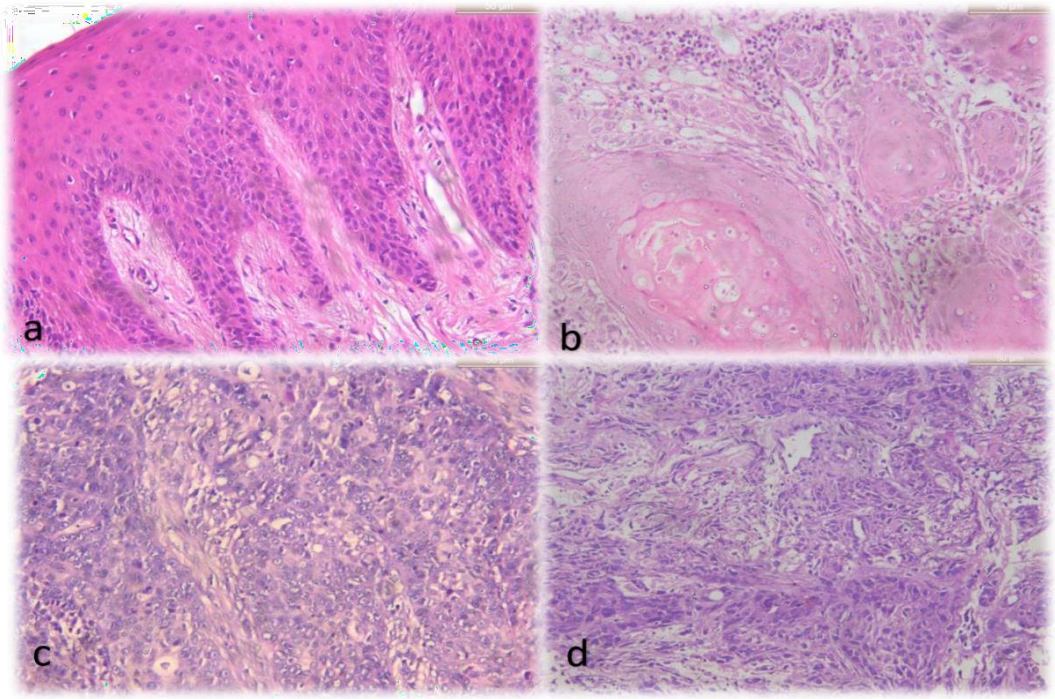


**Figure (1):(a) Photomicrograph showing normal epithelium seen in healthy gingiva (H&EX100).(b)Photomicrograph showing well differentiated OSCC showing infiltrating cell nests some of which showing keratin pearls. (H&Ex 400). (C) Photomicrograph of moderately differentiated OSCC with malignant squamous cells showing pleomorphism and moderate atypia (H&E x 200). (d): Photomicrograph showing poorly differentiated OSCC with diffuse infiltration of connective tissue with malignant cells. (H&E x 200)**

**Reverse transcription quantitative real-time PCR 'RT-qpcr' results.**

All grades of OSCC revealed significant lower mean expression of miR-34c in comparison to normal mucosal tissue samples at p-value (0.000) with the lowest expression noticed in well differentiated OSCC at p-value (0.000). The Moderate and Poorly differentiated OSCC showed higher expression of miR-34c compared to well differentiated OSCC p- value (0.022) and p-value (0.039) respectively with no significance of expression between moderate and poor differentiated OSCC (P-value 1).(Tables 4). Expression of miR-34c was shown as 2^(-ΔCT) values, indicating relative gene expression in all groups (Figure 2).

**Table 4:Pairwise comparison between each 2 groups of OSCC using Post Hoc Test (Bonferroni).**

| Groups → | | Normal | Well | Moderate | Poor |
|---|---|---|---|---|---|
| ↓ | **Mean ± SD** | 0.172 ± 0.036 | 0.059 ± 0.030 | 0.104 ± 0.028 | 0.101 ± 0.032 |
| | **P-Value Bonferroni** | | | | |
| **Normal** | 0.172 ± 0.036 | | 0.000 | 0.000 | 0.000 |
| **Well** | 0.059 ± 0.030 | 0.000 | | 0.022 | 0.039 |
| **Moderate** | 0.104 ± 0.028 | 0.000 | 0.022 | | 1 |
| **Poor** | 0.101± 0.032 | 0.000 | 0.039 | 1 | |

**P-Value Bonferroni**

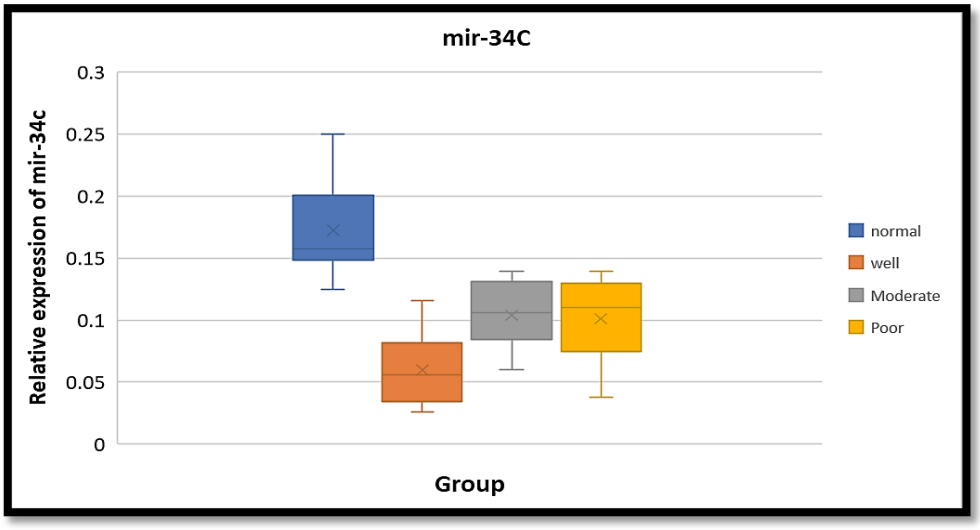


**Figure (2):Box plot graph showing relative gene expression of mir-34c 2^(-ΔCT)**

**Quantification of TAMs and correlation with Mir34c :**

Significant difference of CD68+ Macrophages was observed between the control group and different grades of OSCC.Among different grades of OSCC, no significant difference was found between moderate and well differentiated group. On the other

hand, significant difference was observed between poorly differentiated group compared to the well and moderate groups. **(Table 5).**

**Table 5:Comparison of CD68 mean expression between the study groups**

| **Groups** → | | | **Normal** | **Well** | **Moderate** | **Poor** |
|---|---|---|---|---|---|---|
| ↓ | **Mean ± SD** | | 6.428± 1.397 | 20.571 ±3.823 | 17.142± 3.804 | 10.857± 1.864 |
| | | **P-Value anova** | | | | |
| **Normal** | 6.428± 1.397 | **P-Valueanova** | | 0.0000 | 0.0000 | 0.0437 |
| **Well** | 20.571 ±3.823 | | 0.0000 | | 0.1564 | 0.0000 |
| **Moderate** | 17.142± 3.804 | | 0.0000 | 0.1564 | | 0.0028 |
| **Poor** | 10.857± 1.864 | | 0.0437 | 0.0000 | 0.0028 | |

The comparison of CD68 expressions in the various differentiations of OSCC groups is presented in a histogram. **(Figure 3)**. Photomicrographs representing CD68 Staining in normal oral mucosa and various grades of OSCC are seen in **(Figure 4)**.

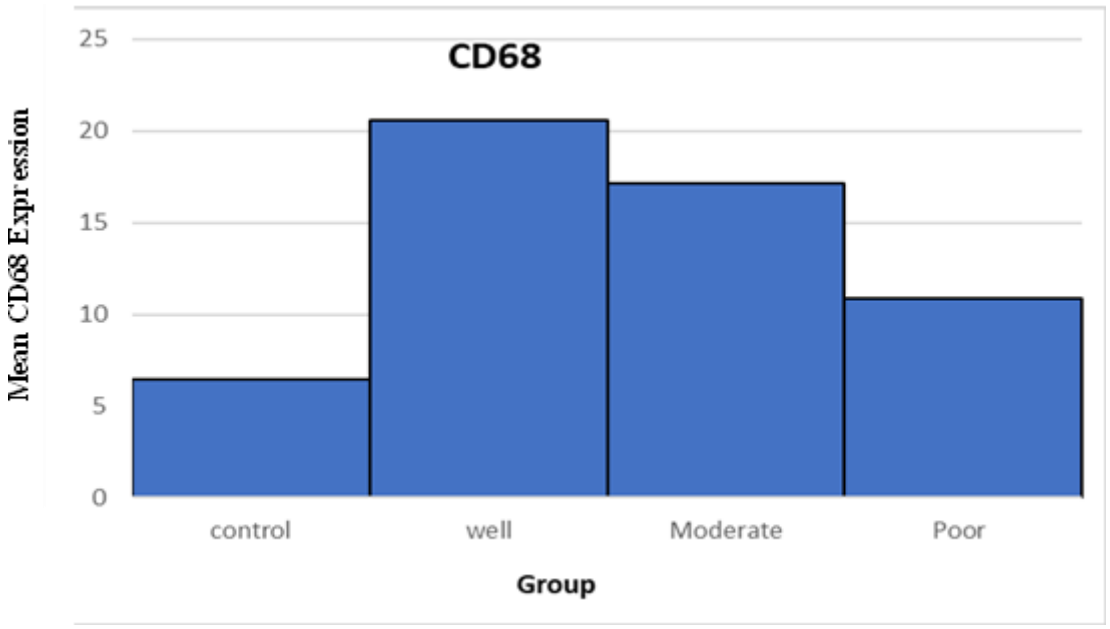


**Figure 3:Histogram showing mean CD68 Expression in Control group and various grades of OSCC.**

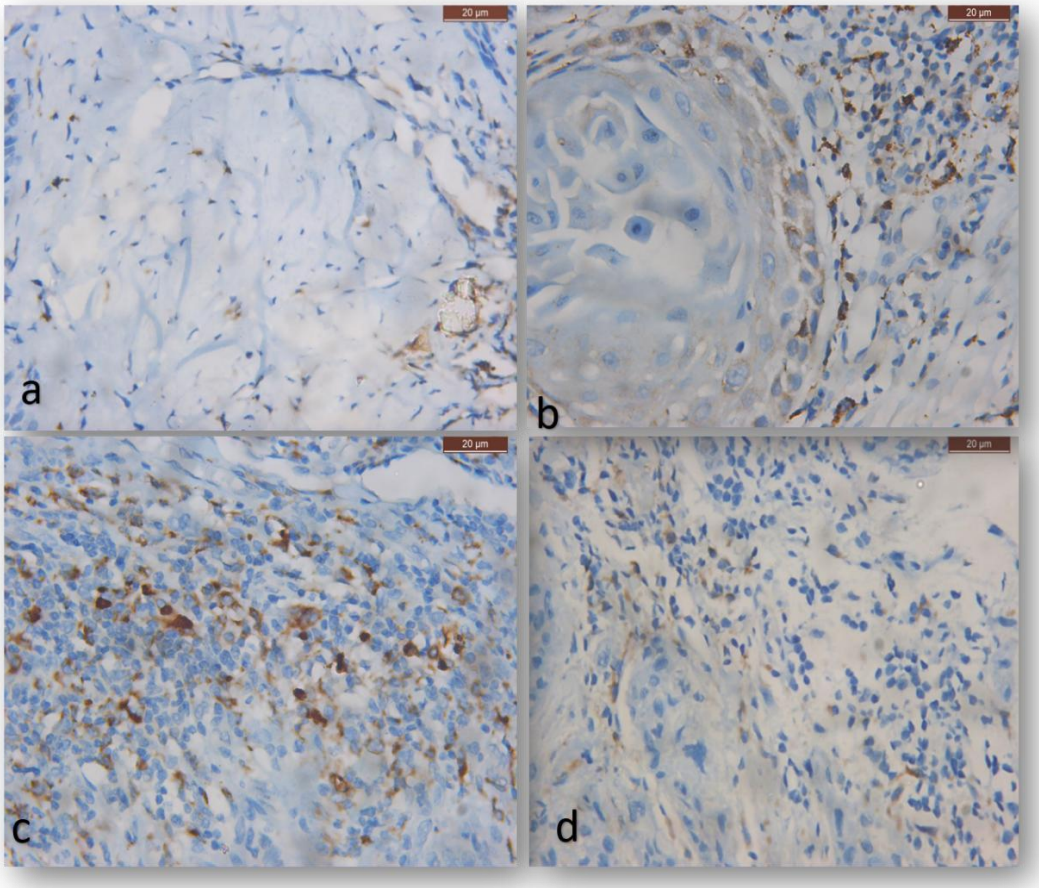

**Figure (4): (a):Photomicrograph showing Immunoreactivity for anti-•CD68 antibodies in normal oral mucosa (original magnification: 400×) only few scatted macrophages were demonstrated in the lamina propria. (b):Photomicrograph showing Immunoreactivity for anti-•CD68 antibodies in well differentiated OSCC (original magnification: 400×) showing a high infiltrate of CD68-positive macrophages in stromal connective tissue around malignant epithelial islands. (c):Photomicrograph showing Immunoreactivity for anti-•CD68 antibodies in moderately differentiated OSCC showing a high CD68-positive macrophage in stromal connective tissue around malignant epithelial islands (original magnification: 400×). (d): Photomicrograph showing Immunoreactivity for anti-•CD68 antibodies in poor differentiated OSCC in stromal connective tissue round discohesive malignant epithelial cells (original magnification400×).**

Relationship between 2^(-ΔCT) values of miR- 34c-5p and CD68 staining showed significant moderate negative association using the Pearson correlation test (r = − 0.690, p = 0.003, R2(0.476). **(Table 6)** and **(Figure 5).**

**Table (6):Pearson correlation between miR-34c expression and Cd68 staining.**

**Correlations**

| | | CD68 | miR-34c |
|---|---|---|---|
| CD68 | Pearson Correlation | 1 | -.690** |
| | Sig. (2-tailed) | | .003 |
| | N | 16 | 16 |
| MiR-34c | Pearson Correlation | -.690** | 1 |
| | Sig. (2-tailed) | .003 | |
| | N | 16 | 16 |

**. Correlation is significant at the 0.01 level (2-tailed).

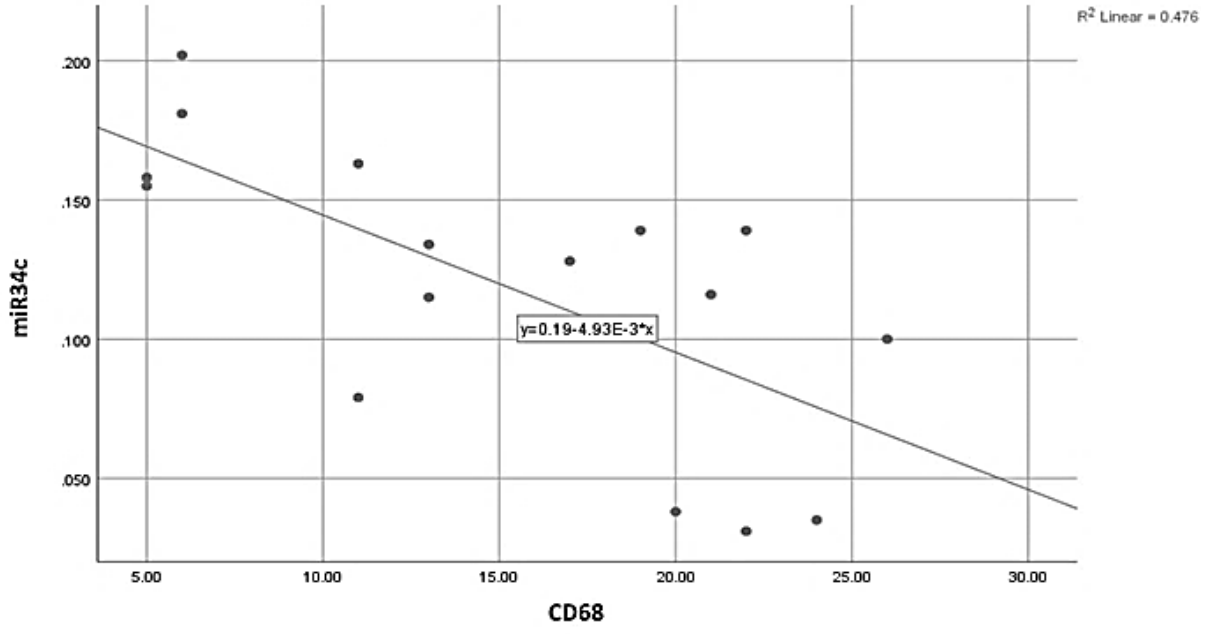


**Figure (5):Scatter plot graph showing negative correlation between miR-34c expression and CD68 staining**

**Discussion:**

Oral cancer is one of the most common cancers worldwide, with squamous cell carcinomas being the most common type, accounting for 90% of all oral cancers. Many studies have demonstrated how clinical and histological staging may explain why some cancers behave aggressively than others, but these staging do not always properly reflect the prognosis of disease **(Lin et al., 2011) .**Gene expression data in combination with OSCC tumor stage information rather than tumor stage information alone has been proposed as better prediction for the patient prognosis **(Méndez et al., 2009)**

The miR‑ 34 family includes (miR‑ 34a, miR‑ 34b and miR‑ 34c), which is considered as a p53 effector, has antiproliferative and pro‑ apoptotic functions. For example, miR-34c was identified to be a pro-apoptotic and anti-proliferative factor in many cell types, including vascular smooth muscle cell **(Choe et al.,**

**2015)**and various cancer cells**(Cai et al., 2010; Hagman et al., 2010; Li et al., 2015).**

In the present study we investigated the expression profile of miR-34c in normal oral mucosal tissues and different grades of OSCC using RT-qPCR. A total of 40 cases were examined, the cases were divided into four groups consisting of 10 cases of normal oral mucosa, 10 cases of well differentiated OSCC, 10 cases of moderately differentiated OSCC and 10 cases of poorly differentiated OSCC. The OSCC cases were retrieved from archival blocks between 2016 to 2020 and were diagnosed according to the current description of WHO classification of head and neck tumors.

Our results revealed that the relative Expression levels of miR-34c were significantly lower in different grades of OSCC compared to normal oral mucosal tissues. This Finding were in agreement with a study by**(Sun et al., 2022)**in which they reported that, the expression of miR-34c- was significantly lower in tissue samples of OSCC cells than in normal tissues and HOK cells (normal cell line).

It has been reported that the miR-34 family is down-regulated in non-small cell lung cancer (NSCLC), hepatocellular carcinoma, and oral squamous cell carcinoma **(Li et al., 2018).**Moreover, miR-34c methylation which induces a repression of miR-34c expression and has been linked to metastasis and poor survival for several cancer types **(Lujambio et al., 2008; Vogt et al., 2011).** Interestingly, the reintroduction of miR-34b/c into cancer cell lines exhibiting miR-34b/c silencing inhibited their motility, reduced tumor growth, and suppressed metastasis in a xenograft model. These effects were accompanied by the downregulation of a number of miR-34b/c target genes, such as MYC, E2F3, and CDK6**(Re et al., 2015)**.

In contrary, other studies by**Metheetrairut et al., 2019; Wong et al., 2008; G. qiang Xu et al., 2019** reported that miR-34c was over-expressed in OSCC and could have oncogenic properties.This debate highlights the potential dual function of Mir-34 family, which might be both tumor-suppressive and oncogenic, depending on the molecular environment, tumour subtype and epigenetic modifications **(Supic et al., 2022**).

**Suzuki et al., 2010**in their study,reported that while assessing the expression of miR-34b/c in normal gastric mucosa from healthy individuals and primary Gastric cancer tissues containing miR-34b/c methylation They have found a considerable downregulation of miR-34b/c expression in the tumor tissues, as compared with normal gastric mucosa.

This was also in agreement by**Jun et al., 2019**and **Kozaki et al., 2008,** They reported that miR-34b/c gene is located in a CpG island region and silenced by DNA methylation in a variety of cancer types including oral cancer. They concluded that in OSCC cell lines, DNA methylation around CpG islands appeared to deregulate the expression of miR-34b/c gene. Hence the downregulated expression of miR-34C in the current study in different grades of OSCC compared to normal healthy oral mucosal tissues could possibly be attributed to high levels of DNA methylation of the tumor tissues, compared to limited/no methylation in normal tissues obtained from a healthy individual.

Furthermore, in the present study revealed that the relative expression of miR-34c in the well differentiated OSCC showed a significantly lower expression compared to moderate and poorly differentiated groups. On the other hand, there was no significant difference between moderately differentiated and Poorly differentiated OSCC. This was in contrast to a study by**Sun et al., 2022**who found a strong correlation between the expression level of miR-34c and clinical traits such TNM stage and histological grade.

**Schmid et al., 2016** also observed a significant negative association between miR-34b/c promoter hypermethylation status and lower miR-34 expression.It has also been stated in a study by **Supic et al., 2022** that there was no significant association found between miR-34b/c promoter methylation and different histological grading of the OSCC. Together these findings may indicate that epigenetic modifications such as miR-34b/c methylation may act independently and could be responsible for variable expression that we observed in the expression of miR-34C in the well differentiated OSCC compared to moderate and poorly differentiated groups.

Moreover, the variable expression of miR-34-family could be attributed to TP-53 status.It was found that lower expression of miR-34-family in TP53-mutated

tumors when compared to tumors with Wild TP53 (functional TP53) in several cancers, such as miR-34a in chronic lymphocytic leukemia**(Dufour et al., 2013)**and miR-34a, miR-34b*, and miR-34c in ovarian cancer**(Corney et al., 2010**)and recently in head and neck squamous cell carcinoma**(Metheetrairut et al., 2019)**.

Additionally, Salzman DW, et al, 2016 observed that numerous mature miR-34 has been found inactive in several kind of cancer cells lines Hela A549 H460 and MCF-7. The inactivity was due to the lack of a 5′-phosphate. However, when given a DNA-damaging stimulus to these cells, inactive miR-34 was activated through 5′-end phosphorylation. This observation could also help explain the relative increase of miR34c expression in moderate and poor differentiated SCC in comparison to well SCC in the present study.

In the current study we also observed a significantly lower relative gene expression ofmiR-34c among the cases with lymph node involvement compared to those without (p0.0084). Our findings were also in agreement with others studies by**Sousa et al., 2016and Supic et al., 2022 i**n which tumors of HNSCC from patients with lymph node metastasis, the levels of miR-34c were lower when compared to those of patients without lymph node involvement.

The previous observation could be connected to the critical function of the miR-34 family in epithelial-mesenchymal transition (EMT) in cancer cells, as the unimpaired p53/miR-34 axis was found to represses SNAIL. which is known to cause EMTand linked to stemness, enhanced motility, and invasion, and hence increases the metastatic potential of malignant cells, so lower expression of miR-34c could promote invasive phenotype of the tumors **(Dong et al., 2016).**

To conclude we suggest that other parameters such as epigenetic alterations and TP53 status are possibly more linked with variable expression levels of miR-34cthan histological grading of the tumor **(Metheetrairut et al., 2019; Supic et al., 2022).** Additionally, a larger sample size is certainly required for confirmation of our findings regarding the differential expression of miR-34c in various OSCC tumour grades.

TAMs constitute a dominant portion of the leukocyte population in tumor stroma (Lee & Liu, 2006).Moreover, there is convincing evidence that they have potential involvement in the progression and metastatic spread of OSCC. Facilitation of angiogenesis, tumor cell invasion, augmentation of cell motility, persistent growth, and suppression of anti-tumor responses are among the most important features associated with their presence in the lesion stroma**.**(Petruzzi et al., 2017). However, sometimes a high number of infiltrating macrophages correlates with better prognosis as reported in several studies**(Forssell et al., 2007; Ohri et al., 2009; Zijlmans et al., 2007).**

In the present study the mean CD68+ Macrophages of each group of OSCC were found to be significantly higher when compared to the control group of normal oral mucosal tissues. We also observed an increased TAM infiltration around the neoplastic and malignant epithelial islands and cells. The previous observation in agreement with a study by Dai et al., 2007 they reported that there were masses of macrophage infiltration in OSCC and the macrophage count was significantly higher in OSCC samples compared to the normal mucosa (42 samples of OSCC and 10 samples normal tissue).

In a study by Mori et al., 2011 and Bagul et al., 2016 they reported that although the CD68-positive cells indicated the total population of macrophages in the OSCC specimens, but there was no association between the number of CD68+ cells and the tumor histological grade. This was in part in agreement with the current study, that didn't show statistically significant difference between the number of CD68+ cells, in the well differentiated group compared to moderately differentiated group. They stated that it is likely that these mixed cell populations are functionally diverse with regard to the development and progression of OSCC because CD68+ cells contain M1, M2, and undifferentiated monocytes/macrophages.

On the other hand, the present study found a significantly lower CD68+ macrophage density in poorly differentiated tumors compared to well and moderately differentiated OSCC. Our finding was in agreement with other studies revealing similar results **(Helal & Wahba, 2016), (Wei & Hu-Jie, 2018) and (Lo et al., 2010).**

This decrease could possibly be attributed to dedifferentiating M1 macrophages during the progression of the high-grade tumors. It was also found that the proportion of M1 cells decreased in specimens from higher grades of the tumor of OSCC, although no statistical significance was obtained. **(Mori et al., 2011).** Moreover, in their research Chang et al., 2013 they observed that a smaller number of M1 macrophages promoted xenograft tumor development whereas a higher number of M1 macrophages hindered tumor growth.

On another hand, in a study by **Komohara et al., 2008** they reported that Infiltration of CD68+ macrophages into gliomas correlated with the grade of histological malignancy and the highest count of Macrophages was seen in higher histological grades (grade 4). However, a meta-analysis by **Kumar et al., 2019** found that although some studies suggested that tumours with poor differentiation may be more likely to have high CD68+ TAM densities, there was no significant association between high density CD68+ TAMs and poorly differentiated tumors.

Moreover, this difference in CD68+ Macrophages count could be attributed to differences between tumor microenvironment and normal oral mucosal epithelial cells. According to a study by **Roca et al., 2009.**They found that production of cytokines and chemokines such IL-13, IL-10, and glucocorticoids by the tumour microenvironment may promote the differentiation of TAMs into the M1 or M2 phenotype.

Additionally,**Ferreira et al., 2008 and L. Lee et al., 2006**Reported that CCL2 (known as monocyte chemoattractant protein-1) and IL-6 promote the survival of human monocytes and induce variable M1/ M2-macrophage differentiation. When compared to normal oral mucosal epithelial cells, several cancer forms, including oral squamous cell carcinoma, were found to express constitutive expression of CCL2 and IL-6.

This may further be attributed due to varying signals from the local microenvironment, tumours can produce a heterogeneous population of tumor-infiltrating myeloid cells that differ at the molecular and functional level (**Kuang et al., 2007 Movahedi et al., 2010; Pettersen et al., 2011).**

The correlation analysis that was conducted in the present study, between the expression of miR- 34c and CD68 staining revealed a significant correlation between the two variables. The correlation showed a moderate negative relationship where the cases that demonstrated a low miR-34c expression, showed an increase in the recruitment of TAMs and hence more aggressive OSCC Phenotype.

On the other hand, as the direct relationship between these two variables isn't strong, we can't reach to a final conclusion regarding their relationship in OSCC. Further studies including epigenetic change controlling miR-34c role and targets are further needed as may serve as a useful therapeutic option in OSCC. In addition, illustrating different signals controlling tumor microenvironment and immune response.

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