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The Expression of The Apoptosis-Regulating miRNAs 16 And 21 In Oral Squamous Cell Carcinoma (In Vitro Study).

Running Title: miRNAs 16 and 21 In Oral Squamous Cell Carcinoma

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Introduction:

Cancer is a leading cause of death in both developed and developing countries, which emerges as a major public health problem. Oral squamous cell carcinoma (OSCC) is the most common form of carcinoma in the oral cavity and ranks as the 12th most common cancer in the world. Unfortunately, many cases of OSCC are clinically detected in a late stage, and the available therapeutic alternatives are highly expensive and disfiguring. The development of reliable biomarkers and more effective therapeutic agents are necessary to improve patient outcome.

Recent studies have identified a class of small non-coding RNA molecules, named micro-RNAs (miRNAs), that negatively regulate gene expression. Recently, these molecules have gained great attention in the research field for their major role in modulating gene expression and controlling various cellular processes, among which the development of human malignancies.MiRNAs are considered as promising molecular biomarkers for diagnosis of cancer and prediction of patient prognosis, as well as being used as targets for cancer therapy.(1)

A hallmark in the process of carcinogenesis is the suppression of apoptosis, which is a distinct mode of programmed cell death. Much of the interest in such process is attributed to its precise regulation by certain oncogenes and tumor suppressor genes. BCL-2 is one of the well-known potent oncogenes that promotes malignant cell survival by attenuating apoptosis.

Overexpression of BCL-2 protein is present in over half of all cancers, regardless of type. This results in tumor cells that are resistant to any intrinsic apoptotic stimuli, including some anticancer drugs.[2]

Several miRNAs have been identified as regulators of BCL-2 expression, among which is the anti-apoptotic miRNA-21, which is one of the early discovered miRNAs in human cells, overexpressed in multiple malignancies as colorectal cancer, lung cancer, pancreas cancers, and breast cancer.[3]On the other hand, microRNA-16 is a newly discovered pro-apoptotic regulator of BCL-2, where its under-expression has been associated with the over-expression of BCL-2 in certain types of cancer cells as in leukemia cells, thus yielding interest in using it as a target for cancer therapies. However, ongoing research is taking place to identify the link between theexpression of these miRNAs and the development and progression of OSCC, in order to address their potential role as early diagnostic markers as well as a novel anti-cancer approach.[4,5]

Material and Methods:

Materials

Tissue Specimens:

Oral squamous cell carcinoma specimens were collected in the form of formalin fixed paraffin embedded blocks retrieved from the archives of Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University, and Surgical Pathology Department, Al-Kasr Al-Einy Hospital, Faculty of Medicine, Cairo University. Specimens were classified into 3 groups: 1. well differentiated OSCC, 2. Moderate/ poorly differentiated OSCC, 3. Normal oral mucosa (control group). Inclusion and exclusion criteria were listed in **Table 1**.

RNA Isolation, Quantification and PCR Analysis

- RNeasy FFPE kit (Qiagen, USA)
- QIAzol lysis reagent.
- Nanodrop (Thermo scientific, USA).
- MiRNeasy Reverse Transcription kit (Qiagen, Valencia, CA, USA).
- **miRNA-21 primer (5'-3'):**
  a.Forward: GCCGCTAGCTTATCAGACTGATGT
  b. Reverse: GTGCAGGGTCCGAGGT
- **miRNA-16 primer (5'-3'):**
  a.Forward: TAGCAGCAGCTAAATA TTGGCG
  b. Reverse: CCAGTATACTATGTGCTCGTGA
- Housekeeping mi-Script PCR control, miRNA SNORD68 (selected as internal control).

Immunohistochemical Reagents

- **Primary Antibody:**Monoclonal anti-human BCL2 oncoprotein (code: M0887, DAKO, Denmark).
- **Universal kit:** iVIEW™ DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). The kit composed ofiVIEW inhibitor (3% H2O2), iVIEW biotinylated Ig secondary antibody, iVIEW SA-HRP, iVIEW DAB substrate, iVIEW Copper, iVIEW H2O2 (<0.08% H2O2).
- Optiview Amplification kit (Ventana Medical Systems, Tucson, AZ, USA).

Study Design

This study was conducted on a total of 32 OSCC cases and 16 normal epithelium control cases. OSCC cases were graded as well, moderately, or poorly differentiated SCC, based on the degree of differentiation, cellular pleomorphism, and mitotic activity. Both moderately and poorly differentiated cases were placed as one group, whereas well differentiated cases were placed as a separate group. Quantitative real time polymerase chain reaction (qRT-PCR) was used to evaluate the expression of miRNA-16 and miRNA-21 in the 3 studied groups. On the other hand, expression of BCL2 protein was evaluated by Immunohistochemical staining**(Table 2).**

Samples Preparation

Paraffin sections (5µm) of each tissue specimen were stained with Hematoxylin and Eosin(H&E) to reconfirm the diagnosis. All specimens were histologically re-evaluated to ensure that all tumor specimens contain at least 80% of cancer cells and that all normal tissue specimens don't have any evidence of dysplasia.

Paraffin sections (4 µm) of each specimen were mounted on positively charged glass slides (Optiplus; BioGenex, Milmont Drive, CA, USA) for immunostaining with anti-BCL-2 antibody. Sections were dried overnight at room temperature for staining procedures.Ten sections of 5-µm thickness were cut from each sample and placed in plastic Eppendorf tubesfor PCR analysis of miRNA-16 and miRNA-21 gene expression.

MicroRNA Extraction

Specimens (paraffin sections) were deparaffinized in xylene and washed with 100% ethanol. 10 µl proteinase K was added and mixed gently by pipetting up and down, then incubated in heating block at 56°C for 15 minutes, then at 80°C for 15 minutes to digest cell walls. Tubes were incubated on ice for 3 minutes, then centrifuged for 15 minutes at 12500 xg (times gravity, which is the unit of relative centrifugal force). The supernatants were then transferred to a new microcentrifuge tube without distortion of the pellet. RNeasy FFPE kit (Qiagen, USA) was used to extract the RNA from the specimens following the manufacturer's instructions. The extracted RNA was treated with DNase I solution to eliminate the residual of DNA in the RNA.

RNA was extracted from pellet using QIAzol lysis reagent and incubated for 5 min at room temperature. Then, 100 uL (Microliters) chloroform was added, vortexed for 15 seconds, and incubated for 2–3 min at room temperature. This was followed by centrifugation at 14000 xg at 4°C for 15 min. The upper watery phase was removed followed by addition of 100 % ethanol. Each 700 uL of this mixture was placed in RNeasy Mini spin column in 2 mL collection tube and centrifuged at 10000 xg at room temperature for 15 seconds.

After the mixture had completely passed the column, Qiagen RWT buffer was added to each column, and again centrifuged at 10000 xg at room temperature for 15 seconds. 500 uLQiagen RPE buffer was added to the column and centrifuged at 10000 xg at room temperature for 15 seconds. After this, another 500 uLQiagen RPE buffer was added to the column and centrifuged at 10000 xg at room temperature for 2 minutes. The column (which had been placed in a new collection tube) was then centrifuged at full speed for 2 minutes. Finally, the column was transferred to a new 1.5 mL collection tube and 50 uL RNase-free water was pipetted directly onto the column and centrifuged for 1 minute at 10000 xg to elute RNA. The extracted RNA was stored at −80 °C until use.

PCR Analysis for miRNA-16 and miRNA-21 Expression

Reverse transcription was carried out on 100 ng of total extracted RNA in a final volume of 20-µL, using the miRNeasy Reverse Transcription Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions:Incubated for 60 min at 37°C.Then for 5 min at 95°C.Finally maintainedat 4°C.

For real-time PCR, diluted reverse transcription products (cDNA template) were mixed with SYBR Green Master Mix (Qiagen, Valencia, CA, USA) in a final volume of 25 uL. Quantitative real time PCR expression of mature miRNAs -16 and 21 were evaluated using an Applied Biosystems 7500 Real Time PCR System (Foster city, CA, USA) according to the manufacturer's protocol. The housekeeping miRNA SNORD68 was used as the endogenous control. Realtime PCR was performed with the following conditions: 95°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds, and 55°C for 30 seconds, and 70°C for 34 seconds.

Immunohistochemical Analysis and assessment

Immunostaining was performed in **National Cancer Institute**using Ventana Benchmark auto-stainer (USA). Lymphoma specimens obtained from national cancer institute was used as apositive control for BCL-2 staining.

Statistical Analysis

Data was analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL). Numerical data was described as mean and standard deviation or median and range. Categorical data was described as numbers

and percentages. Data was explored for normality using Kolmogrov-Smirnov test and Shapiro-Wilk test. Comparisons between two groups for normally distributed numeric variables (as in case of miRNA-16 expression) were done using the Student's t-test, while for non-normally distributed numeric variables (as in case of miRNA-21 expression) were done by Mann-Whitney test. Comparisons between categorical variables were performed using the chi-square test. A p-value less than or equal to 0.05 was considered statistically significant.

Results

Clinical Findings

Among the examined OSCC cases (32 patients), the patient population consisted of 29 males (90.6% of OSCC cases) and 3 females (9.4% of OSCC cases). Patients ranged in age from 20 to 83 years, with a mean age of 59.6 years. The most common anatomical locations included the tongue (lateral border), lower lip, nasopharynx, as well as alveolar ridge. Only one case (3.13% of the studied OSCC cases) presented with regional lymph node metastasis.

Histopathological Findings

The histopathologic examination of all OSCCcases showed **squamous differentiation,** evident by keratinization (with or without keratin pearl formation) and/or presence of intercellular bridges, as well as **invasion**, manifested as interruption of the basement membrane of the surface epithelium and downward growth of tumor cells in the underlying tissue.

Well differentiated OSCC cases revealed easily recognizable squamous epithelium showing abundant keratinization and keratin pearl formation as well as minimal pleomorphism (fig. 1). On the other hand, moderate/poorly differentiated cases showedminimal keratinization(with difficulty to establish squamous differentiation in some cases), in addition to marked cellular pleomorphism and nuclear atypia (fig. 2).

Quantitative Real Time PCR Findings

MiRNA-16 expression

In the well Differentiated OSCC group, the expression levels of miRNA-16 weredecreased compared to the normal control.**In the moderate/poorly differentiated OSCC group**, miRNA-16 expression levelswere also decreased compared to normal control cases. Upon comparing both OSCC groups, the moderate/ poorly differentiated group showed lower expression levels of miRNA-16 compared to the well differentiated group (Table 3).

Statistical Analysis

In case of well differentiated OSCC, miRNA-16 expression showed a significant decrease compared to normal epithelium (table4). Also, the moderate/poorly differentiated OSCC group showed significantly lower miRNA-16 expression compared to normal control group

(table5). As seen in figure (3), comparing the 3 studied groups together revealed a highly significant difference in miRNA-16 expression (table6).

Upon correlation with clinical parameters, no significant difference in miRNA-16 expression was detected between males and females in both well and moderate/ poorly differentiated OSCC groups (table 7). Also, no correlation was found between patient's age and miRNA-16 expression in both OSCC groups (table 8).

MiRNA-21 Expression

In the well Differentiated OSCC group, the expression of miRNA-21 was increased compared to the normal control group. **In the moderate/ poorly differentiated group**, miRNA-21 expression levels were also elevated compared to the normal control group. On comparing both OSCC groups, the moderate/poorly differentiated group showed higher expression levels compared to the well differentiated group (table 9).

Statistical Analysis

In case of well and moderate/poorly differentiated OSCC, miRNA-21 expression showed significant increase compared to normal epithelium. On comparing the 3 studied groups together (figure 4), a highly significant difference in miRNA-21 expression was observed (table 10).

Correlation with clinical parameters revealed a statistically significant relation between miRNA-21 expression and patient's sex only in the moderate/ poorly differentiated OSCC group (figure 5). This relation wasn't detected in the well differentiated group (figure 6). Also, no relation was found between miRNA-21 expression and patients age (table 11). (figure 7)

Immunohistochemical Findings

Normal control cases showed negative BCL-2 immunostaining except for few scattered basal epithelial cells (figure 8 a). All the studied OSCC cases (100%) including both well differentiated and moderate/poorly differentiated groups also showed negative BCL-2 immunoreactivity (figures 8 b&c).

Discussion

OSCC is the most common malignancy in the oral and maxillofacial region, accounting for over 95% of all head and neck cancers. Even with the great advances in therapy and increasing knowledge about the molecular mechanisms behind oral cancer, the 5-year overall survival of patients is still only around 50%. Even though the oral cavity is easily accessible for direct visual examination, most OSCCs are not diagnosed until an advanced stage. Therefore , it is essential to discover new diagnostic and therapeutic targets that are involved in OSCC development and progression[6]. That's why, it has been chosen as a subject of study in the current work.

MiRNAs are small molecules of endogenous origin that recently have gained great attention in the research field for their major role in controlling various essential cellular

processes through the post-transcriptional regulation of various genes. Dysregulated miRNAs were found to be involved in cancer initiation and progression, where they act as either tumor promoters or suppressors. An example of tumor suppressor miRNAs is miRNA-16,which is frequently downregulated or deleted in many cancers, demonstrating its critical role in tumor suppression mainly through its pro-apoptotic effects. On the other hand, miRNA-21was the first miRNA to be described as an ''oncogenic'' miRNA and documented to be upregulated in numerous cancer types, inhibiting apoptosis and promoting cellular proliferation. Several studies have revealed the functional importance of these 2 miRNAs through the various cancer hallmarks.[7]

Being dysregulated in many cancer types, miRNAs 16 and 21 might play a major role in the pathogenesis of OSCC. However, few studies in the available literature focused on assessing their expression and role in the different histologic grades of the most common type of oral cancer. In respect to the previously mentioned points, the present study aimed to examine the expression of miRNA 16 and 21 in the different grades of OSCC compared to normal oral epithelium.

BCL-2 is a well-known anti-apoptotic member of the BCL-2 family, whose dysregulation is detected in over half of all cancers, regardless of type. Overexpression of BCL-2 alters the overall ratio of the apoptotic agonists to antagonists of the BCL-2 family, promoting malignant cell survival by attenuating apoptosis. Being regulated through various mechanisms, BCL-2 is a downstream target for numerous miRNAs, including miRNA 16 and 21. In our study, we investigated the expression levels of BCL-2 in OSCC through immunohistochemical analysisin an attempt to correlate its expression with that of its regulators (miRNA-16 and 21).

In the current work, all OSCC cases showed a significant decrease in the expression levels of miRNA-16 compared to the normal control group. Consistent with our results, low miRNA-16 expression has been reported in many cancers, including: breast cancer [9],osteosarcoma [10]and prostate cancer. Also, Hu et al. [11]reported that miRNA-16 expression levels in OSCC tissues were markedly lower than that in the corresponding adjacent normal tissues. These results indicate that miRNA-16 is downregulated in OSCC and might be involved in the process of its initiation and progression.

However, the previous results are inconsistent with those of Chen et al.[12], who found that miRNA-16 was significantly upregulated in renal cell carcinoma (RCC)tissues compared to their normal counterparts($P<0.05$). Chen results were in accordance with previous studies of miRNA-16 expression profiles in RCC. They found that downregulation of miRNA-16 resulted in decreased cellular proliferation and enhanced apoptosis, while its overexpression resulted in accelerated cellular proliferation and migration, concluding that miRNA-16 may be characterized as an oncogene in RCC. These contradicting findings suggest that miRNA-16 could act as either atumor suppressor or promoter in different cancer types.

On comparing miRNA-16 expression among the studied OSCC groups, a significant expression difference was found between the well differentiated group (mean0.36) and the moderate/poorly differentiated group (mean0.17). This was in line with Zidan et al., who found that the expression levels of serum miRNA-16 decreased gradually from normal control cases (mean 2.15), followed by benign prostate hyperplasia patients (mean1.96), reaching the least levels in case of prostate carcinoma patients (mean 0.55).Qian et al. [13] also found that the status of miRNA-16 expression was closely associated with tumor degree of differentiation in colorectal cancer. These findings suggest that miRNA-16 expression correlates with the degree of OSCC differentiation and tumor grade.

On the contrary, Manikandan et al. [14]didn't find a correlation between miRNA-16 expression and OSCC histological grade.Also Albuquerque et al. [15] didn't find a statistically significant difference in the fold expression of miRNA-16 between low-grade and high-grade anal/perianal squamous intraepithelial lesions. However, more studies should investigate the relation between miRNA-16 expression and tumor histological grade.

Upon correlation with clinical parameters, our results detected no significant difference in miRNA-16 expression between males and females in both well and moderate/ poorly differentiated OSCC groups.Also, no correlation was found between patient's age and miRNA-16 expression in both OSCC groups. This accords with Manikandan et al. [14]who didn't find a relation between miRNA-16 expression in OSCC and patients age, gender, anatomical site, clinical stage (TNM), smoking status or alcohol intake. However, Hu et al. [11] reported significant association of lower miRNA-16 expression with positive lymph node metastasis and higher clinical stage (TNMsubtype) ($p<0.001$).

Regarding miRNA-21, our results revealedsignificant increase inits expression in case of both OSCC groups compared to normal epithelium. These findings indicate that miRNA-21 is upregulated in OSCC and might be involved in the process of its initiation and progression.High miRNA-21 expression has been reported in many cancer studies as studies on breast cancer [16], ovarian cancer [17] and prostate cancer [18]. Also, Gissi et al.[1] found that miRNA-21 was overexpressed in cells exfoliated from OSCC lesions with respect to cells from healthy donors. Interestingly, they found that miRNA-21 showed similar elevated expression levels in clinically normal distant mucosa from OSCC patients, suggesting the presence of a genetically altered field (field cancerization).

Our results showed a significant difference in miRNA-21 expression between well (median expression 3.7) and moderate/ poorly differentiated OSCC (median expression 9.35). This indicates that miRNA-21 expression correlates with tumor histological grade. Similar results were found by Abu-Duhier et al. [19]while comparing the plasma miRNA-21 in different histological grades of lung cancer patients, in which

moderately and poorly differentiated lesions reflected a higher miRNA-21 levels than well differentiated tumors; but the difference didn't reach the value of statistical significance. Meanwhile, these results disagree with those of Mahmood et al. [20], who didn't observe any significant association of circulating miRNA-21 levels with tumor grade in OSCC patients.Therefore, more research investigating the relation between miRNA-21 expression and OSCC histological grade is required.

The present study found a statistically significant relation between miRNA-21 expression and patient's sex, only in the moderate/ poorly differentiated OSCC group, but not in the well differentiated group. Also, our results didn't find a relation between miRNA-21 expression and patients' age. On their study on OSCC, Mahmood et al. [20]didn't observe any significant association between miRNA-21 expression and patients age, sex, ethnicity, as well as smoking status. However, among the various clinical characteristics, theyobserved a significantly higher miRNA-21 expression in case of tumor size > 4 cm (p value <0.001). Also, Singh et al. documented a significant gradual increase in miRNA-21 expression from tumor stage I to IV in OSCC. Sangeetha et al. [21] also found that the expression of miR-21 was associated with tobacco use (smoking) and nodal metastasis in OSCC.

In the current study, all the examined OSCC cases as well as the normal control cases showed negative BCL-2 immunostaining.Thus, no correlation could be achieved between BCL-2 expression and its epigenetic regulators; miRNA-16 and 21. In order to explain their similar results on both well and poorly differentiated OSCC,Loro et al. [22] analyzedtheir samples for mutations that may underlie the loss of BCL-2 expression.Their mutational analyses revealed absence of mutations in *BCL-2* gene in case of OSCC.Consistent with our findings, Liu et al. also found that *BCL-2* gene expression in OSCC tissues had no obvious discrepancy compared with normal tissues, when evaluated by RT-PCR.Many studies revealed that BCL-2 expression isregulated both transcriptionally and post-transcriptionally. Various molecules can downregulate or upregulate BCL-2mRNA levels (transcriptional control)or protein levels (post-transcriptional or translation control).Lymphokines, transforminggrowth factor b, Epstein Barr virus, p53 and retinoids are few contributing factors to be considered that might downregulate or upregulate BCL-2 expression.[23]

Published studies reported variable BCL-2 expression levels in OSCC. Farhadi et al. [24]reported diminished BCL-2 positivity (16.4%), with 10 out of total 25 OSCC samples showing total negative expression. Pallavi et al. [23] reported a higher expression level (37%).Bhattacharya et al. found BCL-2 positive expression in 63.3% of OSCC samples, randomly expressed in all epithelium layers, but no relation was found between BCL-2 expression and degree of tumor differentiation.A similar BCL-2 expression level (63%) was documented bySolomon et al.[25], who also found that as the degree of differentiation decreases, more number of tumor cells express BCL-2 by which they evade apoptosis and achieve unrestrained cell survival.

Despite our negative results, Pekarsky et al. [5]found in their study on CLL that miRNA-16 expression was highest in the CLL samples with lowest BCL-2 expression and was lowest in the CLL samples showing high BCL-2 expression. Their experiments showed that microRNA-16 directly interact with and inhibit BCL-2 expression, concluding that the loss of miRNA-16 is the main cause of BCL-2 overexpression in CLL. Regarding the relation of BCL-2 expression with miRNA-21, Sangeetha et al. [21] reported elevated BCL-2 expression in OSCC and succeeded to find a correlation between its increased expression and miRNA-21 upregulation.Also, results of Liu et al. on large B-cell lymphoma documented a positive correlation between miRNA-21 and BCL-2 expression levels using RT-PCR, indicating that miRNA-21 may decrease cell apoptosis via upregulating BCL-2 gene expression.

Conclusion

In view of the present results and discussion, it can be concluded that:

• MiRNA-16 expression is significantly lower in OSCC compared to normal oral epithelium, and its levels significantly correlates with the degree of tumor differentiation. On the other hand, miRNA-21 expression significantly increases in OSCC compared to normal control, and its level also correlates with tumor histological grade significantly. Such findingssuggest the integration of both miRNAs in both diagnostic and interventional modalities of OSCC.

• MiRNA-21 elevated expression significantly correlates with patients' sex in case of moderate/poorly differentiated OSCC, confirming the male gender as an important adverse prognostic factor.

• BCL-2 immunostaining shows no correlation with miRNA-16 and 21 expression in case of the present study.

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Tables

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Human tissues	Animal tissues
Oral cancer	Lesions other than cancer
	Cancer of different anatomic sites
	Frozen sections

Table 2. Study Design

Study Groups	No. of cases	Parameters Assessed	Assessment Technique
Normal control group	16	miRNA-16 gene expression	Polymerase chain reaction (PCR)
Well-differentiated OSCC group	16	MiRNA-21 gene expression	

Moderate/poorly differentiated OSCC group	16	BCL-2 expression	Immunohistochemistry

Table (3): miRNA 16 expression levels in well and moderate/poorly differentiated OSCC:

Case No.	Well diff. OSCC	Moderate/ poorly diff. OSCC
1	0.5	0.004
2	0.3	0.03
3	0.32	0.06
4	0.71	0.43
5	0.48	0.002
6	0.4	0.003
7	0.82	0.42
8	0.2	0.06
9	0.71	0.08
10	0.49	0.28
11	0.03	0.009
12	0.26	0.17
13	0.072	0.002
14	0.25	0.8
15	0.42	0.36
16	0.14	0.07

Table (4): Significance of miRNA-16 expression difference between well-differentiated OSCC and normal epithelium (Independent t test):

	Type	Mean	Std. Deviation	P-value
miRNA-16 expression	Normal epithelium	0.9283	0.15049	**0.000****
	Well-differentiated SCC	0.3626	0.24923	

** (P value <0.001): highly significant.

Table (5): Significance of miRNA-16 expression difference between moderate/poorly-differentiated OSCC and normal epithelium (Independent t test):

	Type	Mean	Std. Deviation	P-value
miRNA-16 expression	Normal epithelium	0.9283	0.15049	
	Poorly-differentiated SCC	0.1738	0.22726	**0.000****

**(P value <0.001): highly significant.

Table (6) One-way ANOVA post-hoc test of miRNA-16 expression relation among the 3 studied groups:

		Mean Difference	Std. Error	P-value
Normal epithelium	Well-diff. SCC	0.56569*	0.07539	**<0.001****
	Poorly-diff. SCC	0.75456*	0.07539	**0.000****
Well-differentiated SCC	Normal epithelium	-0.56569*	0.07539	**0.000****
	Poorly-diff. SCC	0.18888*	0.07539	**0.041***
Poorly-differentiated	Normal epithelium	-0.75456*	0.07539	**<0.001****
	Well-diff. SCC	-0.18888*	0.07539	**0.041***

**(P value <0.001): highly significant.

* (P value <0.05): significant.

Table (7): Significance of miRNA-16 expression difference between males and females:

Type	Sex	Mean	Std. Deviation	P-value

Well-differentiated SCC	Male	0.3950	0.26668	0.289^{ns}
	Female	0.7100	.	
Poorly-differentiated SCC	Male	0.1623	0.24093	0.458^{ns}
	Female	0.3000	0.18385	

ns: not significant

Table (8): Pearson Correlation between miRNA-16 expression and patient's age:

Well-differentiated SCC	Pearson Correlation	0.209
	P-value	**0.537^{ns}**
Poorly-differentiated SCC	Pearson Correlation	-0.134
	P-value	**0.635^{ns}**

ns: not significant.

Table (9): miRNA 21 expression levels in well and moderate/poorly differentiated OSCC:

Case No.	Well-diff. OSCC	Moderate/poorly diff. OSCC
1	3.8	11.3
2	5.2	9.5
3	4.9	7.4
4	5.7	4.8
5	2.8	10.6
6	3.5	8.6
7	2.2	5.5
8	1.9	10.7
9	4.8	7.3
10	6.9	12.5

11	4.9	15.4
12	3	4.6
13	2.7	7.8
14	5.1	9.2
15	3.6	11.6
16	2.8	20.4

Table (10): Mann Whitney U test correlating miRNA-21 expression among the 3 study groups:

			P-value
Normal epithelium	**Median**	**0.9750**	**0.000****
	Minimum	0.69	
	Maximum	1.23	
Well-differentiated SCC	**Median**	**3.7000**	
	Minimum	1.90	
	Maximum	6.90	
Poorly-differentiated SCC	**Median**	**9.3500**	
	Minimum	4.60	
	Maximum	20.40	
Normal vs. Well differentiated OSCC			**<0.001****
Normal Vs. Moderate/ poorly differentiated OSCC			**<0.001****

**(P value <0.001): highly significant

Table (11): Correlation between miRNA-21 expression and patient age in both OSCC groups:

Well-differentiated SCC	Correlation Coefficient	0.082
	P-value	0.810^{ns}
Poorly-differentiated SCC	Correlation Coefficient	-.368
	P-value	0.177^{ns}

ns: not significant.

Figure legends

Fig. (1). A photomicrograph of well differentiated OSCC showing cohesive epithelial nests with central keratinization and minimal atypia, surrounded by connective tissue stroma showing inflammatory cell infiltration, H&E, x200.

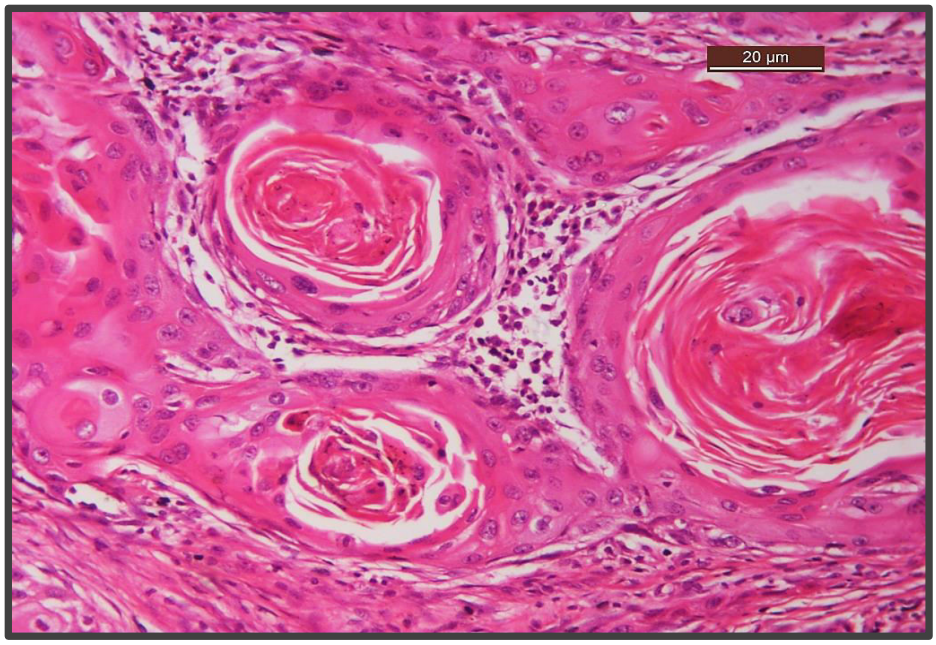

Fig. (2). A photomicrograph of poorly differentiated OSCC showing diffuse epithelial cells with prominent cellular and nuclear pleomorphism as well as high mitotic activity (arrows), H&E, x200.

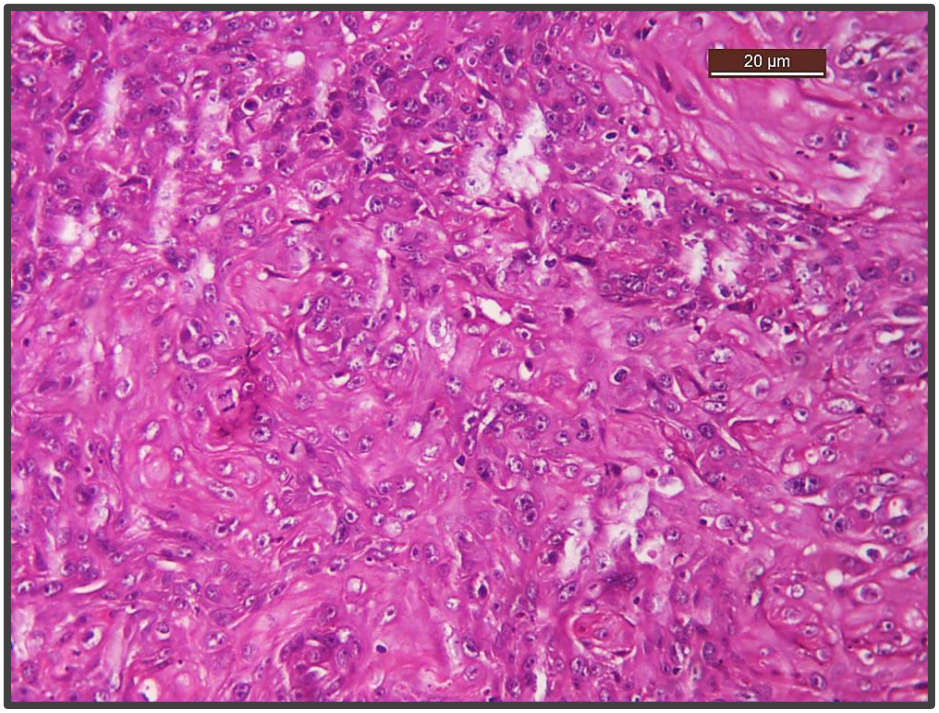

Fig. (3): Line chart illustrating miRNA-16 expression levels among the 3 studied groups.

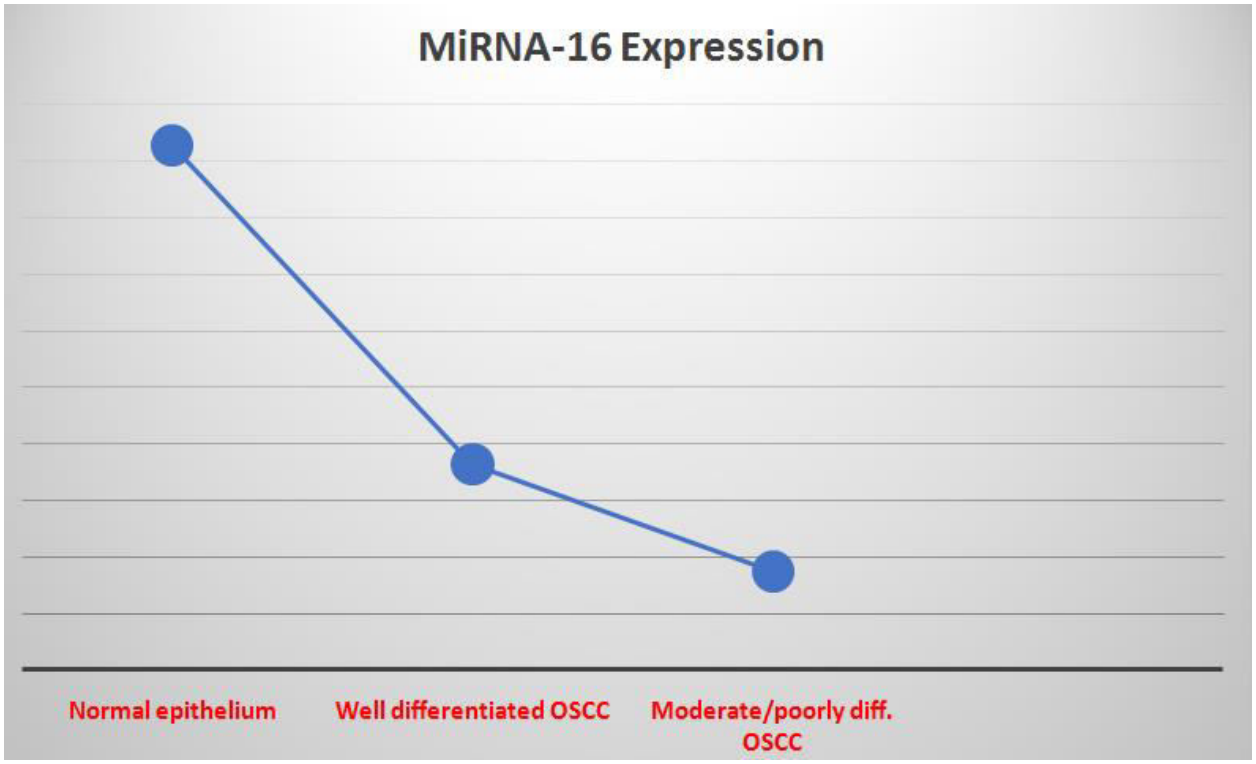

Fig. (4): Column chart illustrating miRNA-21 expression levels among the 3 studied groups.

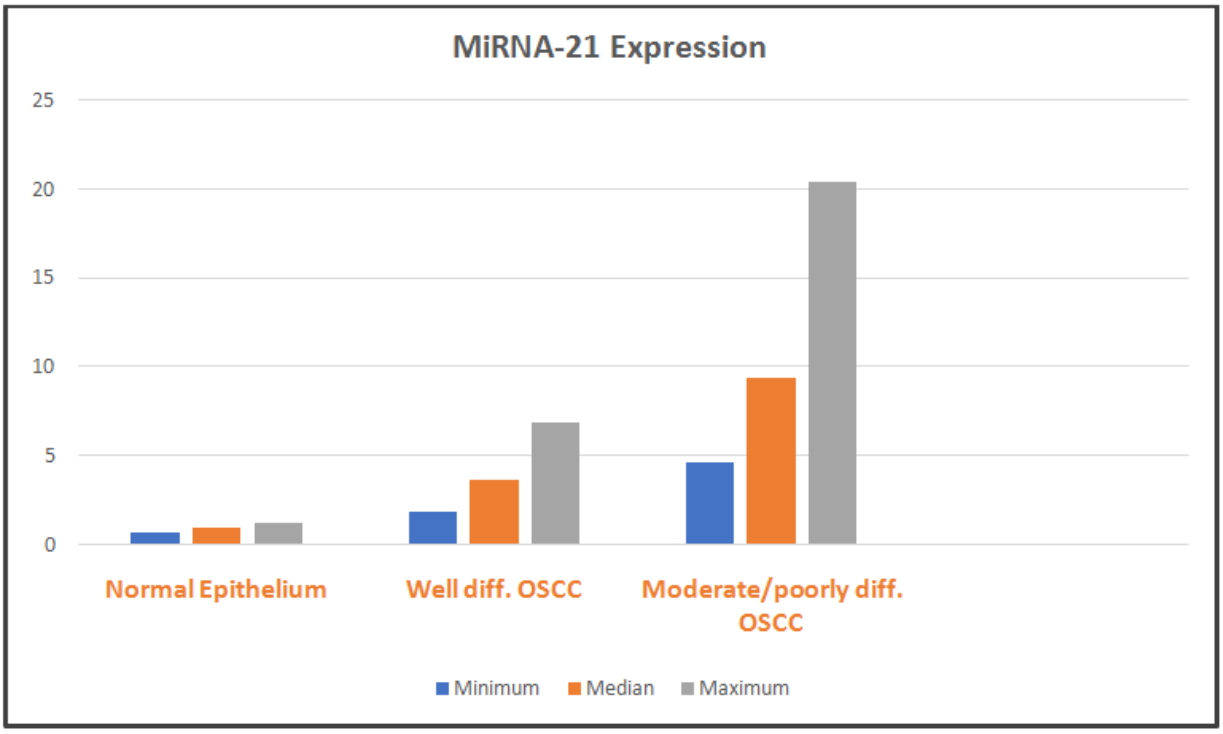

Fig. (5): Column chart illustrating miRNA-21 expression in males and females in both OSCC groups.

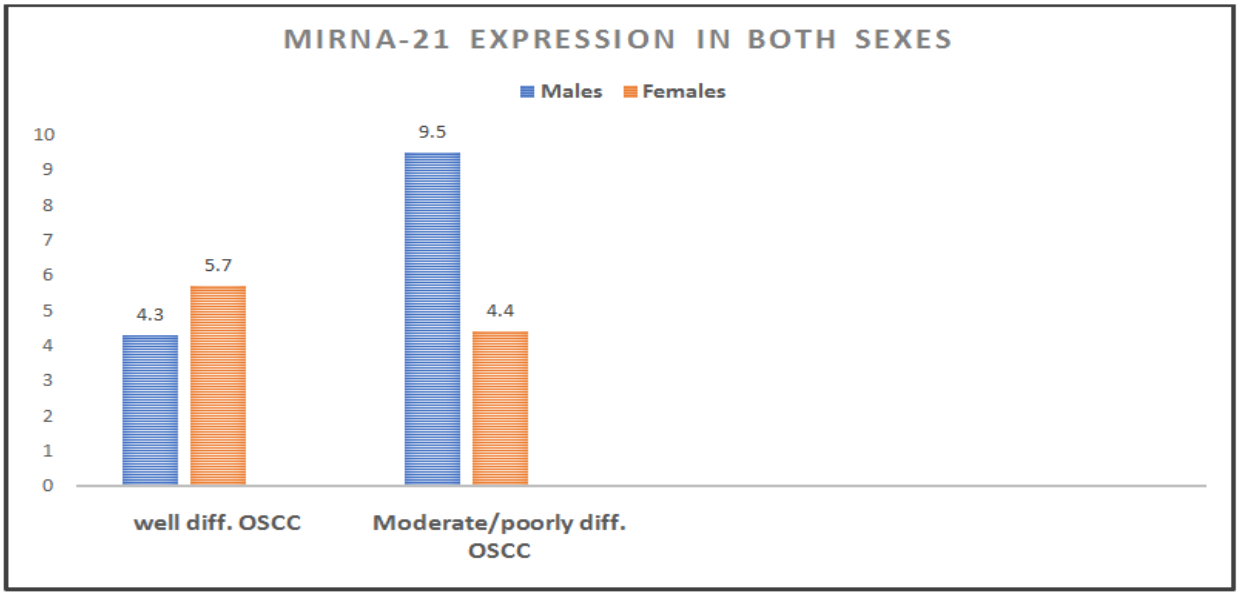

Fig. (6): Caption of display for Mann-Whitney U test result correlating miRNA-21 expression and patient sex in moderate/ poorly differentiated OSCC.

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of miRNA-21 expression is the same across categories of sex21 = 1 (FILTER).	Independent-Samples Mann-Whitney U Test	.019[1]	Reject the null hypothesis.

Fig. (7): Caption of display for Mann-Whitney U test result correlating miRNA-21 expression and patient sex in well-differentiated OSCC.

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of miRNA-21 expression is the same across categories of sex21 = 1 (FILTER).	Independent-Samples Mann-Whitney U Test	.364[1]	Retain the null hypothesis.

Fig. (8). Photomicrograph showing negative BCL-2 expression, a. normal stratified squamous epithelium except for scattered basal and stromal cells, anti-BCL-2 antibody, x200, b. well differentiated OSCC, anti-BCL-2 antibody, x200, c. poorly differentiated OSCC cells. Tumor associated inflammatory cells show positive BCL-2 reaction, anti-BCL-2 antibody, x200.