VALIDATION OF ASSESSMENT OF BMI-1 ON PROTEIN AND MOLECULAR LEVELS IN ORAL DYSPLASIA AND SQUAMOUS CELL CARCINOMA. (A DIAGNOSTIC STUDY)

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ABSTRACT

Background: B-cell–specific moloney murine leukemia virus integration site 1 (BMI-1), a member of the polycomb group (PcG) genes, is considered to be pivotal in regulating stemness-related genes involved in maintaining the self-renewal ability of stem cells by promoting chromatin modifications. BMI-1 also played a significant role in cancer etiology for its involvement in pathological progress such as epithelial–mesenchymal transition (EMT) and cancer stem cells (CSCs) maintenance, propagation, and differentiation.

Aim of the study: is to detect BMI-1 immunohistochemical expression and assess its mRNA level in normal oral mucosa, different grades of oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) as well as to correlate BMI-1 expression on protein and gene levels with different grades of OED and OSCC to detect which of them is more reliable the gene or the protein level.

Materials and Methods: A total of 40 archival paraffin embedded tissue samples divided into 3 groups: control, OED and OSCC. The OED group was further subdivided into (mild, moderate, severe) also the OSCC group was subdivided into (well, moderate, poorly differentiated) for immunohistochemical study and RT-PCR.

Results: we found that the normal oral mucosa group showed scattered nuclear immunoexpression of BMI-1 localized to the basal and parabasal layers. In OED group, BMI-1 showed diffuse nuclear expression in addition to, cytoplasmic immunoexpression among all subgroups. In OSCC group, nuclear and cytoplasmic immunoeexpression was also observed. One-way ANOVA test was used to assess the nuclear count, area percent as well as mRNA level among the groups collectively and within the studied subgroups. High statistical significance was observed among all the groups collectively regarding different techniques. However, within the subgroups only the nuclear count can have statistical significance within the studied subgroups.

Conclusion: Nuclear count assessment of BMI-1 is an accurate and reliable technique that can be used in the early diagnosis of OED and OSCC as well as identification of patients at high risk of malignant transformation.

Key words: BMI-1, oral carcinogenesis, oral potentially malignant disorders, immunohistochemistry, RT-PCR.
I. INTRODUCTION

Globally, oral cancer is considered as a growing public health concern due to its worldwide over-increasing incidence. Early diagnosis is of paramount importance for effective treatment of OSCC. Several studies have revealed that OSCC is often diagnosed in the late stages of the disease and this delayed diagnosis has precluded successful treatment and favorable outcomes. OSCC could arise de novo or from pre-existing potentially malignant disorders, which are referred to as oral potentially malignant disorders (OPMDs). Several studies agreed that the optimal time for clinical intervention is prior to malignancy if patients present with OPMDs, as approximately 50% of patients with OSCC had local or remote metastases at the time of diagnosis (Dalley et al, 2014; Dionne et al, 2015; Brockton et al, 2017; Bray et al, 2018 and Shin & Kim 2018).

Early diagnosis of the OPMDs has represented a continuous challenge. Fortunately, technological advancement in genomics and proteomics has led to identification and detection of different genomic and epigenomic alterations which form a cascading pathway in the formation and progression of tumors (Cecco et al, 2015). These advancements have made investigation of the molecular background and identification of early events for malignant transformation possible. Among these biomarkers, BMI-1 is one of members of the PcG transcription repressors that are involved in pathogenesis of oral cancer. Many researchers revealed the outstanding contribution of BMI-1 to maintain the self-renewal capacity of CSCs (Wang et al, 2017 and Yap et al, 2020).

Accumulating evidence have established important roles for BMI-1 as a transcriptional repressor, a regulator of stem cell function and a DNA damage repair protein (Nacerddine et al, 2012 and Siddique & Saleem 2012). Furthermore, it has been found to regulate cell senescence and proliferation (Benetatos et al, 2014). BMI-1 plays a critical role in the regulation of gene expression and cellular processes, including cell cycle progression and EMT (Bhattacharya et al, 2015; Sahasrabuddhe; 2016 and Soheilifar et al, 2018).

Detection of specific proteins using immunohistochemistry (IHC) is a technically simple and widely accessible test. A trained human eye may achieve an excellent understanding of such images and expression patterns; however, it is less accurate when it comes to quantification. Thus, the use of computer-based image analyzer could represent a solution to this problem by offering standardized image processing and reliable quantification (Di Cataldo et al, 2012). On the other hand, quantitative real time PCR (RT-PCR) is widely known as an effective method to analyze modulations in gene expression because of its efficiency to detect and precisely quantify the target genes, even at low expression levels (Schacht & Kern 2015 and Rocha et al, 2016).

Being highly expressed in many cancers has shed the light on the importance of BMI-1 and encouraged researchers to widely investigate its role. However, its expression levels and actual role in oral lesions were not fully elucidated. Accordingly, it seems interesting to examine the expression of BMI-1 on gene and protein levels using RT-PCR and IHC, respectively and to identify which of them showed more reliable results. In addition, comparing BMI-1 expression levels among normal oral mucosa, different grades of OED and OSCC. Therefore, advancing our understanding about the possible role of BMI-1 in pathogenesis of OED and OSCC as well as its association with different tumor grades. Such an approach may aid in early diagnosis of OPMDs prior to malignant transformation and identification of high-risk patients. Furthermore, developing novel targeted and effective therapies for OSCC which will eventually decrease the morbidity and mortality load carried by OSCC patients (Shin & Kim 2018).

II. MATERIAL AND METHODS:

Materials:

2.1 Study groups:

A. Normal control group:

Archival paraffin blocks of embedded normal tissue samples were obtained from Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University.

B. OED group:

A total of 19 specimens of OED with different grades (classified into: mild, moderate, and severe OED, according to criteria of WHO 2017)

C. OSCC group:
A total of 21 specimens of OSCC with different grades (classified into: well, moderate, and poorly differentiated OSCC, according to criteria of WHO 2017)

2.2 Immunohistochemistry:

Reagents:

1. **Primary antibody:** antibody used was BMI-1 rabbit polyclonal antibody supplied as concentrated antibody 1/30 to be used for staining of formalin-fixed, paraffin embedded tissues. (Catalogue No: abx031610, Abbexa, SNF MEDICAL, Cambridge UK).

2. **Secondary antibodies (Universal Kit):**

The universal immunostaining detection kit (Dako Cytomation Envision+ Dual link system, Peroxidase (DAB+) Code K4065, Carpinteria, CA, USA) was used. It contains the following reagents and materials:

- Dual endogenous enzyme blocks (0.3% hydrogen peroxide containing sodium azide and levamisole).
- Labeled Polymer-HRP
- DAB+ Substrate buffer
- DAB+ chromogen (3,3'-diaminobenzidine chromogen solution).

1) **Buffer:** Phosphate buffer saline (PBS) of pH 7.4 from SNF MEDICAL company.

2) **Image analyzer computer system**

2.3 RT-PCR:

- **Qiagen RNA extraction kit (Qiagen, USA):**
  - lysis Buffer RLT (RNeasy Mini Kit).
  - Buffer RW1 (RNA Wash).
  - Buffer RPE (RNA with 80% Ethanol as important ingredient).
- **Spectrophotometer**

- **cDNA Reagents and equipment:**
  - Moloney murine leukemia virus reverse transcriptase was used for synthesis of cDNA from RNA.
  - Human Placental Ribonuclease Inhibitor (HPRI) for inhibition of RNase activity.
  - First strand buffer: Provides preferred pH and ionic strength for reverse transcription.
  - Deoxynucleotide triphosphate (dNTPs) were used for extension of primers.
  - Random hexamers: primers for reverse transcription of RNA (Stratagene).
  - DEPC (Diethyl pyrocarbonate) - treated water.
  - Thermal cycler (Biometra, Horsham, PA, USA).

- **BMI-1 primer.**
- **SYBR Green mix using Applied Biosystems StepOne™Real-Time PCR.**
III. METHODS:

3.1 Section preparation:
Consecutive sections from paraffin-embedded tissue blocks were cut into 4 microns thick sections for routine histological staining. Tissue sections were stained with Hematoxylin and Eosin stain and re-diagnosed by the candidate and thesis supervisors to confirm the previously made diagnosis and grading according to criteria of WHO 2017. While the OED cases were subclassified into 3 groups: mild, moderate, and severe OED, OSCC was classified into: well, moderate, and poorly differentiated OSCC. For immunohistochemistry, the 4 microns thick sections were mounted on positively charged glass slides (Opti-Plus, Biogenex Laboratory USA from SNF MEDICAL company). The sections were marked by a ring drawn by diamond pencil. The slides were incubated at 37˚C overnight for accurate adhesion of the section to the slide. On the other hand, 30 microns of paraffin section from each sample were cut and preserved in Eppendorf, for procedures of RT-PCR.

Assessment of Histopathological Staining Methods:
The immunostained sections were assessed by two methods:

A. Ordinary Light Microscope:
The ordinary light microscope was used to detect the positive immunoreaction and localization of BMI-1 within the tissues of OED and OSCC.

Controls:
Positive controls: To specify the antibody, tissue sections of lymphoma as datasheet recommended, were included in each assay. Immunohistochemical expression of BMI-1 in the studied cases showed positive brown staining in the nuclei of neoplastic lymphocytes.

Negative controls: Specimens of the studied cases were stained with every assay by the routine immunostaining sequence, but the primary antibody was omitted and replaced by PBS to check for nonspecific staining.

B. Image analysis technique:
In order to provide more precise data on BMI-1 expression in OED and OSCC, all the immunostained sections were examined by the image analyzer computer system using the software Leica Qwin 500 (Germany) which comprise a light microscope (Olympus B x 60 Japan) capable of performing high speed digital image processing for the purpose of cell measurements. It is calibrated automatically to convert the measurement units (pixels) produced by image analyzer program into actual micrometer units, in the Center of Research and Dental Requirements, Faculty of Dentistry, Cairo University. In immunohistochemically stained sections, first immunohistochemical evaluation for nuclear count was performed under high-power magnification (×400). Five different viewing fields were randomly selected, and nuclear staining was considered for positivity, regardless of staining intensity. Then positively stained nuclei were automatically counted within the selected field. Second immunohistochemical evaluation for area percent was performed by selecting five specific fields showing maximum positivity of BMI-1 expression were used in each case; the mean of five values of each slide was calculated and statistically analyzed. For each field of immunostained section, a measuring frame of area 1920000 pixels and then the area % was calculated. The color of immunostaining was automatically selected according to the positive control sections, converted into grey color then masked the red and white to allow automatic measurements by the computer system.

3.2 Quantitative Real time-polymerase chain reaction (RT-PCR) assessment:
RT-PCR assessment was accomplished in Biochemistry Department, Faculty of Medicine, Cairo University to detect the gene expression of BMI-1 in different grades of OED and OSCC.

C. RNA extraction:
Total/ RNA was isolated using Qiagen tissue extraction kit (Qiagen, USA) according to instructions of manufacture.

1. Thirty microns of the tissue sample was excised, disrupted, lysed in lysis Buffer RLT and the lysate was homogenized by tissue homogenizer for 40 seconds.
2. The lysate was centrifuged for 3 min. at full speed and the supernatant was carefully removed and transferred into a new microcentrifuge tube.

3. 1 volume (350 µl) of 70% ethanol was added to the cleared lysate.

4. 700 µl of the sample was transferred to a RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec. at ≥ 8000 rpm.

5. 700 µl Buffer RW1 was transferred to the RNeasy spin column and centrifuged for 15 seconds. at ≥ 8000 rpm then, 500µl Buffer RPE was added and centrifuged for 15 seconds at ≥ 8000 rpm then, 500µl Buffer RPE was added and centrifuged for 2 min at ≥8000 rpm to wash the spin column membrane.

6. RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥8000 rpm to elute the RNA.

7. The eluate was transferred to a new Eppendorf tube and stored at –80 °C for further use. The purity (A260/A280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wavelength Beckman, Spectrophotometer, USA).

D. cDNA synthesis:
The total RNA (0.5–2 µg) was used for cDNA conversion using high-capacity cDNA reverse transcription kit (#K1621, Fermentas, Hanover, MD, USA).

Procedures:
1. Three µl of random primers were added to the 10 µl of RNA which was denatured for 5 min at 65°C in the thermal cycler.

2. The RNA primer mixture was cooled to 4°C.

3. The cDNA master mix was prepared and added according to the kit instructions.

4. Total volume of the master mix was 19 µl for each sample. This was added to the 31 µl RNA-primer mixture resulting in 50 µl of cDNA.

5. The last mixture was incubated in the programmed thermal cycler one hour at 37°C followed by inactivation of enzymes at 95°C for 10 minutes, and finally cooled at 4°C. Then RNA was changed into cDNA. The converted cDNA was stored at –20 °C.

E. RT-PCR for BMI-1 gene using SYBR Green I:
RT-PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The RT-PCR assay with the primer sets were optimized at the annealing temperature. All cDNA including prepared samples (for BMI-1 genes expression), internal control (for β-actin gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate.
Table (1): The primer sequence of the studied gene

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence</th>
<th>Gene bank accession number</th>
</tr>
</thead>
</table>
| BM-I        | F: TGTCTTTTCCGCCCGCTTCG  
              | R: TCTCGTTGTTCGATGCAATTCTGC | 170240                     |
| B-actin     | F: GGCAGCACCACCATGTACCCT  
              | R: AGGGCCGGACTCGTCAACT | NM001101.3                |

Calculation of Relative Quantification (RQ) (relative expression)

After the RT-PCR run, the data were expressed in Cycle threshold (Ct). The RT-PCR data sheet included Ct values of assessed gene (BMI-1) and the housekeeping (reference) gene, the gene that continuously expressed in the cell-(β-actin). To measure the gene expression of certain gene, negative control sample should be used. So, target gene expression was assessed and related to reference (internal control) gene as follows:

Finally, Calculation of Relative Quantification (RQ) (relative expression) according to Applied Bio system software using the following equation

1.  \[ \Delta Ct = Ct \text{ gene test} - Ct \text{ endogenous control} \]
2.  \[ \Delta \Delta Ct = \Delta Ct \text{ sample1} - \Delta Ct \text{ calibrator} \]
3.  \[ RQ = 2^{-(\Delta \Delta Ct)} \]

The RQ is the fold change compared to the calibrator (untreated sample)

Statistical analysis:

Real statistics resource pack version 6.8.2 Add to Microsoft excel version 2016 was used to analyze the results statistically. Results obtained from the immunohistochemistry and PCR tests has all been arranged in excel tables. Descriptive data analysis was performed first then the normality test. All groups for immunohistochemistry, and RT-PCR were distributed normally according to Sharpio Wilk test. Then, the hypotheses testing was done by series of Analysis of variance (ANOVA) single factor (one way) tests with alpha value is 0.05 and confidence intervals 95%. The significance level was set at \( P \leq 0.05 \). Then Tukey’s post hoc test was performed between each two groups.

RESULTS
Fig (1): Photomicrograph of normal oral mucosa showing scattered positive nuclear immunoexpression observed in the basal cells (red arrows) (BMI-1×200). **Fig (2):** Photomicrograph of moderate OED showing diffuse positive nuclear immunoexpression in the basal two thirds of epithelium. The stroma shows positive immunostaining of the chronic inflammatory cells (arrows) (BMI-1 x200). **Fig (3):** Photomicrograph of severe OED showing diffuse nuclear and cytoplasmic immunoexpression involving the whole thickness of epithelium except for the stratum corneum. The stroma shows positive immunoexpression of inflammatory cells (BMI-1 x200). **Fig (4):** Photomicrograph of well differentiated OSCC showing diffuse positive nuclear and cytoplasmic immunoexpression of tumor cells. Note: the positive nuclear reaction in tumor is associated with inflammatory cells (BMI-1 x200). **Fig (5):** Photomicrograph of moderately differentiated OSCC showing positive nuclear and cytoplasmic immunoexpression of tumor cells (BMI-1 x200). **Fig (6):** Photomicrograph of poorly differentiated OSCC showing positive nuclear and cytoplasmic immunoexpression of tumor cells (BMI-1 x400).

**Statistical Results**

**Immunohistochemistry:**

A. **Nuclear Count:**

The highest nuclear count was recorded in OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all groups collectively was statistically significant (P<0.001). Tukey’s post hoc revealed significant difference between each two groups (Table 2, Fig 7).

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>OED</th>
<th>OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>81.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>167.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>314.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Std Dev</td>
<td>24.04</td>
<td>17.1</td>
<td>28.419</td>
</tr>
<tr>
<td>Max</td>
<td>117</td>
<td>189</td>
<td>363</td>
</tr>
<tr>
<td>Min</td>
<td>60</td>
<td>149.667</td>
<td>293.667</td>
</tr>
<tr>
<td>F-value</td>
<td>124.838</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>9.309×10&lt;sup&gt;-09&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at p<0.05

*Sharing the same superscript letter denotes non-significant statistical difference.

**Fig (7):** Column chart showing difference in the mean value of nuclear count among control, OED, OSCC collectively.

Within the studied subgroups, the highest nuclear count was recorded in poorly differentiated OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all subgroups was statistically significant (P<0.001). Tukey’s post hoc revealed no significant difference between mild and moderate OED subgroups (Table 3, Fig 8).

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>Mild OED</th>
<th>Mod OED</th>
<th>Severe OED</th>
<th>Well OSCC</th>
<th>Mod OSCC</th>
<th>Poor OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>81.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133&lt;sup&gt;b&lt;/sup&gt;</td>
<td>167&lt;sup&gt;b&lt;/sup&gt;</td>
<td>202.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>290.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>392&lt;sup&gt;e&lt;/sup&gt;</td>
<td>410&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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Fig (8): Column chart showing difference in the mean value of nuclear count among all studied subgroups

B. Area Percent:
The highest area percent was recorded in OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all groups collectively was statistically significant (P<0.001). Tukey’s post hoc revealed significant difference between each two groups (Table 4, Fig 9).

Table (4): Difference in the area percent of BMI-1 immunoreactivity among collective groups (using one-way ANOVA test):

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>OED</th>
<th>OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.56a</td>
<td>22.96b</td>
<td>32.21c</td>
</tr>
<tr>
<td>Std Dev</td>
<td>1.16</td>
<td>6.76</td>
<td>2.35</td>
</tr>
<tr>
<td>Max</td>
<td>4.37</td>
<td>30.11</td>
<td>34.82</td>
</tr>
<tr>
<td>Min</td>
<td>1.45</td>
<td>15.35</td>
<td>29.53</td>
</tr>
<tr>
<td>F-value</td>
<td>65.895</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>3.44861*10^{-0.07}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at p<0.05

*Sharing the same superscript letter denotes non-significant statistical difference.

Fig (9): Column chart showing difference in the mean value of area percent among control, OED, OSCC collectively.
Within the studied subgroups, the highest area percent was recorded in poorly differentiated OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all subgroups was statistically significant (P<0.001). Tukey’s post hoc revealed no significant difference among mild vs moderate OED, moderate vs severe OED, severe OED vs well OSCC subgroups, and well OSCC vs moderate OSCC (Table 5, Fig 10).

Table (5): Difference in the area percent of BMI-1 immunoreactivity among studied subgroups (using one-way ANOVA):

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>Mild OED</th>
<th>Mod OED</th>
<th>Severe OED</th>
<th>Well OSCC</th>
<th>Mod OSCC</th>
<th>Poor OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.56</td>
<td>15.98</td>
<td>25.74</td>
<td>27.17</td>
<td>27.35</td>
<td>31.73</td>
<td>37.55</td>
</tr>
<tr>
<td>Std Dev</td>
<td>1.16</td>
<td>7.06</td>
<td>9.37</td>
<td>8.46</td>
<td>4.06</td>
<td>3.48</td>
<td>4.09</td>
</tr>
<tr>
<td>Max</td>
<td>4.37</td>
<td>26.09</td>
<td>35.48</td>
<td>41.62</td>
<td>32.37</td>
<td>36.31</td>
<td>44.84</td>
</tr>
<tr>
<td>Min</td>
<td>1.45</td>
<td>9.43</td>
<td>14.61</td>
<td>20.38</td>
<td>23.8</td>
<td>27.23</td>
<td>35.24</td>
</tr>
<tr>
<td>F-value</td>
<td>18.0463</td>
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<td>P-value</td>
<td>1.87328*08</td>
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</tbody>
</table>

*Significant at p<0.05

*Sharing the same superscript letter denotes non-significant statistical difference.

Fig (10): Column chart showing difference in the mean value of area percent among all the studied subgroups

PCR Statistical Results

The highest mRNA level was recorded in OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all groups collectively was statistically significant (P<0.001). Tukey’s post hoc revealed significant difference between each two groups (Table 6, Fig 11).

Table (6): Difference in the mRNA level of BMI-1 among collective groups (using one-way ANOVA test):

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>OED</th>
<th>OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.9</td>
<td>1.4</td>
<td>3</td>
</tr>
<tr>
<td>Std Dev</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Max</td>
<td>1.1</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Min</td>
<td>0.7</td>
<td>1.2</td>
<td>2.8</td>
</tr>
<tr>
<td>F value</td>
<td>235.026</td>
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<tr>
<td>P value</td>
<td>2.07*10^-8</td>
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</table>

*Significant at p<0.05

*Sharing the same superscript letter denotes non-significant statistical difference.
Within the studied subgroups, the highest mRNA level was recorded in poorly differentiated OSCC group, whereas the lowest value was recorded in normal group. ANOVA test revealed that the difference among all subgroups was statistically significant (P<0.001). Tukey’s post hoc revealed no significant difference among mild vs moderate vs severe OED subgroups, as well as between well vs moderate vs poor OSCC subgroups (Table 7, Fig 12).

Table (7): Difference in the mRNA level of BMI-1 among studied subgroups (using one-way ANOVA test).

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Normal</th>
<th>Mild OED</th>
<th>Mod OED</th>
<th>Severe OED</th>
<th>Well OSCC</th>
<th>Mod OSCC</th>
<th>Poor OSCC</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Std Dev</td>
<td>0.158114</td>
<td>0.158114</td>
<td>0.158114</td>
<td>0.158114</td>
<td>0.158114</td>
<td>0.158114</td>
<td>0.158114</td>
</tr>
<tr>
<td>Max</td>
<td>1.1</td>
<td>1.5</td>
<td>1.6</td>
<td>1.7</td>
<td>3.2</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Min</td>
<td>0.7</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
<td>2.8</td>
<td>2.9</td>
<td>2.7</td>
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<td>F-value</td>
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<tr>
<td>P value</td>
<td>5.94*10-21&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>*Significant at p<0.05</sup>

<sup>*Sharing the same superscript letter denotes non-significant statistical difference</sup>

IV. DISCUSSION

Despite wide investigations of OSCC and recent advances in its treatment approaches over the last decades, we have believed that better understanding of the mechanism underlying cancer initiation and growth is urgently needed. Thus, in the current work, we attempted to study one of the markers that may be involved in the early molecular events occurring before frank carcinogenesis can be detected. That’s why, not only OSCC was examined but also OED was included as identification of the fundamental molecular biology of cancer development in the oral cavity through stages may unravel a lot about the process of carcinogenesis (Ghazi et al, 2019 and Givony, 2020).

There is a great interest in developing novel diagnostic biomarkers to enhance early detection and clinical management of OSCC. However, the developmental path towards a clinically valid biomarker remains extremely
challenging (Hussein et al, 2018). The molecular marker of choice in the present work was BMI-1, which has attracted extensive attention as an essential epigenetic repressor of multiple regulatory genes (Althobiti et al, 2020). Controversial results have been obtained from different studies concerning upregulation of BMI-1, where some studies have reported its association with adverse prognosis, however others showed contrasting results (Tamatani et al, 2018 and Althobiti et al, 2020). Moreover, the expression and actual role of BMI-1 in OSCC seemed to be a matter in dispute, asking for further investigation (Ghazi et al, 2019).

In the current study, we assessed BMI-1 expression levels on gene and protein levels. In accordance, Hussein et al (2018) revealed that limiting the focus to protein expression only could be insufficient particularly due to the potential ongoing modifications of proteins by a plethora of post-translation changes. They have recommended that it is highly necessary to integrate all available information using different samples and various molecules and assessed by different assays for better understanding of biological pathways.

In the present study, we assessed the protein level of BMI-1 using IHC as it is an extraordinarily powerful tool in the process of the diagnosis for pathologists. It has been widely used for diagnosis of cancers, in addition to its simplicity and accessibility (Duraiyan et al, 2012). Also, we assessed BMI-1 on the gene level using RT-PCR which has been widely known as the most effective method to analyze modulations in gene expression and to measure mRNA levels because of its efficiency to detect and precisely quantify the target genes, even at low expression levels (Rocha et al, 2016). Moreover, RT-PCR does not require an experienced eye, and results are not affected by subjective interpretations, in addition, standardized protocols and automation ensures accurate performance and fast turn-around (Sinn et al, 2017).

Owing to the fact that BMI-1 is a potent nuclear marker, the nuclear count of positive nuclei was detected in the different studied groups. This technique is widely used for detection of BMI-1 expression in several studies including those of He et al (2015) and Klein et al (2020). However, in addition to the nuclear expression, our results showed positive cytoplasmic immunoreactivity in almost all the studied groups, thus area of positive immunoreactivity was measured, as well. For the best of our knowledge, this is the first study that measures the area percentage of positive BMI-1 immunoreactivity. In accordance with this finding, Reinsch et al (2007) and Guo et al (2011) reported positive BMI-1 cytoplasmic immunoreactivity. This immuno-reaction may be attributed to spread of nuclear BMI-1 into the cytoplasm due to its upregulation during cell proliferation.

Generally, histological diagnosis is considered the gold standard for OSCC. For OED, it is considered as the most important indicator for the risk of progression to oral cancer. In the current work, we used the grading systems adopted by WHO (2017) for both OED and OSCC. This is because they are the most recently used and accepted systems among pathologists. Although histological grading allows uniform reporting and could be used to predict the malignant potential of a lesion, it still has some limitations (Ranganathan &. Kavitha 2019).

The normal control group was used in our study as a reference to compare BMI-1 expression levels in OED and OSCC. As regard BMI-1 pattern of expression in normal control group, it was localized to some basal cells within oral mucosa. Based on the ability of BMI-1 to maintain stemness and self-renewal (Paranjape et al, 2014), these positively stained cells are most suggestive to be stem cells. The basal stem cell layer of normal oral mucosa is a self-perpetuating (self-induction) reservoir of cells that are essential for epithelial homoeostasis. In contrast to the limited BMI-1 expression in normal control group, BMI-1 up-regulation in dysplastic and carcinomatous throughout has been detected. In the same line, Sharma et al (2020) proposed that breakdown in cell-cycle turnover of these cells is antecedent to the development of OPMDs and OSCC.

The possible use of BMI-1 as a stem cell marker is supported by many reports who pointed out the crucial role played by BMI-1 in self-renewal and differentiation of neural stem cells prostate, intestinal, lung epithelial and bronchioalveolar stem cells progenitor cells and adult neural stem cells (Lukacs et al, 2010; Tian et al, 2011; Zacharek et al. 2011 and Bhattacharya et al, 2015). Moreover, inactivation of BMI-1 resulted in impaired stem cell self-renewal (Wang et al, 2015). Owing to the close similarity between stem cells and CSCs, several studies showed that BMI-1 potently promoted the survival and self-renewal of CSCs in different malignancies including HNSCC (Krishnamurthy et al, 2010; Bao et al, 2013 and Ajani et al, 2015).

Concerning BMI-1 expression levels in OED, it showed a significant gradual increase with the severity of grade of OED. This is suggestive that BMI-1 upregulation is associated with epithelial dysplastic changes. Similar results were reported by Kang et al (2007) and Klein et al (2020). Within OED grades, the significant increase of BMI-
1 expression levels in severe OED rather than mild and moderate OED lesions was in accordance with He et al (2015). This reflects the possibility of using BMI-1 as a marker of proneoplastic oral lesions that can detect the early stages of development of oral carcinogenesis and identify patients at high risk to develop malignancy (Klein et al, 2020). In agreement with our results, elevated BMI-1 expression levels were associated with the development of oral cancer in patients with oral leukoplakia (Liu et al, 2012), oral erythroplakia (Feng et al, 2013), oral lichen planus (Ma et al, 2013) and OED Chen et al. (2017). In the same context, Sedassari et al (2015) revealed the role of BMI-1 expression in the process of pleomorphic adenoma malignant transformation into carcinoma ex-pleomorphic adenoma.

In the current study, significant overexpression of BMI-1 in OSCC cells when compared to normal oral mucosa and OED suggests the possible role of BMI-1 in pathogenesis of OSCC. In agreement with our findings, Yang et al (2010), Liu et al (2012) and Xu et al (2018) revealed that BMI-1 strongly acted as an oncogene in several cancers including oral cancer. In the same context, involvement of BMI-1 in initiation and progression was reported in various tumors, including nasopharyngeal (Song et al, 2009), prostate (Lukacs et al, 2010), and oropharyngeal (Huber et al, 2011) cancers. In 2013, Qiao et al reported that BMI-1 overexpression has resulted in senescence bypass and immortalization of normal keratinocytes and malignant progression of precancerous lesions into cancers.

In the current study, the high significant expression of BMI-1 protein in all grades of OED and OSCC in comparison to the normal control group reveals association of BMI-1 levels with occurrence of dysplastic changes. Interestingly, statistical analysis succeeded to differentiate between normal control group, all grades of OED (as one group) and all grades of OSCC (as one group). Accordingly, BMI-1 levels may possibly be used in detection of early events of carcinogenesis as well as identification of patients at high risk of malignant transformation. In addition, its possible role in initiation and progression of OSCC was revealed. These findings were consistent with the studies of Dalley et al (2014) and Klein et al (2020).

Our results revealed increased BMI-1 immunoeexpression in the invasive front of OSCC, this can be attributed to involvement of BMI-1 in EMT process. Along the same lines, several studies believed that BMI-1 upregulation has been significantly connected with the invasive properties of the tumor (Chou et al, 2013; He et al, 2015). In (2015), Kurihara et al found that elevated levels of BMI-1 were accompanied by the downregulation of E-cadherin and the upregulation of vimentin. Accordingly, they concluded that BMI-1 was important factor associated with the promotion of EMT and invasion of tongue SCC.

In the present work, the gradual increase in BMI-1 gene and protein levels is directly correlated with the advanced grades of both OED and OSCC. Higher BMI-1 immunoeexpression in poorly differentiated OSCC than normal control group, OED, well and moderately differentiated OSCC is in the same perspective with Chen et al (2011); Yu et al (2012) and Chou et al (2013) who agreed that increased BMI-1 expression in HNSCC correlated with poor prognosis and poor overall survival in patients. In the same context, Xu et al (2018) reported similar findings in lymphoma, lung cancer, gastric cancer, colon cancer, prostate cancer, cervical cancer, lymphoma, leukemia, liver cancer and gliomas. However, in contradiction with our results, Häyry et al (2010), Yamazaki et al (2013) and Tamatani et al (2018) showed a negative correlation between BMI-1 upregulation and tumor invasion, as well as overall survival rate and poor prognosis of OSCC patients.

One of the remarkable findings in our study is the obvious immunopositivity of BMI-1 in the stroma especially the chronic inflammatory cells as well as endothelial cells suggesting BMI-1 may act as a link between chronic inflammation and tumorigenesis as inflammation is a common and important factor in the pathogenesis of cancer this was in accordance with the study of Yamada et al, (2017) in colitis-associated cancer. In 2014, Shigdar et al proposed that chronic inflammation contributes to the expansion of stem cells, leading to the development of cancer.

Similar to the results of BMI-1 protein levels, upregulation of BMI-1 mRNA levels was detected in OED groups as well as OSCC. These findings are in accordance with Dalley et al (2014), He et al (2015) and Wang et al (2017). Along these lines, Zhu et al (2017) proposed that upregulation of BMI-1 mRNA levels could induce downstream telomerase reverse transcriptase gene expression, resulting in telomerase activation, cell immortality, cell senescence and finally neoplastic transformation. However, detection of BMI-1 mRNA levels failed to differentiate between subgroups of OED and OSCC significantly. This finding is consistent with the study of Dalley et al (2014).
Concerning the used techniques, our results showed that detection of nuclear count, area percentage and mRNA levels of BMI-1 were all able to differentiate significantly between normal control group, OED group and OSCC group. Unlike area percentage of positive BMI-1 immunoreactivity and its mRNA levels, the nuclear count of BMI-1 immuno-positive cells is the only technique in the current work that succeeded to differentiate between the subgroups of both OED and OSCC (i.e: the different grades) significantly. Accordingly, we propose that nuclear count detection is the most accurate and reliable technique that could be used for detection of BMI-1 expression levels.


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