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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Presented By:

Asmaa Mohamed Abou Gabal

I. Title:

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II. Introduction:

Head and neck squamous cell carcinoma (HNSCC) including oral squamous cell carcinoma (OSCC) has been reported as the sixth most common cause of cancer mortality in the world and the fifth most commonly occurring cancer **[1].** Thus a compelling need for investigation of the underlying molecular events associated with OSCC tumorigenesis has emerged for better understanding of such lesion. Moreover, identification of biomarkers for early detection and prediction of prognosis became of extreme importance, as it was reported that early diagnosis has been vital for effective treatment of OSCC and improved the survival rate of OSCC patients **[2, 3].**

OSCC may originate from malignant transformation of the normal oral mucosa, as well as from oral potentially malignant lesions (OPMLs) with different degrees of oral epithelial dysplasia (OED) **[4]**. The approach of a step-wise transition from OPMLs to OSCC was well-established, but it could be difficult to predict if and when an OPML would undergo full transformation and resulted in a tumor**[5].** Thus, using specific molecular biomarkers able to identify OED lesions with higher potential for malignant transformation would be very beneficial**[4]**. Unfortunately, up to date there has been no tools available to monitor OED lesions or HNSCC patients for early stages of local recurrences or distant metastases **[6]**.

Among the recently introduced biomarkers, B-lymphoma Moloney murine leukemia virus insertion region-1 (BMI1), a member of the polycomb group (PcG) genes, was considered to be pivotal in regulating stemness-related genes involved in maintaining the self-renewal ability of stem cells by promoting chromatin modifications. BMI1 was also known to be deregulated in various human types of cancer **[7] [8] [9] [10]**. Previous studies have revealed the capability of BMI1 to be used as a prognostic marker in gastric**[11],** esophageal**[12]**, nasopharyngeal cancer**[7]**, prostate**[13]**, breast **[9]**, cervical **[14]** and ovarian cancer **[10]**, However,

the role of BMI1 in maintaining self-renewal and tumorigenicity in HNSCC or HNSCC-derived cancer stem cells (CSCs) remained to be clarified.

III. Review of literature:

HNSCC often developed within pre-neoplastic fields of genetically-altered cells with different degrees of OED. The presence of OED in OPMLs was generally accepted as one of the most reliable predictors of malignant development [15], however, histopathologic diagnosis was subjective and lacked sensitivity. No approval was reported on which dysplastic features were important in predicting progression. In addition, there was both inter- and intra-observer variation in illustrating the degree of epithelial dysplasia[5].Thus, several studies have been administered to identify objective molecular biomarkers to diagnose and prognose OED using different types of markers such as loss of heterozygosity, DNA ploidy, telomerase activity, methylation, and gene expression analysis [5].

Despite the advances that have been made in HNSCC treatment, prognosis remained discouraging. The major cause of recurrence and metastasis, both local and distant could be attributed to the diverse architecture of this tumor that consisted of functionally heterogeneous lineages of cancer cells that harbour cancer stem cell (CSC) properties [16] [17] [18] [19]. This hypothesis has gained support in a variety of tumors in addition to cultured cancer cell lines [20] [21] [22]. These CSCs have shown to be key contributors to radio- and chemoresistance and were responsible for tumor progression and recurrence after conventional therapy [23] [24].

CSCs were defined by their extensive self-renewal, differentiation and tumor initiation properties [25]. They were considered as the actual tumor initiating subpopulation and could expand depending on its evolutionary landscape during cancer development [16]. Recent findings have suggested that the persistent survival of CSCs, might contribute to the aggression and recurrence of HNSCC [26] [27] [23, 28]. Moreover, published studies suggested a direct link between epithelial-mesenchymal transition (EMT) and the gain of CSC-like properties[29]. The EMT process was considered as a critical step in the induction of tumor metastasis and malignancy [30].

The acquisition of genetic or epigenetic predisposition to tumorigenicity by CSCs has been shown to be a cumulative process **[31]** that might be evident in premalignant lesions **[32] [33]** as well as oral epithelial tumorigenicity **[34]**. This increased the importance of unraveling the underlying molecular events associated with CSC population, as they could serve as early detectors of malignant potential in OED and prognosis of OSCC. Thus, the signaling pathways required for the maintenance of CSCs became candidate targets of recent investigations in this field. Currently, light was shed on **B-lymphoma Moloney murine leukemia virus insertion region-1 (**BMI-1) which has been implicated in the regulation of CSCs.

BMI-1 oncogene is a member of the polycomb-group gene family and a transcriptional repressor. BMI-1 was first recognized in a B-cell lymphoma as a transcriptional repressor that was a member of the PcG transcription factors **[35].** Certain studies have revealed that BMI-1 was involved in the self-renewal, differentiation and tumor initiation of CSCs **[36] [37] [25].** Nevertheless, the molecular mechanisms underlying these biological processes remained vague.

In addition, BMI-1 was known to be upregulated in various human cancer tissues and was important in the regulation of malignant transformation, proliferation, cell cycle, apoptosis and distant metastasis **[38]**. Actually, many studies have reported that Bmi1 was overexpressed in HNSCC cells when correlated to normal epithelium and was thought to impact cell proliferation and survival in HNSCC **[39] [40] [41] [42]. Song et al 2009.,** demonstrated that Bmi-1 mRNA and protein expression levels were found to correlate with the invasion of nasopharyngeal carcinoma **[7].**

In clinical specimens of HNSCC, many researchers have established that the overexpression of Bmi-1 correlated with poor overall survival **[43] [39] [41]**. Likewise, **He et al 2015.,** added that the expression of Bmi1 was correlated with poor overall survival and was also associated with lymph node metastasis and clinical stage.

Association of Bmi1 overexpression, and stem-like properties in tumor cells could be attributed to induction of the EMT that promoted invasion, metastasis, and poor prognosis [41]. In the same background, **Yang et al., 2010** showed that BMI1 was necessary for EMT during tumor development in head and neck cancer patients [8].

Strong evidence suggested that Bmi1 was important to invasive potential and favored to the maintenance and self-renewal of CSCs in several tumor types, including HNSCC [44] [41] [24] [45]. As a stem cell marker, Bmi1 played a key role in the functioning of endogenous stem cells and CSCs [46] [45]. Additionally, it has been found to be related to many other CSC markers such as SOX2 [47], Oct4 [48], Nanog [48], ALDH1[32], ABCG2 [2] and CD44 [49] [50]. **Seo et al., 2011** found a connection between BMI-1 and Sox2 in maintaining self-renewal and identified BMI-1 as a key mediator of Sox2 function [47].

Moreover, recent studies have revealed that Bmi-1 expression was associated not only with the development of oral cancer, but also in patients with oral leukoplakia (LP)[40], oral erythroplakia[32], and oral lichen planus[42]. Furthermore, Bmi-1 overexpression was found in LP, and increased significantly from mild to moderate and severe LP. Consistent results were reported in Kang`s research, which showed that Bmi-1 overexpression was observed in preneoplastic oral mucosal tissues, including mild, moderate or severe OED [51]. **Ma L et al., 2013** suggested that Bmi1 may act as a beneficial marker for the recognition of high risk of malignant progression of OLP [42].

Wang et al., 2015 studies have indicated that silencing BMI-1 can decrease the malignant biological behavior of cancer, in addition to the self-renewal and differentiation of CSCs. Therefore, BMI-1 was hypothesized to affect the malignant biological behavior of human tumors by regulating the self-renewal and differentiation of CSCs. In conclusion, BMI-1 might represent a hopeful target for the prevention and therapy of various types of human cancer. Further understanding the molecular mechanism underlying the regulation of BMI-1 in human cancer and CSCs is of extremely huge clinical value [52].

PIRT

P→ Diagnostic validity of Bmi-1 levels in archival paraffin embedded tissue samples of oral dysplasia and squamous cell carcinoma.

I 1 → Expression of Bmi-1 protein level by immunohistochemistry in oral dysplasia and squamous cell carcinoma.

I 2→ Expression of Bmi-1 molecule at RNA level by PCR in oral dysplasia and squamous cell carcinoma.

R→ biopsy embedded in paraffin blocks.

T→oral dysplasia and squamous cell carcinoma.

IV. Researh Question:

Does the expression of Bmi-1 vary from oral epithelial dysplasia to different grades of squamous cell carcinoma at protein and molecular levels? Which one of them is more sensitive and specific for Bmi-1 assessment, the protein or molecular levels?

V. Methods:

Source of data:

Convenient consecutive sampling; it will include all archived blocks in the past 5 years (2012-2017).

Study design: Prospective cohort (In vitro validation study test)

Participants:

The archival paraffin embedded tissue samples which will be included in this diagnostic study are 30 samples of oral dysplasia and squamous cell carcinoma. We will apply a convenient consecutive sampling; it will include all archived blocks in the past 5 years (2012-2017) for this validation study. Excluded are samples of sarcomas or benign tumors or any carcinoma other than squamous cell carcinoma or any other techniques rather than immunohistochemistry or PCR. Samples that fulfilled the eligibility criteria will be included consecutively (consecutive sampling) until the target sample size will be reached. Data regarding Bmi-1 antibody expression in oral dysplasia and different grades of squamous cell carcinoma will be collected prospectively from both the reference and index tests independently.

Predictors:

Biopsy embedded in paraffin blocks was selected as reference standard method to assess the expression of Bmi-1 antibody at protein and RNA levels by immunohistochemistry and PCR respectively.

FIRST , PCR ANALYSIS

According to **Borbély et al 2007** tissue blocks will be prepared as follow:

1- Tissue of 30 microns will be cut from paraffin blocks using scalpel and was put in eppendorf.

2- 200 ml of tissue lysis buffer (ATL) + 0.6 ml of DNAs inhibitors + 10 ml proteinase K will be added to the sample**.**

3- Sample will be incubated at 55 ˚C for 20 min.

4- 250 micron of ethanol will be added and the sample will be left for 5min at room temperature.

5- Sample will be collected by micropipette into spin column and will be centrifuged at 8000/ rpm for 1 min.

6- 500 microlitre of wash buffer 1 will be added and the spin   column will be centrifuged at 8000/ rpm for 1 min.

7- 500 microlitre of wash buffer 2 will be added and the spin column will be centrifuged at 8000/ rpm for 1 min.

8- 500 microlitre of elusion buffer will be added and the spin column will be centrifuged at 14,000/ rpm for 3 min.

9- 490 microlitre of distilled water will be added for each of DNA extracted sample.

10- Spectrophotometer assessment will be done for concentration and purification of extracted nucleic acid (measurement of extracted nucleic acid 260/280 absorbance UV.

11- The sample will be divided into two portions in 200 ml eppendorfs.

12- The first portion will be added to SYBERGREEN for amplification and quantitation of Bmi-1 gene by real time PCR using APPLIED BIO SYSTEM (U S A).

13- The second portion will be added to SYBERGREEN for amplification and quantitation of Bmi-1 receptor gene by real time PCR using APPLIED BIO SYSTEM (U S A).

SECOND, IMMUNOHISTOCHEMISTRY

A-Reagents:

1- **Primary antibody** BMI-1 rabbit polycolonal antibody
2- **The universal immunostaining detection kit** (Dako Cytomation Envision+ Dual link system, Peroxidase (DAB+) Code K4065, Carpinteria, CA,USA).
3- **Buffer:** Phosphate buffer saline (PBS) of pH 7.4 (1.48 gm dibasic sodium phosphate anhydrous, 0.43 gm monobasic potassium phosphate anhydrous and 7.2 gm sodium chloride in one liter of distilled water).

B-Procedures:

1- Deparraffinization: The paraffin sections will be deparraffinized in three changes of xylene, and will be placed for 10 minutes in each change at room temperature.
   The excess liquid will be drained and the slides will be transferred to alcohol, 15 minutes for each descending grades of concentrations (100%, 70% then 50%). Then the slides will be rinsed under tab water for 5 minutes.
   Immersion of slides in PBS (2changes),5 minutes for each.
2- Epitope retrieval: Citrate Buffer Epitope Retrieval method will be used in this technique. Briefly, a steamer for water bath will be preheated with staining dish containing citrate buffer (ph 6.0) until temperature will be reached 95-100°C. Slides will be immersed in the staining dish and the lid will be loosely placed then will be incubated for 20 minutes and will turn off the steamer. The

staining dish will be placed at room temperature and will be allowed to cool for 20 minutes.

3- Primary antibody: sections will be incubated in rabbit anti-Bmi-1 individually for 1 hour at room temperature. Sections will be rinsed in 2 changes of washing buffer, 2 minutes each.

4- Serum block: sections will be incubated with normal horse serum blocking solution for 30 minutes to block non-specific binding of immunoglobulin. The best results will be obtained with dilution 1:25. Slides will be rinsed in washing buffer for 2×2 min

5- Peroxidase blocking: sections will be incubated in peroxidase blocking solution for 10 minutes to block endogenous peroxidase activity.

6- Slides will be rinsed in washing buffer 3×2 min

7- Secondary antibody: sections will be incubated with biotinylated horse Anti-Mouse IgG diluted in secondary antibody dilution.

8- Slides will be rinsed in washing buffer 3×2 min.

9- Chromogen/substrate: sections will be incubated in DAB peroxidase substrate solution for 5-10 min and then will be rinsed with distilled water

10- Slides will be counter stained with Mayer's haematoxylin solution, then will be rinsed in running tap water for 5 minutes

11- Slides will be dehydrated through 95% ethanol for 2 minutes, 100% ethanol for 2×3 minutes

12- Finally, they will be cleared in xylene for 2-3 minutes and cover slipped with permanent mounting medium.

Since BMI-1 level is measured objectively then no blnding is required.

First: Calculation of Relative Quantification (RQ) (relative expression):

After the RT-PCR will run, the data will be expressed in Cycle threshold (Ct).PCR data sheets will include Ct values of assessed gene and the house keeping (reference) gene which will be continuously expressed in the cell- (β-actin).To measure the gene expression of certain gene, -ve control sample shall be used. So target gene expression will be assessed and related to reference (internal control) gene as follows:

Finally, RQ was calculated according to the following equation:

1. Δ Ct (Cycle threshold) = Ct assessed gene – Ct reference gene
2. ΔΔ Ct = Δ Ct sample – Ct control gene
3. RQ = $2^{-(\Delta\Delta Ct)}$

Second: Image analysis:

In order to provide more précised data on Bmi-1 immunoexpression in OSCC, image analyzer will be used.

The data will be obtained using the software (SIS, Germany), which comprise a light microscope (Olympus B × 60 Japan) capable of performing high speed digital image processing for the purpose of cell measurements. It will be calibrated automatically to convert the measurement units (pixels) produced by image analyzer program into actual micrometer units. In immunohistochemical sections, and by using the image analyzer, five specific fields showing maximum positivity of Bmi-1 expression will be used in each case; the mean of five values of each slide will be calculated and statistically will be ananlyzed. For each field of immunostained section, a measuring frame of area 1920000 pixels and then the area % will be calculated. The color of immunostaining will be 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. Data obtained will be subjected to statistical analysis using Student-t test.

Since BMI-1 level is measured objectively then no blnding is required.

Sample size
The aim of the current study is to assess the validation of Bmi-1 detection at both protein and molecular levels in oral epithelial dysplasia and oral squamous cell carcinoma as a biomarker for early cancer detection versus biopsy embedded in paraffin blocks. We are going to apply a convenient consecutive sampling; it will include all archived blocks in the past 5 years (2012-2017) about 30 samples.

Statistical analysis methods:

Data will be analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL). Numerical data will be described as mean and standard deviation or median and range. Categorical data will be described as numbers and percentages. Correlation analysis will be used. Agreement between both methods will be analyzed with the use of kappa statistics.

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