The Expression Pattern Of Bmi 1 Can Predict The Therapeutic Potential Of Imiquimod And Piperine In Experimentally Induced Hamster Buccal Pouch Carcinoma (Histological And Immunohistochemical Studies)

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Abstract: This study aimed to investigate the expression pattern of Bmi1 of treated hamsters with imiquimod and piperine either alone or in combination with each other, after experimentally induced HBP carcinoma. **Material and methods**: 50 Syrian male hamsters were divided into 5 equal group$_{(s)}$ ($G_{(s)}$), 10 each. The animals in GI were left untreated for 22 weeks (ws), while the right hamster buccal pouch (HBP) of those in (GII, GIII, GIV and GV) were painted with 7, 12-dimethylbenz (a) anthracene (DMBA), three times a week for 14 ws. After that, the animals in GII (positive control) were left with no additional treatment for other 8 ws (total period 22 ws), whereas those in GIII were painted with imiquimod three times a week for other 4 ws, while those in GIV were treated by piperine (50 mg/kg, orally) daily for 4ws, and those in GV painted with imiquimod three times a week and in the same time were given piperine (50 mg/kg, orally) daily for 4ws then, (GIII, GIV and GV) were left with no additional treatment for other 4 ws (total period 22 ws). After termination of the experiment, the animals were euthanized, and all right HBP were surgically excised, prepared, fixed and processed for hematoxylin and eosin (H&E) stain examination for histological examination and classification, and immunohistochemical (IHC) staining utilizing B-lymphoma moloney murine insertion region (Bmi1) cancer stem cell(CSC) antibody. **Results**: There was a high significant difference between GII and the treated Gs (GIII, GIV& GV) (p value < 0.001) regarding to tumor volume, depth of invasion(DOI) and regarding nuclear expression of Bmi1which revealed that, GI had recorded the lowest mean area percentage (9.71%), while GII had recorded the highest mean area percentage (65.88 %),followed by GIII and then GIV **Conclusions:** imiquimod and piperine could inhibit HBP squamous cell carcinoma (SCC) formation either alone or in combination with each other, in addition to downregulation of Bmi1 can predict the therapeutic potential of imiquimod and piperine in HBP SCC.

Keywords: HBP carcinoma, imiquimod, piperine, Bmi1.
DOI:10.48047/ecb/2023.12.si10.00118

1-Introduction:

Globally, oral cancer is the most common head and neck malignancy in 2020 an estimated 377,713 cases of OSCC are newly diagnosed and 177,757 deaths annually[1]. Oral cancer refers to any cancerous growth in the oral cavity. This cancer includes tumors of the lips, tongue, cheeks, gum, floor of the mouth, soft and hard palate, sinuses, tonsils, salivary glands and throat that can be fatal if left untreated [2]. OSCC may develop from premalignant dysplastic lesions, which are clinically present as erythroplakia, leukoplakia, lichen planus, or combinations of these conditions[3]. In such cases with premalignant lesions, frequent exposure to well-established carcinogens such as alcohol, tobacco, betel nut and human papillomavirus (HPV) infection may promote OSCC formation. After OSCC are generated, tumor cells can deeply invade the local structures and lymph nodes of the neck, leading to further distant metastases. These adverse biological features strengthen the recurrence propensity of OSCC[4, 5]. OSCC is generally divided into histological categories: superficially invasive or deeply invasive carcinoma, with additional modifies based on histologic grading including well, moderate or poorly differentiated[6]. Well-differentiated cells almost perfectly resemble normal squamous epithelium, but indicating basement membrane destruction by nests of tumor cells. OSCC shows the following features: disorganized growth, dyskeratosis, loss of polarity, keratin pearls, an increased nuclear-to-cytoplasmic ratio, distinct eosinophilic nucleoli, nuclear chromatin irregularities, and increased mitotic figures (including atypical forms)[7]. In poorly differentiated OSCC, the number of mitotic figures and necrosis tends to increase. The poorly differentiated lesions only have a very little resemblance to squamous epithelium. A rich inflammatory infiltrate comprising mainly lymphocytes and plasma cells is seen at the epithelium to connective tissue junction, with a positive correlation to metastatic potential [7]. It has been found that, oral carcinogenesis induced by DMBA is an effective site specific carcinogenic agent, generally used to induce buccal pouch carcinoma in golden Syrian hamsters[8], which was found to be closely resemble the human OSCC and commonly accepted model for exploring the therapeutic potentiality of natural entities and synthetic drugs[9]. Despite technological advances and improvements in OSCC diagnosis and treatment modalities, its 5-years survival rate remains low[10]. The low immunogenicity of tumor cells and the immunosuppressive microenvironment constitute the key protective factors for cancer cells to evade immune surveillance, including decreased levels of tumor specific-antigens or tumor associated-antigens, and the release of immunosuppressive factors in the tumor microenvironment [11]. Thus enhancing the immunogenicity of tumor antigens[12], improving the maturation efficiency of antigen- presenting cells (APCs)[13] , and activating cytotoxic T lymphocytes (CD8)[14] lead to relief of evasion the immune surveillance. The immune checkpoint blockade therapy has made significant advances in the past decade. However, not all patients show positive responses to the treatment[15, 16] In this context, toll like receptor(TLR) agonists have become the focus for finding adjuvants of immunotherapies, modulating innate immunity and forming adaptive immune responses[17]. The discovered TLRs have ten different types in human and are mainly expressed on tumor cells and immune cells[18]. Imiquimod, trade name is Aldara considered as an immune response modifier acting as a TLR7 agonist which stimulates innate and cell-mediated immunity to induce antitumor effects [19] [20]. Medicinal plant constitute as a resource for drug discovery, with 80% of all synthetic drugs deriving from them[21]. It has been proved that these plant-derived active compounds significantly improved to treat diseases such as cancer[22]. In this context, the anticancer effect of numerous natural compounds, including piperine, was extensively studied, and several research papers and reviews have appeared in the past few years [23-26]. These articles support the use of piperine as chemopreventive and anticancer agents. piperine is isolated from several members of the piperaceae family including piper nigrum (black pepper), piper longum, piper chaba, piper guineense and piper sarmentosum[27, 28]. The anti-cancer activities of piperine are based on its ability to regulate multiple signaling molecules such as. inhibition of cellular proliferation, [27] arrest of cell

cycle,[28] induction DNA damage of tumor cells, [29] induction of apoptosis,[29, 30] alterations in redox homeostasis,[29] modulation of angiogenesis[31] and degradation of extracellular matrix [32]. CSCs are known as cells that show self-renewal ability and asymmetric division. These cells are also associated with cancer cell growths and metastasis and tumor recurrence following treatment. Studies have recently shown that targeting CSCs can be an effective treatment strategy for cancer treatment**[33, 34]**. CSC markers that could have potential usefulness within therapeutic, diagnostic, and prognostic approaches are pointed out and focus on deadliest tumors as OSCC**[35]**.

Bmi1 acts in the self-renewal ability of stem cells. High expression of Bmi1 in cancer was related to epithelial mesenchymal transition (EMT) and poor prognosis **[36, 37]**. Many studies have shown that Bmi1 expression is frequently unregulated in various types of human cancers, including OSCC, acute myeloid leukemia, nasopharyngeal carcinoma and many other types of cancer which indicates that Bmi might play important roles in cancer initiation and progression **[38-50]**.
Hence, the main target of the present study was to assess the effects of imiquimod and/or piperine on DMBA induced HBP carcinoma. The assessment was based on the HBP gross observations, histological tumor tissue changes and IHC expression of Bmi1.

2-Material and methods:

2.1 Chemicals:

DMBA (0.5%) was obtained from Sigma-Aldrich, company, USA and it was prepared by dissolving 0.5gm DMBA powder in 100 ml paraffin oil (Sigma-Aldrich) [51]. A topical cream formulation containing 5% imiquimod marketed by 3M Pharmaceuticals as Aldara supplied in single-use packets each of which contains 250 mg of the cream [52]Piperine was obtained from Sigma-Aldrich Corporation, Steniheim, Germany and prepared by dissolving in corn oil [53].

2.2 Animals:

50 Syrian male hamsters, 5 ws old, weighing 80-120g were obtained from private animal house, Cairo, Egypt.

2.3 Sample size:

Based on, Gkoulioni et al (2010) [25] research and Annie W et al, (2017) [54][26] reported that, a sample size of 10 in each group, in the current study, will have 85% power to detect a difference between means of 0.53 with a significance level (alpha) of 0.05 (two-tailed) at 95% confidence intervals. In 85% (the power) of those experiments, the p value will be less than 0.05 (two-tailed) so the results will be deemed "statistically significant". In the remaining 15% of the experiments, the difference between means will be deemed "not statistically significant". Report created by GraphPad StatMate 2.00.

2.4 Experimental design:

After a week of adaptation, the animals were divided randomly into five equal Gs, 10 each. While the animals in GI) were, left untreated, the right pouches of those in GII, GIII, GIV and GV were painted with the 0.5% DMBA in liquid paraffin using a number 4 camel hair brush, three times a week for 14 ws**[55]**. After that, the animals in GII (positive control) were left with no additional treatment for other 8 ws (total period 22ws), while those in GIII (imiquimod), painted with imiquimod to the right HBP three times a week for other 4 ws[56], then, left with no additional treatment for other 4 ws (total period 22 ws), GIV were treated by piperine (50 mg/kg, orally) daily for 4ws[57], then, left with no additional treatment for other 4 ws (total period 22 ws) and those in GV painted with imiquimod three times a week and in the same time were given piperine (50

mg/kg, orally) daily for 4ws then, left with no additional treatment for other 4 ws (total period 22 ws).

2. 5 Histopathological examinations:

The tissues specimens were cut and fixed in 10% formalin, then washed and dehydrated in an ascending ethanol series, embedded in paraffin wax to form paraffin blocks. Tissue sections of 4µm thickness were cut using rotary microtome, mounted on glass slides, processed, and stained with H&E for histological examination.

2. 6 Immunohistochemical examination:

Other tissue sections were cut at 3µm and put on positive charged slides for the application of standard labeled streptavidin- biotin method to demonstrate the expression of Bmi1 antibodies. The sections were deparaffinized in xylene and rehydrated through graded ethanol (100%, 95 % and 70 %) each run for 5 minutes. Slides were washed in distilled water then in phosphate buffered saline (PBS), each for 5 minutes. Endogenous peroxidase activity was blocked using 3% solution of hydrogen peroxide (H_2O_2) in methanol for 30 minutes at room temperature. Slides were then washed in PBS. Slides were then immersed in plastic jars containing 200 ml of citrate buffer (pH 6). The jars were put in microwave at maximum power at 100°C for 3 intervals, each one 5 minutes. Slides were left at room temperature to coal gradually. Slides were then washed in distilled water followed by PBS for 5 minutes. Tissue sections were received one or two drops of the primary antibodies Bmi1 in a dilution of 1:100 and incubated in a humid chamber at room temperature overnight. Slides were then washed in distilled water, followed by PBS for 5 minutes. Biotinylated secondary antibody was added and incubated at room temperature for 30 minutes. Tissue sections were then washed in PBS for 5 minutes. One or two drops of peroxidase-labeled streptavidin were applied for 30 minutes at room temperature then washed in PBS. The tissue sections were received DAB for 2-4 minutes to develop color, followed by putting in distilled water. Tissue sections were counterstained using Mayer hematoxylin for one minute and then washed in tap water. The slides were placed in two changes of 95% alcohol followed by two changes of absolute alcohol, each for 3 minutes then mounted with DPX and covered with plastic covers in order to be examined. Negative controls were prepared by omitting the primary antibody. Breast carcinomawere used as positive controls for Bmi1.

The immunostained sections were examined using light microscope to assess the prevalence of positive cases and the localization of immunostaining within the tissues. In addition, image analysis computer system was used to assess mean area percentage of IHC stainability of cells with Bmi1 antibody. Assessments were selected under a low power lens (10×10) and a mean value was chosen for statistical analysis[(58)].This was done in oral and dental pathology department, Faculty of Dental Medicine (Boys-Cairo), Al-Azhar University, Egypt.

2. 7 Statistical analysis:

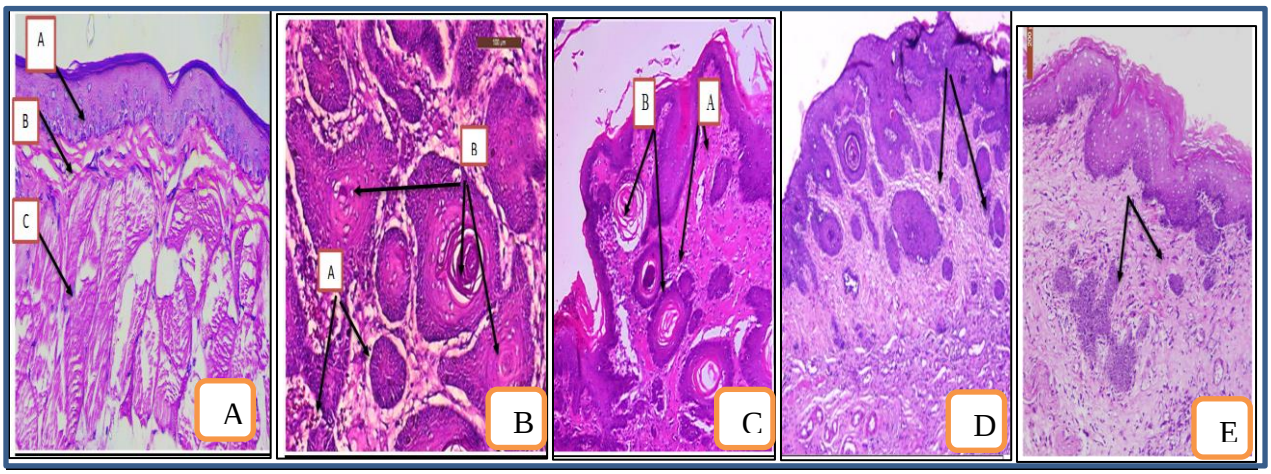
The results were recorded as the mean ± standard deviation (SD) and statistically analyzed. A one-way analysis of variance (ANOVA) was performed using SPSS version 17.0 for windows. The comparison between more than two independent groups with quantitative data and parametric distribution was done by using ANOVA followed by post hoc analysis using LSD test. The p value was considered significant as the following: $p > 0.05$: non-significant, $p < 0.05$: significant and $p < 0.001$: highly significant.

3-Results: 3-Results:

3. 1 Histological findings:

Histological sections of GI, using H&E stain, revealed normal features of the mucosa, which is composed of a thin stratified squamous epithelium, consists of four layers of squamous cells exhibiting slight keratinization (i.e.; one layer of basal cells and two or three layers of spinous and thin keratinized cells with lacking rete ridges. Subepithelial connective tissue (C.T)and muscular layer (Fig.4A),while **GII:** revealed that, 7 hamsters out of 10 exhibited well differentiated SCC(Fig.4B) and 3 hamsters exhibited moderately differentiated SCC .The invading epithelial islands into underlying C.T are variable in size, dysplastic features and keratin formation which extend deeply in C.T with mean DOI=10.02 mm (Table .3), **GIII** showed that, 2 hamsters out of 10 showed severe epithelial dysplasia, while2 hamsters showed moderate epithelial dysplasia and the other 6 hamsters exhibited SCC with superficial invasion of malignant cells in the form of well differentiated SCC which was limited to the sub epithelium area not extended to deeper areas within C.T (Fig. 4C), with mean DOI=2.58 mm (Table .3), **GIV** revealed that, 3 hamsters out of 10 showed moderate epithelial dysplasia, 1 hamster showed mild epithelial dysplasia, 2 hamsters showed carcinoma in situ, and 4 hamsters exhibited less invasive SCC with superficial invasion of malignant cells in the form of well differentiated SCC which was limited to the sub epithelium area not extended to deeper areas within C.T (Fig. 4D),with mean DOI= (0 – 2.81) mm (Table .3),and finally**GV:** revealed that, 7 hamsters out of 10 exhibited different degrees of epithelial dysplasia 1 mild, 3 moderate and 3 severe epithelial dysplasia, while 3 hamsters out of 10 showed less invasive SCC which were ranged from just destruction of basement with invading epithelial cells very close to surface epithelium which was present in 1 out of 3while the invading dysplastic epithelial cells of the other 2 less invasive SCC were limited to sub-epithelial and not extended to the deeper C.T (Fig. 4E) with DOI= (0 – 2.4mm) (Table .3).

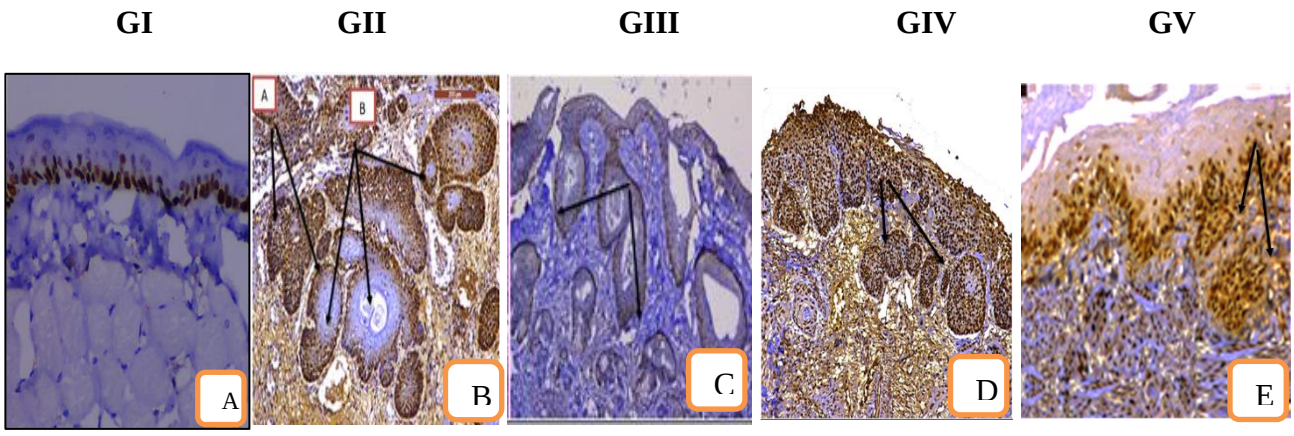
GI **GII** **GIII** **GIV** **GV**



(Fig.4): (**A**) H&E stain of GI showing keratinized stratified squamous epithelium with flattened rete ridges (arrow A), sub-epithelial CT layer (arrow B)and muscular layer(arrow c). (X200); **(B)** H&E stain of GII showing well differentiated SCC, with deeply invading multiple epithelial islands into the underlying C.T (arrows A) and keratin pearls (arrows B). (X 200); **(C)** H&E stain of GIII showing well differentiated SCC, with multiple tumor islands into the underlying C.T (arrows A) and keratin pearl (arrow B). (X 200); **(D)** H&E stain of GIV showing well differentiated SCC, with few and small epithelial islands into the underlying CT (arrows). (X 200); **(E)** H&E stain of GV showing less invasive SCC, with the invading dysplastic epithelial cells limited to sub-epithelial and not extended to the deeper C.T (arrows) (X 200).

3.2 Bmi-1 IHC results:

The IHC staining in GI using Bmi-1 antibody exhibited positive nuclear expression (9.71 %) which limited to basal and suprabasal epithelial layer(Table.4)(Fig.5A),while in GII exhibited positive nuclear expression (65.88 %)of the invading epithelial islands and negatively stained keratin pearl(Table.4) (Fig.5B),also in GIII(Fig.5C) ,GIV(Fig.5D) and GV (Fig.5E) Bmi-1 antibody exhibited positive nuclear expression throughout the dysplastic and invading malignant cells (37.02%)(31.09%)(25.89%) (Table.4).



(Fig.5): (A) Bmi1 IHC staining of GI showing positive nuclear expression limited to the basal and suprabasal epithelial layer (arrow) (Streptavidin biotin peroxidase, X 200); **(B)** Bmi-1 IHC staining of GII showing positive nuclear expression of the invading epithelial islands(arrows A) and negatively stained keratin pearl(arrows B) (Streptavidin biotin peroxidase, X 200); **(C)** Bmi-1 IHC staining of GIII showing positive nuclear expression throughout the

epithelial nests, islands and invading tumor cells (arrows) (Streptavidin biotin peroxidase, X 200); **(D)** Bmi-1 IHC staining of GIV showing positive nuclear expression throughout the epithelial nests, islands and invading tumor cells (arrows) (Streptavidin biotin peroxidase, X 200); **(E)** Bmi-1 IHC staining of GV showing positive nuclear expression throughout dysplastic epithelial cells, and invading tumor cells (arrows) (Streptavidin biotin peroxidase, X 200).

Statistical analysis results of IHC staining of Bmi-1:

Statistical analysis results, were revealed that, GI had recorded the lowest mean area percentage (9.71%), while GII had recorded the highest mean area percentage (65.88 %).There were high significant difference between GII and the treated Gs (GIII, GIV& GV) (p value < 0.001). Table (3), Chart (3), while by comparing the various treated Gs (GIII, GIV, and GV) there were significant difference between GIII and GV (P-value <0.05), while no significant difference between GIV and GV (P-value >0.05). Table (4), Chart (3).

Table (4): Comparison between the studied groups regarding the mean area percentage of IHC stainability of cells with Bmi1 antibody.

	Bmi-1		**Post Hoc analysis by LSD**				
	Mean ± SD	**Range**	**G I**	**G II**	**G III**	**G IV**	**G V**
G I	9.71 ± 1.87	7.12 – 12.8	--	0.000	0.000	0.000	0.000
G II	65.88 ± 10.59	45.98 – 77.02	0.000	--	0.000	0.000	0.000
GIII	37.02 ± 13.35	19.75 – 58.41	0.000	0.000	--	0.162	0.011
G IV	31.09 ± 7.94	20.78 – 44.98	0.000	0.000	0.162	--	0.219
GV	25.89 ± 8.84	15.86 – 40.89	0.000	0.000	0.011	0.219	--
Test value	**48.539**						
P-value	**<0.001 (HS)**						

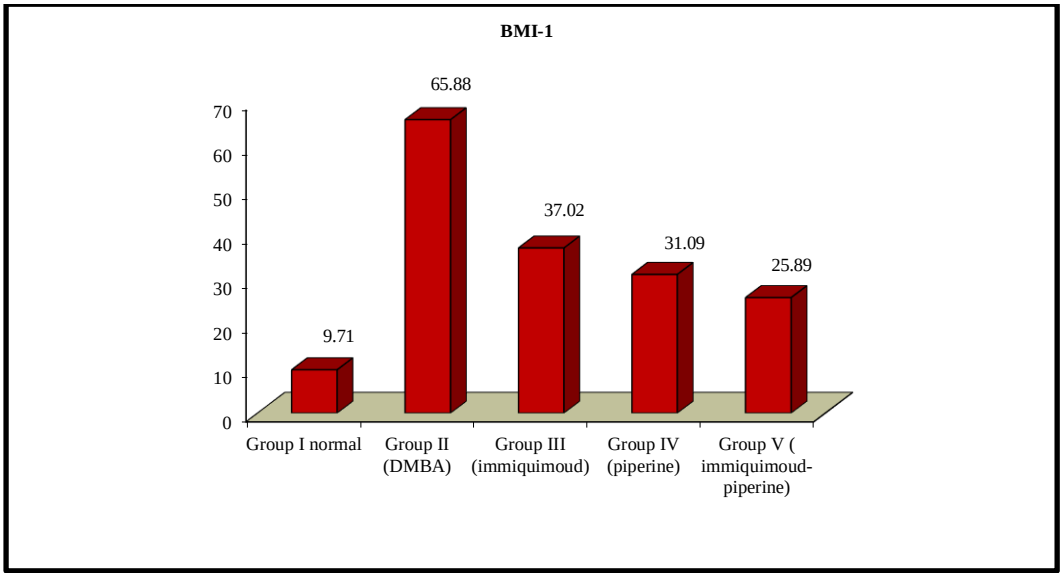


Chart (3): Bar chart representing the mean area percentage of IHC stainability of cells with Bmi-1 antibody in the studied groups.

4-Discussion:

Oral cancer considered as one of the most common cancers worldwide. Despite easy access to the oral cavity and significant advances in treatment, the morbidity and mortality rates for oral cancer

patients are still very high. The model of hamster buccal pouch system of oral carcinoma is useful and important in deeper understanding of cancer biology, diagnosis and treatment modalities. In the current work, the histopathological results of GI are in line with that of other investigators [59-61].In the current study, IHC staining of GI showed positive nuclear expression of Bmi1 (9.71 %) that was seen to be restricted to the basal and supra-basal epithelial layers and almost negative in the remaining epithelial cell layers. This result is in agreement with that of other investigators[37, 40]. Kalish et al (2020)[40] stated that, Bmi1 was found to be necessary for the self-renewal of both normal and CSCs.

Balakrishnan et al (2022)[62] found that, 100% tumor formation after 14 weeks of painting DMBA alone on the hamster cheek pouches. These results did not differ much from other studies done by Selvasundaram et al(2018)[63] and Manimaran et al(2017)[64]. This may be attributed to procarcinogenic nature of DMBA, which metabolized by phase I enzymes such as cytochrome P450 to its ultimate carcinogenic metabolite, dihydrodiol epoxide, which binds to and damage DNA, contributing to mutation and carcinogenesis. Also, ROS has been implicated in all the three stages (initiation, promotion, and progression) of carcinogenesis. ROS-mediated DNA damage could cause structural modifications in DNA, activation of proto-oncogene and inactivation of tumor suppressor genes, which eventually leads to neoplastic transformation[65].

In contrast to the present study, Yang et al (2006) [66], Li et al (2002) [67]and Hussein et al (2018) [68]reported that OSCC induced by DMBA was seen in the examined HBPs was 53.5%,76.9% and 66.67% respectively. This discrepancy may be due to the using of different type of carcinogenic agent or using short period of DMBA application.

In the current study, IHC staining of GII had recorded the highest mean area percentage of IHC stainability of cells with Bmi1 antibody (65.88 %),that was seen throughout the epithelial layers and invading tumor cells, with highly significantly difference compared to GI (p value < 0.001). This result is in agreement with many studies [69-72].Herzog et al (2021)[43] showed that, Bmi1 was necessary for EMT during tumor development in head and neck carcinomas particularly OSCC patients. 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[42]. 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 OSCC[43].Patel et al (2018)[45] indicated that silencing Bmi1 can inhibit the malignant biological behavior of cancer, in addition to the self-renewal and differentiation of CSCs. Bmi1 expression has been noted to increase in the preneoplastic lesions such as esophageal adenocarcinomas and OED, this could imply its role in the malignant transformation of OED[70, 73] .Curtarelli et al (2018)[36] added that, the expression of Bmi1 was correlated with poor overall survival and was also associated with lymph node metastasis and clinical stage.

In contrast to our results , Hayry`s et al (2010) [74],study found that, Bmi1 negative tumors showed a correlation with a poor prognosis in tongue SCC. Thus, more evidence is needed to confirm the relationship between Bmi1 and the development of tongue SCC.

In the present study, the histopathological results of GIII revealed that, 60% hamsters exhibited SCC with superficial invasion of malignant cells in the form of well differentiated SCC, which was limited to the sub epithelium area not extended to deeper areas within C.T, while 20% hamster showed severe epithelial dysplasia and the other 20% hamsters showed moderate epithelial dysplasia. This is in line with other studies[75, 76], which were showed that, imiquimod

had some success in the management of varying degrees of dysplastic skin lesions and superficial skin cancers , also it has been shown to reduce the degree of dysplasia in a mouse model of oral cancer [(77)] and multiple BCCs seen in xeroderma pigmentosum have demonstrated a favorable outcome following application of 5% imiquimod [(78)].Mane et al, (2021)[(79)] concluded that, topical imiquimod 5% is the best alternative to conservative management of moderate to severe dysplasia cases where we can offer more to the patient than just observation, and it is also better than a surgical option where surgery leads to more morbidity, in recurrent cases.

Mullin et al,(2016) [(80)] reported treatment of OED on the soft palate with 5% imiquimod cream for 6 weeks showed complete disappearance of dysplasia post treatment. Hu et al, (2015) [(81)] have demonstrated the efficacy of 5% imiquimod in superficial infantile hemangioma.

Ly et al (2011) [(82)] have done a clinicopathologic evaluation of lentigo maligna regression after 5% imiquimod cream usage and have demonstrated complete clearance in 53–75% of the treated patient. Patinote et al, (2020)[(83)].Indicates that the antitumor effect of topical imiquimod, at least within the first 1 or 2 weeks, can be entirely mediated by plasmacytoid dendritic cells that acquire CD8 and lytic function. Also another study by Vola et al (2018)[(84)] described that, tumor-specific CD8+ T cells were increased in imiquimod-treated mice, and local tumor control was associated with decreased spontaneous lung metastases, an effect that could result from decreased seeding from the treated tumor and/or inhibition by antitumor T cells that are active systemically. Inhibition of tumor growth for topically applied imiquimod were attributed in another studies to induction of apoptosis via modulation of the expression of Bcl-2/Bax in cells of various cancer types, including renal cell carcinoma, [(85)] SCC[(77)], basal cell carcinoma, [(86)] and malignant melanoma[(87)]. Another studies were attributed inhibition of tumor growth to enhancement of the immune response activated by dendritic cells (DCs) [(88)].

In contrast to those studies and our present study Larson et al,(2010) [(89)]showed topical imiquimod has been shown to induce temporary regression of breast cancer in an animal model, which were contributed to expansion of Tregs within the tumor environment, which may enhance tumor progression.

In the present study, IHC staining using Bmi1 antibody of GIII showed positive nuclear expression throughout the overlying dysplastic and invading malignant epithelial cells with (mean%= 37.02), that showed highly significant difference (p value < 0.001) compared to GII. To our knowledge, in the open English literatures, this was the first study to evaluate therapeutic efficacy of imiquimod on IHC staining using Bmi1 as a one of CSCs, but there are several studies on another CSCs which were shown to be down regulated by imiquimod treatment. A study done by Ren et al, (2016)[(90)] explore the functions of imiquimod on hepatocellular carcinoma stem cells, when the cells were treated with imiquimod for 48 h, and the self-renewal ability was determined using mammosphere formation assay. Imiquimod (100 µl) significantly inhibited the self-renewal of hepatocellular carcinoma stem cells. Flow cytometry-based ALDEFLUOR fluorescent sorting, which aims to separate aldehyde dehydrogenase+ cells, also demonstrated the influence of imiquimod on stem cell numbers. Gruber et al,(2014) [(91)] had been investigated that, imiquimod blocks the activation of the Hedgehog /glioma-associated oncogene signaling pathway, it has been suggested that an aberrant activation of this CSCs pathway plays an important role in the pathogenesis of BCC[(92)]. Imiquimod binds to the adenosine receptors and activates protein kinase A, which in turn phosphorylates glioma-associated oncogene, heralding down-regulation of glioma-associated oncogene mRNA and protein levels in BCC cells. Because of this, there is a

negative effect on the Hedgehog /glioma-associated oncogene signaling pathway thereby thwarting its oncogenic potential.

In the current study, the histopathological results of GIV showed that, 40% hamsters exhibited SCC with superficial invasion of malignant cells, which was limited to the sub epithelium area not extended to deeper areas within C.T, with the mean DOI (0 – 2.81) mm, while 20% hamster showed carcinoma in situ, 30% hamsters showed moderate epithelial dysplasia and 10%hamster showed mild epithelial dysplasia. This is findings in consistence with that shown by other researchers in different tumors [(93)]. Selvendiran et al,(2005) [(93)] concluded that, after they had studied oral administration of piperine in mice model of lung carcinogenesis, piperine had a significant activity reduction of phase-I enzymes, and increase in glutathione-metabolizing enzymes including glutathione peroxidase and glutathione reductase, similar finding were detected by Gunasekaran et al, (2017) [(94)] in nitrosamine induced hepatocellular carcinoma, in which it has been showed that piperine mediates deficiency of benzo pyrene metabolism through a direct interaction with cytochrome P450 enzyme and consequently, it abolishes cancer aggressiveness .Siddiqui et al,(2017) [(28)] demonstrated that, a high amount of ROS synthesis, which induces cell apoptosis by activating the caspase-3 pathway in cancer cells and cell cycle arrest in the G2/M phase ,which were obtained, when treated KB cell lines (human epithelial carcinoma cells) by Piperine at a concentration of 100 and 200 µM for 12 while piperine were reported to arrest cell cycle at G0/G1 phase in human prostate cancer cell lines, both in androgen dependent cells and androgen independent cells lines, by interfering the expression of the CDK inhibitors p21 and p27 [(95)].

Doucette et al,(2013) [(96)] by using cultures of human umbilical vein endothelial cells, showed that piperine inhibited cell proliferation, migration, angiogenesis, and, it also stopped cellular cycle at G1/S phase.

In contrast to our present study which didn't show complete inhibition of SCC formation Manoharan et al, (2009) [(97)] evaluated the chemopreventive efficacy of piperine in DMBA induced carcinogenesis in Syrian golden hamsters showed that, piperine totally inhibited the oral carcinoma formation. This may be attributed to protective efficacy of piperine against gradual effect of carcinogenic agent while in our study we treat already established SCC.

The IHC staining using Bmi1 antibody of GIV exhibited positive nuclear expression throughout the dysplastic and invading malignant cells with (mean%= 31.09), and highly significant difference (p value < 0.001) compared to GII. To our knowledge, in the open English literatures, this was the first study to evaluate therapeutic efficacy of piperine on IHC staining using Bmi1 as a one of CSCs, but there are several studies on another CSCs which were shown to be down regulated by piperine treatment[(98)].

Thao et al, (2021) [(98)] fabricated nanoliposomal complexes of piperine and anti-CD133 monoclonal antibodies,they proved piperine very effective in inhibiting the growth of CSCs while being nontoxic to normal cells.Almeida et al, (2020) [(99)] reported piperine had been inhibited the Wnt/ catenin pathway in colorectal cancer cell lines by piperine, as well as suppressed β-catenin nuclear localization. Similar findings were detected in breast CSCs especially when piperine combined with curcumin, which had been inhibited Wnt/ β -catenin signaling pathway and, as a consequence, it hinders the mammospheres formation, thus afecting their self-renewal ability[(100)].

In the present study, the histopathological results of GV showed that, 30% hamsters showed superficial invasion of well differentiated SCC limited to sub-epithelial and not extended to the deeper C.T, which DOI had recorded the lowest value DOI (0 – 2.4) with highly significantly

difference compared to GII (p value < 0.001), while the other seven hamsters exhibited different degrees of epithelial dysplasia 10% mild, 30% moderate and 30% severe epithelial dysplasia.

To our knowledge, in the open English literatures, this was the first study to evaluate combinatory therapeutic efficacy of imiquimod and piperine. These findings reflected the beneficial effect of combining imiquimod with piperine in order to achieve these positive results. These results may be attributed to the synergetic anti-tumor effect between imiquimod and piperine by combining direct approach to SCC by topical application of imiquimod and systemic action by oral administration of piperine, which lead to targetion tumor cells by different integrated mechanisms as anti-carcinogenic effect of piperine which mediates deficiency of benzo pyrene metabolism through a direct interaction with cytochrome P450 enzyme,[(94)] reduction of phase-I enzymes, and increase in glutathione-metabolizing enzymes including glutathione peroxidase and glutathione reductase [(93)]

In addition to stimulation of immune cells by imiquimod through inducing the release of various cytokine as IL-12, tumor necrosis factor-alpha, and interferon-gamma. These in turn increase the levels of cytotoxic T cells and natural killer cells [(101)]. These cytokines stimulate a cell-mediated immune response, and this may be of value in the drug's anti-tumor activity [(102)]. Such a strategy is through a mechanism by preventing the escape of tumor cells from the immune surveillance and restoring the function of the host's immune system to attack the tumor cells[(103)]. In addition to previous integrated mechanisms of piperine and imiquimod they induce apoptosis of malignant cells by different mechanisms as mentioned before [(30)]. Also combined treatment of piperine and curcumin alters the circulating levels of IL-10 and miR-21 in hepatocellular carcinoma patients[(104)].

In the current study, IHC staining of GV using Bmi1 antibody exhibited positive nuclear expression throughout the overlying dysplastic and invading malignant epithelial cells with (mean%= 25.89) ,with the lowest mean area percentage compered to GII,GIII and GIV, which mean that, the highest inhibitory effect of Bmi1 on SCC was reflected on H&E results this may be attributed to targeting different pathways of CSCs as demonstrated by another separate studies for imiquimod [(90)] and piperine[(98)]. Salazar et al,(2017) [(105)] reported that a combination of topical imiquimod plus nab-paclitaxel for breast cancer cutaneous metastases, showed robust T-cell infiltrates, which had been associated with superior response rates to chemotherapy and increased progression free and overall survival. Pachauri et al, (2015)conjugated piperine nanoparticles and paclitaxel showed remarkable decline in needed paclitaxel dosages for suppressing the growth of paclitaxel resistant breast cancer cells [(106)]. In another study piperine is able to suppress, or at least, down-regulate P-gp dependent drugs-resistance by competing with its ATP binding site when co-administered with vincristine or colchicine or paclitaxel could reverse drug resistance in vitro in cervical and colon cancer cells overexpressing P-gp [(107)].Greenshields et al, (2015) [(108)] reported that a combination of piperine and γradiation had higher cytotoxicity and effectiveness in stopping the growth of tripe negative cancer cells than radiation alone in immune-deficient mice.

5-conclusions

This study demonstrated that, imiquimod and piperine could inhibit HBP SCC formation either alone or in combination with each other, our results demonstrated that combination of imiquimod and piperine has an inhibitory effect on tumor progression in OSCC, which was found to be more efficient than either agent alone.

Bmi1 downregulation has an inverse proportion to increased degree of malignancy of HBP SCC, in addition to downregulation of Bmi1 can predict the therapeutic potential of imiquimod and piperine in OSCC.

Funding:
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Institutional Review Board Statement:
The National Research Council's Guide for the care and use of laboratory animals have been followed. All experiments were approved by ethical committee of Faculty of Dental Medicine, Al-Azhar University, Egypt (Ethical Code No. 147/163, 25-05-2019).

Informed Consent:
Statement: Not applicable.

Data Availability Statement:
The data sets used during current study are available from the corresponding author on reasonable request.

Conflicts of Interest:
The authors have no conflict of interest to declare.

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