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SYNERGISTIC EFFECT BETWEEN CETUXIMAB AND CURCUMIN ON EXPERIMENTALLY INDUCED HAMSTER BUCCAL POUCH CARCINOMA

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ABSTRACT

The aim of the present study was directed to investigate the therapeutic efficacy of cetuximab and curcumin either in combination or alone as anticancer treatment mmodality on 7,12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch(s) (HBP(s)) carcinoma. **Material and methods:** Twenty-Five Syrian male hamsters, five weeks old, weighing 80-120g were divided into three group(s) (G(s)): A, B, and C. G-A (negative control): HBP mucosa of 5 animals were painted with liquid paraffin alone three times a week for 18 weeks. G-B: 20 right HBP animals were painted with 0.5% DMBA in paraffin oil 3 times a week for 18 weeks. Then animals were divided randomly into four sub-groups. G-B1 (DMBA treated group): 5 animals were left untreated for 6 weeks after DMBA painting. G-B2 (cetuximab treated group): 5 animals were injected intraperitoneally with cetuximab 1 mg/animal at 3 days intervals for 6 weeks following DMBA-treatment. G-B3 (curcumin treated group): Right HBP of 5 animals were directly injected with curcumin 80 mg/kg body weight every day for 6 weeks following DMBA-treatment. G-B4 (curcumin-cetuximab treated group): 5 animals were directly injected with curcumin followed by intraperitoneal injection of cetuximab for 6 weeks following a DMBA-treatment. The assessment was based on the gross observation, histological and immunohistochemical examination utilizing anti-apoptotic Bcl-2 antibody. **Results:** Gross observation revealed variation in reduction of the tumor size in the treated groups (G-B2, G-B3, and G-B4) compared to that observed in group G-B1. Histopathological findings revealed variations among the treated groups. Immunohistochemical results revealed that Bcl-2 expression has a variability in the area % throughout the groups used. Groups G-B1, G-B2, G-B3 and G-B4 were 70.7 %, 54.8 %, 42.7% and 24.5% respectively. **Conclusion:** The synergistic effect of both cetuximab and curcumin resulted in promising therapeutic effect in regression of DMBA induced HBP carcinoma.

KEYWORDS: HBP carcinoma, cetuximab, curcumin.

INTRODUCTION

Oral cancer refers to a subgroup of head and neck malignancies, is located within the top 10 ranking incidence of cancers worldwide [(1)]. The annual incidence is higher around the world, which is over 300.000 diagnosed cases, and the annual mortality is about 145,000 deaths [(2)]. Oral squamous cell carcinoma (OSCC) represents over 90% of oral cancer [(3)]. It has been found that 7,12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch (HBP) carcinogenesis closely mimics with human OSCC on biochemical, morphological and histological aspects as well as at molecular level[(4)]. The unfavorable survival outcomes coupled with the toxicity of current treatments (chemo- and radiotherapy) underscore the importance of incorporating targeted therapies within the treatment paradigm particularly against the epidermal growth factor receptor (EGFR) which expressed in more than 90% of all cases of

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head and neck squamous cell carcinoma (HNSCC) and associated with advanced stage of the disease, poor survival and resistance to chemo- and radiotherapy[5]. With its targeted mechanism of action and synergistic activity with current treatment modalities, cetuximab is a potentially valuable treatment option for patients with HNSCC which competitively binds to the extracellular domain of the EGFR, preventing activation of the receptor by endogenous ligands. The antibody-receptor complex is internalized and degraded, resulting in a downregulation of the EGFR expression [6]. The alternative natural compounds had been shown to enhance the effects of the chemotherapeutic drugs, reduce drawbacks of chemotherapy, and increase the sensitivity of cancer cells[7]. One of these alternatives is curcumin, a natural compound that is derived from turmeric[8]. It has been shown that curcumin alone or in combination with other drugs increase cell death in a wide variety of tumor cells, including HNSCC[9]. One of the most compelling reasons for continued interest in exploring the cancer chemopreventive and chemotherapeutic uses of curcumin has been curcumin's ability to influence a diverse range of molecular targets within cells[10]. Indeed, curcumin has been shown to induce apoptosis via the up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins of the Bcl-2 family in cancer cells which results in cell death [11]. Hence, the main target of the present study was to assess the therapeutic efficacy of cetuximab and curcumin either in combination or alone as anticancer treatment modality in DMBA induced HBP carcinoma. The assessment was based on the gross observation, histological tumor tissue changes and immunohistochemical examination utilizing anti-apoptotic Bcl-2 antibody.

MATERIAL AND METHODS

The Experimental animals used in the current study were golden Syrian hamsters. They were used as model for OSCC induction utilizing DMBA as chemical carcinogen. Then, intraperitoneal injection of cetuximab and lesional injection of curcumin depending on type of each group, were employed. After tissue preparation, various investigations: hematoxylin and eosin (H&E) stain and immunohistochemical staining utilizing Bcl-2 antibody, were done.

Material used: ***Animals:***

Twenty-Five Syrian male hamsters five weeks old, weighing 80-120g were obtained from the animal house, Cairo University (Cairo, Egypt). The experimental animals were housed in standard cages with sawdust bedding under controlled environmental conditions of humidity (30-40%), temperature (20 ± 2°C), and light (12-h light/12-h dark). All experimental animals were supplied with standard diet and water ad libitum. **7,12 DMBA** (0.5%) was obtained from Sigma-Aldrich company, dissolved in paraffin oil and used thrice a week for 18 weeks via Painting of right HBP using a number 4 camel's hair brush [12]. **Cetuximab** was obtained from Imclone Systems Company and used via intraperitoneal injection as 1 mg/animal at 3 days intervals for 6 weeks [13]. **Curcumin** was obtained from Sigma-Aldrich Company, dissolved in dimethyl sulfoxide (DMSO), and used via intralesional injection as 80 mg/kg body weight, every day, for 6 weeks [14].

Experimental design:

Twenty-Five Syrian male hamsters were divided into three G(s). G-A (negative control): HBP mucosa of 5 animals were painted with liquid paraffin alone three times a week for 18 weeks. G-B: 20 right HBP animals were painted with 0.5% DMBA in paraffin oil using 3 times a week for 18 weeks. Then animals were divided randomly into four sub-Gas. G-B1 (DMBA treated group): 5 animals were left untreated for 6 weeks after DMBA painting. G-B2 (cetuximab treated group): 5 animals were injected intraperitoneally with cetuximab 1 mg/animal at 3 days intervals for 6 weeks following

DMBA-treatment. G-B3 (curcumin treated group): Right HBP of 5 animals were directly injected with curcumin 80 mg/kg body weight every day for 6 weeks following DMBA-treatment. G-B4 (curcumin-cetuximab treated group): 5 animals were directly injected with curcumin followed by intraperitoneal injection of cetuximab for 6 weeks following a DMBA-treatment.

Investigations: After termination of the experiment, the animals were sacrificed by cervical dislocation, the cheek pouches were everted, and the diameter of each tumour was measured with a Vernier caliper. The tumour volume was calculated by the formula, $Vmm^3 = (4/3)\ \pi\ [(D1/2)\ (D2/2)\ (D3/2)]$, where D1, D2 and D3 are the three diameters (mm) of the tumour [4]. Then, the cheek pouches were excised and fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin blocks for preparation in order to be examined histologically and immunohistochemically.

For histopathological examination: The fixed specimens were dehydrated in an ascending ethanol series, embedded in paraffin wax to form paraffin blocks. Tissue sections of 4μm thickness on rotary microtome were cut, mounted on glass slides, processed, and stained with H&E for light microscopic examination.

For immunohistochemical examination: Other tissue sections were cut at 5 μm thickness for the application of standard labeled streptavidin-biotin method to demonstrate the expression of mouse monoclonal Bcl-2 antibody. The Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H2O2 in methanol for 10 min. The antigen retrieval was achieved by adding citrate buffer solution (pH 6.0) and keeping in microwave for 10 min, followed by washing with Tris-buffered saline (pH 7.6). The tissue sections were then incubated with the universal proteinaceous blocking reagent power BlockTM for 15 min at room temperature to block non-specific binding, and further with the respective primary antibody Bcl-2 at 4°C. The bound primary antibody was detected by incubation with their corresponding secondary antibodies, conjugated with horseradish peroxidase for 30 min at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3-diaminobenzidine (Sigma, USA), the substrate of horseradish peroxidase. When acceptable colour intensity was reached, the slides were washed, counter stained with Mayer's haematoxylin and covered.

The immunostained sections were examined using light microscope to assess the prevalence of positive cases and the localization of immmunostaining within the tissues. In addition, image analysis computer system was used to assess area percentage of positive cells of the immunostaining. This was done at the Oral and Dental Pathology Department - Faculty of Dental Medicine - Boys- Cairo - Al-Azhar University. The degree of positive staining for antibody was evaluated by a well-established quantitative scoring on a scale rang from negative to strong positive staining as follow: Strong staining (more than 50% stained), moderate staining (between 25 and 50% stained), weak staining (between 5 and 25% stained), and negative (less than 5% stained) [15].

RESULTS

The gross observation results of HBP mucosa of G-A was pink in color with smooth surface with no observable abnormalities (Fig.1:A). In G-B1, HBP mucosa showed various changes including variable sized nodular elevations and exophytic papillary tumor masses. Vernier caliper revealed that the mean tumor volume of tumor-bearing animals was 827.7mm^3 with 100% tumor formation. Furthermore, eroded and ulcerative areas were seen throughout the tumor growth, with spontaneous bleeding (Fig.1:B). In G-B2, HBP mucosa showed multiple different

sizes of exophytic masses. Vernier caliper revealed that the mean tumor volume of tumor-bearing animals was 589.9 mm^3 (Fig.1:C). In G-B3, HBP mucosa showed different exophytic tumor masses with decrease in tumor volume. Vernier caliper revealed that the mean tumor volume of tumor-bearing animals was 209.9 mm^3 (Fig.1:D). In G-B4, HBP mucosa showed that injection of curcumin and cetuximab for 6 weeks after 18 week of DMBA treatment led to marked improvement in general health of animals. Vernier caliper revealed that the mean tumor volume of tumor-bearing animals was 39.492 mm^3 (Fig.1:E).

Histopathological and immunohistochemical results: The tissue sections of HBP mucosa of experimental groups showed variable results in regard to the histopathological results and immunohistochemical results. In G-A, histological sections, using H&E stain, revealed normal HBP mucosa, with thin stratified squamous epithelium without rete ridges with submucosal dense fibrous connective tissue and layer of longitudinal striated muscle fibers (Fig.2:A). The immunohistochemical staining using Bcl-2 antibody exhibited weak positive (5.74%) cytoplasmic expression of basal and supra-basal layers and negative in the remaining epithelial cell layers (Fig.2:B). In G-B1, histological sections, using H&E stain, showed moderately to poorly differentiated SCC with deeply invading islands of tumor cells into the underlying connective tissue. The surface epithelium showed severe epithelial dysplasia (Fig.2:C). The immunohistochemical staining using Bcl-2 antibody exhibited strong positive (70.71%) cytoplasmic expression throughout the epithelial layers and invading tumor cells (Fig.2:D). In G-B2, histological sections, using H&E stain, showed well differentiated SCC with deeply invading multiple epithelial islands of tumor cells into the underlying connective tissue (Fig.2:E). The immunohistochemical staining using Bcl-2 antibody exhibited strong positive (54.81%) cytoplasmic expression throughout the epithelial layers and invading tumor cells (Fig.2:F). In G-B3, histological sections, using H&E stain, revealed well differentiated SCC which with superficial invasion of tumor islands and keratin pearls. The surface epithelium showed severe epithelial dysplasia with hyperkeratosis (Fig.2:G). The immunohistochemical staining using Bcl-2 antibody exhibited moderate positive (42.71%) cytoplasmic expression throughout the epithelial layers and invading tumor cells (Fig.2:H). In G-B4, histological sections, using H&E stain, revealed smaller size of invasion of well differentiated SCC which was Juxta-epithelial and not extended to the deeper connective tissue with increased amount of keratin formation (Fig.2:I). The immunohistochemical staining using Bcl-2 antibody exhibited weak positive (24.57%) cytoplasmic expression throughout the epithelial layers and invading tumor cells (Fig.2:J).

Fig.1(A): HBP mucosa of G-A was pink in color with no observable abnormalities (arrow). **Fig.1(B):** HBP mucosa of G-B1 showed variable sized nodular elevations and exophytic papillary tumor masses (arrow). **Fig.1(C):** HBP mucosa of G-B2 showed solitary large exophytic tumor mass (arrow). **Fig.1(D):** HBP mucosa of G-B3 showed small exophytic tumor masses (arrow). **Fig.1(E):** HBP mucosa of G-B4 showed marked decrease in distribution and size of tumor masses with absence of bleeding and ulceration (arrow).

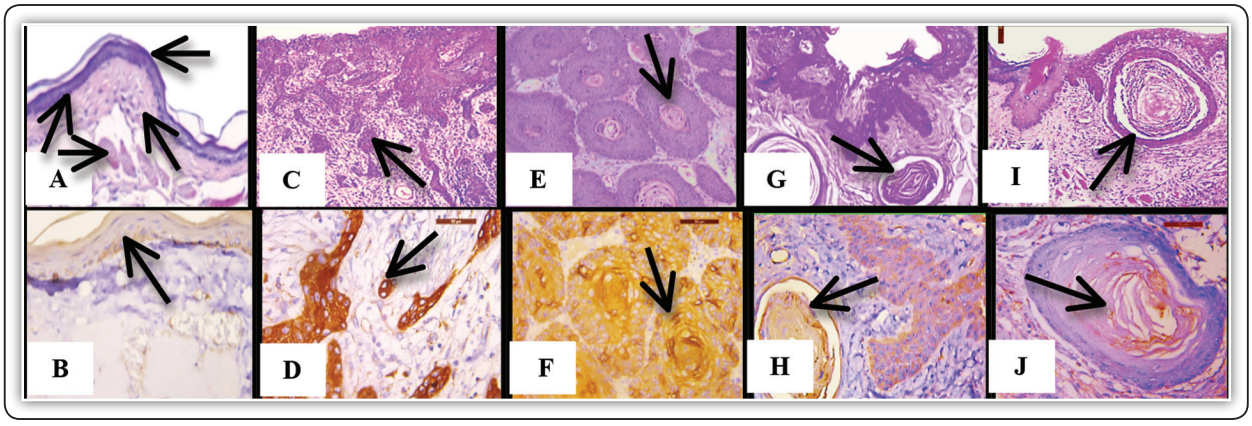

Fig.2(A): Photomicrograph of G-A showed normal HBP mucosa, composed of thin stratified squamous epithelium without rete ridges with submucosal dense fibrous connective tissue and layer of longitudinal striated muscle fibers (arrows). **Fig.2(B):** Bcl-2 expression in G-A showed weak positive cytoplasmic expression of basal and supra-basal layers (arrow). **Fig.2(C):** Photomicrograph of G-B1 showed moderately differentiated SCC with deeply invading islands of tumor cells into the underlying connective tissue (arrow). **Fig.2(D):** Bcl-2 expression in G-B1 showed strong positive cytoplasmic expression throughout the epithelial layers and invading tumor cells (arrow). **Fig.2(E):** Photomicrograph of G-B2 showed well differentiated SCC with deeply invading multiple epithelial islands of tumor cells into the underlying connective tissue (arrow). **Fig.2(F):** Bcl-2 expression in G-B2 showed strong positive cytoplasmic expression throughout the epithelial layers and invading tumor cells (arrow). **Fig.2(G):** Photomicrograph of G-B3 showed well differentiated SCC which with superficial invasion of tumor islands and keratin pearls (arrow). **Fig.2(H):** Bcl-2 expression in G-B3 showed moderate positive cytoplasmic expression throughout the epithelial layers and invading tumor cells (arrow). **Fig.2(I):** Photomicrograph of G-B4 showed smaller size of invasion of well differentiated SCC which was Juxta-epithelial and not extended to the deeper connective tissue with increased amount of keratin formation (arrow). **Fig.2(J):** Bcl-2 expression in G-B4 showed weak positive cytoplasmic expression throughout the epithelial layers and invading tumor cells (arrow).

Statistical analysis results of Bcl-2 expression were obtained by comparing its area % in the groups used. Statistical analysis results revealed that G-A has recorded the lowest mean area % (5.74%), while G-B1 had the highest mean area % (70.71%). Comparing the various treated groups (G-B2, G-B3, and G-B4) with G-B1, G-B4 showed statistically lower mean value than the G-B2, G-B3, and G-B1 (Fig.3).

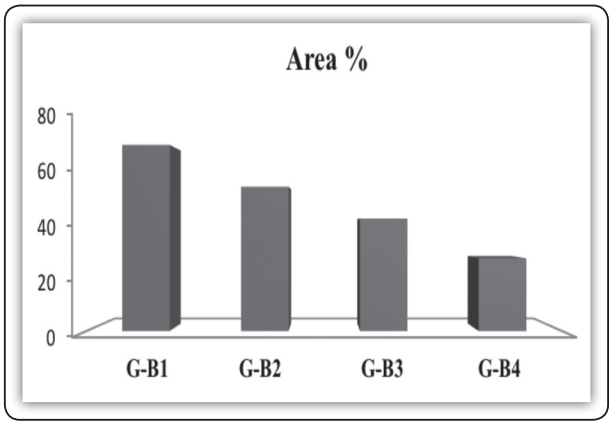

FIG (3) Bar chart representing mean area % results in the B sub-Gs.

DISCUSSION

Oral cancer, one of the most disfiguring types of cancer and despite the significant advancement in oral cancer treatment strategy; it remains as a major cause of morbidity in human populations. The use of hamster cheek pouch system of oral carcinogenesis model is beneficial for deeper understanding of cancer biology, prevention and treatment. To our knowledge, in the English open literatures, the current study was the first study to evaluate the combinatorial anticancer effect of both curcumin and cetuximab on DMBA induced HBP carcinoma. In the present study, the gross observation findings in G-A showed no observable abnormalities of the HBP. After sacrificing, the buccal pouches length was about 5cm for all hamsters with normal histological structures. Other studies reported the same findings [16, 17]. Immunohistochemical staining of normal hamster mucosal tissue showed that weak positive (5.74%) Bcl-2 localization was restricted to the basal and supra-basal layers and negative in

the remaining epithelial cell layers. This result is in agreement with that of other investigators[18,19]. This observation might be attributed to that up regulation of Bcl-2 in basal and supra-basal cells serve to maintain the keratinocyte stem cells from apoptosis, and that negative Bcl-2 expression in the remaining epithelial layers concomitant with terminal cell differentiation (keratinization) [20].

In the present study, the gross observation of HBP in G-B1 revealed variable sized nodule and exophytic papillary tumor masses with mean tumor volume of tumor-bearing animals were 665.42 and 100% tumor formation. Furthermore, eroded and ulcerative areas were seen throughout the tumor growth, with spontaneous bleeding. These results are almost the same with that shown by other investigators [21-24]. Histopathological results of G-B1 showed moderately to poorly differentiated SCC with deeply invading islands of tumor cells into the underlying connective tissue. The surface epithelium showed hyperkeratinization, and elongated drop-shaped rete ridges with areas of variable degrees of dysplasia. These results are in agreement with those of other investigators [25,25]. These observations might be interpreted as DMBA induced over production of reactive oxygen species, chronic inflammation, oxidative modification of DNA bases, impairment in antioxidant defense system, defect in the activities of detoxification cascade and deregulated expression pattern of molecular markers are implicated in the promotion and progression of oral carcinogenesis[17]. The Bcl-2 immunohistochemical results of G-B1 showed strong positive (70.71%) cytoplasmic expression throughout the epithelial layers and invading tumor cells. These results are in line with those of other investigators[17,26,27]. This suggests that overexpression of Bcl-2 indirectly inhibits the process of apoptosis and thus plays a role not only in the onset of tumorigenesis, but also influences the progression of the disease because it increases the survival rate of neoplastic cells, allowing new genetic mutations to occur and granting them higher resistant to treatment [27].

In the present work, the gross observation of HBP in G-B2 revealed variable sized nodules and exophytic papillary tumor masses with mean tumor volume of tumor-bearing animals were 589.9 mm^3. Furthermore, there was no improvement in general health of the animals. Histopathological results showed well differentiated SCC with deeply invading islands of tumor cells into the underlying connective tissue. These results were in line with other studies[28,29]. The non-responsiveness of cetuximab as a single agent may be caused by multiple intrinsic and extrinsic/acquired resistance mechanisms. In the case of HNSCC, many tumors remain non-responsive to cetuximab in which the single-agent response rate of this drug is less than 15%. Nevertheless, cetuximab is known to provide a clinical benefit when used either in conjunction with radiation or in combination with chemotherapy[30,31]. From a clinical point of view, **Lu et al.** [32] reported that acquired resistance occurs after an initial response to therapy and eventually all HNSCC patients will relapse or become insensitive to further cetuximab therapy. The Bcl-2 immunohistochemical results of G-B2 showed strong positive cytoplasmic expression throughout the epithelial layers and invading tumor cells. However, the mean area % of Bcl-2 expression in G-B2 was lower than G-B1. This observation might be attributed to that cetuximab decrease the expression of Bcl-2, that consequently increases the Bax/ Bcl-2 ratio and then promotes the progression of cells to apoptosis [33,34].

In the present work, the gross observation of HBP in G-B3 showed slight improvement in general health of animals. An exophytic tumor mass showing decrease in tumor volume which was statistically significant lower than G-B2 and G-B1 with mean tumor volume of tumor-bearing animals was 209.9 mm^3. These results are in agreement with other studies which report the growth suppressive effects of curcumin on HNSCC [8,35-37]. **Guha et al.**[38] reported that growth suppression effect was

represented primarily by the effect of curcumin on the nuclear factor kB (NF-kB) signaling pathway. Curcumin caused a reduction in the expression of NF-kB and, in addition, inhibited its nuclear localization. The activity of curcumin on the NF-kB in this type of tumor is due to inhibition of IkB kinase, resulting in NF-kB sequestration in the cytoplasm. As a result, curcumin has been shown to suppress the expression of a variety of NF-kB regulated gene products involved in carcinogenesis and tumor growth.

Histopathological results of G-B3 revealed invasion of well differentiated SCC which was juxta-epithelial only and not extended to the deeper connective tissue. These results were in line with **Hung et al.** [39] in which they reported that curcumin evidently decreased cell number migrating through the connective tissue. The observed effect of curcumin on cell invasion may be partially caused by decreasing activity of extracellular matrix proteases which play key roles in local invasion. The Bcl-2 immunohistochemical results of G-B3 showed moderate positive cytoplasmic expression throughout the epithelial layers and invading tumor cells. This result is in agreement with **Ravindran et al. (2009)** [40] who was postulated that curcumin induces downregulation of anti-apoptotic proteins and up regulation of pro-apoptotic proteins that lead to a loss of the mitochondrial membrane potential, opening of the transition pore, releasing cytochrome c, activating caspase-9 and caspase-3, and ultimately DNA fragmentation and apoptosis.

In the present work, the gross observation of HBP in G-B4 showed that injection of curcumin and cetuximab for 6 weeks after 18 week of DMBA treatment resulted in marked improvement in general health of animals. Furthermore, there was marked decrease in tumor volume of exophytic masses which was lower than other groups treated with cetuximab or curcumin only with mean tumor volume of tumor-bearing animals was 39.492mm^3. These observations might be attributed to a synergistic effect between curcumin and cetuximab in which cetuximab synergizes the efficacy of curcumin and vice versa that allowed for G-B4 to be the best treated group in the current study. Several studies postulated that cetuximab and curcumin can be synergized the action of other chemotherapeutic agents as well as the action of radiotherapy in different types of cancers including HNSCC [41-44].

In the present study, G-B4 was the best treated group, not only in the gross observation results but also in histopathological and immunohistochemical results. The histopathological results of G-B4 revealed invasion of well differentiated SCC which was juxta-epithelium only and not extended to the deeper connective tissue with increase amount of keratin formation. Bcl-2 immunohistochemical results showed weak positive cytoplasmic expression throughout the epithelial layers and invading tumor cells which was lower than G-B2 and G-B3. These results might be attributed to the dual effect of both curcumin and cetuximab on the Bcl-2 protein in which they downregulate the Bcl-2 expression in tumor cells that increase the Bax/Bcl-2 ratio and promotes the progression of cells to apoptosis in a higher level than other groups that treated with cetuximab or curcumin only. In conclusion, the synergistic effect of both cetuximab and curcumin could be a promising treatment of that DMBA induced HBP carcinoma model not only in the gross observation results but also in histopathological and immunohistochemical results. This was enhanced by inducing apoptosis via downregulation of the Bcl-2 protein.

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