International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Therapeutic efficacy of capecitabine in combination with lenalidomide and/or doxycycline on experimentally induced hamster buccal pouch carcinoma

Mohammed Hamdy Saad[1], Khaled Mohamed Afify[2], Kamal Abd El Rahman Kamal[3], Mohamed Mahmoud Ahmed[4]

[1]*Assistant lecturer, Oral and Dental Pathology department, Faculty of Dental Medicine, Assiut, Al-Azhar University, Egypt.*

[2]*Assistant Professor, Department of Oral and Dental Pathology Faculty of Dental Medicine, Assiut, Al-Azhar University, Egypt.*

[3]*Assistant Professor, Department of Oral and Dental Pathology, Faculty of Dental Medicine, (Boys-Cairo), Al-Azhar University, Egypt*

[4]*Professor and head of Oral and Dental Pathology department, Faculty of Dental Medicine, (Boys-Cairo), Al-Azhar University, Egypt.*

Abstract

The current research has directed to investigate the therapeutic efficacy of capecitabine (CAP) in combination with lenalidomide (LEN) and/or doxycycline (DOX) on experimentally induced hamster buccal pouch (HBP) carcinoma. Sixty Syrian male hamsters 5weeks old, weighing80-120g had been split into 6groups (10 in each). GI: Animals left untreated. GII: Animals had painted with seven, 12-dimethylbenz (a) anthracene (DMBA) (3times/week/14 weeks). GIII: DMBA applied as in GII to induce cancer followed by treatment with CAP only. GIV: DMBA applied as in GII followed by treatment with CAP and LEN. GV: DMBA applied as in GII followed by treatment with CAP and DOX. GVI: DMBA applied as in GII followed by treatment with CAP +LEN + DOX. At the end of the experiment, animals were euthanized, & then all right pouches were grossly examined, excised, & divided into three parts for further histologic and immunohistochemical examination, and RT-qPCR for evaluation of CD44 and caspase 3 gene expression. Gross observation of the right HBP mucosa showed a complete disappearance of lesions leaving only wrinkled surfaces in three, five, five, and six animals in GIII, GIV, GV, and GVI respectively. Gross observation also showed some decrease in the size and number of lesions in five, five, five, and four animals in GIII, GIV, GV, and GVI respectively. Two animals in GIII showed no clinical improvement. Histologic observations demonstrated that major pathologic response (MPR) found in three, five, five, and six animals in GIII, GIV, GV, and GVI respectively. Histologic observations also showed partial pathologic response (PPR) in five, five, five, and four animals in GIII, GIV, GV, and GVI respectively. Two animals in GIII showed no pathologic response (NPR). Caspase 3 gene expression analysis demonstrated that apoptosis decreased in GII compared to GI. Upon treatment, apoptosis increased and reached its maximum in GVI. According to immunohistochemical staining of ALDH1A1 and CD44 gene expression analysis, cancer stem cells (CSCs) increased in GII and reached the highest level in GIII. The addition of LEN or DOX to CAP in GIV and GV decreased the CSCs population, but the maximum reduction of CSCs found in GVI when both LEN and DOX added to CAP. The addition of both LEN and DOX increased the therapeutic efficacy of CAP and improved the outcome of treatment through induction of apoptosis and targeting CSCs more effectively than CAP alone.

Keywords: Capecitabine, Immunotherapy, lenalidomide, doxycycline, HBP carcinoma, CSCs.

Full-length article **Corresponding Author*, e-mail: *Mohammedhamdy.9@azhar.edu.eg.*

1. Introduction

Oral cancer is a challenging public health problem, which causes 3–10% of cancer mortality worldwide [1]. Oral squamous cell carcinoma (OSCC) subtype represents more than ninety percent of all head and neck malignancies. Generally, chemotherapy may be needed in addition to surgery in patients with resectable OSCC but it remains the standard treatment for patients with unresectable OSCC [2]. Capecitabine (CAP) is a chemotherapeutic prodrug that generates 5-Fluorouracil (5-FU) through three enzymatic steps. The first two steps require 2 enzymes (carboxylesterase, cytidine deaminase) which are present in

the liver that convert CAP to 5'-deoxy-5- fluorocytidine & later to 5'-deoxy-5'fluorouridine, respectively. The third step occurs only in the tumor site by utilizing the higher activity of the thymidine phosphorylase (TP) enzyme in tumor tissue more than in other tissues [3].

5-FU belongs to the antimetabolite class of chemotherapy and has a wide activity against a variety of neoplasms [4]. 5-FU has the ability to block the enzyme thymidylate synthase (TS), which plays a role in the synthesis of both DNA and RNA. 5-FU also can act though replacing thymidine within DNA or replacing uracil within RNA leading to induction of apoptosis [5]. Orally administered CAP prodrug is preferable to infusion 5-FU, which may require hospitalization and continuous administration for 5 days. In addition to the more favorable oral route, CAP has a better safety profile with at least comparable efficacy [6]. Despite the increasing development of chemotherapy, a large number of OSCC patients still show resistance to chemotherapeutic drugs including 5-FU [7]. One of the causes of resistance is the multiple pathways & mechanisms used by tumor cells to sustain their continuous proliferation & resist apoptosis [8]. So it is rational to combine drugs of differing classes to maximize cell-killing rate and prevent or decrease resistance [9].

Lenalidomide (LEN) is a cytotoxic, immunomodulatory, and antiangiogenic compound that targets both cancer cells and their tumor microenvironments (TME) [10-11]. Regarding direct tumor cytotoxicity LEN causes concentration-dependent cell cycle arrest in G_0-G_1 non-small cell lung cancer cells [12]. The immunomodulatory effects include T-cell activation [13], inhibition of regulatory T cells [14] and augmentation of natural killer cell function [13-15]. LEN also alters the host microenvironment through an antiangiogenic effect [11]. In combination therapy, LEN was capable of enhancing the anti-tumor activity of docetaxel, gemcitabine, cisplatin, and 5-FU [10-15-17]. Although the clinical results for studied cases with OSCC have improved after using combination therapies, the prognosis remains relatively unfavorable, with a 5-year overall survival rate ranging between 60-80% even for early-stage I and II tumors [18]. Current research on the cause of recurrence and metastasis is focused on a small subpopulation of cells which has been called CSCs [19-20]. It has found that conventional chemotherapies mainly target the highly proliferating & mature cancer cells, but the quiescent & less differentiated population of CSCs can avoid the therapeutic insults & survive to reestablish their numbers leading to cancer recurrence [21-22].

In head & neck cancer, the existence of CSCs identified using CSC-specific markers in several kinds of literature [20-23-24]. The most common markers used were surface markers as cluster of differentiation (CD) 44, CD 133, and functional enzyme as Aldehyde dehydrogenase 1A1 (ALDH1A1) [20-25]. Conventional cytotoxic chemotherapy is able to eradicate the majority of tumor cells, but not CSCs. Therefore, combining CSC-targeted medicines with chemotherapy may aid in achieving the objective of eliminating the tumor [26-27]. Several studies confirmed that CSCs are sensitive to mitochondrial targeting antibiotics like Doxycycline (DOX) [28-29]. DOX is an FDA-approved antibiotic used in the treatment of several types of bacterial infections. It kills bacteria by inhibiting protein synthesis via binding to bacterial ribosomes. However, its anti-cancer activities have been recently demonstrated in various studies [30].

This was predicated on the idea that bacterial ribosomes and mitochondrial ribosomes are connected through evolution [28]. DOX inhibited tumor-sphere formation in a broad panel of cancer cell lines derived from 6 different tumor types [28]. DOX also inhibits metastasis by inhibiting matrix metalloproteinase (MMPS) [31]. In addition to its chemo sensitizing effect [30] and radio sensitizing effect [32], DOX increases the efficacy of antiangiogenic therapies by targeting the surviving hypoxic CSCs [33]. One of the most widely acknowledged animal tumor models for studying multistage oral carcinogenesis & assessing the effectiveness of chemotherapy is the hamster buccal pouch (HBP) carcinogenesis model [34]. 7, 12-dimethylbenz (a)anthracene (DMBA), is utilized as a potent carcinogen to induce OSCC in the buccal pouches of golden Syrian hamsters [35]. In this regard, the main target of the current research will be to investigate the therapeutic efficacy of CAP in combination with LEN and/or DOX on experimentally induced HBP carcinoma.

2. Materials and Methods

2.1. Experimental animals

Sixty male Syrian hamsters, aged 5 to 10 weeks and weighing between 80 and 120 grams, acquired from the animal house at the Theodor Bilharz Research Institute in Giza, Egypt. The experimental animals kept in standard cages with sawdust bedding under controlled temperature (22 ± 2°C), humidity (thirty to forty percent), & light (12 hours of light & 12 hours of darkness). Every experimental animal fed a regular meal & had access to water. The Animal Research Unit of the Faculty of Pharmacy, Cairo, Boys, and Al-Azhar University is where the experiment carried out. The experiment was conducted in compliance with worldwide criteria for biomedical research with animals as well as the guidelines of the Medical Research Institute [36]. The procedures were approved by the ethical & research committee protocol of the Faculties of Dental Medicine and Pharmacy, Cairo, Boys, Al-Azhar University.

2.2. Materials

DMBA had been obtained from the **(**Sigma-Aldrich) company then dissolved in paraffin oil at a concentration of .5%. CAP purchased from the pharmacy as a Xeloda tablet 500 mg (Xeloda®, Roche, and Basel, Switzerland). LEN purchased from the pharmacy as a Revlimid 50 mg (Celgene Inc., Warren, NJ, USA). DOX purchased from the pharmacy as a Doxycost tablet 50 mg (Penta Pharma, Egypt).

2.3. Experimental design

After a week of adaptation, the animals divided randomly into 6 groups of 10 hamsters in each group: GI (normal group) animals, had been fed & watered only & served as negative controls. GII (DMBA) in which the right HBP had painted with DMBA in liquid paraffin using a number four camel's hairbrush three times a week, for 14weeks, and served as positive controls. GIII (CAP) in which DMBA was applied as in GII to induce cancer followed by administration of CAP orally by modified syringe, 600mg/kg/day, daily for a week followed by a week of rest, then a week of treatment [37-38]. GIV (CAP+LEN) animals were painted with DMBA as in GII then CAP was

administered as in GIII in addition to LEN which was administered orally by modified syringe, 50 mg/Kg/ day, daily for 3 weeks [17]. GV (CAP+DOX) animals were painted with DMBA as in GII then CAP was administered as in GIII in addition to DOX which was administered orally by modified syringe, 60 mg/Kg/ day, daily for 3 weeks. GVI (CAP+LEN+DOX) animals painted with DMBA as in GII then CAP +LEN+DOX administered as in GIII, GIV, and GV respectively.

2.4. Samples collection and preparation

After the end of the experiment, animals euthanized, & the right HBPs grossly examined & excised and each HBP divided into 2 specimens. One specimen fixed in ten percent neutral buffered formalin for forty-eight hours to prepared for examination histologically and immunohistochemically. Then the second part of the specimen was snap-frozen in liquid nitrogen & kept at -80°C' to be used for RNA extraction & RT-qPCR.

2.5. Histological examination

After formalin fixed, tissue samples washed in xylene, dehydrated with a series of alcohol solutions, & infiltrated with paraffin wax before embedded in paraffin blocks. After cutting tissue sections to a thickness of 5µ, they regularly stained with H&E to facilitate histological evaluation & recording of the results. Pathological response had been determined on H&E-stained sections according to Vos et al. as follows: The portion of the tumor bed that could be identified histologically was identified first [39]. The area occupied by viable tumor cells along with necrosis, keratinous debris, scarring & fibrosis, & multinucleated giant cell reaction referred to as the tumor bed area. Tumors with ≤ten percent residual viable tumor cell percentage in the resected tumor bed had a major pathological response (MPR). Tumors with ≤ 50% residual viable tumor cells had a partial pathological response (PPR), & tumors in which the residual viable tumor cells percentage represent > fifty percent had no pathological response (NPR).

2.6. Immunohistochemical examination

Paraffin blocks were cut into 4µm thick sections and mounted on positively charged glass slides [40]. After deparaffinizing & rehydrating the slides, a 0.01 M citrate buffer (pH 6.0) used to extract the antigen. The slides treated for fifteen minutes in three percent aqueous hydrogen peroxide to quench endogenous peroxidase activity, then for twenty minutes. At room temperature in Protein Block Serum-Free (Dako, CA, USA) to reduce nonspecific binding of following reagents. The tissue sections received 1 or 2 drops of the primary antibodies (polyclonal goat anti-mouse ALDH1A1 antibody (Catalog# AF5869) R&D systems, Biotechne) in dilution of 1:15 & incubated in a humid chamber at 4°C overnight. (Sequence homology of anti-mouse and anti-hamster proteins is 86.23 % according to NCBI BLAST tool. After that, the slides cleaned with distilled water and then PBS for five minutes. After adding the biotinylated anti-goat secondary antibody, the mixture incubated for thirty minutes at room temperature. After applying 1 or 2 drops of peroxidase-labeled streptavidin for 30 minutes at room temperature, the samples cleaned in PBS. Finally, the slides stained with DAB & hematoxylin, observed under a microscope, & photographed.

2.7. Evaluation of caspase 3 and cluster of differentiation 44 expression using Real-time quantitative reverse transcriptase polymerase chain reaction

The total RNA content had been isolated from tumor tissues using the RNeasy Mini Kit (cat. nos. 74104 and 74106) supplied by (Qiagen®, Germany) according to the manufacturer's instructions. The concentration & purity of extracted RNA were assessed using a NanoDrop™ ultraviolet spectrophotometer (Thermo Fisher Scientific™, USA), and total RNA was employed for the gene expression analysis. Isolated total RNA (one µg) had been reverse transcribed to cDNA with random primers from Quantitect reverse transcription kit (Cat # 205311) (Qiagen®, Germany). SensiFAST™ SYBR No-ROX Kit (Cat # BIO-980050, Bioline, Germany) on Piko-Real real-time PCR (Thermo Fisher Scientific Co., USA) used for detection of target and reference gene. The PCR conditions were as follows: 95° C for two min, 40 cycles of thirty s at 95° C, 30 s at 62° C (based on the target) & 60 s at 72° C. The fold change in the expression of target genes calculated for each time point using the Livak method ($2^{-\Delta\Delta CT}$ method) [41] sequences of the oligonucleotides used in this research were as follow

2.8. Statistical Analysis

Data collected from each (experimental & control) group had been expressed as mean ± SEM. One-way analysis of variance (ANOVA) & post hoc tests were performed to analyze the differences among groups using GraphPad Prism 5 program & $p<0.05$ had been denoted as a statistically significant difference among experimental & control groups.

3. Results and discussion

3.1. Results

GI (normal group): There were no apparent changes in animal health or activity. The right HBP mucosa did not show any abnormalities and appeared normal in color with a smooth surface. The HBP can everted completely outside the mouth. GII (DMBA painted group): Gross observation of the right HBP mucosa of GII showed multiple exophytic nodules of variable size accompanied by areas of ulceration & bleeding. These large lesions had also spread to contiguous tissue so that the buccal HBP fixed and could not [40] everted with the tumor. Animals revealed some weight loss and marked perioral hair loss in all hamsters. In addition, some lesions invaded the skin side of the mouth. GIII (CAP): Gross observation of the right HBP mucosa of GIII showed a complete disappearance of lesions in three animals leaving only a wrinkled surface. Five animals showed some reduction in the size & number of exophytic nodules as compared to animals painted with DMBA. The remaining two animals showed no clinical improvement.

GIV (CAP + LEN): Gross observation of the right HBP mucosa of GIV showed a complete disappearance of lesions in five animals leaving only a wrinkled surface. The remaining five animals also showed a reduction in the size & number of exophytic nodules as compared to animals painted with DMBA. Erosive and bleeding surfaces have disappeared. GV (CAP +DOX): Gross observation of the right HBP mucosa showed a complete disappearance of lesions in five animals leaving only a wrinkled surface. The HBP of the remaining 5animals showed a marked reduction in the size of exophytic nodules rather than animals painted with DMBA with the absence of ulceration and bleeding.

GVI (CAP+LEN+DOX): Gross observation of the HBP mucosa showed a complete disappearance of lesions in six animals leaving only a wrinkled surface. The HBP of the remaining 4animals showed a marked reduction in the size of exophytic nodules rather than animals painted with DMBA with the absence of ulceration & bleeding (fig.1).

3.1.1. Histopathological and immunohistochemical results

GI: Histological sections, using H&E stain exhibited that the HBP mucosa consists of thin, regular, keratinized stratified squamous epithelium, flat epithelium-connective tissue junction (no rete ridges), sub-epithelial connective tissue, & muscular layers seen. The IHC staining with anti-ALDH1A1 antibody showed low expression (mean = 6.48%) & only detected in the basal & suprabasal cell layers. ALDH1A1 detected in the cytoplasm with a granular pattern. GII: H&E stain showed that 8animals exhibited well differentiated SCC & 2animals showed moderately differentiated SCC. The IHC staining with anti ALDH1A1 antibody showed low expression (mean = 13.47) but highly significantly increased if compared to GI (P value<.000). Expression of ALDH1A1 had mainly observed in the cytoplasm of tumor cells, mainly in scattered cells and sometimes in focal areas especially in the invasive front but it always decreased in the center of keratin pearls. GIII: H&E stain showed that only three animals exhibited MPR, five animals showed PPR, and two animals showed NPR. The IHC staining with anti-ALDH1A1 antibody showed high expression (mean = 27.58) which represents the highest value among all groups. The expression of ALDH1A1 had been mainly present in scattered cells but focally increased expression had also detected in tumor cells near necrotic foci & around blood vessels.

GIV: H&E stain showed that five animals exhibited MPR & 5 exhibited PPR. The IHC staining with anti-ALDH1A1 antibody showed low scattered and patchy expression (mean = 18.17). In tumor cells surrounding blood vessels & close to necrotic foci, there was a focally elevated expression. It is worth noticing that ALDH1A1 expression in this group showed a highly significant decrease if compared to GIII (P value<.000) however it remained highly significantly stronger than GII (P value<.000). GV: H&E stain showed that five animals exhibited MPR & 5 exhibited PPR. The IHC staining with anti-ALDH1A1 antibody exposed low expression (11.76) in scattered cells. ALDH1A1 expression in this group showed a highly significant decrease if compared to both GIII and GIV (P value<.000) and showed non-significant difference if compared to GII (P value=.074). GVI: H&E stain showed that six animals exhibited MPR & 4 exhibited PPR. The IHC staining with anti-ALDH1A1 antibody showed low expression (7.73) in scattered areas which represents the lowest value among all groups except GI (fig.2&3).

3.1.2. Caspase 3 expression results

This investigation] carried out to evaluate apoptosis in all groups. Thus, the RT-qPCR method used to detect caspase 3 gene expression levels among groups by measuring the amount of mRNA fold change in comparison to GI (normal group). It was found that the expression of caspase 3 decreased significantly after induction of OSCC in GII and its ratio was .18-fold of the GI (P value=.042). A single treatment with CAP only in GIII has induced the expression of caspase 3 with a significant increase if compared to GII (mean 2.46-fold of GI, P value=.002). The combination between CAP and LEN in GIV resulted in more apoptosis induction, in comparison to GIII as evidenced by the expression level of caspase 3, which had increased to reach 3.2-fold of GI. The combination between CAP and DOX in GV resulted in more apoptosis induction, in comparison to GIII as evidenced by the expression level of caspase 3, which was 3.3-fold of GI. Caspase 3 expression in GVI showed a highly significant increase in comparison to all other groups (7.68-fold of GI) (fig.3).

3.1.3. Cluster of differentiation 44 expression results

This investigation carried out to assess the efficacy of the treatment in different groups on the cells expressing the CSCs marker CD44 in all groups. Thus, the RT-qPCR method used to detect CD44 gene expression levels among groups by measuring the amount of mRNA fold change in comparison to GI. It was found that the expression of CD44 was increased significantly after induction of OSCC in GII and its ratio was 2.3 of the GI (P value=.002). A single treatment with CAP only in GIII has significantly induced the expression of CD44 which was increased to reach 3.87 of GI (P value=.006). The combination of CAP and LEN in GIV resulted in decreased expression of CD44 in comparison to GIII (3.15 of GI). In addition, the combination of CAP and DOX in GV resulted in a highly significant decrease in CD44 expression in comparison to GIII and a significant decrease in comparison to GIV (2.03 of GI). The best result found in GVI in which the three drugs were combined (1.46 of GI) (fig.3).

3.2. Discussion

OSCC cases represent 90 % of all head and neck malignancy, which in turn represents the seventh most common cancer worldwide [42]. Despite the continuous research and trials to understand the molecular bases and pathways behind this type of cancer, still the prognosis of this type is not favorable, especially in the advanced stages. In other words, stage III and stage IV OSCC treated by surgical resection with pathologically proven free margins, in combination with chemo radiotherapy, and still, the tumor may recur in 70 % of cases [43]. During the last 50 years, frequent studies have proved that the cells that form the tumor mass are not the same, instead, they found that cells that populate the same tumor are heterogeneous, and multiple pathways and mechanisms interact with each other to help the cancer persistence and progression [44-46]. More specifically they also found that there is a definite subpopulation of cells called CSCs that have stem cells-like features in terms of self-renewal, asymmetric division, chemotherapeutic drug resistance, anchorage-independent growth, and immature dedifferentiated state [47].

The previous facts taken into consideration during the selection of the drugs that the study aimed to investigate their anticancer effects and accordingly LEN was added to CAP to combine the antimetabolite action of CAP and the antiangiogenic and immunomodulation action of LEN for targeting highly proliferating cells by multiple mechanisms [16-48]. Also, DOX was added to the drug combination to target the specific subpopulation called CSCs [29]. Since cancer cells' main defense mechanism is to resist

apoptosis, effective anticancer therapies would activate the apoptotic pathways to destroy cancer cells [49]. As the ultimate purpose of the drug combination used in the present study is to induce cancer cell death so, investigations dedicated to detecting apoptosis are the first choice to assess the therapeutic efficacy of the selected drug combination. A widely used assay is the evaluation of caspase 3 gene expression. Caspase 3 is one of the effector caspases and its expression decreases in malignant & benign tumors than the level in normal tissue. Conversely, caspase 3 had more expressed in malignant tumors, than in benign tumors [50].

The advantage of using caspase 3 as a marker of apoptosis is that caspase 3 activation occurs either in intrinsic, extrinsic, or alternate pathways of apoptosis [49-51]. The untreated normal group in the current investigation, represented by GI, had gross observation findings that demonstrated no abnormalities of the HBP. The buccal pouch mucosa had a smooth, pink complexion and no visible anomalies. These findings are consistent with previous research [52-53]. The HBP epithelium consists of a thin, regular, keratinized stratified squamous epithelium, the epithelium-connective tissue junction being relatively flat. These are the same observations of other investigators [54-55]. The right HBP mucosa of GII in the current investigation had several exophytic lesions of varying sizes accompanied by areas of bleeding & ulceration. Moreover, animals suffered from debilitation. These findings are nearly identical to those of other researchers who used the same methodology [36].

Histopathological findings of GII showed that eight animals exhibited well-differentiated OSCC and two animals exhibited moderate differentiated OSCC. These results are consistence with other studies that used DMBA as a carcinogenic agent for 14 weeks [53-56]. The occurrence of OSCC attributed to the potent carcinogenic action of DMBA. DMBA carcinogenic action mediated by three mechanisms, DNA adducts formation, induction of chronic inflammation, and production of free radicals [57]. This study has shown that treatment with CAP only in GIII resulted in the complete clinical disappearance of the lesions accompanied by MPR of the lesions in 3 out of 10 hamsters. MPR means that viable tumor cells ≤ 10 % of all tumor beds. Five animals showed clinical reduction in size and number of lesions accompanied histologically by PPR. PPR means that the residual tumor cells are ≤ 50% of all tumor beds. This is in line with other studies that showed that CAP treatment significantly decreased the number of lesions induced in hamsters and mice [58-59].

To investigate whether CAP administration alone was able to induce apoptosis of malignant cells, apoptosis induction assessed by evaluating the expression of caspase 3 gene by RT-qPCR, which showed that the caspase 3 expression was significantly increased after administration of CAP alone. The previous observations are consistence with other studies that used either CAP alone or other members of the chemotherapeutic drugs [43-60]. This can be explained by the antimetabolite effect of CAP which is efficiently able to target highly proliferating cells [61]. Although the induction of apoptosis by CAP-only treatment was evident the fact that cancers contain heterogeneous populations of cells and cancer cells switch among molecular pathways & mechanisms to ensure their proliferation, invasiveness, & resistance to chemotherapeutic agents necessitates the use of combination treatment with different mechanisms to increase the efficacy of treatment & decrease the possibility of developing resistance [43].

Several studies suggested that anti-angiogenic therapy, targeting angiogenic growth factors, & genes related to TME shows better outcomes when combined with chemotherapy and especially 5-FU-based chemotherapy [10-21-62]. Antiangiogenic therapy may inhibit angiogenic signaling & consequently neo vasculature formation but, results in normalization of the residual tumor vasculature [17]. To increase the effectiveness of cancer therapy by reducing the immunosuppression that immunosuppressive cells (such as regulatory T cells, tumor-associated macrophages, & myeloid-derived suppressor cells) exert, as well as by blocking the expression of programmed death-1 and controlling apoptotic pathways in cytotoxic CD8+ T cells [63]. Additionally, it was found that immunotherapy is more effective when combined with chemotherapy [64]. In the present study LEN was combined with CAP for achieving both antiangiogenic, & immunomodulatory in addition to its direct cytotoxicity actions [10].

This study has shown that treatment with CAP + LEN in GIV resulted in the complete clinical disappearance of the lesions accompanied by MPR of the lesions in 5 out of 10 hamsters. The remaining animals showed clinical reduction in size and number of lesions accompanied by PPR. This is in line with other studies that showed LEN enhances the clinical and pathological outcome of chemotherapy [16-65]. As regards head and neck cancer, LEN enhanced the clinical and pathological response of cetuximab [66]. The previous result is not consistence with the results reported by Rabih et al. who found that when LEN combined with 5-Fu, leucovorin, and oxaliplatin no patients showed complete or partial pathological response and only some patients expressed stable disease. But this can be attributed to the criteria of patients who underwent this study who were with histologically confirmed advanced and metastatic cancer that had been refractory to standard therapy, recurred after standard therapy, or for which there had been no standard therapy available [67].

In GIV, the addition of LEN in combination with CAP further increased the induction of apoptosis as evidenced by the increased expression of the caspase 3 gene. This synergistic effect of LEN has reported in other studies that used LEN in combination with chemotherapeutics. Yin et al. reported that the combination of LEN and cisplatin treatment on breast cancer cells in vitro led to increased apoptosis induction, which had measured by annexin V /propidium iodide assay. Being in vitro means that this therapeutic effect of LEN is not mediated by either antiangiogenic or immunomodulatory effect. Instead, Yin et al. found that the therapeutic impact of LEN had mediated by increased caspase 3 induction, which is the same in our study [10]. Leuci et al. also reported that a combination of LEN and 5-FU treatment on colon cancer enhanced the chemotherapeutic effect of 5-FU through its antiangiogenic, immunomodulatory, and direct cytotoxicity actions [17].

This study has shown that treatment with CAP + DOX in GV resulted in the complete clinical disappearance of the lesions accompanied by MPR of the lesions in 5 out of 10 hamsters. The remaining animals showed clinical reduction in size and number of lesions accompanied by PPR. This is in line with other studies that showed that DOX

enhanced the clinical and pathological outcome of chemotherapy [29-68]. The addition of DOX to CAP in GV also increased apoptosis induction as evidenced by the expression level of caspase 3. This observation is consistent with research done by Liu et al. who investigated the therapeutic effect of DOX in combination with different chemotherapeutics and documented that the best findings had been obtained when DOX was combined with CAP [68]. This synergistic effect can be explained by the direct cytotoxic action of DOX [69].

The best result obtained when both LEN and DOX added to CAP in the treatment regimen, as complete clinical disappearance of the lesions accompanied by MPR of the lesions in 7 out of 10 hamsters. The remaining animals showed clinical reduction in size and number of lesions accompanied by PPR. The expression level of the proapoptotic caspase 3 gene was the highest among groups and this ensured that the maximum apoptosis induction obtained when both LEN and DOX added to CAP in the treatment regimen. Although the induction of apoptosis may be great to the degree that the tumor mass may completely disappear, this found to be unable to eradicate cancer [22-70]. In contrast, a small subpopulation of cells called CSCs can resist chemotherapy and either repopulate the tumor or re-establish a new one in the metastatic site [25]. So, one of the main targets of the current research is to specifically target this subpopulation of cells to eradicate the tumor. For this purpose, DOX added to the treatment regimen as it has been used successfully for targeting CSCs in multiple types of cancer [27-71].

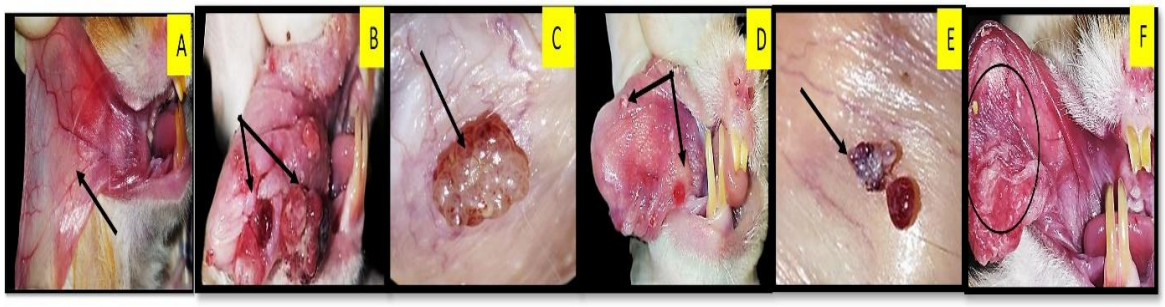
Certain indicators used to identify CSCs, and these markers differ based on the type of tumor and the origin tissue. Since there is not a single marker that can used to identify CSCs with certainty, CSCS may distributed among overlapping subsets of the cell population [72-74]. It's interesting to note that multiple CSC subpopulations exist and can result in processes with varied functions [22]. As a result, different marker combinations have been employed to characterize CSCs [75]. Because ALDH1A1 and CD44 have documented to be markers of CSCs in head and neck SCC, the effect of the therapeutic regimen on CSCS evaluated at the protein level by the immunehistochemical expression of ALDH1A1 enzyme and at the mRNA level by the expression of CD44 mRNA. The same markers have used for the same purpose in several studies [20-76].In the current research, the expression of CD44 was the least in GI-containing normal hamsters. This result was consistent with other studies that assessed CD44 in normal tissues [76].

After induction of OSCC in HBP, the level of CD44 expression increased to reach a 2.3-fold increase of the normalized level. The same observation recorded by other studies [74-76]. The highest level of CD44 expression found in GIII that treated with CAP alone. The increased expression of CD44 in GII can be explained by the CSCs theory of cancer origin, which reveals that cancer, can arise from mutations in stem cell populations, as; they are the only cells permanently resident in the tissue without turnover, making them subjected to the carcinogens for longer periods than other cells [25]. The additional increase of CD44 expression in GIII is the result of CSCs resistance to chemotherapy, so CAP alone treatment induced apoptosis but in contrast, it enhanced the population of CSCs which was evidenced by higher CD44 expression than GII [77].

The addition of LEN to the treatment regimen in GIV was associated with decreased CD44 expression but this reduction was non-significant if compared to GIII. In contrast, the addition of DOX to CAP as in GV significantly decreased the expression of CD44 if compared to GIII yet still non-significant if compared to GII. These observations are in agreement with the previous studies [78-79]. And can be explained by the ability of DOX to target CSCs in different tumor types mainly by preventing mitochondrial biogenesis [80]. When combining the three drugs in GVI the lowest level of CD44 expression obtained. In the present study, the expression ALDH1A1 was the least in GI-containing normal hamsters. ALDH1A1 showed low diffuse positivity only in the cytoplasm of the cells present in the basal and suprabasal layers. These results were consistent with other studies that assessed ALDH1A1 in normal tissues [81-82] . These results are predicted since ALDH1A1, a molecule linked to stemness, is necessary for basal cell function & continuing epithelial turnover [81].

After induction of OSCC in the HBP of GII the level of ALDH1A1, expression showed a highly significant increase. This result is consistence with other studies and also, this low expression is compatible with the concept that CSCs comprise a small population of cancer cells with multiple differentiation & long-term repopulation capabilities [82-83]. The increased expression of ALDH1A1 in GII could be attributed to the role of ALDH1A1 in regulating various pathways that contribute to tumorigenesis & stem cell signaling [84-85]. The highest expression among all groups found in GIII, which was treated, with CAP alone. The additional increase of ALDH1A1 expression in GIII is the result of CSCs resistance to chemotherapy, so CAP alone treatment-induced apoptosis but in contrast, it enhanced the population of CSCs, which evidenced by higher ALDH1A1 expression than all other groups [77].

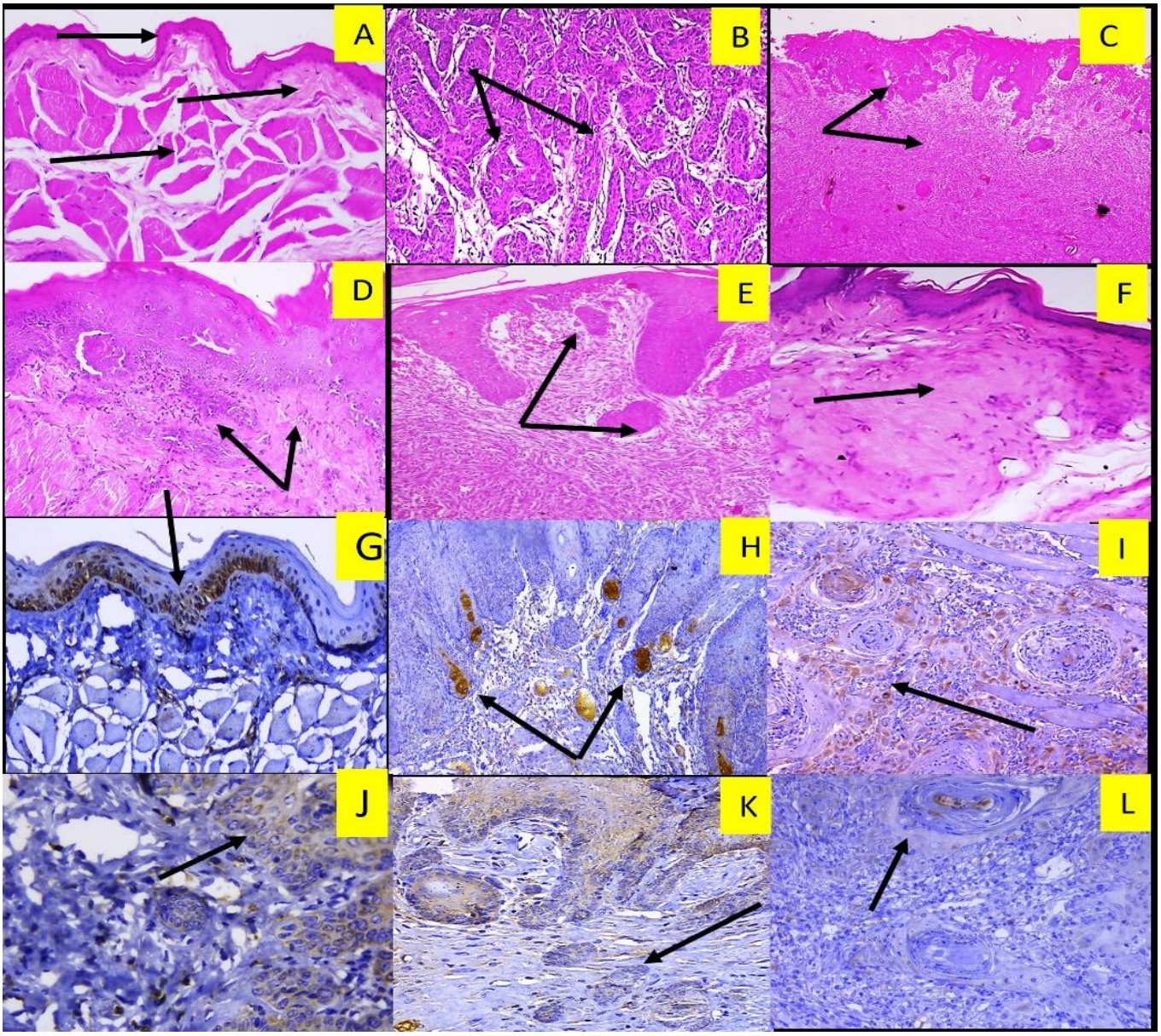
The addition of LEN to the treatment regimen in GIV was associated with a decrease in ALDH1A1 expression if compared to GIII but still higher than that of GII. The addition of DOX to CAP in GV decreased the expression of ALDH1A1 in comparison to GIII and GII, however, this reduction in expression was statistically significant in comparison to GIII but not significant when compared to GII. Paclitaxel therapy produced around four times more ALDH+ breast cancer stem cells than the vehicle control, which is consistent with the earlier finding. Nonetheless, when cells first treated with DOX & then co-treated with paclitaxel, the enrichment of ALDH+ CSCs brought about by the drug greatly reduced [85]. Also, Liu et al. have reported that treatment with DOX effectively enhanced the impact of CAP in comparison with the outcomes obtained when CAP only was used and this improvement was accompanied by decreased expression of ALDH1A1 [68]. This can explained by the ability of DOX to target ALDH+CSCs in different tumor types [80-85]. When combining the three drugs in GVI the expression decreased significantly in comparison to all groups except the normal group.



(Fig.1A): Photograph of GI displaying a rosy, smooth buccal pouch mucosa (arrow). **(Fig.1B):** Photograph of GII showing number of polypoid papillary masses surrounded by bleeding zones (arrow). **(Fig.1C):** Photograph of GIII showing exophytic papillary nodule, erosive and bleeding surfaces have disappeared (arrow). **(Fig.1D):** Photograph of GIV great reduction in lesion size, erosive and bleeding surfaces have disappeared. **(Fig.1E):** Photograph of GV showing only irregular surface remaining after treatment. **(Fig.1F):** Photograph of GVI showing very small lesion.

Table 1. Sequences of the oligonucleotides used in this research

Gene	Forward primer	Reverse primer5`- 3`
GAPDH Reference gene	5’CACCACCACCCCAGTTTCTAT-3’	5’-TAACCAGGCGTCCAATACGG-3’
Caspase 3	5’TGAAGCTAGAAACCCCGCTG-3’	5’CCTCCGACTCCACCTTTTGG-3’
CD 44	5’TTCCACACCTACCTTCCCAC-3’	5’GAGTCTTGGTCTCTGGTGGC-3’



(Fig.2A): Photomicrograph of GI HBP mucosa indicating stratified squamous epithelium with flattened rete ridges, connective tissue, & striated muscle fibers layers (arrow) (H&E stain X400). **(Fig.2B):** Photomicrograph of GII HBP showing well differentiated SCC with deeply invasive tumor islands into the connective tissue (arrow) (H&E stain X200). **(Fig.2C):** Photomicrograph of GIII HBP indicating shrinkage of epithelial islands and fibrous stroma (arrow) (H&E stain X100). **(Fig.2D):** Photomicrograph of GIV HBP showing residual epithelial islands and fibrous stroma (arrow). **(Fig.2E):** Photomicrograph of GV HBP mucosa showing very small islands in fibrous stroma (arrow) (H&E stain X400). **(Fig.2F):** Photomicrograph of GVI HBP showing MPR with absence of islands and hyalinized stroma (arrow) (H&E stain X200). **(Fig.2G):** Photomicrograph of GI HBP mucosa showing low cytoplasmic expression of ALDH1A1 in basal & suprabasal epithelial layers (arrow). **(Fig.2H):** Photomicrograph of GII HBP mucosa showing (a) low cytoplasmic expression of ALDH1A1in scattered area (arrow). **(Fig.2I):** Photomicrograph of GIII HBP mucosa showing high cytoplasmic expression of ALDH1A1 in scattered area with absence in keratin pearls (arrow) (Streptavidin biotin peroxidase, X 100). **(Fig.2J):** Photomicrograph of GIV HBP mucosa showing (a) CAP + LEN treated SCC which revealed residual epithelial islands and fibrous stroma **(Fig.2K):** Photomicrograph of GV HBP focal cytoplasmic expression of ALDH1A1 in the same area (Streptavidin biotin peroxidase) **(Fig.2L):** Photomicrograph of GVI HBP mucosa showing low cytoplasmic expression of ALDH1A1in diffuse area (Streptavidin biotin peroxidase X 200).

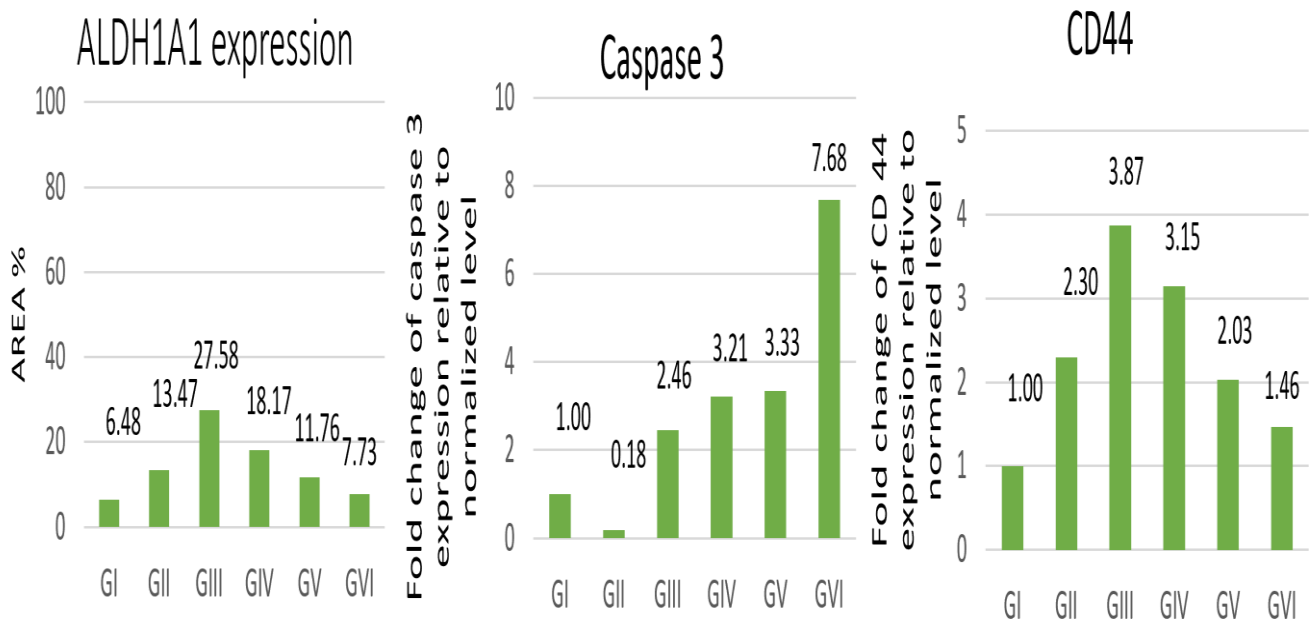


Fig. (3): Bar charts representing the mean results of ALDH1A1 protein expression, caspase three gene expression and CD 44 gene expression in studied groups.

4. Conclusions

Combination therapy and targeting of multiple pathways is necessary for the eradication of cancer. The addition of both LEN and DOX increased the therapeutic efficacy of CAP and improved the outcome of treatment through induction of apoptosis and targeting CSCs more effectively than CAP alone.

References

[1] J. Ferlay, M. Colombet, I. Soerjomataram, D.M. Parkin, M. Piñeros, A. Znaor, F. Bray. (2021). Cancer statistics for the year 2020: An overview. International journal of cancer. 149(4): 778-789.

[2] R. Tabrizi, A. Garajei, E. Shafie, S. Jamshidi. (2016). Outcome of neoadjuvant chemotherapy on local recurrence and distant metastasis of oral squamous cell carcinoma: a retrospective study. Journal of Dentistry. 17(3): 207.

[3] N. Jootun, T. Evans, J. Mak, G. Makin, C. Platell. (2018). Comparing pathological complete response rate using oral capecitabine versus infusional 5-fluorouracil with preoperative radiotherapy in rectal cancer treatment. ANZ Journal of Surgery. 88(1-2): 62-65.

[4] M.R. Eskandari, F. Moghaddam, J. Shahraki, J. Pourahmad. (2015). A comparison of cardiomyocyte cytotoxic mechanisms for 5-fluorouracil and its pro-drug capecitabine. Xenobiotica. 45(1): 79-87.

[5] J. Fernando, R. Jones. (2015). The principles of cancer treatment by chemotherapy. Surgery (Oxford). 33(3): 131-135.

[6] P.M. Manohar, E. Sapir, E. Bellile, P.L. Swiecicki, A.T. Pearson, M.E. Prince, A.G. Shuman, C.R. Bradford, D.B. Chepeha, G.T. Wolf. (2017). Capecitabine after surgical salvage in recurrent squamous cell carcinoma of head and neck. Otolaryngology–Head and Neck Surgery. 157(6): 995-997.

[7] B.M. Robert, M. Dakshinamoorthy, B. Ganapathyagraharam Ramamoorthy, M. Dhandapani, R. Thangaiyan, G. Muthusamy, R.M. Nirmal, N.R. Prasad. (2018). Predicting tumor sensitivity to chemotherapeutic drugs in oral squamous cell carcinoma patients. Scientific reports. 8(1): 15545.

[8] L. Bolm, L. Kaesmann, A. Paysen, C. Karapetis, D. Rades, U.F. Wellner, T. Keck, D.I. Watson, R. Hummel, D.J. Hussey. (2018). Multimodal anti-tumor approaches combined with immunotherapy to overcome tumor resistance in esophageal and gastric cancer. Anticancer research. 38(6): 3231-3242.

[9] A. Hosokawa, T. Ando, K. Ogawa, A. Ueda, H. Yoshita, H. Mihara, H. Fujinami, S. Kajiura, K. Yabushita, N. Horikawa. (2018). Phase I/II study of S-1 plus cisplatin alternating with S-1 plus docetaxel in patients with advanced gastric cancer. American Journal of Clinical Oncology. 41(10): 977-981.

[10] L.L. Yin, X.M. Wen, Q.H. Lai, J. Li, X.W. Wang. (2018). Lenalidomide improvement of cisplatin

antitumor efficacy on triple-negative breast cancer cells in vitro. Oncology letters. 15(5): 6469-6474.

[11] J.B. Zeldis, R. Knight, M. Hussein, R. Chopra, G. Muller. (2011). A review of the history, properties, and use of the immunomodulatory compound lenalidomide. Annals of the New York Academy of Sciences. 1222(1): 76-82.

[12] K. Kim, S. An, H.J. Cha, Y.M. Choi, S.J. Choi, I.-S. An, H.G. Lee, Y.H. Min, S.-J. Lee, S. Bae. (2013). Lenalidomide induces apoptosis and alters gene expression in non-small cell lung cancer cells. Oncology letters. 5(2): 588-592.

[13] A. Segler, A.-M. Tsimberidou. (2012). Lenalidomide in solid tumors. Cancer chemotherapy and pharmacology. 69: 1393-1406.

[14] C. Galustian, B. Meyer, M.-C. Labarthe, K. Dredge, D. Klaschka, J. Henry, S. Todryk, R. Chen, G. Muller, D. Stirling. (2009). The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. Cancer Immunology, Immunotherapy. 58: 1033-1045.

[15] D. Zhu, L.G. Corral, Y.W. Fleming, B. Stein. (2008). Immunomodulatory drugs Revlimid®(lenalidomide) and CC-4047 induce apoptosis of both hematological and solid tumor cells through NK cell activation. Cancer Immunology, Immunotherapy. 57: 1849-1859.

[16] G.J. Ullenhag, F. Mozaffari, M. Broberg, H. Mellstedt, M. Liljefors. (2017). Clinical and immune effects of lenalidomide in combination with gemcitabine in patients with advanced pancreatic cancer. Plos one. 12(1): e0169736.

[17] V. Leuci, F. Maione, R. Rotolo, E. Giraudo, F. Sassi, G. Migliardi, M. Todorovic, L. Gammaitoni, G. Mesiano, L. Giraudo. (2016). Lenalidomide normalizes tumor vessels in colorectal cancer improving chemotherapy activity. Journal of Translational Medicine. 14: 1-12.

[18] S.M. Smith, N. Hijiya, K.M. Sakamoto. (2021). Chronic myelogenous leukemia in childhood. Current Oncology Reports. 23: 1-10.

[19] F.N. Chowdhury, J. Reisinger, K.E. Gomez, T.-S. Chimed, C.M. Thomas, P.N. Le, B. Miller, J.J. Morton, C.M. Nieto, H.L. Somerset. (2019). Leading edge or tumor core: Intratumor cancer stem cell niches in oral cavity squamous cell carcinoma and their association with stem cell function. Oral oncology. 98: 118-124.

[20] N. Cirillo, C. Wu, S.S. Prime. (2021). Heterogeneity of cancer stem cells in tumorigenesis, metastasis, and resistance to antineoplastic treatment of head and neck tumours. Cells. 10(11): 3068.

[21] C. Sethy, C.N. Kundu. (2021). 5-Fluorouracil (5-FU) resistance and the new strategy to enhance the sensitivity against cancer: Implication of DNA repair inhibition. Biomedicine & Pharmacotherapy. 137: 111285.

[22] K. Sudo, K. Kato, H. Kuwabara, Y. Sasaki, N. Takahashi, H. Shoji, S. Iwasa, Y. Honma, N.T. Okita, A. Takashima. (2018). Patterns of relapse after definitive chemoradiotherapy in stage II/III (Non-T4) esophageal squamous cell carcinoma. Oncology. 94(1): 47-54.

[23] Z. Zhang, Z. Dong, I.S. Lauxen, M.S.A. Filho, J.E. Nör. (2014). Endothelial cell-secreted EGF induces epithelial to mesenchymal transition and endows head and neck cancer cells with stem-like phenotype. Cancer research. 74(10): 2869-2881.

[24] J.M. Kalish, X.-H. Tang, T. Scognamiglio, T. Zhang, L.J. Gudas. (2020). Doxycycline-induced exogenous Bmi-1 expression enhances tumor formation in a murine model of oral squamous cell carcinoma. Cancer Biology & Therapy. 21(5): 400-411.

[25] P. Singh, D. Augustine, R.S. Rao, S. Patil, K.H. Awan, S.V. Sowmya, V.C. Haragannavar, K. Prasad. (2021). Role of cancer stem cells in head-and-neck squamous cell carcinoma–A systematic review. Journal of Carcinogenesis. 20.

[26] P. Jagust, B. de Luxán-Delgado, B. Parejo-Alonso, P. Sancho. (2019). Metabolism-based therapeutic strategies targeting cancer stem cells. Frontiers in pharmacology. 10: 203.

[27] R. Lamb, M. Fiorillo, A. Chadwick, B. Ozsvari, K.J. Reeves, D.L. Smith, R.B. Clarke, S.J. Howell, A.R. Cappello, U.E. Martinez-Outschoorn. (2015). Doxycycline down-regulates DNA-PK and radiosensitizes tumor initiating cells: Implications for more effective radiation therapy. Oncotarget. 6(16): 14005.

[28] R. Lamb, B. Ozsvari, C.L. Lisanti, H.B. Tanowitz, A. Howell, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti. (2015). Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. Oncotarget. 6(7): 4569.

[29] T. Matsumoto, T. Uchiumi, K. Monji, M. Yagi, D. Setoyama, R. Amamoto, Y. Matsushima, M. Shiota, M. Eto, D. Kang. (2017). Doxycycline induces apoptosis via ER stress selectively to cells with a cancer stem cell-like properties: importance of stem cell plasticity. Oncogenesis. 6(11): 397.

[30] Q. Tan, X. Yan, L. Song, H. Yi, P. Li, G. Sun, D. Yu, L. Li, Z. Zeng, Z. Guo. (2017). Induction of mitochondrial dysfunction and oxidative damage by antibiotic drug doxycycline enhances the responsiveness of glioblastoma to chemotherapy. Medical science monitor: international medical journal of experimental and clinical research. 23: 4117.

[31] W.C. Duivenvoorden, S.V. Popovic, S. Lhoták, E. Seidlitz, H.W. Hirte, R.G. Tozer, G. Singh. (2002). Doxycycline decreases tumor burden in a bone metastasis model of human breast cancer. Cancer research. 62(6): 1588-1591.

[32] G.D. Kao, Z. Jiang, A.M. Fernandes, A.K. Gupta, A. Maity. (2007). Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. Journal of Biological Chemistry. 282(29): 21206-21212.

[33] E.M. De Francesco, M. Maggiolini, H.B. Tanowitz, F. Sotgia, M.P. Lisanti. (2017). Targeting hypoxic cancer stem cells (CSCs) with Doxycycline:

Implications for optimizing anti-angiogenic therapy. Oncotarget. 8(34): 56126.

[34] A. Vinoth, R. Kowsalya. (2018). Chemopreventive potential of vanillic acid against 7, 12-dimethylbenz (a) anthracene-induced hamster buccal pouch carcinogenesis. Journal of Cancer Research and Therapeutics. 14(6): 1285-1290.

[35] R. Selvasundaram, S. Manoharan, R. Buddhan, M. Neelakandan, R. Murali Naidu. (2018). Chemopreventive potential of esculetin in 7, 12-dimethylbenz (a) anthracene-induced hamster buccal pouch carcinogenesis. Molecular and cellular biochemistry. 448: 145-153.

[36] P.V. Turner, C. Pekow, J.M. Clark, P. Vergara, K. Bayne, W.J. White, T.M. Kurosawa, S.-H. Seok, P. Baneux. (2015). Roles of the international council for laboratory animal science (ICLAS) and international association of colleges of laboratory animal medicine (IACLAM) in the global organization and support of 3Rs advances in laboratory animal science. Journal of the American Association for Laboratory Animal Science. 54(2): 174-180.

[37] K. Kolinsky, B.-Q. Shen, Y.-E. Zhang, J. Kohles, U. Dugan, T.F. Zioncheck, D. Heimbrook, K. Packman, B. Higgins. (2009). In vivo activity of novel capecitabine regimens alone and with bevacizumab and oxaliplatin in colorectal cancer xenograft models. Molecular cancer therapeutics. 8(1): 75-82.

[38] S. Anand, A. Yasinchak, T. Bullock, M. Govande, E.V. Maytin. (2019). A non-toxic approach for treatment of breast cancer and its metastases: capecitabine enhanced photodynamic therapy in a murine breast tumor model. Journal of cancer metastasis and treatment. 5.

[39] J.L. Vos, J.B. Elbers, O. Krijgsman, J.J. Traets, X. Qiao, A.M. van der Leun, Y. Lubeck, I.M. Seignette, L.A. Smit, S.M. Willems. (2021). Neoadjuvant immunotherapy with nivolumab and ipilimumab induces major pathological responses in patients with head and neck squamous cell carcinoma. Nature communications. 12(1): 7348.

[40] W.T. Liu, W.B. Liu, M. Gao, Y.Y. Zhang, K.S. Gu. (2019). Expression of ALDH1A1 and CD133 is associated with the prognosis and effect of different chemotherapeutic regimens in gastric cancer. Oncology letters. 18(5): 4573-4582.

[41] K.J. Livak, T.D. Schmittgen. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 25(4): 402-408.

[42] D. Nandini, R.S. Rao, J. Hosmani, S. Khan, S. Patil, K.H. Awan. (2020). Novel therapies in the management of oral cancer: An update. Disease-a-Month. 66(12): 101036.

[43] J. Sha, Y. Bai, H.X. Ngo, T. Okui, T. Kanno. (2021). Overview of evidence-based chemotherapy for oral cancer: focus on drug resistance related to the epithelial-mesenchymal transition. Biomolecules. 11(6): 893.

[44] C. Denise, P. Paoli, M. Calvani, M.L. Taddei, E. Giannoni, S. Kopetz, S.M.A. Kazmi, M.M. Pia, P. Pettazzoni, E. Sacco. (2015). 5-fluorouracil resistant colon cancer cells are addicted to OXPHOS to survive and enhance stem-like traits. Oncotarget. 6(39): 41706.

[45] J. Kaur, S. Bhattacharyya. (2021). Cancer stem cells: metabolic characterization for targeted cancer therapy. Frontiers in Oncology. 11: 756888.

[46] J. Chen, S. Liu, Y. Su, X. Zhang. (2020). ALDH1+ stem cells demonstrate more stem cell-like characteristics than CD44+/CD24–/low stem cells in different molecular subtypes of breast cancer. Translational Cancer Research. 9(3): 1652.

[47] M. Simple, A. Suresh, D. Das, M.A. Kuriakose. (2015). Cancer stem cells and field cancerization of oral squamous cell carcinoma. Oral oncology. 51(7): 643-651.

[48] Z. Qu, C. Jiang, J. Wu, Y. Ding. (2016). Lenalidomide induces apoptosis and inhibits angiogenesis via caspase-3 and VEGF in hepatocellular carcinoma cells. Molecular Medicine Reports. 14(5): 4781-4786.

[49] P. Yadav, R. Yadav, S. Jain, A. Vaidya. (2021). Caspase-3: A primary target for natural and synthetic compounds for cancer therapy. Chemical biology & drug design. 98(1): 144-165.

[50] R. Dwivedi, R. Pandey, S. Chandra, D. Mehrotra. (2020). Apoptosis and genes involved in oral cancer-a comprehensive review. Oncology reviews. 14(2).

[51] H.A. Hassan, M.L. Salem, M.S. Gouida, K.M. El-Azab. (2018). Comparative expression of caspases and annexin V in benign and malignant ovarian tumors. Journal of Cancer Research and Therapeutics. 14(5): 1042-1048.

[52] A. Manimaran, S. Manoharan. (2018). Tumor preventive efficacy of emodin in 7, 12-Dimethylbenz [a] Anthracene-induced oral carcinogenesis: a histopathological and biochemical approach. Pathology & Oncology Research. 24: 19-29.

[53] M. Goldberg, A. Manzi, A. Birdi, B. Laporte, P. Conway, S. Cantin, V. Mishra, A. Singh, A.T. Pearson, E.R. Goldberg. (2022). A nanoengineered topical transmucosal cisplatin delivery system induces anti-tumor response in animal models and patients with oral cancer. Nature communications. 13(1): 4829.

[54] V.K. Nimbalkar, J. Gangar, S. Shai, P. Rane, S.K. Mohanta, S. Kannan, A. Ingle, N. Mittal, S. Rane, M.B. Mahimkar. (2022). Prevention of carcinogen-induced oral cancers by polymeric black tea polyphenols via modulation of EGFR-Akt-mTOR pathway. Scientific reports. 12(1): 14516.

[55] W.M. Darwish, A.S. Abdoon, M.S. Shata, M. Elmansy. (2020). Vincristine-loaded polymeric corona around gold nanorods for combination (chemo-photothermal) therapy of oral squamous carcinoma. Reactive and Functional Polymers. 151: 104575.

[56] Y. Sun, J. Ren, F. Wang. (2021). [6]-Gingerol impedes 7, 12-dimethylbenz (a) anthracene-induced inflammation and cell proliferation-associated hamster buccal pouch carcinogenesis through

modulating Nrf2 signaling events. Journal of Biochemical and Molecular Toxicology. 35(4): e22689.

[57] I.S. Santa Cruz, M.A. Garabalino, V.A. Trivillin, M.E. Itoiz, E.C. Pozzi, S. Thorp, P. Curotto, J.S. Guidobono, E.M. Heber, D.W. Nigg. (2020). Optimization of the classical oral cancerization protocol in hamster to study oral cancer therapy. Oral Diseases. 26(6): 1175-1184.

[58] Á. Arjona-Sánchez, J. Ruiz-Rabelo, M.D. Perea, R. Vázquez, A. Cruz, M.d.C. Munoz, I. Túnez, J. Muntané, F.J. Padillo. (2010). Effects of capecitabine and celecoxib in experimental pancreatic cancer. Pancreatology. 10(5): 641-647.

[59] J. Ruiz-Rabelo, R. Vázquez, A. Arjona, D. Perea, P. Montilla, I. Túnez, J. Muntané, J. Padillo. (2011). Improvement of capecitabine antitumoral activity by melatonin in pancreatic cancer. Pancreas. 40(3): 410-414.

[60] M. Wisniewska-Jarosinska, T. Sliwinski, J. Kasznicki, D. Kaczmarczyk, R. Krupa, K. Bloch, J. Drzewoski, J. Chojnacki, J. Blasiak, A. Morawiec-Sztandera. (2011). Cytotoxicity and genotoxicity of capecitabine in head and neck cancer and normal cells. Molecular biology reports. 38: 3679-3688.

[61] V. Ciaffaglione, M.N. Modica, V. Pittalà, G. Romeo, L. Salerno, S. Intagliata. (2021). Mutual prodrugs of 5-fluorouracil: From a classic chemotherapeutic agent to novel potential anticancer drugs. ChemMedChem. 16(23): 3496-3512.

[62] J. Zhang, J. Lee, S. Urba, J. Foster, F. Worden. (2010). A phase II trial evaluating weekly docetaxel and capecitabine in patients with metastatic or advanced, locally recurrent head and neck cancers. Cancer Investigation. 28(9): 910-916.

[63] W.-t. Ju, R.-h. Xia, D.-w. Zhu, S.-j. Dou, G.-p. Zhu, M.-j. Dong, L.-z. Wang, Q. Sun, T.-c. Zhao, Z.-h. Zhou. (2022). A pilot study of neoadjuvant combination of anti-PD-1 camrelizumab and VEGFR2 inhibitor apatinib for locally advanced resectable oral squamous cell carcinoma. Nature communications. 13(1): 5378.

[64] M.-C. Vo, T.-N. Nguyen-Pham, H.-J. Lee, T.J. Lakshmi, S. Yang, S.-H. Jung, H.-J. Kim, J.-J. Lee. (2017). Combination therapy with dendritic cells and lenalidomide is an effective approach to enhance antitumor immunity in a mouse colon cancer model. Oncotarget. 8(16): 27252.

[65] R.A. Madan, F.H. Karzai, Y.M. Ning, B.A. Adesunloye, X. Huang, N. Harold, A. Couvillon, G. Chun, L. Cordes, T. Sissung. (2016). Phase II trial of docetaxel, bevacizumab, lenalidomide and prednisone in patients with metastatic castration-resistant prostate cancer. BJU international. 118(4): 590-597.

[66] E.M. Bertino, E.L. McMichael, X. Mo, P. Trikha, M. Davis, B. Paul, M. Grever, W.E. Carson, G.A. Otterson. (2016). A phase I trial to evaluate antibody-dependent cellular cytotoxicity of cetuximab and lenalidomide in advanced colorectal and head and neck cancer. Molecular cancer therapeutics. 15(9): 2244-2250.

[67] R. Said, Y. Ye, D.S. Hong, A. Naing, G. Falchook, S. Fu, J.J. Wheler, S. Piha-Paul, A.-M. Tsimberidou. (2016). Phase I clinical trial of lenalidomide in combination with 5-fluorouracil, leucovorin, and oxaliplatin in patients with advanced cancer. Cancer chemotherapy and pharmacology. 77: 575-581.

[68] H. Liu, H. Tao, H. Wang, Y. Yang, R. Yang, X. Dai, X. Ding, H. Wu, S. Chen, T. Sun. (2021). Doxycycline inhibits cancer stem cell-like properties via PAR1/FAK/PI3K/AKT pathway in pancreatic cancer. Frontiers in Oncology. 10: 619317.

[69] A. Markowska, J. Kaysiewicz, J. Markowska, A. Huczyński. (2019). Doxycycline, salinomycin, monensin and ivermectin repositioned as cancer drugs. Bioorganic & Medicinal Chemistry Letters. 29(13): 1549-1554.

[70] P.-X. Liw, Y.-W. Wen, C.-Y. Tsai, H.-K. Chang, C.-K. Tseng, T.-M. Hung, Y.-K. Chao. (2018). Pretreatment clinical stage predicts locoregional recurrence in patients with esophageal cancer who achieved a complete clinical response to chemoradiotherapy. The Journal of thoracic and cardiovascular surgery. 155(5): 2233-2242. e2.

[71] B. Bhattacharya, S. Mukherjee. (2015). Cancer therapy using antibiotics. Journal of Cancer Therapy. 6(10): 849.

[72] V. Masciale, G. Grisendi, F. Banchelli, R. D'Amico, A. Maiorana, P. Sighinolfi, A. Stefani, U. Morandi, M. Dominici, B. Aramini. (2019). Isolation and identification of cancer stem-like cells in adenocarcinoma and squamous cell carcinoma of the lung: a pilot study. Frontiers in Oncology. 9: 1394.

[73] X. Qian, S. Wagner, C. Ma, J.P. Klussmann, M. Hummel, A.M. Kaufmann, A.E. Albers. (2013). ALDH1-positive cancer stem-like cells are enriched in nodal metastases of oropharyngeal squamous cell carcinoma independent of HPV status. Oncology reports. 29(5): 1777-1784.

[74] R.C. Ortiz, N.M. Lopes, N.G. Amôr, J.B. Ponce, C.K. Schmerling, V.S. Lara, R.A. Moyses, C.O. Rodini. (2018). CD 44 and ALDH 1 immunoexpression as prognostic indicators of invasion and metastasis in oral squamous cell carcinoma. Journal of Oral Pathology & Medicine. 47(8): 740-747.

[75] B. Varun, P. Jayanthi, P. Ramani. (2020). Cancer stem cells: A comprehensive review on identification and therapeutic implications. Journal of oral and maxillofacial pathology. 24(1): 190.

[76] T. Tamatani, N. Takamaru, G. Ohe, K. Akita, T. Nakagawa, Y. Miyamoto. (2018). Expression of CD44, CD44v9, ABCG2, CD24, Bmi-1 and ALDH1 in stage I and II oral squamous cell carcinoma and their association with clinicopathological factors. Oncology letters. 16(1): 1133-1140.

[77] K.A. Finkel, K.A. Warner, S. Kerk, C.R. Bradford, S.A. McLean, M.E. Prince, H. Zhong, E.M. Hurt, R.E. Hollingsworth, M.S. Wicha. (2016). IL-6 inhibition with MEDI5117 decreases the fraction of

head and neck cancer stem cells and prevents tumor recurrence. Neoplasia. 18(5): 273-281.

[78] L.-C. Shen, Y.-K. Chen, L.-M. Lin, S.-Y. Shaw. (2010). Anti-invasion and anti-tumor growth effect of doxycycline treatment for human oral squamous-cell carcinoma–in vitro and in vivo studies. Oral oncology. 46(3): 178-184.

[79] P.X. Mouratidis, K.W. Colston, A.G. Dalgleish. (2007). Doxycycline induces caspase-dependent apoptosis in human pancreatic cancer cells. International journal of cancer. 120(4): 743-752.

[80] B. Ózsvári, L.G. Magalhães, J. Latimer, J. Kangasmetsa, F. Sotgia, M.P. Lisanti. (2020). A myristoyl amide derivative of doxycycline potently targets cancer stem cells (CSCs) and prevents spontaneous metastasis, without retaining antibiotic activity. Frontiers in Oncology. 10: 1528.

[81] S. Javed, S. Sood, B. Rai, S. Bhattacharyya, R. Bagga, R. Srinivasan. (2021). ALDH1 & CD133 in invasive cervical carcinoma & their association with the outcome of chemoradiation therapy. Indian Journal of Medical Research. 154(2): 367-374.

[82] R. Chatterjee, B. Ghosh, M. Mandal, D. Nawn, S. Banerjee, M. Pal, R.R. Paul, S. Banerjee, J. Chatterjee. (2021). Pathophysiological relationship between hypoxia associated oxidative stress, Epithelial-mesenchymal transition, stemness acquisition and alteration of Shh/Gli-1 axis during oral sub-mucous fibrosis and oral squamous cell carcinoma. European Journal of Cell Biology. 100(1): 151146.

[83] M. Poturnajova, Z. Kozovska, M. Matuskova. (2021). Aldehyde dehydrogenase 1A1 and 1A3 isoforms–mechanism of activation and regulation in cancer. Cellular Signalling. 87: 110120.

[84] R.S. Rao, L. Raju K, D. Augustine, S. Patil. (2020). Prognostic significance of ALDH1, Bmi1, and OCT4 expression in oral epithelial dysplasia and oral squamous cell carcinoma. Cancer Control. 27(1): 1073274820904959.

[85] C.C. Lin, M.C. Lo, R.R. Moody, N.O. Stevers, S.L. Tinsley, D. Sun. (2018). Doxycycline targets aldehyde dehydrogenase-positive breast cancer stem cells. Oncology reports. 39(6): 3041-3047.