Curcumin Enhanced the Anti-proliferative Effect of Cetixumab Through TRPV1 Channels in Human Larynx Cancer Cells

Original Article

Günizi Hüseyin¹, Günizi Özlem Ceren², Övey İshak Suat³

Department, of ¹Otolaryngology, ²Medical Pathology, ³Medical Physiology, Alaaddin Keykubat Üniversity Medical School, Antalya, Turkey

ABSTRACT

Objective: Curcumin (Cur) has been shown to modulate biological processes. It has been reported that apoptosis is induced by stimulation of transient receptor potential protein channels in numerous cancer cells. We aimed to explain the synergistic effects and underlying molecular mechanism and immunohistochemical (İHC) evaluate of combined treatment of and Cur and Cetuximab (Ctx) in squamous cell cancer of Larynx.

Patients and Methods: Human epidermoid larynx carcinoma cell line (Hep2/An3) were cultured and cells were divided into seven main groups. Cells were incubated with Ctx and Cur separately and together performed on Hep2/An3 cell cultures. The effects of Ctx and Cur were investigated on all molecular pathways of apoptosis. We evaluated the combined treatment of Ctx and Cur in laryngeal squamous cell cancer immunohistochemically.

Results: ROS levels and apoptotic levels were statistically increased in the Ctx and Ctx+curc group compared with the control ($p < 0.001$). Caspase-3 and caspase-9 levels were statistically higher in the Ctx and Curc group ($p < 0.001$). P53 and Bcl-2 showed a moderate staining rate in the control and Curc group in İHC staining. The rate of Ki 67 positive cell percentage was lower in Ctx, Cur and Ctx+Cur group.

Conclusion: TCtx significantly increases apoptosis in larynx cancer cells and Curc increased apoptosis in larynx cells and enhance the apoptotic effect of Ctx on these cells.

Key Words: Cetuximab, curcumin, larynx, molecular, squamous cell cancer.

INTRODUCTION

Head and neck tumors have a high incidence of malignant carcinomas. They are the sixth most common type of cancer around the world. Laryngeal cancer is the most common cancer in the head and neck region. About 25% of head and neck tumors are laryngeal carcinomas. They account for approximately 3% of all malign tumors seen in adults. It is detected five times more in men than in women. 85-90% of laryngeal cancers are squamous cell carcinoma[1]. Surgery, radiotherapy, and chemotherapy are the treatments planned according to the stage of the disease. Surgery (stage 1 or 2) or radiotherapy can be applied alone in early-stage tumors, or combined therapy is required in advanced stages. Neck metastasis rate is approximately 20-30% in laryngeal tumors. Although factors such as tumor histology, T stage, and local spread of the primary tumor are associated with spread to the cervical lymph nodes, the results are generally variable[1]. Cervical metastasis reduces the chance of treatment by 50%, so the condition of the cervical lymph nodes is the most important prognostic factor[2]. Chemotherapy is a treatment option in patients with extracapsular spread and neck metastasis.

Epidermal growth factor receptor (EGFR) is a membrane receptor showing tyrosine kinase activity with intracellular, extracellular, and α-helix transmembrane segments. EGFR signal increases proliferation and metastasis while decreasing apoptosis. An increase in EGFR has been observed in laryngeal tumor cells. High expression of EGFR protein in larynx cancer is associated with poor prognosis, reduced survival time, and increased metastatic potential Cetuximab is a monoclonal antibody used in larenx ca and is a targeted treatment[3]. Targeted drugs block the growth of cells in cancer cells by binding to specific areas. Cetuximab binds to cancer cells with EGFR expression with affinity 5-10 times stronger than endogenous ligands, inhibiting tumor proliferation, angiogenesis, metastasis process, and blocking tumor progression by increasing apoptosis. In combination with chemotherapeutic agents, it has been demonstrated to improve the overall survival rate of patients with head and neck squamous cell cancer and to have less toxicity[3].

EGFR inhibitors cause fewer local or systemic side effects seen with other cytotoxic drugs, they are generally considered a safe drug group and well-tolerated. It has been reported that its combined use with other chemotherapeutic agents increases its antitumor effectiveness and it has become the new standard advanced treatment[4].

Curcumin is a polyphenolic compound derived from the turmeric plant. It is a member of the Zingiberaceae (ginger) family[5]. Curcumin is lipophilic in nature, showing low solubility and stability in an aqueous solution. Curcumin, a yellow-colored polyphenol, is another polyphenol that has attracted great interest in recent years due to its biofunctional properties such as antitumor, antioxidant, and anti-inflammatory activities. It has been found to increase apoptosis in cells and prevent tumor proliferation by affecting cellular signaling pathways. The combination of curcumin with a chemotherapeutic exhibited inhibited proliferation and induced apoptotic cell death of head and neck squamous cell carcinoma cells[6].

Ca^{2+} has several essential roles in physiological and pathophysiological functions in body cells and plays an important role in activating Ca^{2+} dependent enzymes and TRP channels[7]. Increased intracellular free calcium ion (Ca^{2+}) concentration triggers apoptosis induced by reactive oxygen species (ROS). The increase of Ca^{2+} concentration in the cell is essential for killing the tumor cells. Ca^{2+} concentration is considerably high outside of body cells (1–1,5 mM) compared to the inside of cells (50–100 nM). Intracellular free Ca^{2+} ($[Ca^{2+}]i$) concentration is increased in the cytosol by activating voltage-gated calcium channels and ligand-gated ion channels. The transient receptor potential (TRP) superfamily contains six subgroups in mammals, and one subgroup of the TRP superfamily is TRP melastatin (TRPM)[7].

There is not enough research about the molecular mechanism of the suppression of cell proliferation and apoptotic induction of squamous cell Larynx carcinoma following co-incubation with cetuximab and curcumin. Therefore, we aimed to explain the synergistic effects and underlying molecular mechanism and immunohistochemical (İHC) evaluate the combined treatment of cetuximab and curcumin in squamous cell cancer of the Larynx.

PATIENTS AND METHODS:

Cell culture

The human epidermoid larynx carcinoma cell line (Hep2/An3) was maintained from the Culture Collection of Animal Cells, Foot and Mouth Disease (ŞAP) Institute, Ankara, Turkey. Hep2 cells were cultured in Eagle's Minimum Essential Medium (EMEM). Mediums were used for cell cultures containing 10% fetal bovine serum

(FBS) (Fisher Scientific, and 1% pen./strep. Antibiotic combination (Thermo-Fischer). Hep2 cells were evenly distributed as 1×10^6 cells in each 8-10 flasks (filter cap, sterile, 5 ml, 25 cm^2). Cells were incubated at 37°C at 5% CO_2 in a humidified incubator. After cells had reached 75–85% confluence, the cell was incubated with the chemical compounds described in the groups' section. Cells were examined daily for evidence of contamination. After treatments, the cells were detached with % 0.25 Trypsin–EDTA for analysis and split into the sterile falcon tubes for analysis.

Reagents/Dyes

Pluronic® F-127 was obtained from Biovision (San Francisco, USA). Dihydrorhodamine-123 (DHR 123), was obtained from Sigma Aldrich (St. Louis, MO), Fura 2 (AM) calcium florescent dye was obtained from Calbiochem (Darmstadt, Germany). APOPercentage assay with releasing buffer were obtained from Biocolor (Belfast, Northern Ireland). Caspase-3 (AC-DEVD-AMC) and Caspase-9 (AC-LEHD-AMC) substrates were obtained from Enzo (Lausen, Switzerland). A mitochondrial stain 5,50, 6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and Probenecid were obtained from Santa Cruz (Dallas, Texas, USA).

Groups

The study was planned as 7 main groups below,

Group 1 (Control): Non of the study drugs were used and Human epidermoid larynx carcinoma cells (Hep2) were preserved in a flask with the same cell culture condition.

Group 2 (CTX): Cells in the group were incubated with for 20 μg/ml cetuximab for 24 hrs[6].

Group 3 (CTX+Capz): Cells in the group were incubated with 20 μg/ml cetuximab for 24 hrs and then incubated with Capsazepin (TRPV1 Channel Blocker) (Capz, 0.1 mM, 30 min).

Group 4 (CTX+CURC): Cells in the group were incubated with 20 μg/ml cetuximab combined with 20 μM curcumin, for 24 hrs[6].

Group 5 (CTX+CURC+Capz): Cells in the group were incubated with 20 μg/ml cetuximab combined with 20 curcumin, for 24 hrs and then incubated with Capsazepin (TRPV1 Channel Blocker) (Capz, 0.1 mM, 30 min).

Group 6 (CURC): Cells in the group were incubated with 20 μM curcumin, for 24 hrs[6].

Group 7 (CURC+Capz): Cells in the group were incubated with 20 μM curcumin, for 24 hrs and then

incubated with Capsazepin (TRPV1 Channel Blocker) (Capz, 0.1 mM, 30 min).

In Capz (s) incubated groups, Hep2 cells were also blocked by TRPV1 blocker Capz (0.1 mM, 30 min) before related analysis in the existence of 1.2 mM calcium in extracellular environment. For all experiments (except for calcium signaling), the cells were further treated with Capsaisin (Cpsn, 0.1 mM, 10 min) for activation of TRPV1 channel before related analysis. During calcium signaling analysis (Fura-2/AM), cells were stimulated on 20th cycles with 0.1 mM Cpsn in the existence of 1.2 mM calcium and calcium free buffer in extracellular environment.

Measurements of Intracellular Calcium Concentration

Fluorescent Fura-2 AM (acetoxymethyl ester) dye was used for measuring intracellular calcium level in Hep2 cells. The cells were incubated with HEPES-buffered saline [HBS; 5 mM KCl, 145 mM NaCl, 10 mM D-glucose, 1 mM MgCl2, 1,2 mM $CaCl_2$, 10 mM HEPES and 0.1% (w/v) bovine serum albumin (BSA); pH 7.4] containing 5 μM Fura-2 AM and 0.05% (w/v) Pluronic F-127 for 60 min at 37˚C in the dark. The loaded cells were washed twice with HBS and covered with 1ml of HBS supplemented with 2.5 mM probenecid for at least 20 min at 37˚C in the dark to allow for Fura-2 AM de-esterification. Fluorescence intensity at 510 nm (emission) was determined in individual wells using a plate reader equipped with an automated injection system (Synergy™ H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3s for 50 acquisition cycles. During the measurement of intracellular calcium signaling, TRPV1 channels were stimulated by automatic injector with Capsaisin (Cpsn, 0.1 mM) on 20th cycle. Measurement of $[Ca^{2+}]i$ including staining process modification was performed to according to method of Martinez *et al*.[8].

Apoptosis assay

The APOPercentage™ cell apoptosis assay was used for the detection and quantification of apoptosis. The APOPercentage dye is actively bound to phosphatidyl-serine lipids and transferred into the cells and apoptotic cells are stained red. The apoptosis analyzes procedure was performed according to the manufacturer instruction of Öz and Celik 2017[9]. The Hep2 cells were analysed for apoptotic cells detection by spectrophotometry (multiplate reader) at 550 nm (Synergy™ H1, Biotek, USA).

Intracellular ROS production measurement.

Dihydrorhodamine 123 (DHR 123) is a non-charged and non-fluorescent dye which easily go through the cell membrane. Inside the Hep2 cells, DHR123 is oxidized to cationic rhodamine 123 (Rh 123) which is localized in the mitochondria and demonstrates green fluorescence. The cells (10^6 cells/ml for per group) were incubated with 20 μm DHR 123 as florescent oxidant dye at 37 °C for 25 min[9].

Synergy™ H1 automatic microplate reader device was used for determining Rh 123 fluorescent intensities. The analyzes were performed at 488 nm (excitation) and 543 nm (emission) wavelengths. We presented the data as fold increase over the level before treatment.

Caspase 3 - 9 activity assays and Mitochondrial membrane potential (JC-1) analyses.

Caspase 9 and Caspase 3 activity evaluation methods were based on previously reported[10]. Caspase 9 (AC-LEHD-AMC) and Caspase 3 (ACDEVD-AMC) substrates cleavages were calculated with Synergy™ H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths (excitation/emission). The values were evaluated as fluorescent units/mg protein and shown as fold change from the level before treatment (experimental/control).

JC1 (1 μM) which is a mitochondrial membrane potential fluorescence dye intensity was evaluated by 485 nm (green) excitation wavelength and the emission wavelength of 535 nm, the red signal at the 540 nm (excitation) and 590 nm (emission) the wavelengths (Synergy™ H1, Biotek, USA)[9]. Data are presented as emission ratios (590/535). Mitochondrial membrane potential changes were quantified as the integral of the decrease in JC1 fluorescence ratio of experimental/control.

Immunohistochemistry

Immunostaining was performed using the streptavidin-biotin amplification method using a Histofine Kit (Nichirei Co. Ltd., Tokyo, Japan) for Bax and Ki-67 and EnVision_ (DAKO Corp., Carpinteria, CA). Endogenous peroxidase in deparaffinized sections was blocked with 3% hydrogen peroxide for 10 min. The sections were then washed with phosphate-buffered saline (pH 7.4) for 5 min, placed in 20% zinc sulfate aqueous solution, and heated at 90°C by a microwave oven for 15 minutes. A mouse monoclonal antibody against human p53 protein, clone DO-7 (Dako, Glostrup, Denmark), was applied at a dilution of 1:100 according to the manufacture's instructions. The conventional avidin-biotin complex method was carried out for the following reaction. The DAB was used for coloration. To confirm that immunohistochemical(İHC) staining findings with anti-mutant type p53 are consistent with those with anti-p53, we made serial sections and performed in situ hybridization and immunohistochemical study, using specimens obtained from several patients. As we expected, positive cells obtained by the two different studies were the same. (These data are not shown.)

The same method examined the immunohistochemistry of bcl-2, bax, and ki-67. The Mouse monoclonal antibodies against human bcl-2 protein, clone 124 (Dako), anti-bax protein, clone (N-20)-G (Santa Cruz Biotechnology, Inc.), and anti-ki-67 protein, clone MIB-1 (Dako) were applied at a dilution of 1:40, 1:100, and the prepared solution respectively according to the manufacture's instructions. More than 5000 tumor cells were counted to calculate percentages of p53 and bcl-2 positive cells in randomly selected high-power fields. Ki-67 labeling index (KI) values were determined by counting more than 5000 tumor cells in randomly selected high-power fields, and KI was calculated as a percentage[11].

Immunohistochemistry sections were evaluated for nuclear and cytoplasmic staining presence or absence. Since most cells were diffusely positive for bax (Fig. 2E) in all cases, expression levels of bax were analyzed based on the intensity of immunoreactivity[11,12]. Specimens were classified stained with strong intensity, moderate intensity (similar to normal laryngeal squamous epithelium), and weak intensity.
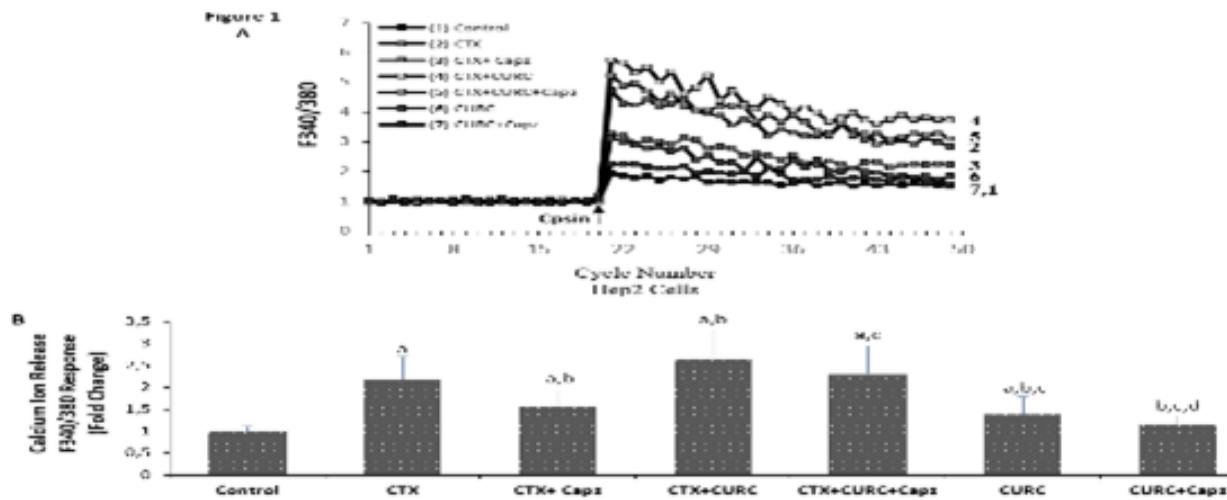

Fig. 1: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on cytosolic calcium levels. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=3). *ap<0.001* vs control, *bp<0.001* vs CTX, *cp<0.001* vs CTX+CURC and *dp<0.05* vs CURC.
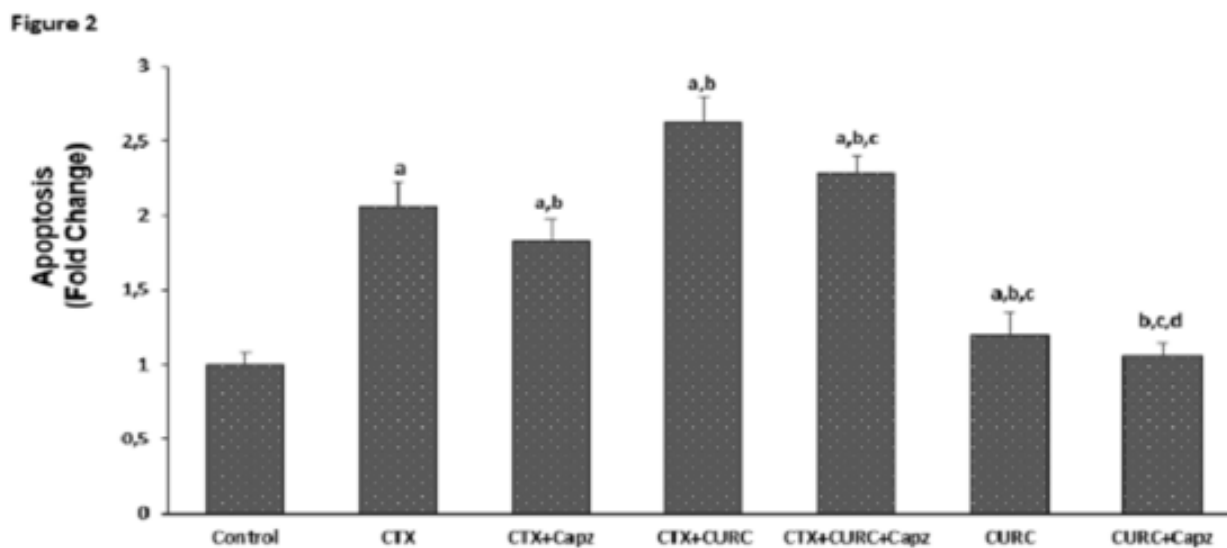

Fig. 2: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on apoptosis levels in the Hep2 Cells. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=10). *ap<0.001* vs control, *bp<0.001* vs CTX, *Cp<0.001* vs CTX+CURC and *dp<0.05* vs CURC.
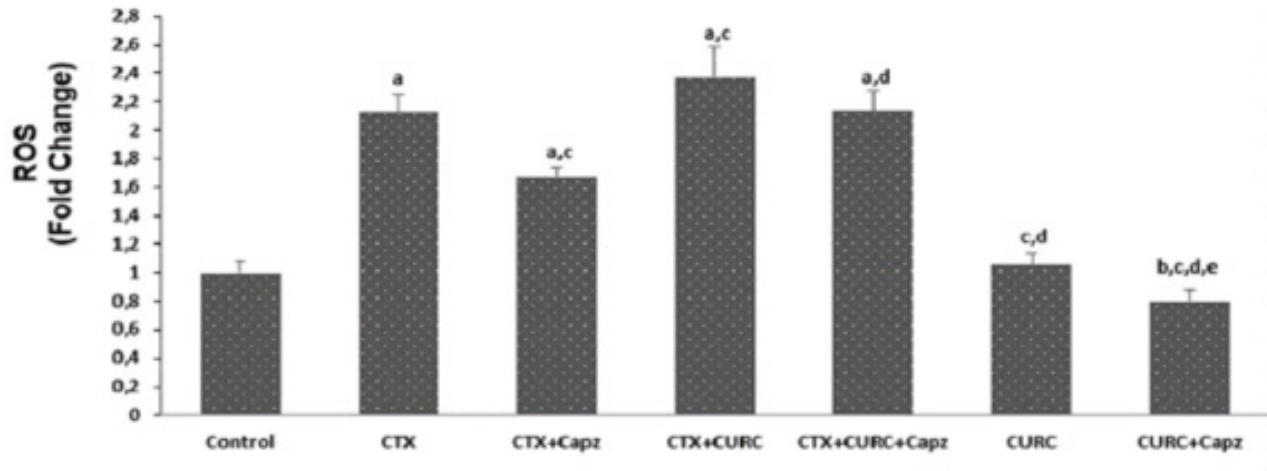
Figure 3

Fig. 3: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on ROS levels in the Hep2 Cells. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=10). *ap<0.001* vs *bp<0.05* control, *cp<0.001* vs CTX, *dp<0.001* vs CTX+CURC and *ep<0.001* vs CURC.
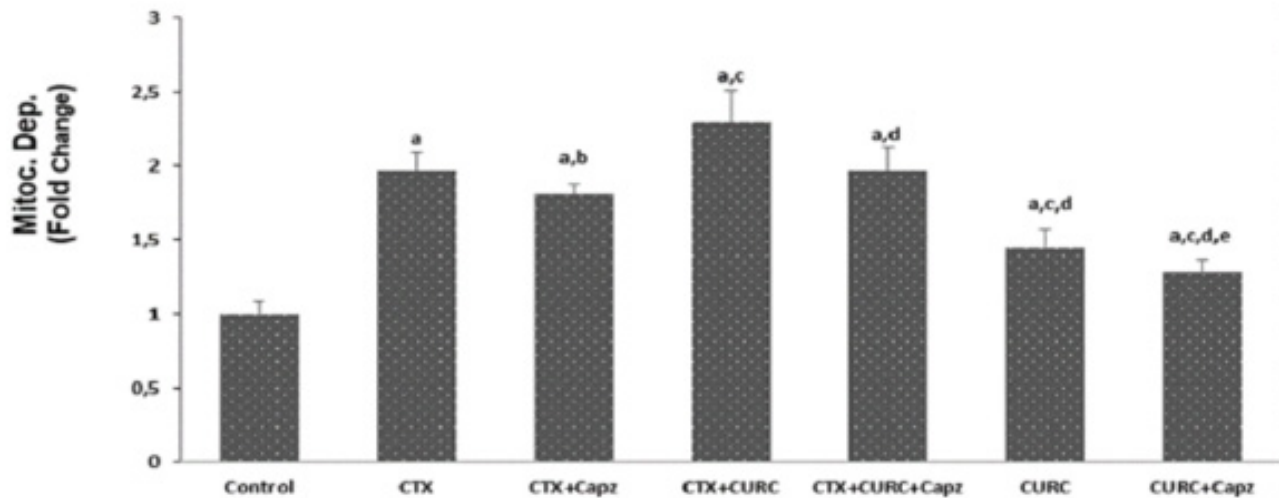
Figure 4

Fig. 4: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on mitocondrial depolorisation levels in the Hep2 Cells. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=10). *ap<0.001* vs control, *bp<0.05* and *cp<0.001* vs CTX, *dp<0.001* vs CTX+CURC and *ep<0.05* vs CURC.
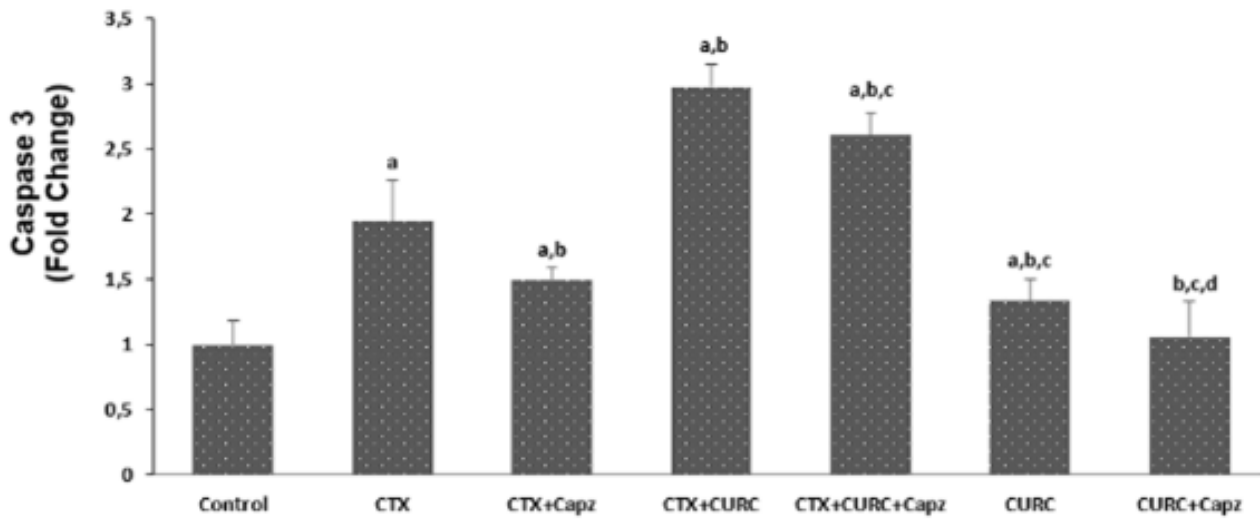
Figure 5

Fig. 5: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on caspase-3 levels in the Hep2 Cells. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=10). *ap<0.001* vs control, *bp<0.001* vs CTX, *cp<0.001* vs CTX+CURC and *dp<0.05* vs CURC.
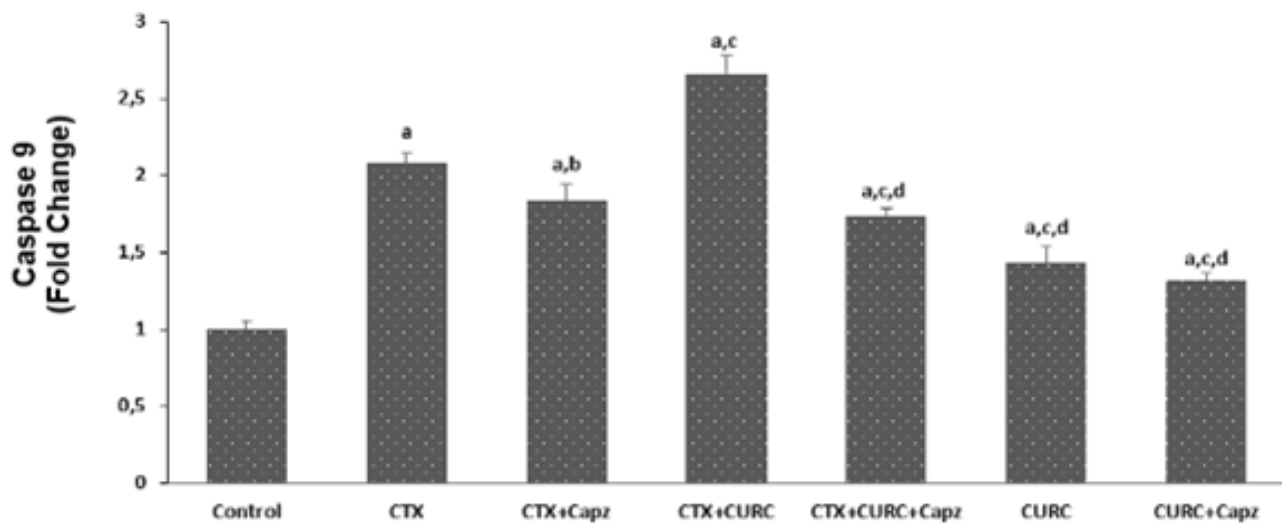
Figure 6

Fig. 6: The effect of Cetuximab (CTX, 20 µg/ml, 24 hrs) and CURC (20 µM, 24 hrs) on caspase-9 levels in the Hep2 Cells. Cells are stimulated by Capsaisin (Cpsin, 0.1 mM for 10 min) but they were inhibited by Capsazepine (Capz 0.1 mM, 30 min) (mean ± SD and n=10). *ap<0.001* vs control, *bp<0.05* and *cp<0.001* vs CTX, *dp<0.001* vs CTX+CURC and *ep<0.05* vs CURC.

Statistical analyses

All results were expressed as means±standard deviation (SD). Significant values in the groups were assessed with one-way ANOVA. Statistical analyses were calculated using GraphPadPrism version 7.04 for Windows (GraphPad Software, San Diego California, the USA). *P <0.05* was considered significant. IHC staining results in groups were evaluated with the Kruskall-Wallis and Bonferroni post-hoc test using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). *P <0.001* was considered significant.

RESULTS:

Effects of CTX and CURC on F340/380 calcium concentration

The calcium signaling results shown as line plots and columns are presented in Fig. 1A and B. TRPV1 antagonist Capz and agonist Cpsin were used for the evaluation of the activities of CURC and CTX in Hep2 cell culture. When intracellular calcium ion levels were examined, it was observed that they increased significantly in the Cetuximab group compared with the control ($p < 0.001$). Intracellular Ca^{2+} levels decreased in the cetuximab+Capz group using Capz, a TRPV1 channel inhibitor, compared with the CTX group ($p < 0.001$). Also, when the CTX+CURC group was compared with the CTX group, intracellular calcium levels were determined to be statistically higher in the CTX+CURC group than the CTX group ($p < 0.001$). When the CURC group and control group were compared, the calcium level was determined to be statistically higher in the CURC group ($p <0.001$). It was also determined that the calcium level was lower in the CURC+Capz group compared with the CURC group ($p < 0.05$).

Effects of CTX and CURC on reactive oxygen species and apoptosis levels

The ROS levels and apoptotic activities of TRPV1 channel mediators CURC and CTX in Hep2 cell culture are presented in Figs. 2 and 3, respectively. When the ROS and apoptotic levels were examined, they were determined to statistically increase in the cetuximab group compared with the control ($p < 0.001$). The ROS and apoptosis levels were determined to significantly decrease in the t cetuximab+Capz group with TRPV1 channel inhibitor Capz compared with the CTX group ($p < 0.001$). In addition, when the CTX+CURC group and the CTX group were compared, the apoptotic levels were determined to be statistically higher in the CTX+CURC group compared with the CTX group ($p < 0.001$). When the CURC group and the control group were compared and the ROS and apoptosis levels were examined, they

were determined to be statistically higher in the CURC group ($p < 0.001$). The ROS and apoptosis levels were determined to decrease in the CURC+Capz group compared with the CURC group ($p < 0.001$).

Effect of CTX and CURC on mitochondrial membrane potential levels

The mitochondrial membrane potential levels are presented in Fig. 4. When the mitochondrial membrane potential levels were examined, they were determined to statistically increase in the CTX and CURC group compared with the control group ($p < 0.001$). When the CTX+CURC group and the CTX group were compared, the mitochondrial membrane potential was determined to be statistically higher in the CTX+CURC group ($p < 0.001$). When the CURC group and the control group were compared, the mitochondrial membrane potential was determined to be statistically higher in the CURC group ($p < 0.001$). When the CTX, CTX+CURC, and CURC groups and the CTX+Capz, CTX+CURC+Capz, and CURC+Capz groups were compared, the mitochondrial membrane potential levels of inhibitor groups were found to be statistically lower ($p < 0.001$ and $p < 0.05$).

Effect of CTX and CURC on caspase-9 and caspase-3 activities

Caspase-9 and caspase-3 levels are presented in Fig. 5a and Fig. 6. It was determined that caspase-3 and caspase-9 levels increased statistically in the CTX and CURC group compared with the control ($p < 0.001$). When the CTX group and the CTX+CURC group were compared, they were observed to statistically increase in the CTX+CURC group ($p < 0.001$). When the CURC group and the control group were compared, caspase-3 and caspase-9 levels were determined to be statistically higher in the CURC group ($p < 0.001$). When the CTX and CTX+Capz, CTX+CURC and CTX+CURC+Capz, and CURC and CURC+Capz groups were compared, caspase-3 and caspase-9 levels were determined to be statistically lower in the groups in which an inhibitor was used ($p < 0.001$ and $p < 0.05$).

Results of immunohistochemistry evaluation

P53 showed a moderate (40%) staining rate in the control and Curcumin (%35) group in immunohistochemical staining. Weaker staining rate was observed in the cetux (%30) and Cetux + curcumin (%20) group (Figure 7). İt was determined that apoptosis increased in cells with the synergistic effect of cetux+curcumin, and the İHC staining of wild-p53 in these cells was decreased, and this finding was statistically significant ($p<0.001$). Bcl-2 showed a moderate staining rate in the control (80%) and Curcumin (%70) group in immunohistochemical

staining. Weaker staining rate was observed in the cetux (%50) and Cetux + curcumin(%20) group (Figure 8). As the staining rates in bcl-2 decreased in the groups, an increase in the apoptosis index was detected, which was statistically significant. (*p<0.001*). The rate of Ki 67 positive cell percentage in the control, curcumin, cetuximab, cetuximab+curcumin group were %90, %70, %80 and %40 (Figure 9). Cetuximab+curcumin group's rate was lower than the control group (Table 1).
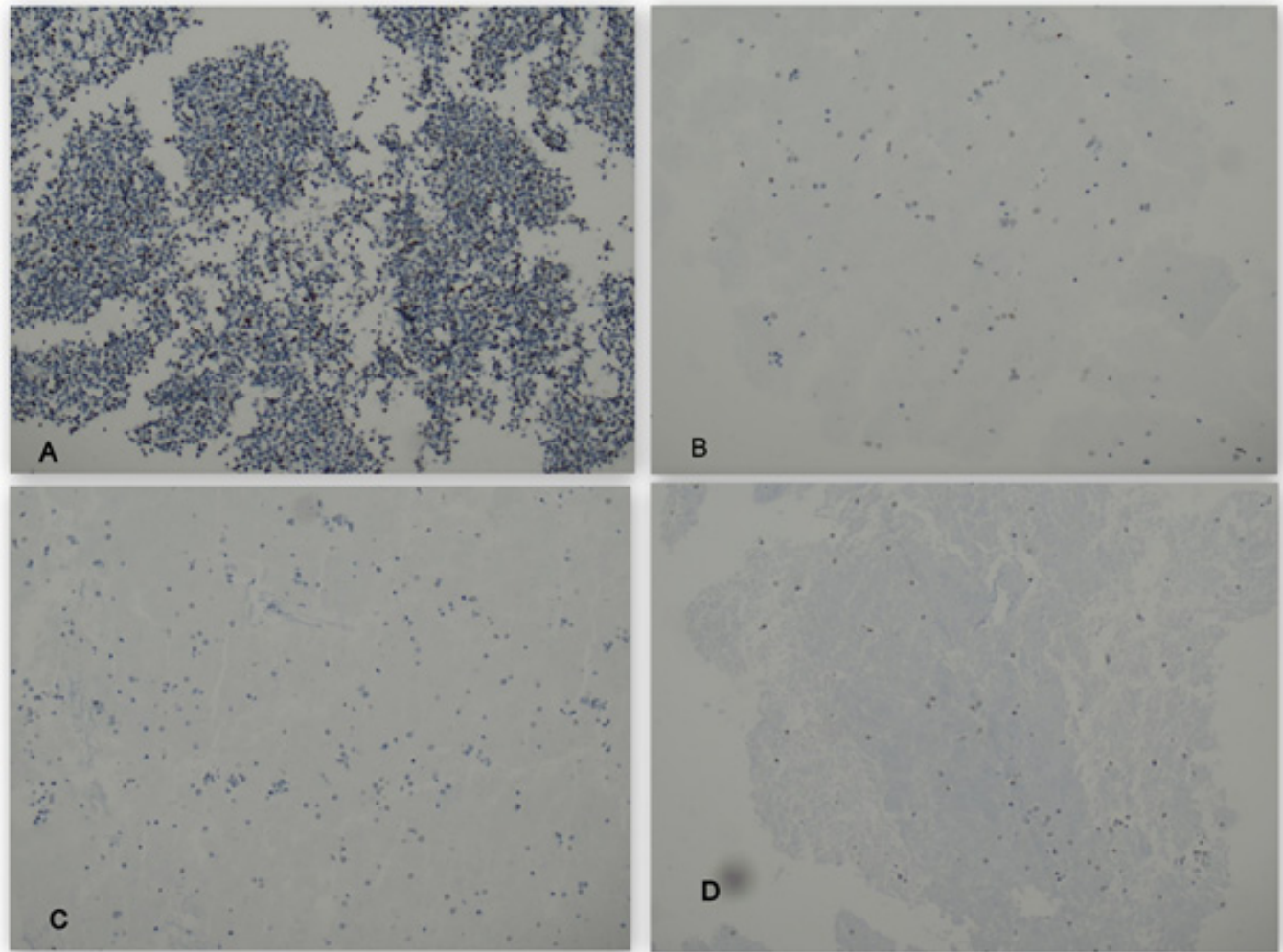

Fig. 7: İmmunohistochemical staining of all groups with P 53. (A: Control Group: %40   B: Cetuximab Group: %30
C: Curcumin Group: %35   D: Cetuximab+Curcumin Group: %20)
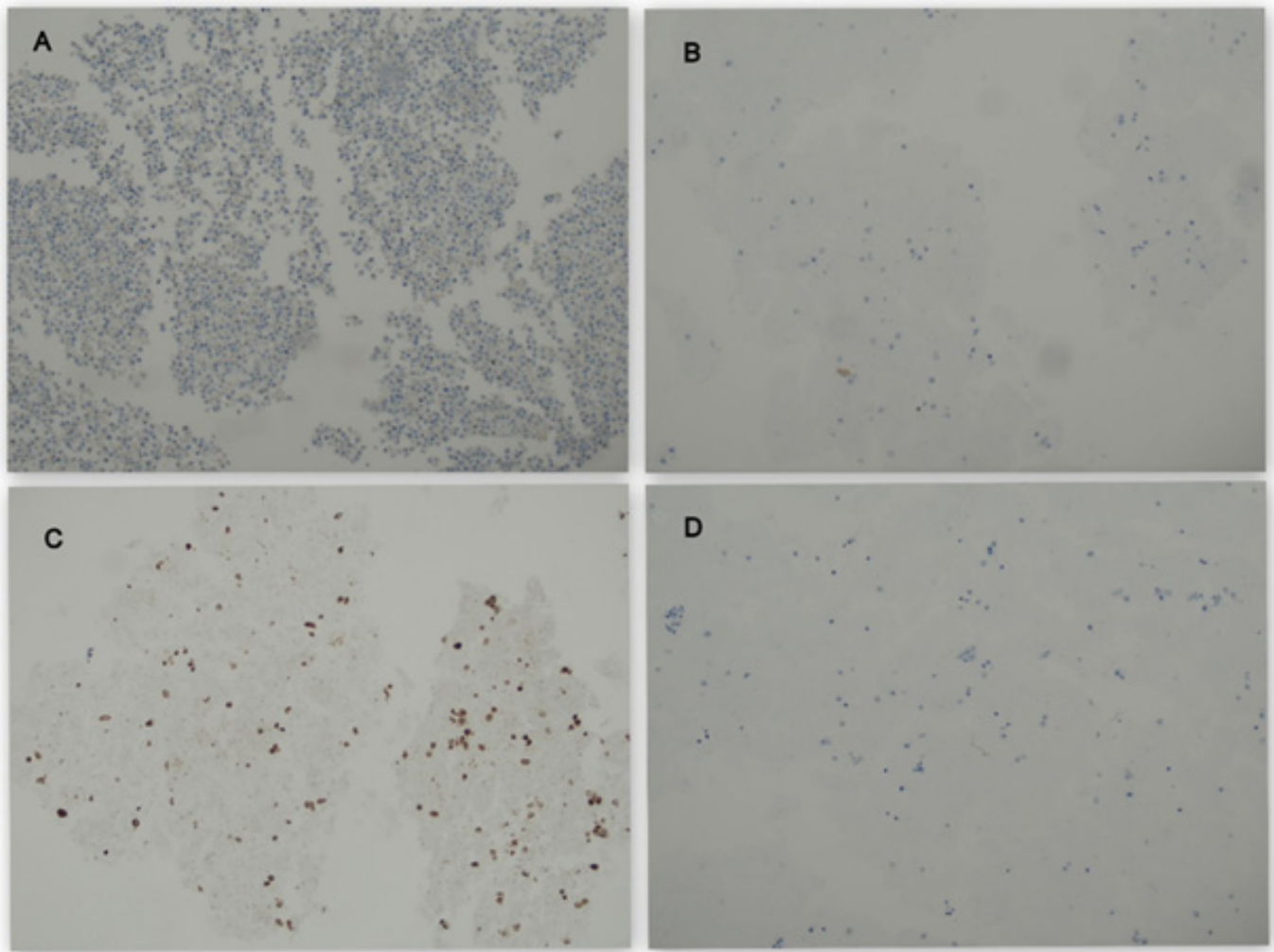
Fig. 8: İmmunohistochemical staining of all groups with Bcl-2 (A: Control Group: %80   B: Cetuximab Group: %50 C: Curcumin Group: %70   D: Cetuximab+Curcumin Group: %20)
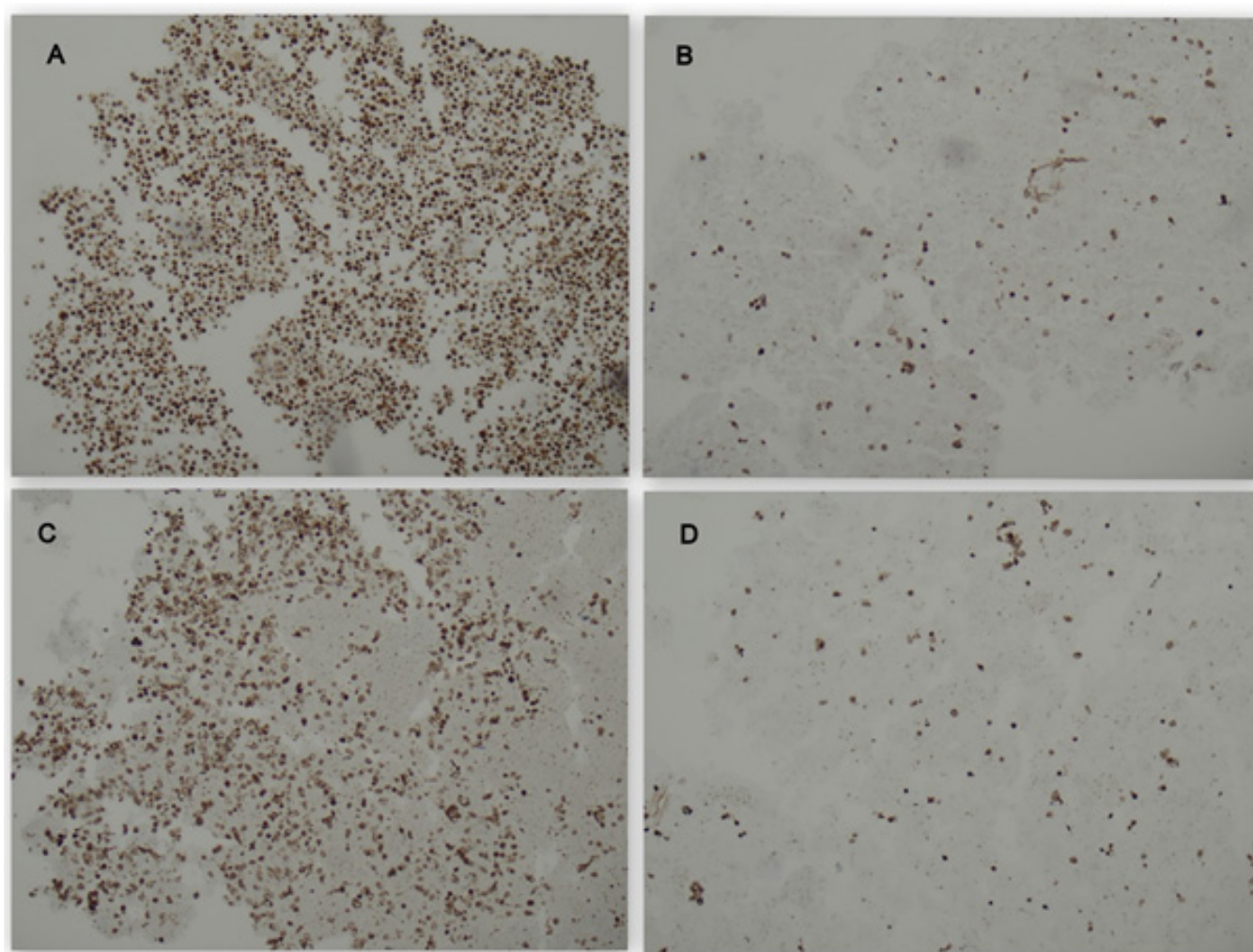
Fig. 9: İmmunohistochemical staining of all groups with Ki-67 2 (A: Control Group: %90   B: Cetuximab Group: %70
C: Curcumin Group: %80   D: Cetuximab+Curcumin Group: %40)

Table 1: İmmunohistochemical staining intensity of p53 and immunohistochemical staining ratio of p53, Bcl-2 and Ki-67

Group	p53	Bcl-2	Ki-67
I (control)	%40 (moderate)	%80	%90
II (curcumin)	%35 (moderate)	%70	%70
III (Cetuximab)	%30 (weak)	%50	%80
IV (Cetuximab+ Curcumin)	%20 (weak)	%20	%40

DISCUSSION

Chemotherapy and radiotherapy are preferred treatment for patients who do not undergo surgery or are too sick for surgery, and chemotherapeutic agents represent an important impact, even though they also have several adverse effects in normal cells. The targeted drug cetuximab is often used for people who can not tolerate chemoradiation. İt may be combined with radiation therapy for early-stage cancers. For more advanced cancers, such as those that have spread or come back after treatment, Cetuximab may be combined with chemo drugs like cisplatin and 5FU, or it may be used by itself. Cetuximab is a monoclonal anticor used to treat many cancer types, including squamous cell Larynx ca. It is a targeted drug. Targeted drugs block cell growth in cancer cells by binding to specific areas. Cetuximab neutralizes the receptor by binding to EGFR-expressed cancer cells with affinity 5-10 times stronger than endogenous ligands. It blocks tumor progression by inhibiting tumor proliferation, angiogenesis, apoptosis, and metastasis process. Cetuximab suppresses phosphorylated EGFR formation and increases ROS and apoptosis in laryngeal carcinoma cells[4], and it is known that its combined use with other chemotherapeutic agents increases the antitumor efficacy[13]. Since there is no research in the literature regarding the effect of Cetuximabon TRPs channels, the general effects of cetuximab are mentioned.

Many recent studies have shown abnormal TRP channel expression in various cancer types[14]. In several studies, the effects of subtypes of TRP channels on many different cancer cells and the relationship between TRP channel expression and surveillance in these cancers have been clearly demonstrated. It is known that intracellular Ca^{2+} levels significantly impact cancer cells and TRP channels[14], and they have important roles in intracellular oxidative stress and apoptosis[15]. Various chemotherapy protocols are widely used during preoperative and postoperative periods in cancer treatments[14], and these treatment protocols indirectly increase the intracellular calcium ion level through TRP channels[15]. Following the increase of intracellular Ca^{2+}, the increase in the amount of intracellular ROS triggers the molecular pathway of apoptosis, resulting in irreversible changes in components such as intracellular lipid, protein, and nucleic acid due to the oxidative stress induced by activation of caspase three and caspase nine associated with an increase in mitochondrial depolarization, leading to apoptosis[16]. Our study investigated the role of TRPV1 channels on chemotherapeutic prophylaxis and larynx cell death in cancer chemotherapy. We also examined the role of Cetuximab, alone and with Curcumin in apoptosis phases. As a result of the

investigations, it was observed that the intracellular calcium levels in TRPV1 channel activation and the related mitochondrial depolarization and intracellular ROS levels were significantly increased, and the apoptosis levels in the oxidative stress-related cancer cells were significantly increased in Cetuximab and Cetuximab+curcumin groups compared to the control group. As a result of TRPV1 channel inhibition, these effects were significantly reduced in the same related groups.

In cancer cells, oxidative stress is triggered by the increase in the amount of ROS, resulting in irreversible changes in components[4,13]. ROS regulates critical cellular events, such as proliferation, gene expression, and protein phosphorylation. Apoptosis is one of the most important functions affected in this process. It has been shown that inhibition of the caspase-dependent pathway of apoptosis has an important role in chemotherapeutic effect[17]. Caspase-3, a critical driver of apoptosis, is responsible for the breakdown of skeletal proteins that cause the typical morphological changes observed in cells undergoing apoptosis. Chemotherapeutic agents show their cytotoxic effects by stimulating apoptosis. A study reported that the activity of caspase-3, one of the key enzymes of the apoptotic pathway, increased three-fold in liver cancer cells at the end of cetuximab application compared to control[18]. Caspase-3 activation and cellular lysis were increased by cetuximab in the head and neck squamous cell carcinoma (HNSCC) cell line (HSC-3 and HSC-4). Chen C *et al*.[6] reported that a combined treatment of cetuximab and curcumin synergistically inhibited cell viability, induced cell death, and stimulated caspase 3 – and 9. Our findings in curcumin group and the combination of cetuximab and curcumin increased caspase 3, caspase 9, mitochondrial depolarization, intracellular reactive oxygen species level, and apoptosis compared to the control group. Our results are also consistent with previous studies showing that curcumin is effective against various types of cancer via intrinsic apoptotic function.[6,9,18]

The beginning of cancer is a process involving genetic mutations, like activation of oncogenes and inactivation of tumor suppressors. p53, more exactly a suppressor gene, is the transcription factor that increases the synthesis of many different gene products that trigger apoptosis. Disruption of the pathways regulated by p53 leads to increased cell proliferation and additional genetic damage[19,20]. Since its half-life is very short, immunological methods cannot detect it. However, it becomes detectable in immunohistochemistry studies when it undergoes a genetic change. Overexpression of p53 is related to poor prognosis, and It is an indicator of disease-free survival and overall survival for patients with

squamous cell carcinomas[19,20]. The studies describe a relation between the P-53 overexpression and the prognosis for the laryngeal location, a relation that is stronger than in any other location of cancer[19]. The p53 gene product can block the cell cycle in G1 after encountering genotoxic stress. İt has the ability to integrate different signals and induce arrest cell cycle. Cetuximab inhibited the proliferation of wild-type p53 in the HCC cells (Hep G2) by up to 57%[20]. The essential function of P53 as a mediator of cell growth and apoptosis control is already reported in the literature[21] with p53 gene transfer in head and neck cell lines. Otherwise, In some other studies[22] has been reported that p53 expression was not a significant prognostic predictor in laryngeal SCC. İn our study, ıt was determined that apoptosis increased in cells with the synergistic effect of cetux+curcumin, and the İHC staining of wild-p53 in these cells was decreased finding was statistically significant. (*p<0.001*)

The pro-apoptotic/anti-apoptotic factor ratio determines the path to apoptosis. Wang *et al.* reported that Bcl-2 protein was overexpressed in drug-resistant human esophageal squamous carcinoma (ESCC) cells[23]. Ciardiello *et al.*[24] observed that there was no change in Bcl-2 expression after cetuximab in mouse colon adenocarcinoma cell tumor cells in their study. It has been found that the combined application of Cetuximab in liver cancer cells compared to its alone application is more effective in decreasing Bcl-2 expression[18]. İn our study weaker staining rate was observed in the cetux (%50) and Cetux + curcumin (%20) groups. As the staining rates in bcl-2 decreased in the groups, an increase in the apoptosis index was detected, which was statistically significant.(*p<0.001*)

Ki-67 is a nonhistone protein, and its expressions in all phases of the cell cycle except the resting stage (G0), so it has been used as a proliferation marker in cancer cells[12]. The prognostic value of Ki-67 has been demonstrated in the squamous cell carcinoma of the larynx. Rodriguez Tojo *et al.*[11] Reported that all cases that showed a low proliferation index were related to the best prognosis. And the overexpression of Ki-67 could be a prognostic factor in Larynx cancer. The Ki 67 positive cell percentage rate in the control, curcumin, cetuximab, cetuximab+curcumin group were %90, %70, %80, and %40. The findings support the hypothesis that cet+curc reduces carcinogenesis by significantly reducing mitosis with a synergistic effect.

CONCLUSION

In conclusion, the apoptotic effects of Cetuximab on tumor cells are indirectly associated with TRPV1 channels. It has been found that TRPV1 channels play an essential role in the intrinsic molecular pathway of apoptosis by increasing intracellular Ca^{2+} level and enhancing mitochondrial depolarization, Caspase 3 & 9. Cetuximab significantly increases apoptosis in larynx cancer cells, and Curcumin increases apoptosis in larynx cells and enhances the apoptotic effect of Cetuximab on these cells. Immunohistochemically, it was determined that curcumin increased apoptosis synergistically with cetuximab.

CONFLICT OF INTEREST

There are no conflicts of interest.

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