Antiproliferative effects of cetuximab on triple negative breast cancer cell line MDA-MB-231

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Abstract
In this study, antiproliferative effects of the anti-EGFR monoclonal antibody Cetuximab were evaluated using MDA-MB-231 cell line originated from triple negative breast cancer. As cell kinetic parameters, we evaluated Cell index, mitotic index, labeling index and apoptotic index. For this purpose, 20 μM, 45 μM and 60 μM Cetuximab concentrations were applied to the cells using the real-time cell analysis system (xCelligence DP) and IC50 values were determined. IC50 concentrations were used for all other parameters. According to experimental results, Cetuximab administration inhibited cell kinetics of MDA-MB-231 cells. xCelligence DP instrument detected IC50 concentrations of Cetuximab for cell line. These values were 45 μM for MDA-MB-231 cells. When these IC50 value applied to cells, significant decrease was detected in mitotic index, labelling index and significant increase was detected in apoptotic index for experimental groups. Student’s t tests for paired samples were used to assign statistical significance. p<0.05 level of significance was accepted. According to the results obtained, Cetuximab has the potential to slow down the prognosis of the triple negative breast cancer subtype.

1. Introduction
Although cancer seems to be a single disease, it can be described as a disease group formed by the combination of many diseases. Breast cancer, which is the second species of this disease group that causes death in women, has an important place among cancer diseases. Triple-negative breast cancer (TNBC) known as an aggressive cancer type is characterized by its appearance at younger ages, higher average tumor size, high-grade tumors, and sometimes higher rate of node positivity [1]. In addition, this group is known for the occurrence of metastases in the internal organs such as lung and brain, although early peak of relapse and less in bone between the first and third years after diagnosis [2].

One of the member of the class I superfamily of receptor tyrosine kinases (RTKs) is the epidermal growth factor receptor (EGFR). These RTKs include different signaling proteins. All members consist of three regions: 1- Ligand binding site, 2- single membrane-spanning site, 3- site containing a cytoplasmic intracellular tyrosine-kinase. Receptors of this superfamily are expressed in various tissues such as neuronal, epithelial and mesenchymal tissues under normal conditions [3].

EGFR is a tyrosine kinase receptor and is overexpressed in different types of cancer. For instance, breast, ovary, colorectal and head and neck cancer. It plays an important role in the work of mechanisms necessary for tumor formation and progression [4]. EGFR is associated with advanced disease, poor prognosis, and resistance to therapy and therefore EGFR is an ideal candidate for various types of cancer [5].

A monoclonal antibody Cetuximab binds to the extracellular domain of EGFR. In many human cancers, including head and neck and colorectal cancers, EGFR is overexpressed. Binding of EGFR with its endogenous ligand is prevented by this process and this blocks the receptor-dependent transduction pathway, and provides many antitumor agents [6, 7].

In this study, it was aimed to evaluate the effects of the anti-EGFR monoclonal antibody Cetuximab on MDA-MB-231 cell line originated from triple negative breast cancer at the cellular level by using different cell kinetics parameters.
2. Materials and Methods

2.1. Cell culture

The MDA-MB-231 cells used in the experiments were purchased by our research laboratory from American Type Culture Collection (ATTC Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100-unit ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Cells were maintained at 37 ºC in a atmosphere containing 95% humidity and 5% CO₂.

2.2. Preparation of drug concentration

Application doses of 20 µM, 45 µM and 60 µM concentrations were prepared by diluting a total of 1 mM Cetuximab (Sigma) stock solution.

2.3. Cell index (CI)

For cell index analysis, xCELLigence DP system was used. In experimental process for the impedance background measurement 100 µL of appropriate medium was added to each well. Then 5000 cell/well were seeded for MDA-MB-231 cell line. The final volume was 200 µL. 16 well E-Plates were incubated at 37°C with 95% humidity and 5% CO2 and monitored on the RTCA system at 15-minute time intervals for up to 24 hours without treatment and following 72 hours with treatment. The DP unit is wired to an external laptop runing the xCELLigence software (version 1.2.1).

2.4. Mitotic index (MI)

For evaluation of MI, cells were planted in 24-well plates. Each well contained 3x10⁴ cells for both cell lines. Cells were incubated 24 hrs after cell seeding. Cells were treated with optimum Cetuximab concentration 0-72 hrs. At the end of these experimental period, for fixatation, Carnoy fixative was used and the cells were made clear with the Feulgen method and stained with Giemsa. For analysing MI, approximately 3000 cells were counted.

2.5. Apoptotic index (AI)

DAPI (6-diamidino-2-phenylindole) was used to determine the apoptotic nucleus. It is a blue fluorescent dye. Cells were cultured in 6-well culture dishes and fixed with methanol: FTS mixture after the experimental process until staining was performed.

3. Results

3.1. Cell index

Cell index values obtained by applying Cetuximab to MDA-MB-231 cells at 20 µM, 45 µM and 60 µM concentrations were compared with the standard curves; while no significant antiproliferative effect can be seen at a concentration of 20 µM, it is believed that DNA damage occurs at concentrations of 45 µM and 60 µM (Figure 1). In addition, after 24 h Cetuximab applying, the IC₅₀ value of MDA-MB-231 cells was determined as 45 µM with the xCelligence DP device's own system.

3.2. Mitotic Index

In order to assess the change in mitotic index values by applying Cetuximab on MDA-MB-231 cells; cultured cells were treated at a concentration of 45 µM for 24 h, 48 h and 72 hours. The results showed that this concentration decreased the mitotic index from 4.24 % to 2.76 % at 24 h; from 5.56 % to 1.98 % at 48 h and 6.18 % to 0.93 % at 72 h (Figure 2).
3.3. Labelling index

Labelling index values belonging to MDA-MB-231 cell line after administration of 45 μM concentration Cetuximab was shown in Figure 3. Labelling index values decreased from 7.81 to 3.57 at 24 h; from 8.18 to 2.26 at 48 h and from 8.32 to 1.19 at 72 h for MDA-MB-231 cell line. Labelling index values of MDA-MB-231 cells decreased significantly with time as a result of Cetuximab administration. This decrease was statistically significant (p<0.05).

3.4. Apoptotic Index

Administration of 45 μM concentration of Cetuximab to MDA-MB-231 cells caused apoptotic cell death. Apoptotic index values increased from 7.23 to 15.26 at 24 h; from 10.62 to 21.22 at 48 h and from 11.22 to 29.43 at 72 h for MDA-MB-231 (Figure 4). Apoptotic index values of MDA-MB-231 cells increased significantly with time as a result of Cetuximab administration. This decrease was statistically significant (p<0.05).

4. Discussion

Because of the toxic side effects of traditional drugs used in cancer treatment and the emergence of resistant cells after treatment, cancer treatment is not fully successful and the disease-free survival is reduced [8]. Many studies have shown that triple negative breast cancer has a worse prognosis than hormone receptor positive breast cancers [9-14]. Current treatment options are limited especially for TNBC among the different types of breast cancers. Therefore discovery and development of novel molecules is very important in terms of treatment options. Among breast cancers, TNBC subtype metastatic progression is characterized by poor prognosis and also. Metastatic progression is one of the poor prognosis indicators of TNBC. In addition, the absence of biomolecules that can be targets for targeted therapies is the main feature of TNBC [15]. Therefore, there are currently no FDA-approved targeted therapies for breast cancer subtypes. At first triple negative breast cancer is very sensitive to chemotherapy, but a lot of triple negative breast cancer patients develop resistance to chemotherapy rapidly, at which point metastatic disease can be quite fatal [16].

In many human malignancies, EGFR is abnormally activated by many mechanisms, such as receptor overexpression, overexpression of receptor ligands, gene amplification [17]. 72% of patients diagnosed with TNBC have overexpression of EGFR [18-22]. Studies have shown that EGFR mRNA levels are higher in TNBC than luminal A type breast cancer, which is positive for the hormone receptor [23, 24]. Cetuximab activates cellular immunity and antitumor mechanisms while inhibiting the downstream signal of EGFR [25]. In our study the effects of EGFR inhibitor Cetuximab were evaluated MDA-MB-231 cell line which expressed EGFR more. Experimental findings have led to the several clinical studies’ initiation to evaluate the effect of EGFR.
inhibition (EGFRi) on triple negative breast cancer with metastatic potential. In the Phase II study, patients who had advanced breast cancer and who were not previously selected for chemotherapy evaluated EGFR kinase inhibitors [26]. Clinical studies also required the evaluation of the combination of DNA alkylating agent carboplatin with ligand blocking monoclonal antibody Cetuximab in addition to kinase inhibitors [27].

A study by Wang et al using MTT showed that Cetuximab is effective in many cell lines and increases the chemotherapeutic efficacy of other drugs on the cells [28]. In the study with MDA-MB-231 and SUM159 cell lines to evaluate breast cancer stem cells, fluorescence-activated cell sequencer analysis (Aldefluor + or CD44 + and CD24- / low) and mammosphere formation efficacy were measured. In this study, in vitro conditions, cells were treated alone with the combination of Cetuximab or Ixabepilone and Cetuximab. This study using Cetuximab alone or in combination showed that Cetuximab, applied in vitro conditions in breast cancer cells, decreases mammosphere formation efficiency and cancer stem cell population [29].

Brand et al. have demonstrated that after the EGFR expression is deactivated, it remains dependent on EGFR for proliferation of triple negative breast cancer cell types [30]. Liao et al. investigated the efficacy of paclitaxel and cetuximab and conjugated Nanodiamond on the MDA-MB-231 cell line. They found that this combination increased the anticancer effects in this cell line [31]. The study by Oliveras-Ferraros et al. showed that the use of Cetuximab alone inhibits cell viability in MCF10A and MDA-MB-468 cell lines where EGFR is over-expressed compared to other cell lines which EGFR is under-expressed in [32]. In the current study, the use of Cetuximab alone caused an increase in apoptotic index values in triple negative breast cancer cell line MDA-MB-231 which overexpress EGFR.

As a result, in our study; when antiproliferative effects of Cetuximab on MDA-MB-231 cells originated from triple negative breast cancer was evaluated, Cetuximab decreased cell proliferation at an optimum concentration of 45 μM concentration for MDA-MB-231 cells. It caused a significant decrease in cell index, mitosis phase and cell percentages in synthesis and also caused an increase in apoptotic index.

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Conflicts of interest
The authors state that they did not have conflict of interests

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