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Investigation of Sensitivity to AZD7762 in Triple-Negative Breast Cancer (TNBC) with *RBFOX2* Gene Expression as a Biomarker

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ABSTRACT

Objective: Triple negative breast cancer (TNBC) is one of the most metastatic, aggressive with poor prognosis types of breast cancers. There is currently no standard molecular-targeted treatment for TNBC. Therefore, new therapeutics should be developed. The aim of this study was to determine the effect of AZD7762 on several breast cancer cell lines and evaluate the *RBFOX2* gene expression levels as a marker to show sensitivity to this drug.

Materials and Methods: The cytotoxic effect of AZD7762 on breast cancer cell lines was determined by sulforhodamine B method. The expression levels of *RBFOX2* gene were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The association between the IC50 values of the selected drug AZD7762 and *RBFOX2* expression levels was evaluated by using Pearson r correlation analysis.

Results: Although there was no significant difference between the IC50 values of TNBC and non-TNBC groups, it was determined that TNBC cell lines tended to be more sensitive to AZD7762. In addition, it was obvious that increasing levels of *RBFOX2* expression were detected in cells that showed more sensitivity to AZD7762.

Conclusion: It was concluded that the *RBFOX2* gene can be used as a biomarker to show AZD7762 efficiency. Further studies are needed to investigate the potential signaling mechanisms that are associated with the effect of AZD7762.

Keywords: Triple negative breast cancer, AZD7762, *RBFOX2*, biomarker

INTRODUCTION

Breast cancer was the most frequently diagnosed cancer type (2.26 million) among women worldwide in 2020. Moreover, according to a 2022 report, 290,560 new cases were diagnosed in USA resulting in roughly 43,780 deaths (1, 2). Breast cancer is also the most frequently detected cancer type among Turkish women, and according to Globocan 2020 data, 24,175 new cases were reported while the incidence was 46.6/100,000 (3). TNBC is a form of breast cancer in which the progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth receptor-2 (HER-2) are lacking. TNBC accounts for 15-20% of all invasive breast cancer patients (4). 13.1 per 100,000 new TNBC cases among women were reported in 2021 around the world (5). 10.6% (22,375) of breast cancer cases were determined as TNBC in 2018 in Turkey (6). Compared to other breast cancer subgroups, TNBC is characterized by its unique molecular profile, aggressive behavior, differential propensity for metastasis, and unsuitability for targeted therapies (7). A specific treatment approach against TNBC is not yet available. Therefore, different combinations of current chemotherapeutic agents and the same dose of ionizing radiation (IR) are used (8).

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AZD7762 is a check point kinase inhibitor. It is applied in combination with chemotherapeutic (gemcitabine, topotecan, irinotecan, cisplatin, paclitaxel) or radiotherapeutic agents that damage DNA (9-11). In addition, there are many studies that have tried to overcome treatment resistance in triple negative breast cancers that are resistant to chemotherapeutics or radiotherapy, and significant results have been achieved (12-15).

In this study, it was aimed to find a drug with a specific effect against TNBC and to define a potential biomarker to determine the effectiveness of this drug.

MATERIALS AND METHODS

In Silico Drug and Biomarker Selection

Garnett et al. have tested 130 anticancer drugs in 368 different cancer cell lines, such as breast cancer, renal cell carcinoma, pancreatic cancer, etc. (16). Among these, we selected 24 breast cancer cell lines and classified them as TNBC and non-TNBC, according to the clinicopathological classification of Kao et al. (17). Moreover, we compared IC50 values of these 130 anticancer drugs between these 24 TNBC and non-TNBC cell lines. To determine the marker gene, we performed correlation analysis between Garnett's gene expression data and IC50 values of selected drugs in 24 breast cancer cell lines.

Cell Culture

MDA-MB-231, MDA-MB-157, and BT-20 cell lines were used for TNBC trials, and T47D, MCF-7, and MDA-MB-453 cell lines were used for non-TNBC trials. Cell lines were kindly provided by Bilkent University, Department of Molecular Biology and Genetics. There was no need to obtain ethics committee approval.

DMEM was used for cell lines MDA-MB-231, MCF-7, BT-20, and MDA-MB-453; for MDA-MB-157 and T47D cell lines, media prepared with RPMI 1640, 1% penicillin/streptomycin antibiotic, and 10% FBS was used, and all cells were incubated at 37°C and 5% CO_2.

96-well plates were used for ionizing radiation (IR) and cytotoxicity tests, and optimization was performed to determine the number of cells to be seeded in each well. As a result, 6400 cells/mL for BT-20, 10000 cells/mL for MDA-MB-157, 8000 cells/mL for MDA-MB-231, 6000 cells/mL for MCF-7, 2500 cells/mL for MDA-MB-453 /mL, and 9000 cells/mL for T47D were seeded into the wells.

Ionizing Radiation (IR) Application

Eight 96-well plates were used for each cell line. Each plate was stained with sulforhodamine B (SRB) at time zero (24 hours after cell cultivation) and measured spectrometrically. One plate was used as a control group and incubated for 96 hours without any drug application. The remaining 6 plates were given 1Gy/1min IR 24 hours after the cells were seeded. A dose of 1 Gy causes less than 10% apoptosis in MCF-7 and MDA-MB-231 cell lines (18). Many studies stated that, to increase the effectiveness of AZD7762, which is a Chk1/2 inhibitor, it was necessary to give an IR or chemotherapeutic agent that creates DNA breaks (19-21).

AZD7762 Drug Application

Different drug concentrations (0.63μM, 1.25μM, 2.5μM, 5μM, 10μM, 20μM) were applied 24 hours after IR was given to 6 plates, and 72 hours after drug addition, they were stained with SRB together with the control group (no drug added) and their IC50 values were determined.

Cytotoxicity Assay (sulforhodamine B method) and IC50 Calculation

After drug and IR applications, fixation was performed with 10% trichloroacetic acid (TCA). After washing with distilled water, the plates were left to dry for 1 day to remove all the water. SRB dye dissolved with acetic acid was applied to each well and then washed with 10% acetic acid. The plates were then allowed to dry for a short time. Then, after 30 minutes of incubation with 300 mM Tris base in a shaker, OD 515 values were measured, and standard deviations were calculated.

RNA Isolation and cDNA Synthesis

RNA isolations were performed using TRIzol (Thermo Fisher Scientific, USA) 72 hours after application of IR and drug. Nanodrop (Thermo Fisher Scientific, USA) was used to assess RNA quality and quantity. cDNA synthesis was performed using 100 ng total RNA and iScript cDNA synthesis kit (Biorad, USA) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA binding protein fox-1 homolog 2 (RBFOX2) gene expression levels were analyzed by qRT-PCR method using TaqMan Gene Expression Assays (Applied Biosystems (AB by life technologies)) in a Biorad T100 thermalcycler device. The relative expression levels of *RBFOX2* and the β-actin reference gene were calculated using the ΔΔCt method.

Statistical Analyses

TNBC and non-TNBC cell lines were classified by using a one way ANOVA test. Drug selection was performed by Student's t-test. Candidate biomarker gene was determined by Pearson r correlation analysis. All statistical calculations were done using Graphpad prism 5 (GraphPad Software, USA). The statistical significance of the data determined as $p<0.05$ was acceptable.

RESULTS

In Silico Drug and Biomarker Selection

AZD7762 and RDEA119 were found significantly more effective on TNBC than non-TNBC (p: 0.01 and p: 0.04, respectively). AZD7762 (AstraZenecaDrug 7762) was selected as a potential biomarker due to the more significant p-value.

As a result of the correlation analysis between the IC50 values of the AZD7762 of all breast cancer cell lines used by Garnett et al. (16), and the gene expression levels of these cell lines, the *RBFOX2* gene, which was found to be 70% inversely correlated with the IC50 values of AZD7762, was selected as a potential biomarker candidate gene (p: 0.02607).

RBFOX2 Gene Expression

For *in vitro* validation studies, the ΔΔCt method was used together with the Ct values of the beta-actin gene in order to calculate the expression value of the *RBFOX2* gene in cell lines according to the qRT-PCR results, and the relative quantification calculation is given in Figure 1. When the *RBFOX2* gene expression levels of the two groups: TNBC and non-TNBC were compared, no significant difference was found (p: 0.5947; Figure 1).

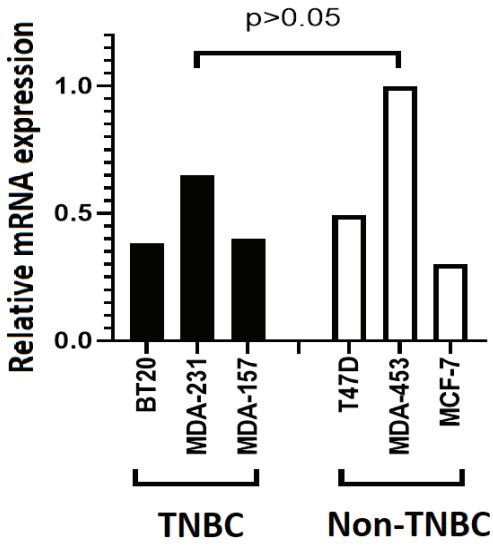

Figure 1. Relative quantification results of *RBFOX2* among TNBC and non-TNBC groups. TNBC: Triple negative breast cancer.

In addition, MDA-MB-231 and MDA-MB-453 cell lines were found to have the highest *RBFOX2* expression among both TNBC and non-TNBC groups. Significant differences in *RBFOX2* expression between two groups are shown in Figure 1.

SRB Cytotoxicity and IC50 Values

The drug concentrations of each cell line and the cytotoxicity values obtained after the SRB method and IC50 values were calculated. There was no statistically significant difference between the IC50 values of the cell lines in the TNBC and non-TNBC groups in our investigation (p: 0.6965; Figure 2). However, on average, a sensitivity to this drug was observed in TNBC cell lines.

Correlation Analysis

There was no significant correlation between *RBFOX2* expression levels and IC50 values in our study (p: 0.8101; Figure 3). However, as *RBFOX2* expression increases, drug resistance increases; in other words, there is an inverse correlation between IC50 and *RBFOX2*.

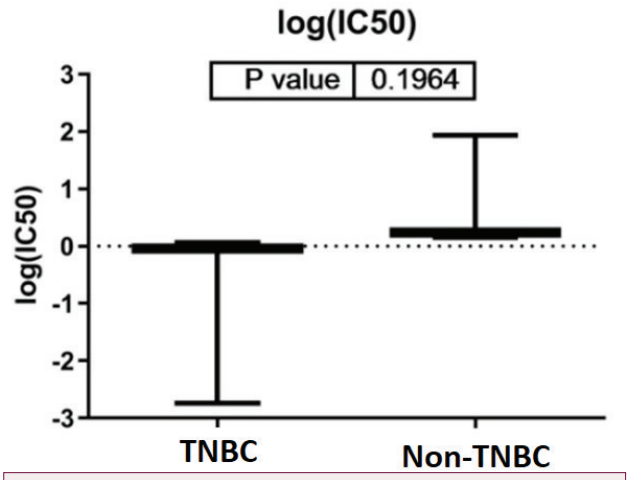

Figure 2. Comparison of log (IC50) between TNBC and non-TNBC groups. TNBC: Triple negative breast cancer.

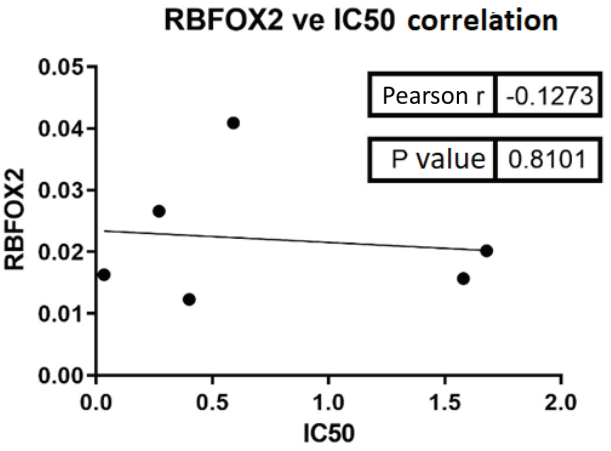

Figure 3. Correlation analysis results between *RBFOX2* gene expression and IC50 values. TNBC: Triple negative breast cancer, *RBFOX2:* RNA binding fox-1 homolog 2, IC50: half maximal inhibitory concentration.

DISCUSSION

DNA breaks created by ultraviolet (UV) and chemotherapeutic agents used in cancer treatments lead the cell to apoptosis. However, breast cancer and especially TNBC cells develop resistance to chemotherapeutic agents. They provide this resistance by evading checkpoints and by DNA repair. Therefore, in order to break drug resistance in TNBC, that is, to increase sensitivity to chemotherapeutic agents and UV, it has been an important strategy recently to eliminate kinase activity in these control contacts and direct the cell to apoptosis without giving DNA repair opportunity. Thus, when Chk1/2 inhibitors are used in combination with agents that cause DNA damage (radio and chemotherapeutic agents), they increase the anti-tumor effect and response. The drug AZD7762 is also a Chk1/2 inhibitor and, therefore, has an anti-tumor effect (22-25).

In many studies, it was shown that AZD7762 in combination with gencitabine or cisplatin in solid tumors, urethral carcinoma, lung carcinoma, and osteocarcinoma causes increased sensitivity to radiation or chemotherapeutic agents by inhibiting the G2/M transition, thus promoting apoptosis and mitotic catastrophe. In addition, AZD7762 has been shown to inhibit growth in radiotherapy-resistant breast cancer cells (21, 25-29).

Moreover, TNBC frequently harbors *TP53* mutations, resulting in the loss of the G1 checkpoint, thereby relying on Chk1 to recruit cells in response to DNA damage. Therefore, agents targeting Chk1 might be more specific for TNBC in particular (16, 23, 26, 30).

Studies have been focused on indication of whether AZD7762 is a suitable therapeutic option that might be used in the treatment of TNBC, and that it might also be used as a biomarker at the expression level of pChk1 (s296), showing a therapeutic synergy when used in combination with gemcitabine (23, 26, 28, 30).

According to the literature, many studies have strong statements about AZD7762 that it is a candidate drug that can be used in combination in the treatment of breast cancer. However, associating the usability of this drug with a genetic marker has not yet been specified in the current literature. In this study, it was aimed to find a drug that has a specific effect on TNBC and to define a potential marker to determine the effectiveness of AZD7762 drug, which was predicted to be sensitive to TNBC cell lines, as a result of the analysis made using the raw data of Garnett et al. (16). In consideration of this study, the drug AZD7762, which is predicted to be sensitive to TNBC cell lines, was selected, and its cytotoxic effect on MCF-7, T47D, BT-20, MDA-MB-157, MDA-MB-231, and MDA-MB-453 cell lines was investigated. In addition, the expression levels of *RBFOX2*, which can be a candidate marker, was examined by correlation analysis.

The fact that *RBFOX2* is associated with TNBC cells in preliminary analysis and its expression is relatively higher in TNBC cells in the experimental results reveals the relationship of this gene with breast cancer cells. In breast cancer, the alternative splicing mechanism plays a very important role in tumor progression by the Endothelial mesenchymal transition (EMT) and is associated with metastasis (24-27). In early prognostic marker identification and association studies, it has been shown that *RBFOX2*, one of the alternative splicing factors, is associated with EMT (especially in the formation of EMT in breast cancer) (24-26). In a polymorphism study of *RBFOX2*, it was shown that *RBFOX2* is highly associated with estrogen negative (ER-) breast cancer subtype, and it has been reported that *RBFOX2* plays a role in the development and progression of breast cancer (27). According to this information, the *RBFOX2* gene was selected as the biomarker candidate in our study, which showed a 70% correlation with the IC50 values of the AZD7762 drug (p: 0.02607) as a result of our correlation analysis.

In our experiments, we applied IR to the cells. This is because Chk1/2 is a checkpoint kinase that is activated (triggered) by breaking the DNA by chemical or IR agents (28,29). For this reason, in order to show the effectiveness of AZD7762, which is a Chk1/2 inhibitor, the amount of IR that would not kill the cells but would cause DNA breakage was given (1 Gy/min), and the Chk1/2 kinase was activated (A dose of 1 Gy causes less than 10% apoptosis in MDA-MB-231 and MCF-7 cell lines (18)). Then, the Chk1/2 inhibitor, AZD7762, was added. Thus, the cells were sensitized to IR.

Although there was no significant difference in the IC50 values of the TNBC and non-TNBC groups in the validation studies (p: 0.6965), it was determined that TNBC cells tended to be more sensitive to AZD7762 compared to non-TNBC. In addition, although there was no significance in the correlation analysis between *RBFOX2* gene expression and IC50 values (p: 0.8101), a relationship between increased *RBFOX2* expression and decreased IC50 value was observed between TNBC and non-TNBC groups.

As a conclusion, although no significant results were found between cytotoxicity tests and gene expression levels in our study, it was determined that *RBFOX2* gene expression and IC50 values were different between the TNBC and non-TNBC groups, that TNBC cells tended to be more sensitive to the drug, and that *RBFOX2* gene expression was higher. Thus, the *RBFOX2* gene expression for TNBC was associated for the first time with AZD7762 activity in the preliminary analysis and experiments performed in our study.

There may be several reasons why *in silico*-detected relationships could not be verified *in vitro*. One of the most important reasons may be the transcript variants of the gene of interest in microarrays used for *in silico* studies can be different from those produced by qRT-PCR primers.

Studying with the various breast cancer cell lines could support the usage of *RBFOX2* gene as a biomarker to detect the effect of cancer drugs. Moreover, the protein levels of *RBFOX2* gene can be analyzed. Further studies are needed to detect the association between *RBFOX2* gene and applicability of AZD7762 to support our results.

Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - İ.M.A., A.O.G., B.V.; Data Acquisition - M.İ.; Data Analysis/Interpretation - İ.M.A., M.İ., B.K.; Drafting Manuscript - İ.M.A., M.İ., B.V.; Critical Revision of Manuscript - İ.M.A., M.İ., B.İ., B.K., A.O.G., B.V.; Final Approval and Accountability - İ.M.A., A.O.G., B.V.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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