



TMEM97 Expression Level Related to Tamoxifen Resistance in Breast Cancer Cells

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OBJECTIVE

Breast cancer is the most diagnosed cancer in female gender worldwide and the primary cause of cancer deaths. TMEM97 or MAC30 is a transmembrane protein, and it has been shown to be important in cholesterol metabolism. In this study, we aimed to investigate whether TMEM97 expression is related to tamoxifen resistance in breast cancer.

METHODS

Protein expression was examined through western blotting in the cell lines. TMEM97 expression was silenced using the CRISPR/Cas9 method. To determine the resistance of cell lines to tamoxifen, cells were incubated with different doses of the drug and cell viability was assessed using the CellTiter-Glo luminescent solution. The AmplexRed cholesterol assay was used to determine intracellular cholesterol levels.

RESULTS

TMEM97 expression was higher in MCF7 cells and more sensitive to tamoxifen. TMEM97 knock-out isogenic cells were determined more resistant to tamoxifen. In addition, total cholesterol level was increased upon tamoxifen in isogenic cell lines at a similar ratio.

CONCLUSION

In this study, we found that high TMEM97-expressing cells are resistant to tamoxifen. It could be speculated that TMEM97 expression could be an important indicator for tamoxifen sensitivity. Further studies will elucidate the mechanisms behind this association between gene expression and tamoxifen resistance.

Keywords: Breast cancer; gene expression; tamoxifen resistance; TMEM97/MAC30.

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INTRODUCTION

Breast cancer is the most diagnosed cancer in female gender worldwide and the primary cause of cancer deaths in women.[1] Breast cancer generally develops

from hyperproliferation of ductal cells and then evolves into benign or metastatic tumors. There are multiple risk and causative factors for breast cancer development such as aging, smoking, reproductive factors such as early menstruation, late menopause, and family history.

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[2] In addition to the role of tumor microenvironment, many genes have been associated in the pathogenesis of the disease.[3] Mutations and/or abnormal amplification of these genes have been related to tumor initiation and progression. Breast cancer is categorized into 3 subtypes based on the presence or absence of molecular markers.[4] Among the molecular markers responsible for breast cancer development, estrogen receptor alpha (ER) expression is found nearly 70% of the invasive breast cancer cases.[5] ER is a steroid hormone receptor (HR) and when is induced by estrogen, activates downstream oncogenic signaling pathways. In addition to ER expression, progesterone receptor (PR) is also a marker of ER signaling. Tumors expressing either ER or PR are categorized as HR positive and primary therapy method is the use of endocrine agents. The second category of breast cancer is defined with the positivity of epidermal growth factor 2 (ERBB2, previously HER2). The third category of breast cancer is called triple-negative tumors which are characterized by the lack of expression of molecular markers mentioned above ER, PR, or ERBB2. Triple-negative breast cancer makes up nearly 15% of all types and has a high risk of relapse within the 3–5 years following diagnosis.[6] As majority of the breast tumors are HR+, the main anti-cancer agent group used in the therapy is selective estrogen receptor modulators (SERMs). Tamoxifen is a SERM and inhibits estrogen hormone from entering cells, therefore inhibits breast cancer cell development.[7] 5 years' tamoxifen treatment of patients with HR+ breast cancer reduces recurrence rate 30–50% depending on the anatomic stage of the disease.[8] Thus, clinical studies have evaluated the longer use tamoxifen, but these studies showed a small improvement in the recurrence rate.[8] Furthermore, large number of patients develops resistance to tamoxifen in years.[9] Tamoxifen resistance has been related to number of genes, other molecular factors, and activation or downregulation of signaling various signaling pathways.[10,11] Benefits from the studies investigating resistance factors to clinical use are highly limited.

Cholesterol is a non-essential metabolite required for normal cell growth and proliferation. For new membranes to be made in proliferating cells and for the cell signal transmission system to continue actively, endogenous cholesterol synthesis and the intake of lipid particles from the outside are needed.[12] Altered cholesterol metabolism has been reported in various cancer cells.[13,14]

Transmembrane protein 97 (TMEM97), also known as MAC30, is member of TMEM proteins that span the lipid bilayer of membranes such as plasma membrane

and organelle membranes. Recently, TMEM97 was identified as the sigma receptor 2 which acts as opiate receptor and gained more attention.[15] Furthermore, TMEM97 has been related to cholesterol metabolism as it has been shown to interact with LDLR.[16] TMEM97 gene expression varies among cancer types. In pancreatic and prostate cancers, low TMEM97 expression has been reported.[17] On the other side, high TMEM97 expression has been found in breast, gastric, colon, ovarian, oral squamous, and non-small cell lung cancer cells, and thus, it is defined as presumptive oncogene.[18] Ro48-8071 is a cholesterol-lowering agent by inhibiting oxidosqualene cyclase, an enzyme in the cholesterol biosynthesis steps. Ro48-8071 and tamoxifen have been shown to have higher affinity for TMEM97 binding suggesting the protein as pharmacologically druggable.[19] This data highlight the possible role of TMEM97 in cholesterol metabolism.

Identifying new drug targets could be beneficial in understanding of the molecular basis of tamoxifen resistance. Based on these observations in the literature, this project was aimed to investigate whether TMEM97 could be a target for tamoxifen.

MATERIALS AND METHODS

Cell Culture and Medium

MCF7, MDA-MB-231, and MCF10A cell lines are kind gifts by Dr. Ceren SARI of Karadeniz Technical University, Institute of Health Sciences. All cell lines except from MCF10A were cultured in DMEM medium (Gibco, ThermoFisher,) supplemented with 10% FBS, 1% pen/strep, and 1% l-glutamine. MCF10A cells were cultured in DMEM/ Ham's F-12 (Gibco, ThermoFisher) supplemented with 100 ng/ml cholera toxin, 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5% chelex-treated horse serum at 37°C with 5% CO₂. MCF7/TAMR-1 (ATCC, CRL-3435) cell line was cultured in phenol red-free DMEM medium (ThermoFisher cat no: 11054–020) supplemented with 10% FBS, 1% pen/strep, 1% non-essential amino acids, and 0.1% insulin (Sigma cat no: I0516–5ML). When first thawed, the cell line was exposed to 5 µM tamoxifen (Sigma-Aldrich 4-Hydroxy-tamoxifen cat no: T176) 2–3 times.

Generating TMEM97 Knockout MCF7 Cells

Two different sets of single guide RNA (sgRNA) for TMEM97 gene and one set control sgRNA were determined (Table 1) using UCSC genome browser and crispr.mit.edu web pages. Annealed oligonucleotides were cloned into a plentiCRISPR-Cas9 v2 vector using T4

Table 1 sgRNA list

sgRNA1 forward	caccGAGGAAGGCATACGTTGCAA
sgRNA1 reverse	aaacTTGCAACGTATGCCTTCCTC
sgRNA2 forward	caccgCATTTACCAACCTTTGAGGA
sgRNA2 reverse	aaacTCCTCAAAGGTTGGTAAATGc
Control sgRNA1 forward	caccGGCATCGCCTCCTTTGTCA
Control sgRNA1 reverse	aaacTGACAAAGGAGGGCGATGCC

sgRNA: Sets of single guide RNA

ligase (NEB). Transfection-grade plasmids were used for viral packaging in Hek293T cells. Cell supernatant was collected to obtain virus particles which were then used to transfect MCF7 cells through spinfection method in the presence of 8 µg/ml polybrene. After antibiotic selection, knockout clones were selected with single-cell cloning. When clones reached enough confluency, TMEM97 expression was evaluated by western blotting by selecting different clones obtained.

Immunoblotting

Cultured cells were collected by centrifugation to obtain a cell pellet and rinsed with ice-cold PBS. Protein isolation from the cell lines was done with RIPA buffer supplemented with protease inhibitor (Complete, Roche). The protein concentration in the lysates was determined with the BCA (bicinchoninic acid, Pierce, ThermoFisher) kit. After the protein concentrations were adjusted to 1 mg/mL, the protein loading dye was added to the final concentration of 1X. The prepared protein samples were denatured by boiling for 5 min. Denatured protein samples were run on a 12% tris-glycine gel by SDS-PAGE electrophoresis. After electrophoresis, the gel was transferred to PDVF membrane. Samples were treated with appropriate primary and secondary antibodies. Images were taken on Azure C300 gel imaging system.

Drug Treatment and Cell Viability

Cells were plated in 96-well plates at a concentration of 15.000 cells/mL, and then, cells were treated with desired concentrations of tamoxifen. Cells were cultured at 37°C for 96 h in an incubator at 5% CO₂. At the end of incubation, cell viability was determined using CellTiter GLO Luminescent Reagent (Promega). Relative fold change of cell viability was presented as (%) comparing luminescence measurements to normal medium.

Cholesterol Assay

MCF7 isogenic cells for TMEM97 expression were cultivated in medium with or without 10 µM tamoxifen for 96 h. After incubation, the cells were collected and

intracellular cholesterol level was determined using the Amplex Red Cholesterol Assay Kit (Invitrogen) according to the manufacturer's instruction. Fluorescence was measured on a multimode spectrophotometer (BioTek Cytation 5) with excitation at 530 nm and emission detection at 590 nm. The values of cholesterol level were calculated from the standard curve. The results were presented as µg/ml of cholesterol per mg of protein.

Statistical Analysis

Graphs and statistical analysis were done using Graph-Pad V7. Student's t-test with Welch's correction was applied to compare two groups. TCGA Target GTEx data set was analyzed on Genome Browser Xena.[20] TMEM97 mRNA expression of breast cancer cell lines was obtained from cancer cell line encyclopedia.[21] All experiments were performed at least two independent times with similar results. Cell viability assay was performed with quad technical replicates.

RESULTS

TMEM97 Expression in Breast Cancer Cells

TMEM97 mRNA expression was detected significantly higher in tumor samples compared to normal tissue samples as it was analyzed in Genome Browser Xena. In addition, high TMEM97 mRNA expression (log fold >10.5) was found associated with poor survival rate (Fig. 1). Protein expression was evaluated with immunoblotting in all cell lines. MCF7 cells were found to have higher expression of TMEM97, compared to other malignant cell lines and MFC10A cells (Fig. 2a).

Tamoxifen Sensitivity of Breast Cancer Cells

MCF7, MDA-MB2331, and MCF10A cells treated with tamoxifen at increasing concentrations for 3 days. After treatment, MCF7 cells were determined more sensitive to tamoxifen at all doses. MCF10A non-tumorigenic breast cell line was found more resistant to tamoxifen at 2.5 and 5 µM. However, MDA-MB2331 cells were detected more resistant after 10 µM tamoxifen dose compared to MCF10A and MCF7 cells. All these differences were found statistically significant (Fig. 2b).

TMEM97 mRNA Expression is Lower in Tamoxifen Resistant MCF7 Cells

To demonstrate the relation between TMEM97 expression and tamoxifen resistance, TMEM97 mRNA was evaluated by qRT-PCR in MCF7 cells and MCF7/TAMR-1 cells. It was found that TMEM97 mRNA expression is significantly low in tamoxifen-resistant cells as compared to MCF7 cell line (p<0.01) (Fig. 3).

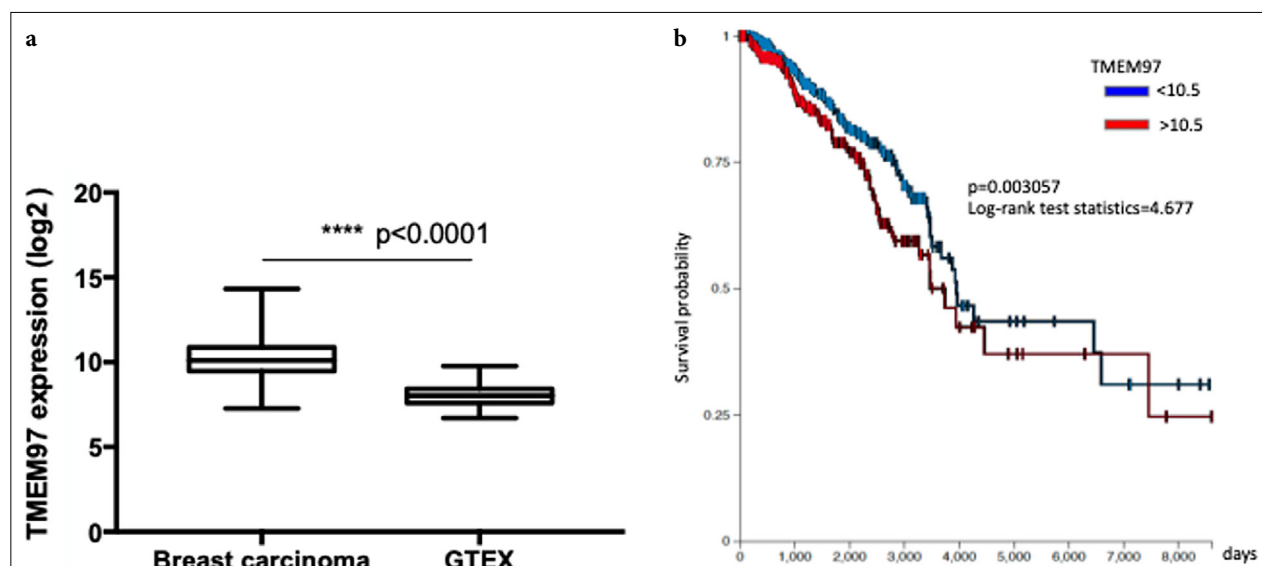


Fig. 1. (a) TMEM97 mRNA expression in breast carcinoma and normal breast tissue samples. TCGA GTEx dataset was analyzed on Genome Browser Xena. A p value <0.0001 was found with one-way ANOVA test. (b) Kaplan–Meier survival analysis. High TMEM97 expression could be related to decreased survival rate. TMEM97: Transmembrane protein 97; GTEx: Genotype-Tissue Expression Portal; TCGA: The Cancer Genome Atlas Portal; ANOVA: Analysis of variance.

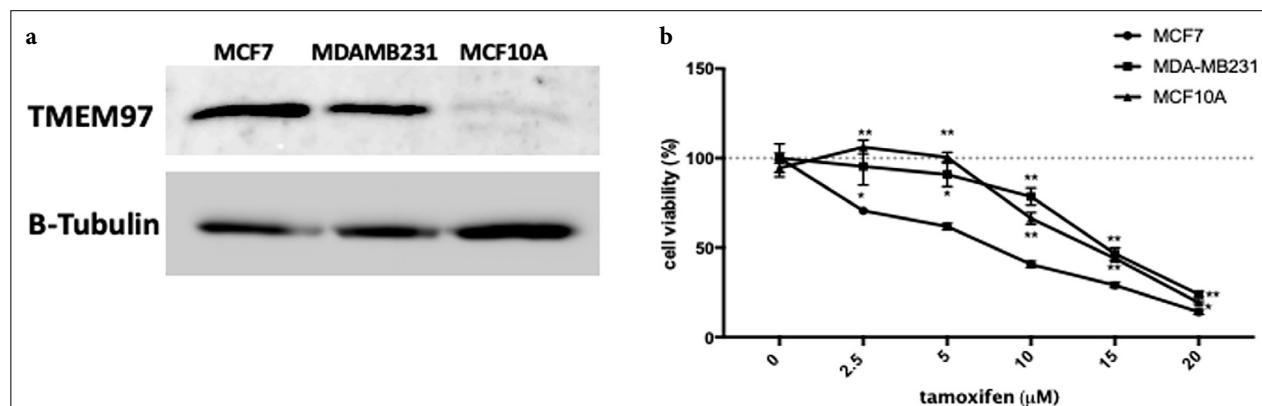


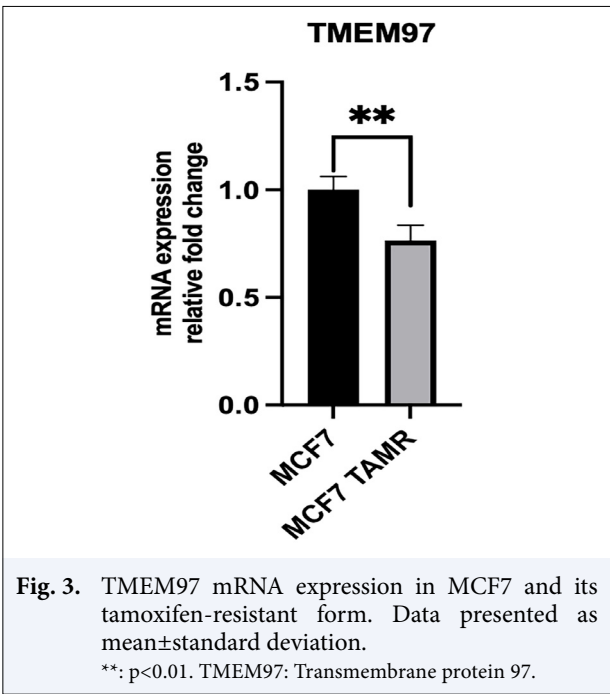
Fig. 2. (a) TMEM97 protein expression of MCF7, MDA MB231, and MCF10A cells lines. (b) Cell viability of cell lines upon tamoxifen treatment. Data presented as mean±standard deviation. *: p<0.05; **: p<0.01. TMEM97: Transmembrane protein 97.

TMEM97 Knockout MCF7 Cells are more Resistant to Tamoxifen

As TMEM97 protein expression was observed higher in MCF7 cells and this cell line was found more sensitive to tamoxifen, we further evaluated the role of TMEM97 expression in tamoxifen response by knocking out the gene using CRISPR/Cas9 genome editing system (Fig. 4a). TMEM97 knockout two isogenic and one control cell lines were cultured with tamoxifen for 3 days. It was found that TMEM97 knockout cells were significantly more resistant compared to control cell line at all doses (Fig. 4b).

Tamoxifen Treatment Increased Total Cholesterol Level in all Cells

Knocking out TMEM97 gene slightly decreased total cholesterol in MCF7 cells. In the control group, the amount of total cholesterol was 41 μg/mL per mg protein, whereas in TMEM97 knockout cells, 36 μg/mL per mg protein was detected (Fig. 5). We also found that tamoxifen treatment was increased total cholesterol level in all isogenic cells at a similar trend (almost doubled). However, the amount of total cholesterol was still found higher in TMEM97-expressing cells (Fig. 5).



DISCUSSION

Breast cancer is one of the most common cancer types among women, worldwide.[22] There are three subgroups of breast cancer: ER-positive, ERBB2-positive, and triple-negative breast cancer and ER is expressed in almost 70% of the patients.[9] Tamoxifen is widely used in the treatment of ER-positive breast cancer patients. However, many patients are resistant to tamoxifen at the beginning of the therapy and a greater number of patients develop resistance to the drug later in the therapy.

[8] Up to date, some mechanisms have been associated with tamoxifen resistance in ER+ breast cancer including polymorphisms of several genes, changes in expression of genes that have role in ER signaling.[10,23,24] A recent genome-wide association study identified candidate pathways that could be responsible for tamoxifen resistance in ER+ patients.[25] SIRT1 was shown to be important in breast cancer onset and progression by involving in Akt signaling.[26] Recently, together with SIRT1, high expression of SRC was found in tamoxifen-resistant cells and related to poor prognosis.[27] In addition, tongue cancer resistance-related protein1 was found to induce tamoxifen resistance by activating SGK1-signaling pathway, and thus, it was identified as a novel drug target as it was related to chemo-resistance in several cancer types including breast cancer cells.[28]

In cells, cholesterol levels are dynamically balanced by uptake, biosynthesis, transport, distribution, esterification, and export.[29] In recent years, cholesterol biosynthesis pathway has been identified as a mechanism of estrogen deprivation resistance in ER+ breast cancer.[30] Elevated expression of genes of the cholesterol synthesis pathway has been shown to be involved in poor drug response.[30] Furthermore, increased expression of the genes related to lipid metabolism has been determined.[31] TMEM97 also known as MAC30 is a transmembrane protein and its putative role in cholesterol metabolism has been shown in several studies.[16,32] Recently, TMEM97 has been identified as sigma 2 receptor and suggested to play role in drug response.[17] Furthermore, increased expression of TMEM97 was found in gastric, lung, breast, and ovarian cancer, and high expression level was associated with poor prognosis.

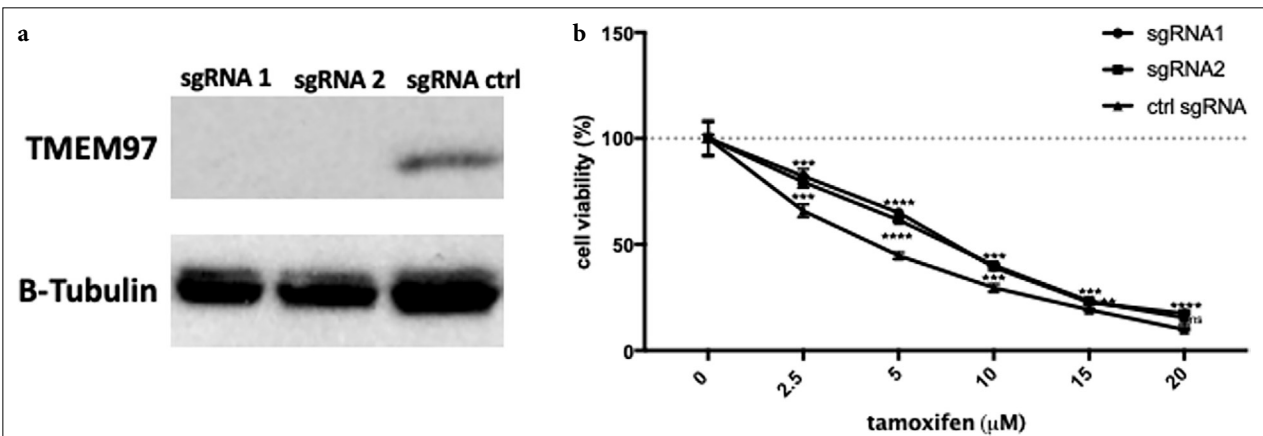
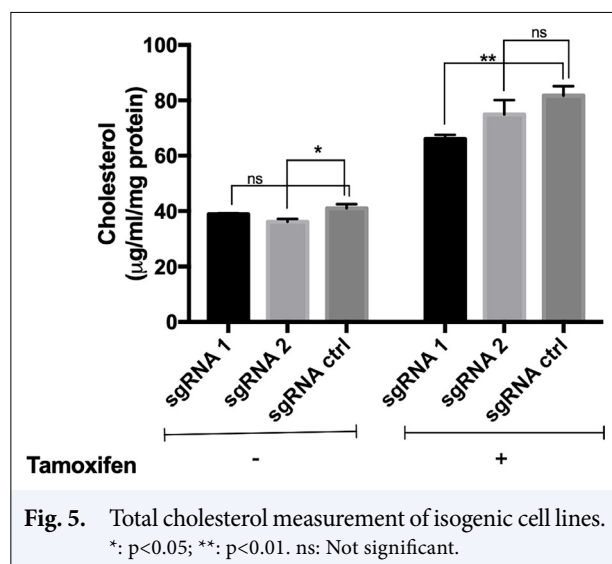


Fig. 4. (a) TMEM97 knockout validation by immunoblotting. (b) Cell viability of isogenic cell lines upon tamoxifen treatment. Data presented as mean±standard deviation.
: $p < 0.01$; *: $p < 0.001$; ****: $p < 0.0001$. TMEM97: Transmembrane protein 97; ns: Not significant.



sis and tumor recurrence.[18] All these data suggested TMEM97 as a drug target for cancer therapy and recently, an orally active ligand for TMEM97 has been developed for pancreatic cancer therapy.[33] In breast cancer samples, TMEM97 expression was shown to be induced by BRCA1 expression.[34] On the other side, downregulation of TMEM97 expression was shown to be controlled by p53.[35] Silencing of TMEM97 expression inhibited cell proliferation of glioma [36] and gastric cancer cell lines.[37] Reduced expression of TMEM97 inhibited cell growth and reduced colony formation of MCF7 cells.[38] In addition, downregulation of TMEM97 inhibited breast cancer cell invasion through suppression of Wnt/ β catenin and PI3K/Akt pathways.[39] These data suggest that TMEM97 could be important for tumor growth. However, knockdown or knockout of TMEM97 did not change proliferation of HeLa cells.[40] In addition, TMEM97 did not mediate cytotoxicity through sigma 2 ligand in HeLa cells. [40] In addition, it has been demonstrated that silencing of TMEM97 through siRNA-mediated knockdown leads to a decrease in cell proliferation in colorectal cancer cells, while its overexpression positively affects cell proliferation.[41] This data should be further evaluated with a more comprehensive approach in a broad range of cancer types with the use of various known ligands.

In this study, TMEM97 gene expression was found higher in breast tumor samples compared to normal breast tissue samples as analyzed with TCGA GTEx dataset.[20] Furthermore, we showed that ER+ MCF7 cell line has higher TMEM97 protein expression compared to benign breast tumor cell line MCF10A and triple-negative breast carcinoma cell line MDA-MB-231.

Although triple-negative cell lines are presumed to be tamoxifen resistant, there are multiple studies in the literature indicating tamoxifen is cytotoxic and induces apoptosis through different signaling pathways in breast cancer cells rather than ER signaling.[42–44]

It was found that MCF7 cells express higher TMEM97 protein, and they were more sensitive to tamoxifen treatment. We, therefore, hypothesized that tamoxifen sensitivity could be related to TMEM97 expression. To confirm this hypothesis, TMEM97 expression was controlled in tamoxifen-resistant MCF7 cell line. Indeed, it was shown that TMEM97 expression is decreased in tamoxifen-resistant MCF7 cells. To further demonstrate the effect of TMEM97 expression on tamoxifen resistance, TMEM97 KO isogenic MCF7 cells were generated by CRISPR/Cas9 editing. TMEM97 KO cells were found more resistant to tamoxifen treatment. One hypothesis regarding to the role of TMEM97 in tamoxifen resistance is that the protein has a domain called EXPERA which is found in sterol isomerase protein family members such as emopamil binding protein (EBP).[45] Tamoxifen is known to inhibit EBP and its homolog TM6SF.[46] Furthermore, tamoxifen was found among the ligands of TMEM97.[19] Therefore, it could be speculated that tamoxifen creates a signaling through TMEM97 protein, and if the protein abundance is low, tamoxifen cytotoxicity is not applicable for cells.

In this study, tamoxifen increased total cholesterol level in both KO and control cells at a similar ratio. However, to clarify the role of TMEM97 in cholesterol metabolism upon tamoxifen treatment, this experiment should be done in cholesterol/lipid-free media and level of lipids should be determined at subcellular level. In a study with T47D cell line, it was reported that 1 μ M tamoxifen treatment increased both free cholesterol and cholesterol ester level, which was consistent with our findings.[47] Altered lipid metabolism upon tamoxifen treatment suggests that genes responsible in the metabolism could be important for tamoxifen resistance.

CONCLUSION

To conclude, TMEM97 is a new player in cholesterol metabolism and has been shown to be an important factor for tumor development in different cancer cells. In this study, we showed that for the first time, breast cancer cells that have higher TMEM97 expression are resistant to tamoxifen. TMEM97 expression could be an important indicator for tamoxifen sensitivity as knockout cells are more resistant to the drug. To fur-

ther confirm our results, clinical studies should be conducted with high number of patients to obtain more reliable results regarding the relation of TMEM97 expression level and tamoxifen resistance.

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