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Research Article

BRCA1 is Overexpressed in Breast Cancer Cell Lines and is Negatively Regulated by E2F6 in Normal but not Cancerous Breast Cells

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ABSTRACT
This study focused on the expression and regulation of *BRCA1* in breast cancer cell lines compared to normal breast. *BRCA1* transcript levels were assessed by real time quantitative polymerase chain reaction (RT-qPCR) in the cancer cell lines. Our data show overexpression of *BRCA1* mRNA level in all the studied breast cancer cell lines: MCF-7, T47D, MDA-MB-231 and MDA-MB-468 along with Jurkat, leukemia T-lymphocyte, the positive control, relative to normal breast tissue. To investigate whether a positive or negative correlation exists between *BRCA1* and the transcription factor *E2F6*, three different si-RNA specific for *E2F6* were used to transfect the normal and cancerous breast cell lines. Interestingly, strong negative relationship was found between *BRCA1* and *E2F6*, in which depletion of *E2F6* in MCF-10A, the normal breast cell line, resulted in more than four-fold increased expression of *BRCA1* transcript level. On the other hand, our findings suggest that *E2F6* might lost control on *BRCA1* in breast cancer cells. *E2F6* knockdown by either of two specific siRNA (i.e. si-E2F6#1 or si-E2F6#2) had no influence on the *BRCA1* expression in MCF-7 cells. Although, transfecting these cells with si-E2F6#3 showed overexpression of *BRCA1* compared to cells transfected with scrambled negative control.

Keyword: Breast cancer; *BRCA1*; gene expression; RT-qPCR; *E2F6* knockdown

INTRODUCTION

Breast cancer is the most common cancer-related mortality in women in both developing and developed countries. It is well known that breast cancer susceptibility (*BRCA*) genes are tumour suppressors that preserve the structural and numerical stability of chromosomes during cell division [1]. *BRCA1* gene, which was mapped in 1990 and was subsequently cloned in 1994 [2], is located on chromosome 17q21 and encodes for a 220 kDa multifunctional nuclear phosphoprotein composed of 1863 amino acids [3]. BRCA1 protein contains two highly conserved domains in the N- and C-terminal regions that interact with numerous molecules [3-6].

The molecular pathways in which BRCA1 functions and how disturbance of these functions foster breast carcinogenesis remains anonymous so far [12]. Functionally, BRCA1 has been involved in numerous cellular functions, including ubiquitination [13-15], regulation of the G1/S [16], intra-S and G2/M-phase cell cycle

checkpoint control [17-19], control of spindle pole body duplication [1], transcription [20,21], sex chromosome inactivation [22-25] and DNA double stranded break repair via homologous recombination [26,27]. Taken together, these individual roles suggest a function for BRCA1 in the maintenance of genomic integrity [4]. Furthermore, BRCA1 has been suggested to play a role in the differentiation of breast epithelial cells, with loss of BRCA1 function resulting in reduced acini formation and an accumulation of less differentiated cells with different proliferation properties [28-30].

Increased *BRCA1* mRNA levels were noticed in rapidly proliferating cell lines in mice [31]. BRCA1 is a cell cycle regulated protein, and its mRNA level was observed to be highly expressed in late G1 phase of the cell cycle, whereas conditions that cause cell cycle exit downregulate its level [32]. Furthermore, BRCA1 protein was shown to be overexpressed during S phase of the cell cycle [33]. Expression of BRCA1 has been reported to affect the choice of chemotherapeutic agents used in the breast cancer treatment [34,5].

Concerning E2F6, it has been demonstrated to act as both transcriptional repressor as well as activator [35]. A specific link exists between E2F6 and carcinogenesis, this link comes from studies demonstrating that overexpression of E2F6 can change cell growth parameters [36]. E2F6 was found to be highly expressed at the mRNA and protein levels in breast carcinoma cell lines as well as primary breast tumours (our unpublished data). The present study focused on detecting the *BRCA1* expression status in some breast cancer cell lines compared to normal breast tissue. Additionally, in order to see if depletion of E2F6 influences the expression of *BRCA1*, three different small interfering RNA (si-RNA) mediated E2F6 depletion were used to transfect MCF-7 cells, which are known to express more *E2F6* [our unpublished data] and *BRCA1* [this study; 37] as well as MCF-10A cells, the normal breast cell line were transfected as well.

MATERIALS AND METHODS

Cell culture

Breast cancer cell lines including MCF-7, T47D, MDA-MB-231 and MDA-MB-468, normal breast cell line, MCF-10A, and Jurkat leukemia T-lymphocytes were used in this study. The breast cell lines were obtained from Sigma-Aldrich, UK. Breast cancer cells were grown in Dulbecco's Modified Eagles medium (DMEM; Lonza, UK) containing 4.5 g/L glucose with L-glutamine, and supplemented with 10% fetal calf serum (FCS; Seralab, UK) and 1x non-essential amino acids (NEAAs; Bio Whittaker, UK). Regarding MCF-10A cells, they were grown in DMEM containing 4.5 g/L glucose with L-glutamine with addition of 1x NEAAs, 5% horse serum (Invitrogen, UK), insulin 10 µg/ml (Sigma-Aldrich, UK), epidermal growth factor 10 µg/ml (EGF; Sigma-Aldrich, UK), hydrocortisone 50 µM final concentration (Sigma-Aldrich, UK), and cholera toxin 0.1 µg/ml (Calbiochem, UK).

Jurkat cells were presented as a gift from Prof. Matthew Holley, Department of Biomedical Science, The University of Sheffield, Sheffield, UK. They were grown in RPMI 1640 (Roswell Park Memorial Institute medium; Lonza, UK) containing L-glutamine, 10% FCS and 1x NEAAs were added. Before use, all the aforementioned media along with Trypsin-Versene (EDTA) and phosphate buffered saline (PBS) were warmed in a 37˚C water bath for at least 30 min.

SiRNA transfection

E2F6 knockdown in MCF-7 and MCF-10A cells has been achieved by using three different siRNA specific for *E2F6* including: si-E2F6#1 sense 5'-AAGGAUUGUGCUCAGCAGCUG, si-E2F6#2 sense 5'-AGUUAAAGCUCCAGCAGAA and si-E2F6#3 sense 5'-CUUAAGAAGUGCUCAAUAA. As a negative control, non-specific siRNA (scrambled) was used to transfect the cells whose sequence was 5'-UAAUGUAUUGGAACGCAUA. All siRNAs used in this study were purchased from Eurofins, Germany. DharmaFECT®#4 (Thermo Scientific, UK) was the transfection reagent. Reverse transfection protocol was optimized for the above cell lines using 6-well plate format. Briefly, 20 µM siRNA made in 1x siRNA buffer provided was mixed by pipetting with serum free medium (SFM) in an Eppendorf tube, and was left for 5 min at room temperature. Synchronously, the appropriate amount of DharmaFECT® #4 was added to SFM in an Eppendorf tube and left for the same time as before. Afterwards, siRNA-SFM was mixed by pipetting carefully up and down with the DharmaFECT-SFM and incubated at room temperature for a further 30 min. During that time, healthy cell lines

approximately 80% confluence and grown overnight in a medium without antibiotic were trypsinized, counted and diluted in an antibiotic-free complete medium. Appropriate amount of the mixture (siRNA-DharmaFECT-SFM) was then pipetted to the bottom of the well. Finally, cells were added at the optimized density into each well and mixed with the previously added complex of siRNA-DharmaFECT-SFM. Plates were incubated at 37°C with 5% CO_2 and humidity for 48h.

RNA Extraction

To analyze *BRCA1* gene expression at the mRNA level in the cell lines under study, after harvesting the cell pellets, RNA was extracted using mammalian total RNA miniprep kit (Sigma-Aldrich, UK). Manufacturer's instructions were followed to obtain the whole cell RNA, which was eluted in 50µl RNase free water. RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, UK) and was converted immediately to cDNA.

Reverse Transcription

Following extraction, total cell RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems, UK) in a total volume of 20µl. The recommendations of the manufacturer were applied. The cDNA was kept at -20˚C until use.

Primers

Oligonucleotide primers designed for *BRCA1* and *18S* were bought from Eurofins, Germany. The oligonucleotide sequences were forward 5'-CCCTTCACCAACATGCCCACA and reverse 5'-CTGCCCAATTGCATGGAAGCC for *BRCA1* amplification, while for 18*S* the primer set was forward 5'-AGAAACGGCTACCACATCCA and reverse 5'-CACCAGACTTGCCCTCCA. Each primer set was checked for its specificity to its target gene with no homology to other sequences using National Centre for Biotechnology Information (NCBI; BLAST). The primers were used in the real time quantitative polymerase chain reaction (RT-qPCR) to detect the expression of those genes in the above cell lines. 18S primers were used in 5 µM concentration, whereas primers specific for the target gene were diluted to 10 µM using deionized water prior to use and stored at -20°C.

RT-qPCR

The expression of *BRCA1* and *18S* was studied by RT-qPCR using a Corbett Robotics Rotor-Gene™ 6000 (Qiagen, UK). PCR reaction consisted of 20 µl of the following: 2× SensiMix (10 µl) containing a mixture of: buffer, dNTP, SYBR Green, HiRox and modified Taq polymerase. Furthermore, the reaction included: primers (2 µl forward and reverse), cDNA (5 µl) as a template and deionized sterile water (3 µl). Reactions were carried out in triplicate technical repeats.

Melt curve analysis was applied to check for presence of primer dimmers and to confirm amplification of a single product. The cycling conditions were 95°C for 10 min followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 58°C and extension at 72°C for 30 s. PCR product quantity gained is proportional to the fluorescence signal. Using Rotor-Gene 6000 software, C_T values were determined for each cDNA sample in every reaction. Relative gene-expression value was calculated using the formula: $\Delta C_T = C_{T\ (\text{target gene})} - C_{T\ (\text{reference gene})}$, allowing for the comparison of samples independently of the amount of total cDNA input. Furthermore, $\Delta\Delta C_T$ equals to $\Delta C_{T\ (\text{treatment or cancer})} - \Delta C_{T\ (\text{control or normal})}$ [38]. The average amplification efficiency was 1.9, so that $1.9^{-\Delta\Delta CT}$ parameter was applied to compare the expression level among genes in the same cell line or relative to the expression of same gene in a normal breast tissue or MCF-10A cells. This represents the expression fold of one gene with regard to a reference gene. Concerning gene knockdown in RNA interference (RNAi) experiments, the remaining mRNA of the silenced gene was measured using the method described by Haimes and Kelley (2010) [39].

Statistical Analysis

SPSS 22.0 software (IBM) for Windows was used to analyse the data. The non-parametric Mann-Whitney U test was used to analyse the differences between the gene expression levels of each sample compared to the normal, and was considered significant at confidence levels larger than 95% ($P < 0.05$). Correlation between the mRNA level remaining after E2F6 depletion and BRCA1 transcript level was determined using Pearson's R Correlation Test.

RESULTS

BRCA1 mRNAs are overexpressed in breast cancer cell lines compared to normal breast tissue.

Our findings show statistically significant high mRNA levels for BRCA1 in breast cancer cell lines: MCF-7, T47D, MDA-MB-231 and MDA-MB-468 in addition to Jurkat leukemia T-cells in comparison with the normal breast tissue (Fig. 1).

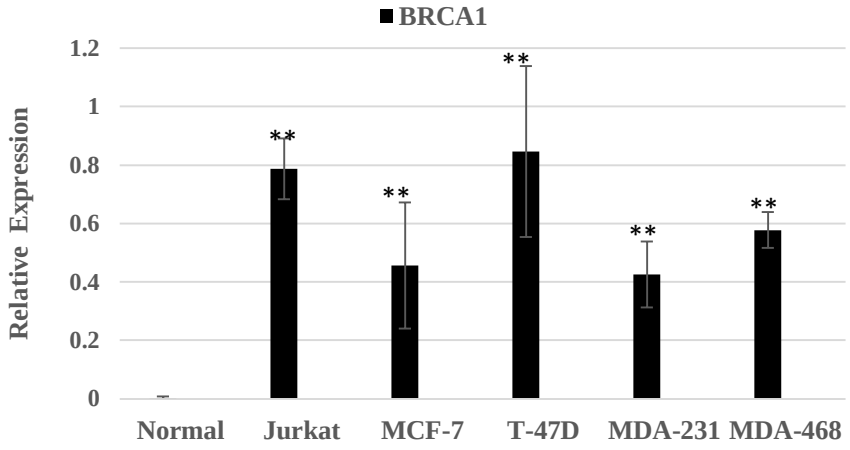

Fig. 1: BRCA1 is overexpressed in cancer cell lines versus normal breast tissue

E2F6 knockdown has different impacts on BRCA1 expression in breast cancer cells.

Interestingly, the three different si-*E2F6* revealed reduced *E2F6* cDNA level to various degrees. While si-E2F6#1 was the best in decreasing *E2F6* level followed by si-E2F6#3, the *E2F6* cDNA level remaining after its knockdown by si-E2F6#2 was in between that of the other two si-RNAs. MCF-7 cells transfected with either of si-E2F6 showed different responses. Concerning the correlation between *E2F6* mRNA depletion and *BRCA1* expression, our data show no correlation exists between their expression. Although si-E2F6#1 caused approximately more than 70% decrease in *E2F6* cDNA level *BRCA1* expression remained similar to that of scrambled control. However, si-RNA#3 resulted in 25% increase in the cDNA level of *BRCA1*. Regarding si-E2F6#2, the *BRCA1* expression decreased 25% when compared to the scrambled control (Fig. 2).

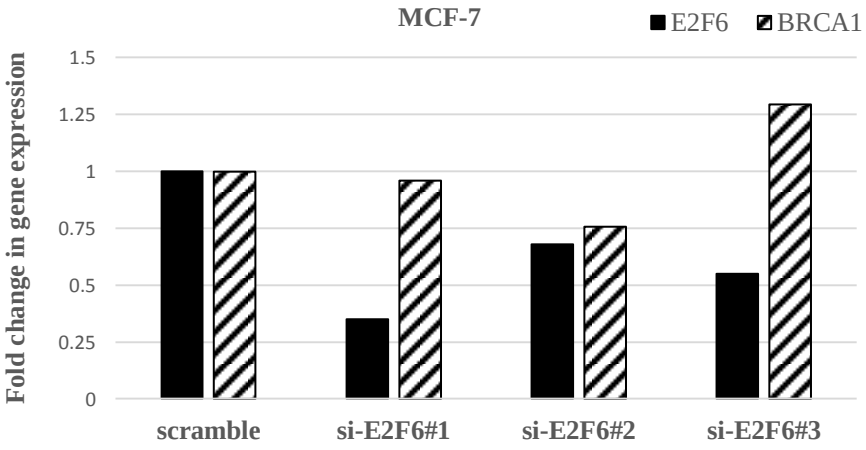

Fig. 2: Each si-E2F6 has different influence on the expression of BRCA1 in MCF7 cells

***E2F6* knockdown increases *BRCA1* expression in the normal breast cell line.**

The three different si-RNAs mediated *E2F6* knockdown successfully inhibited its expression at the mRNA level. The transfected MCF-10A cells showed more than four-fold increase in *BRCA1* cDNA level when transfected with either si-E2F6#1, si-E2F6#2 or si-E2F6#3.

Interestingly, those si-E2F6 were consistent in their influence on *E2F6* by reducing its mRNA to approximately similar levels (approximately 60% decrease), and by enhancing *BRCA1* cDNA level to several folds relative to its level in the scrambled negative control (Fig. 3).

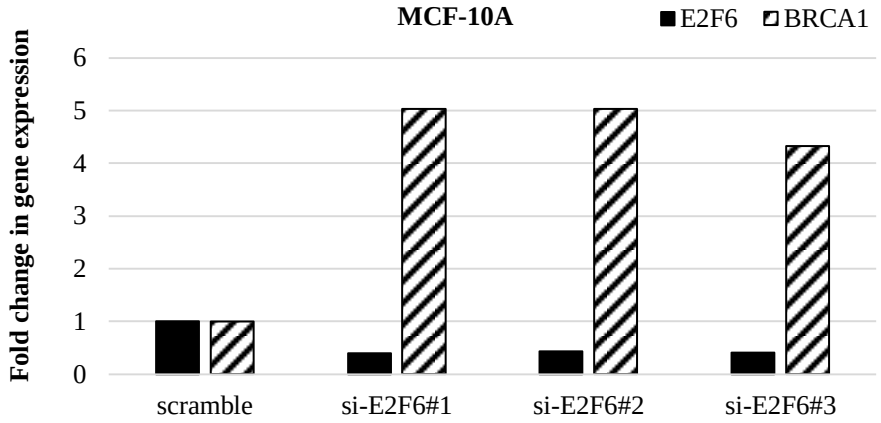

Fig. 3: E2F6 knockdown increases the expression of BRCA1 in the normal breast cell line

DISCUSSION

The results of the present study demonstrate that *BRCA1* is upregulated in the breast cancer cell lines including: MCF-7, T47D, MDA-MB-231 and MDA-MB-468 as well as leukemia T-lymphocytes, Jurkat cells, paralleled to normal breast tissue. These findings in part are consistent with that of Gudas et al. (1996) [32] and Blagosklonny et al. (1999) [40], in which they found that the immortalized, non-tumourigenic breast epithelial cells (MCF-10A) expressed relatively low levels of *BRCA1* mRNA. In comparison, *BRCA1* mRNA was overexpressed in MCF-7, T47D (both oestrogen-receptor positive) [41] and MDA-MB-468 (oestrogen-receptor negative), whereas MDA-MB-231 cells expressed relatively low levels of this mRNA [32]. At the protein level, MCF7, MDA-MB-468 and MDA-MB-231 cells expressed high basal levels of BRCA1 protein, whereas in MCF-10A cells BRCA1 protein was barely detectable in untreated cells [40]. In contrast, our findings do not support the data of [9] where it was reported that *BRCA1* mRNA levels are higher in normal mammary epithelial tissue relative to invasive breast cancers [9]. This discrepancy in *BRCA1* transcript level in different cancer cell lines may be because of the oligonucleotide primers used for its amplification. In our study, the primers used in the real-time PCR detects all the known six *BRCA1* mRNA variants. The detection of novel BRCA1 transcript variants and proteins raises the possibility that various species could play different biological roles, and the maintenance of an appropriate ratio between them is possibly essential for normal function [32].

It was suggested that stable/overexpressed BRCA1 may be functionally impaired. However, functional impairment of BRCA1 is difficult to identify. Increased transcript levels of *BRCA1* might mirror an endeavour of a cell to compensate for loss of BRCA1 function. An example to support this hypothesis is what has been found that the expression of an exogenous *BRCA1* in MCF-7 cells inhibited their growth, although MCF7 cells express a high steady-state level of endogenous BRCA1 protein [42]. High expression of *BRCA1* mRNA has been shown to correlate with increased proliferation [32]. Furthermore, increased BRCA1 expression in human breast cancer cell lines has been reported to increase resistance to DNA-damaging chemotherapeutic agents [43-45]. Thus, our finding suggests targeting the *BRCA1* gene in the tumours expressing it via using drugs or specific si-RNAs that interfere with its expression in cancer but not normal tissues.

BRCA1 has been shown to contain several well-defined functional domains. An N-terminal RING finger domain interacts with some proteins including E2F transcription factor family members [46]. The E2F family members have been verified in many reports to have important roles in regulating normal and tumour cell proliferation. Among those E2Fs,

E2F6 has been shown to be an effective transcriptional repressor [47-49]. Overexpression of E2F6 in mouse embryonic fibroblast cell line has been demonstrated to inhibit entry into S phase [48] and can delay the exit from S phase as well [49].

E2F6 was proposed to negatively regulate BRCA1 in HEK293 (human embryonic kidney 293) cell line [36]. Therefore, in our study, *E2F6* mRNA level was transiently depleted from MCF-7 and MCF-10A cells using three different si-RNAs specific for *E2F6*, and the correlation between *E2F6* and *BRCA1* cDNA levels was studied. Our data demonstrate no correlation was found between *E2F6* knockdown and *BRCA1* expression in MCF-7 cells. However, it has been shown that not all E2F6 target genes are affected by reduction of the levels of E2F6 protein, and many of those targeted genes were found not to bind by E2F6 [36]. For instance, E2F6 was found to bind to the *art27* (Androgen receptor trapped clone 27) gene promoter in HEK293 cells but not in HeLa cells, despite E2F6 protein is overexpressed in the latest cells [50]. These findings show that E2F6 has target gene selectivity and cell type specificity [36]. This suggests that E2F6 might not bind *BRCA1* promoter in breast cancer cell lines. On the other hand, if E2F6 protein occupies *BRCA1* promoter in breast cancer cells (this needs to be investigated), then E2F6 might lost its control on *BRCA1* in cancer cells. However, our data clearly indicate that E2F6 negatively regulates *BRCA1* in the immortalized non-tumourigenic cell line using three different si-RNAs (Fig. 3).

A larger study focusing on investigating the correlation between E2F6 depletion and BRCA1 expression at the protein level in both normal and cancerous breast cell lines employing different BRCA1 antibodies that can match the different BRCA1 splice variants would be very helpful in verifying this correlation at the protein level. Because *BRCA2* mRNA has a tissue-specific expression pattern similar to that of *BRCA1* mRNA and that those two genes are coordinately controlled during mammary epithelial proliferation and differentiation, therefore; we suggest studying the expression of BRCA2 at both the mRNA and protein levels in different breast cancer cell lines and to check its relationship with E2F6 expression.

Overall, we can conclude that *BRCA1* is overexpressed in breast cancer cell lines relative to the normal breast, and E2F6 could be a negative regulator of BRCA1 in normal breast but not cancerous cell lines.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: IJL. Performed the experiments: IJL and NAMA. Analyzed the data: IJL. Wrote the paper: IJL and NAMA.

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