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FLI1 Expression in Breast Cancer Cell Lines and Primary Breast Carcinomas is Correlated with ER, PR and HER2

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ABSTRACT

FLI1 is a member of ETS family of transcription factors that regulate a variety of normal biologic activities including cell proliferation, differentiation, and apoptosis. The expression of FLI1 and its correlation with well-known breast cancer prognostic markers (ER, PR and HER2) was determined in primary breast tumors as well as four breast cancer lines including: MCF-7, T47D, MDA-MB-231 and MDA-MB-468 using RT-qPCR with either 18S rRNA or ACTB (β-actin) for normalization of data. FLI1 mRNA level was decreased in the breast cancer cell lines under study compared to the normal breast tissue; however, Jurkat cells, which were used as a positive control, showed overexpression compared to the normal breast. Regarding primary breast carcinomas, FLI1 is significantly under expressed in all of the stages of breast cancer upon using 18S as an internal control. This FLI1 expression was correlated with ER, PR and HER2 status. In conclusion FLI1 can be exploited as a preliminary marker that can predict the status of ER, PR and HER2 in primary breast tumors.

Keywords: *FLI1*, RT-qPCR, Breast cancer cell lines, Primary breast tumors, ER, PR, HER2

Abbreviations: ER: Estrogen Receptor; *FLI1*: Friend Leukemia Virus Integration 1; HER2: Human epidermal Growth Factor Receptor 2; IHC: Immunohistochemical; PR: Progesterone Receptor; ETS: E26 transformation-specific

INTRODUCTION

The genes of ETS family of transcription factors express in a variety of tissues and their proteins either positively or negatively regulate transcription depending on the cellular and promoter situation [1,2]. Friend leukemia virus integration 1 (*FLI1*) is a member of the ETS family. *FLI1* plays a critical role in normal development, differentiation, proliferation, homeostasis, apoptosis, oncogenesis and functioning as both a transcriptional activator and repressor [1,3-5].

The *FLI1* gene was first recognized as a proto-oncogene as it is aberrantly expressed in mouse erythroleukemia induced by retrovirus known as Friend murine leukemia virus (F-MuLV) [6,7]. *FLI1* has been shown to encode two proteins, *p48* (419aa) and *p51* (452aa) [7], and this gene consists of nine exons that extend over about 120 kb [8]. In humans, *FLI1* is involved in the development of Ewing sarcoma and related primitive neuroectodermal tumor subtypes [9]. *FLI1* makes fusion genes with the EWS gene in human Ewing sarcoma by chromosomal translocations. In this translocation, the C-terminal region of *FLI1* including the ETS domain and the N-terminal region of EWS are fused [10]. The fusion protein is characterized by bigger transactivation relative to wild-type *FLI1*, thereby contributing to malignant transformation of the cells [11].

It has been shown that *FLI1* expression promotes the progression of human breast cancer and associate with breast cancer malignancy by controlling the anti-apoptotic bcl-2 gene, thereby inhibiting apoptosis in invasive breast cells [12]. Another study showed that *FLI1* binds to an ETS consensus site within the retinoblastoma (*Rb*) gene resulting in the transcriptional repression of *Rb* and cell growth conservation [13]. *FLI1* functions as a potent transcriptional repressor via chromatin remodeling along with competition with the transcriptional activator, ETS1 [14]. Moreover, other studies performed in erythroleukemic cells demonstrated that *FLI1* negatively controls *p53*, the tumor suppressor,

via direct binding to and up-regulating MDM2 [15]. Loss of *FLI1* expression was found to be associated with shorter survival, accelerated tumor growth and more aggressive breast cancer phenotypes [16].

In the present study, we measured the expression of *FLI1* in breast cancer cell lines and primary breast tumors using RT-qPCR. Two internal control reference genes were used separately to normalize the data of *FLI1* gene expression to obtain a clear picture of *FLI1* mRNA level. Furthermore, we examined the possible correlation between ER, PR and HER2/neu/ErbB2 status and *FLI1* transcript level. For our knowledge, no reports illustrating the relation between *FLI1* expression and the aforementioned breast cancer prognostic markers are available so far.

MATERIALS AND METHODS

Cell culture

All cell lines, except Jurkat, used in this study were obtained from Sigma-Aldrich, UK. The breast cell lines included: MCF-7, T47D, MDA-MB-231 and MDA-MB-468. The 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). Jurkat cells, the positive control, were a kind gift from Prof. Matthew Holley, Department of Biomedical Science, The University of Sheffield, Sheffield, UK. These cells were grown in RPMI 1640 (Roswell Park Memorial Institute medium; Lonza, UK) containing L-glutamine, 10% FCS and 1x NEAAs were added.

Mycoplasma infection for cell culture was checked periodically for each cell line used. This test was performed using PCR Mycoplasma Test Kit (EZ-PCR Mycoplasma Test Kit, Geneflow, UK) in the Department of Oncology, Medical school, the University of Sheffield, Sheffield, UK.

Primary breast tumors and normal mammary tissues

To further explore the expression of *FLI1* in primary tumor samples, a cDNA panel of 48 female dried samples (including 43 breast tumors; representing four different TNM stages of breast cancer) and 5 normal breast tissues were obtained from Origene Technologies (TissueScan Breast Tissue qPCR Array, Cat. No. BCRT302, USA). All the patient information is stated regarding patient's age, tissue of origin, tumor stage, and pathology report including ER, PR and HER2/neu using immunohistochemistry (IHC) for each, along with using FISH for HER2/neu weakly positive by IHC. However, for some of the tumor samples, the above prognostic markers (i.e., ER, PR and HER2) were not detected.

Making cell pellets

Breast cancer cell lines were grown to about 80-90% confluence. After removing the media from the flask, washing three times with phosphate-buffered saline (PBS), and discarding PBS, 1 ml trypsin-versene (EDTA) was added to the cells, and left in an incubator at 37°C, 5% CO_2 until the cells detached from the flask. Fresh DMEM described above (9 ml) was added to the cells to inhibit trypsin, and mixed with the cells in about 10 times by up and down pipetting. Then, 10 ml cells with medium were centrifuged at 1500 rpm using (Heraeus MegaFuge 16) for 3 minutes to get cell pellets. Regarding Jurkat cells, they were collected into a suitable tube by taking the volume from the cultured medium that is equivalent to ~30×10^6 cells. Cells were spun as above, and after removing the media, cell pellets were washed twice with PBS at 1500 rpm for 3 min each as before. The supernatant was removed, and the pellets were re-suspended in 1 ml PBS in an Eppendorf tube and spun as above using (Mini Spin Eppendorf centrifuge). Finally, after discarding PBS, the pellets were stored at -80°C for RNA extraction.

RNA extraction

Total RNA from breast cancer cell line pellets was extracted using RNeasy® Mini Kit (Qiagen, UK). Manufacturer's instructions were applied to obtain total RNA. RNA concentration was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific), and was converted immediately to cDNA as below.

Reverse transcription

Following extraction, 1 µg of total RNA was converted to cDNA using a High Capacity RNA-to-cDNA kit (AB

Applied Biosystems) in a total volume of 20 µl reaction. The recommendations of the manufacturer were followed, and the cDNA was kept at -20°C until further use. Concerning primary breast tumors, cDNA panel of dried samples were obtained from Origene Technologies, as mentioned earlier.

Primers for RT-qPCR

Oligonucleotides used in this study were bought from Eurofins, Germany. Two sets of primer pairs were used for *FLI1* termed *FLI1*#1 and *FLI1*#2 to amplify two different regions of its mRNA sequence. Either 18S or ACTB was used as an internal control to detect the expression of *FLI1* in breast cancer cell lines and primary tumors using RT-qPCR. The forward or reverse primer of each gene was put at the junction between two exons to avoid contamination with amplified genomic DNA. Amplicons were approximately 100 nucleotides long. All the oligonucleotides supplied were dried, and suitable amounts of deionized distilled water (ddH_2O) were added to make a stock concentration of 100 µM as stated by the manufacturer. The 18S and ACTB primers were used in 5 µM concentration, whereas *FLI1* oligonucleotides were diluted to 10 µM concentration using ddH_2O prior to use and stored at -20°C. Table 1 shows primers and their sequences used for RT-qPCR amplification.

Table 1 Primer sequences of the studied genes

Primer	Forward Primers	Reverse Primers
FLI1#1	5'-GAATTCTGGCCTCAACAAAAG-3'	5'-CCCAGGATCTGATACGGATCT-3'
FLI1#2	5'-ATCCAGCTGTGGCAATTCCT-3'	5'-CATCGGGGTCCGTCATTTTG-3'
18S	5'-AGAAACGGCTACCACATCCA-3'	5'-CACCAGACTTGCCCTCCA-3'
ACTB	5'-CAGCCATGTACGTTGCTATCCAGG-3'	5'-AGGTCCAGACGCAGGATGGCATG-3'

RT-qPCR

The RT-qPCR assay was conducted using a Corbett Robotics Rotor-Gene™ 6000 (Qiagen) to study the expression of *FLI1* and the reference genes in breast cancer cell lines and primary breast carcinomas. Concerning cancer cell lines, each reaction consisted of 20 µl of 2× SensiMix (10 µl), 3 µl of ddH_2O, 2 µl of 10× forward and reverse primers with 5 µl of cDNA template. For primary tumors where each well contained an average amount of 2-3 ng dried cDNA, adjusted amounts of SensiMix and ddH_2O mixture were added to each well of the cDNA plate, mixed with the dried cDNA by pipetting up and down using filter tips, and left for 15 min on ice to dissolve the dried cDNA. Afterwards, 13 µl of the above mixture was moved to each qPCR tube and then mixed with 2 µl of 10× suitable primers. Finally, the qPCR tubes, without air bubbles, were sealed and placed in the 72 well rotor of the RT-qPCR. Reactions were carried out in duplicate or triplicate technical repeats for each cDNA sample. The cycling conditions were 95°C for 10 minutes for *Taq* polymerase activation followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 30 s. Amplification was followed by melt analysis, which was achieved to check for presence of primer dimmers as well as amplification of a single product. Melting program was 72°C to 95°C, hold secs on the first step and hold 5 secs on next steps.

Quantity of the PCR product is proportional to the fluorescence signal. Using the software provided by the instrument manufacturer, Ct values were quantified for each gene in every reaction. Each experiment included a non-template control. Data were normalized to either 18S or ACTB transcript levels.

Statistical analysis

Standard deviation was included, which shows how much the data are spread out around the mean or average. The results were represented by median to avoid the influence of outliers. SPSS 22.0 software (IBM) for Windows was used to analyze the data. Comparisons among different cancer cell lines or among breast tumors and normal breast tissues were analyzed using the non-parametric Mann-Whitney U test. Differences between gene expression levels were considered significant at confidence levels larger than 95% ($P<0.05$).

RESULTS

Expression of FLI1 in breast cancer cell lines

Two sets of primer pairs specific for *FLI1* mRNA were used to amplify this gene and either ACTB or 18S was used for normalization of RT-qPCR data. Our data showed decreased levels of *FLI1* cDNA in breast cancer lines:

MCF-7, T47D, MDA-MB-231 and MDA-MB-468 in comparison to normal breast cDNA. In contrast, Jurkat cells overexpressed *FLI1* compared to other cells (Figure 1). Similar results in breast cancer cell lines and Jurkat cells were obtained when normalizing RT-qPCR findings with 18S or ACTB (data not shown for ACTB normalization).

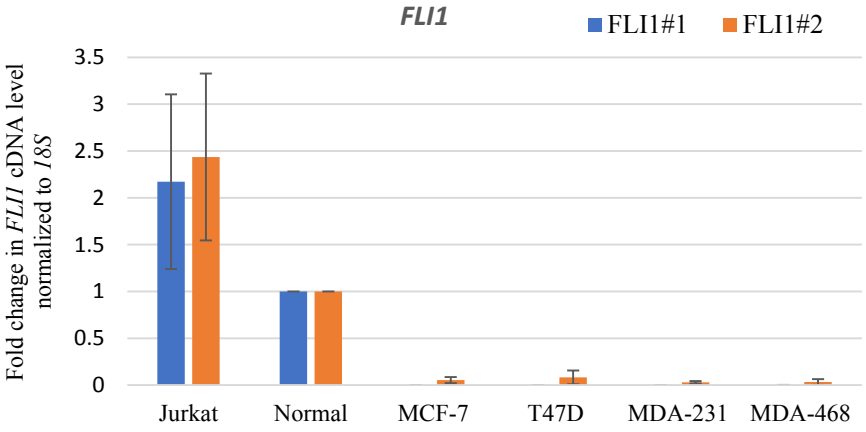

Figure 1 Under-expression of *FLI1* gene in breast cancer cell lines compared to normal breast and Jurkat cells

RNA extracted from the above cancer cell lines in addition to the normal breast tissue RNA was converted to cDNA. RT-qPCR was used to determine the fold change in cDNA levels of *FLI1* gene normalized to 18S and relative to normal breast cDNA. The blue bars represent the mean of *FLI1* cDNA levels amplified with *FLI1*#1 primer, and the yellow bars are the *FLI1* cDNA mean amplified by *FLI1*#2 primer. Error bars denote the standard deviation of at least three independent experiments.

Expression of *FLI1* in primary breast tumors

Forty-three primary tumor cDNAs representing four different stages of breast cancer with five normal breast samples were used to check *FLI1* expression. Mean age of patients was 56.4 years (range 34-84 years). Fold change in *FLI1* expression in primary carcinomas was calculated firstly relative to ACTB and normalized with normal cases. *FLI1* showed significant overexpression (P=0.03) in all the tumor samples relative to normal. However, *FLI1* increased expression was not significant throughout stages I, II and IV (Figure 2). On the other hand, normalization with 18S revealed statistically significant decreased mRNA levels of *FLI1* (P=0.008) in the largest part of the samples regardless of the tumor stage, and significant underexpression of *FLI1* was noticed in all of the stages except stage II (Figure 3).

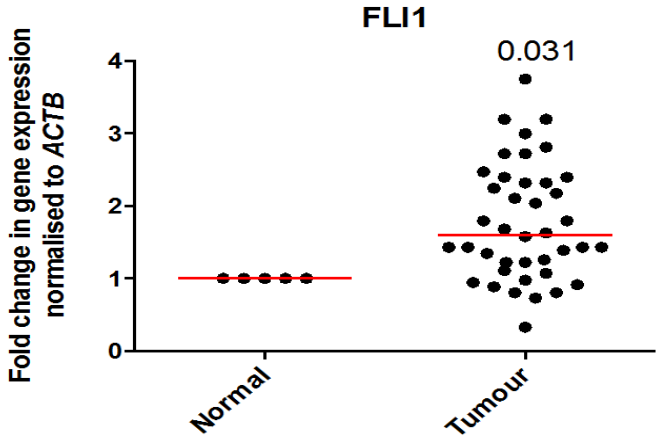

Figure 2 (A) *FLI1* expression in all the studied primary tumors regardless of cancer stage

Figure 2 *FLI1* gene shows statistically significant difference between cDNA levels in breast tumor and normal samples when normalizing with ACTB

Total 48 Breast cDNAs were used in RT-qPCR to determine the fold change in cDNA levels of FLI1 normalized to ACTB and relative to normal breast cDNA. The samples included 43 tumor cDNAs (stages I-IV) and 5 normal cDNAs. Each point represents the mean of two technical repeats. The horizontal lines refer to the median value. The decimal numbers are the P values for the significant difference between the normal and tumor samples using Mann-Whitney U test.

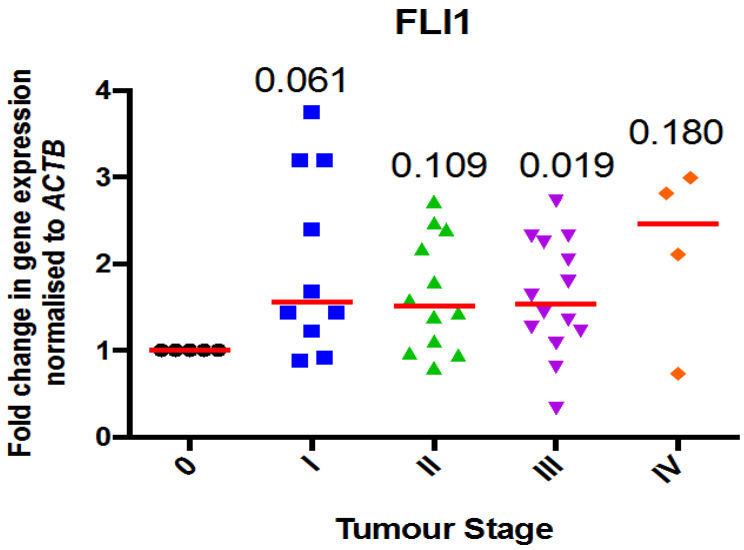

Figure 2 (B) The *FLI1* cDNA level in different stages of breast cancer

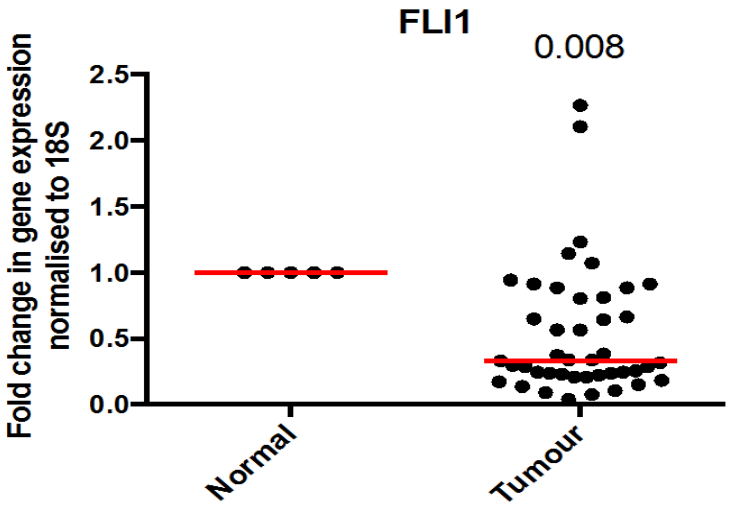

Figure 3 (A) Fold change in the *FLI1* cDNA levels of all samples regardless of the cancer stage

Figure 3 FLI1 gene shows statistically decreased cDNA levels in breast tumor samples relative to normal breast

Total 48 breast cDNAs were used in RT-qPCR to determine the fold change in cDNA levels of *FLI1* normalized to 18S and relative to normal breast cDNA. The samples included 5 normal (Stage 0) and 43 tumor cDNAs (stages I-IV). Each point represents the mean of duplicate technical repeats. The horizontal lines refer to the median value. The decimal numbers are the P values for the significant difference between the normal and tumor samples using Mann-Whitney U test.

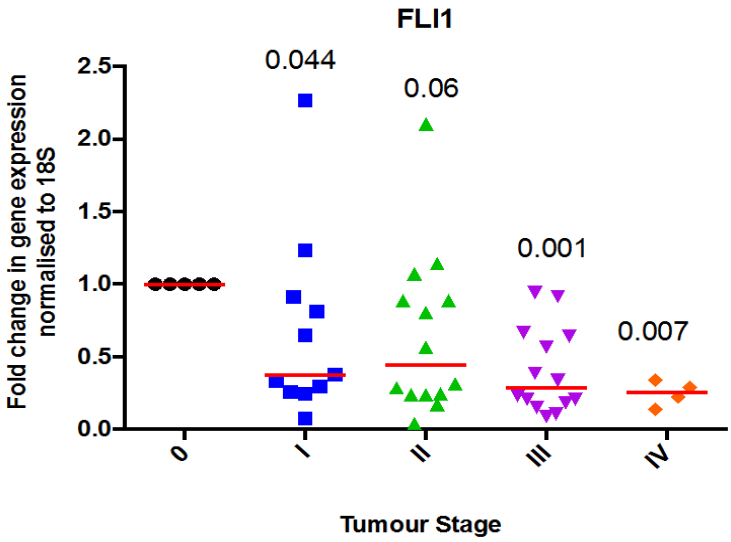

Figure 3 (B) Fold change in the *FLI1* cDNA levels throughout different stages (I-IV) of breast cancer

As shown in Figures 2 and 3 in primary breast tumors, normalization of RT-qPCR data with ACTB revealed unexpected *FLI1* expression where most samples were overexpressed. This result conflicts with that of breast cell lines; therefore, to address this issue and to obtain reliable and accurate results, primary breast tumors of the same Cat. No. were used to examine and compare the Ct values of both ACTB and 18S and to check their expression stability throughout different breast cancer stages. Our data demonstrate that the Ct values of 18S were approximately similar throughout different stages of cancer except for stage IV, which might be due to limited samples (Figure 4). In comparison, ACTB Ct values were fluctuated with less Ct values noticed in normal samples might be that is why most of the tumor samples had increased *FLI1* expression upon normalization with ACTB (Figure 4). Taken together according to the above findings, *FLI1* expression data normalized with 18S were depended in our comparisons in the primary tumors.

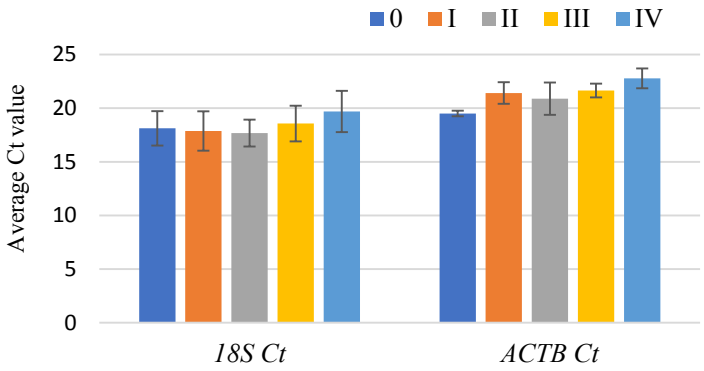

Figure 4 Comparing the Ct values of 18S and ACTB in normal breast and different stages of breast cancer

RT-qPCR was used to check the cDNA levels of ACTB and 18S in normal and cancerous breast from four different stages of breast cancer. The samples included 43 tumor cDNAs (Stages I-IV) and 5 normal cDNAs (Stage 0). The bars represent the average Ct values of the above reference genes in normal and cancer. Error bars represent the standard deviation of the data.

The correlation between *FLI1* expression and the stages of breast cancer along with ER, PR and HER2 expression in primary breast carcinomas

To see whether a relationship exists between cancer stage and *FLI1* expression, patients from different breast cancer stages were included in this study, as mentioned earlier. In this study, 14/43 (32.56%) of patients belonged to Stage II, and the same percentage (i.e., 32.56%) of patients had stage III. While 11/43 (25.58%) belonged to Stage I, 4/43

(9.30%) of cases belonged to stage IV. *FLI1* expression was checked in the above samples, which showed repressed mRNA level relative to 18S in all cancer stages with no difference among them compared to the normal (Figure 3B). Therefore, no correlation exists between *FLI1* transcript level and cancer stage.

To further examine the correlation of *FLI1* expression with the well-known breast cancer prognostic factors including: ER, PR or HER2, the same above primary tumors were used. Our findings showed decreased expression of *FLI1* in 39/43 (90.7%) cases of breast cancer. Furthermore, tumors underexpressing *FLI1* were 25/33 (75.75%) and 8/33 (24.24%) in ER/PR positive group and ER/PR negative group, respectively (Table 2). The decreased expression of *FLI1* was 24/32 (75%) in HER2 negative cases and 8/32 (25%) in HER2 positive patients (Table 3). Then, the relationship between *FLI1* mRNA level and ER, PR and HER2 collectively was estimated in the same samples, we noticed that 21/31 (67.7%) of the cases with reduced *FLI1* level showed ER-positive, PR-positive and HER2-negative. Additionally, 2/31 (6.5%) of the less expressed *FLI1* samples were triple negative and another two out of 31 (6.5%) were positive for each ER, PR and HER2. Finally, 6/31 patients (19.4%) whose *FLI1* mRNA level was decreased revealed ER-negative, PR-negative and HER2-positive (Table 4).

Table 2 Correlation of *FLI1* expression levels with ER/PR in primary breast carcinomas

No. Primary tumors	*FLI1* expression	ER status	PR status
25/33 (75.75%)	decreased	positive	positive
8/33 (24.24%)	decreased	negative	negative
Total tumors 33			

Table 3 Correlation of *FLI1* expression levels with HER2 in primary breast carcinomas

No. Primary tumors	FLI1 expression	HER2 expression
24/32 (75%)	decreased	negative
8/32 (25%)	decreased	positive
Total tumors 32		

Table 4 Correlation of *FLI1* expression levels with known breast cancer prognostic parameters including ER status, PR status and HER2 expression collectively in primary breast carcinomas

No. Primary tumors	*FLI1* expression	ER status	PR status	HER2 expression
21/31 (67.7%)	decreased	positive	positive	negative
2/31 (6.5%)	decreased	negative	negative	negative
6/31 (19.4%)	decreased	negative	negative	positive
2/31 (6.5%)	decreased	positive	positive	positive
Total tumors 31				

DISCUSSION

This study analyzed the expression levels of *FLI1* in breast cancer cells as well as primary breast tumors using RT-qPCR normalized with either 18S or ACTB. Our data show that *FLI1* transcript level was underexpressed in the studied breast cancer cell lines using two different *FLI1* sequence-specific primers and normalizing with either reference gene. This result is consistent with that of Jin et al. [17] who reported that *FLI1* mRNA and protein levels are decreased in the human breast cancer cell lines. Unlike the study by Sakurai [12] who found *FLI1* overexpression in MDA-MB-231cells whereas many studies are consistent with loss of *FLI1* expression in breast cancer, the exact expression pattern could be cancer specific. For instance, *FLI1* may have an oncogenic function in colon cancer [18]. The *FLI1* gene amplification in acute myeloid leukemia (AML) was discovered in two case reports [19,20], and was found to be overexpressed in two acute promyelocytic leukemia patients compared with four normal samples [21]. Expression of *FLI1b*, an alternative spliced form, was identified in two human B-cell leukemias [22]. In this study we report increased transcript levels of *FLI1* in Jurkat cells (leukemia T-lymphocytes) compared with normal breast tissue or other cancer cell lines.

Concerning primary breast tumors, our findings show that the *FLI1* expression pattern was heterogeneous when normalizing with ACTB. Although, the use of 18S for normalization revealed different *FLI1* expression pattern, where almost all tumor samples were significantly down regulated (90.7%) relative to the normal breast tissue. This result obtained with 18S was consistent with the findings of Jin et al. [17], in which it has been found that *FLI1* mRNA

levels were decreased in a small sample cohort comprising of seven human primary breast carcinomas compared with eight normal breast tissues. The conflicting results of normalizing tumor samples of this study with either reference gene were confusing as the main drawback of RT-qPCR is the normalization of the amplified products. Our findings reveal gradual increase in Ct values of ACTB with the lowest Ct values shown in the normal samples, and this is probably the cause behind increased expression of *FLI1* throughout different stages of cancer although it is not significant. In contrast, 18S Ct values look more stable than that of ACTB through the cancer stages.

Furthermore, the correlation of *FLI1* mRNA level with the breast cancer prognostic markers was determined in primary breast tumors. Our data highlight presence of correlation between the decreased expression of *FLI1* with the positivity of both ER and PR, along with existence of a relationship between *FLI1* under-expression and HER2-negative status. Regarding the correlation between *FLI1* expression and the above prognostic biomarkers in cancer cell lines, both MCF-7 and T47D are well-known to be ER-positive, PR-positive and HER2-negative [23,24] and showed decreased *FLI1* transcript. However, MDA-MB-231 and MDA-MB-468 are ER- and PR-positive and their *FLI1* was under-expressed [24]. As all of those cell lines and numerous primary breast tumors showed HER2 negative and reduced *FLI1* mRNA, so there might be a correlation between these genes, therefore, more and more breast cancer cell lines and primary carcinomas need to be examined for their *FLI1* expression and its relationship with HER2. Although in another study *FLI1* overexpression did not affect the expression levels of HER2/neu [12]. *FLI1* has been proven to play a key role as a negative regulator of the *ERα* gene in dermal fibroblasts [25]. In the same study of [25] expression of *ERα* was found to be increased in response to small interfering RNA (siRNA)-mediated *FLI1* knockdown. *FLI1* was shown to bind to the proximal region of the ERa promoter, and detaches from the promoter upon treatment with transforming growth factor-β [25].

CONCLUSION

Overall, our findings reveal that *FLI1* decreased expression correlated with ER-positive, PR-positive and HER2-negative, collectively. Thus, *FLI1* can be exploited as a preliminary marker that can predict ER, PR and HER2 status.

DECLARATIONS

Acknowledgement

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