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DNAJC25 as a tumor suppressor candidate gene in breast cancer

Meme Kanserinde tümör baskılayıcı gen adayı olarak *DNAJC25*

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ABSTRACT

DNAJC25 gene is a member of the HSP40 (DNAJ) family, and it was suggested as a tumor suppressor gene in hepatocellular carcinoma. The aim of this study was to analyze the expression, genetic/epigenetic alterations, and prognostic value of the *DNAJC25* gene in breast cancer. *DNAJC25* transcript levels are upregulated in BT-20 and ZR-75-1 cell lines and downregulated in MDA-MB-231 cell line compared to the non-tumorigenic mammary epithelial cell line (MCF 10A) ($P< 0.05$). According to UALCAN in-silico tool, clinical breast cancer samples show significantly reduced levels of *DNAJC25* mRNA relative to the normal samples (P=1.47e-02). The Kaplan–Meier plotter tool shows that high *DNAJC25* expression is favorable for post-progression survival in breast cancer (P=0.0035). Point mutations or copy number variations of *DNAJC25* are uncommon in clinical breast cancer samples. Combined bisulfite restriction analysis (COBRA) results showed that *DNAJC25* promoter is not methylated in breast cell lines. Promoter hypomethylation was also observed in normal and tumor clinical samples (Beta-value<0.25). In conclusion, *DNAJC25* is suggested as a tumor suppressor candidate having limited biomarker potential in breast cancer. Functional studies are essential to reveal its role in breast carcinogenesis.

Key Words

DNAJC25, HSP40, biomarker, breast cancer.

ÖZ

DNAJC25 geni HSP40 (DNAJ) ailesinin bir üyesidir ve hepatoselüler karsinomada tümör baskılayıcı gen olarak önerilmiştir. Bu çalışmanın amacı, meme kanserinde *DNAJC25* geninin ifadesini, genetik/epigenetik değişimlerini ve prognostik önemini analiz etmekdir. *DNAJC25* mRNA ifadesi meme kanseri hücre hatlarında farklılık göstermektedir. Tümörijenik olmayan meme epitel hücre hattına (MCF 10A) göre, *DNAJC25* transkript seviyesi BT-20 ve ZR-75-1 hücre hatlarında upregüle olurken, MDA-MB-231 hücre hattında downregüle olmuştur (P<0.05). UALCAN in-siliko aracına göre, klinik meme kanseri örnekleri normal örneklere göre anlamlı oranda düşük *DNAJC25* mRNA seviyesi göstermektedir (P=1.47e-02). Kaplan–Meier plot çizerine göre, *DNAJC25* ifadesi meme kanserinde post-progres sağ kalımı için olumludur (P=0.0035). *DNAJC25* nokta mutasyonları veya kopya sayısı değişimleri klinik meme kanseri örneklerinde nadirdir. Kombine Bisülfit Restriksiyon Analiz (COBRA) sonuçları *DNAJC25* promotorunun meme hücre hatlarında metile olmadığını göstermiştir. Promotor hipometilasyonu ayrıca normal ve tümör klinik örneklerinde de gözlenmiştir (Beta-value<0.25). Sonuç olarak, *DNAJC25*, meme kanserinde biyobelirteç potansiyeli düşük bir tümör baskılayıcı adayı olarak önerilmektedir. Meme kanseri karsinogenezindeki rolünü ortaya çıkarmak için fonksiyonel çalışmalar gereklidir.

Anahtar Kelimeler

DNAJC25, HSP40, biyobelirteç, meme kanseri.

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INTRODUCTION

Breast cancer is a leading cause of cancer-related death among women worldwide [1]. New biomarkers or specific drug targets are needed for breast cancwer therapy. A number of Heat Shock Proteins (HSPs) and some DNAJ (HSP40) proteins are considered as biomarkers or selected as drug targets for cancer diagnosis/prognosis and treatment [2, 3]. The aim of this study was to analyze the expression, genetic/epigenetic alterations, and prognostic value of the *DNAJC25* gene in breast cancer.

DNAJ heat shock proteins constitute the biggest and most diverse sub-group of the HSP family. There are 49 DNAJ members which are divided into three subclasses: DNAJA, DNAJB and DNAJC. DNAJ proteins are regarded as "co-chaperones" as they regulate major chaperones; HSP70 and HSP90. Because of these regulatory actions DNAJ members are suggested as more specific and selective drug targets to be used in cancer therapy compared to major chaperones. Many DNAJ members are associated with carcinogenesis or they are regarded as biomarkers or drug targets [2, 3].

DNAJC25 gene is a member of the HSP40 (DNAJ) family, and it was suggested as tumor suppressor gene in hepatocellular carcinoma. *DNAJC25* was shown to be down-regulated in hepatocellular carcinoma (HCC) tissues relative to adjacent normal tissues. Besides, anti-carcinogenic properties of *DNAJC25*, such as colony growth inhibition and enhancement of apoptosis, were also shown [4]. According to the Protein Atlas, high *DNAJC25* mRNA is favorable for endometrial cancer (http://www.proteinatlas.org) [5]. This study provides some pieces of evidence regarding the expression, genetic/epigenetic regulation, and the prognostic value of the *DNAJC25* gene in breast cancer.

MATERIALS and METHODS

Cell lines

Breast cancer cell lines (BT-20, SK-BR-3, MDA-MB-231, ZR-75-1) and non-tumorigenic mammary epithelial cell line (MCF 10A) were kindly provided by Dr. I. Yulug (Bilkent University, Ankara, Turkey) and they cultured according to American Type Culture Collection guidelines (ATCC, Mannassas, VA, USA).

Real-Time qRT-PCR

RNeasy Mini Kit (Cat. No: 74104) (Qiagen, Germany) is used to isolate the total RNAs from the cell lines. RNAs are then used to synthesize cDNA by using Verso cDNA Synthesis Kit (Cat. No: AB1453A; Thermo Fisher Scientific Inc. MA, USA). Comparative CT method (2-ddCT method) [6] was used to measure the relative levels of *DNAJC25* mRNA levels by using iTaq™ Universal SYBR® Green Supermix (Cat. No: 1725120). CFX96 Touch™ Real-Time PCR Detection System was used according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., CA, USA). (*DNAJC25* Forward: 5'-AAC GAG AGC TCT GGA TCA AGG-3', Reverse: 5'-AGG CCC TTC ATT CTT CAT CCA-3'). *TBP* (TATA box binding protein) gene was used as a reference [7] (TBP Forward 5'-TGC ACA GGA GCC AAG AGT GAA-3', Reverse 5'- CAC ATC ACA GCT CCC CAC CA-3). Statistical analyses were performed by Student's t-test. The *P* value<0.05 was considered significant.

In Silico Tools

The relative *DNAJC25* mRNA values and promoter methylation levels of clinical breast cancer samples (TCGA dataset) was analyzed by the UALCAN in-silico tool (http://ualcan.path.uab.edu/index.html) [8]. The Kaplan-Meier plotter (KM plotter) (www.kmplot.com) was used to evaluate the survival values of *DNAJC25* mRNA levels in patients with breast cancer (The probeset Affy ID: 226859_at). The samples were divided according to *DNAJC25* expression as high and low by selecting "Auto select best cutoff" option of the software [9]. The software calculated the Log rank P value and the hazard ratio (HR) with 95% confidence intervals and displayed them on the graph. The *P* value<0.05 was considered significant.

Genetic alterations of *DNAJC25*

Point mutations and copy number variations of *DNAJC25* gene were screened by using Sanger COSMIC (Catalogue of Somatic Mutations in Cancer) database (v91) [10].

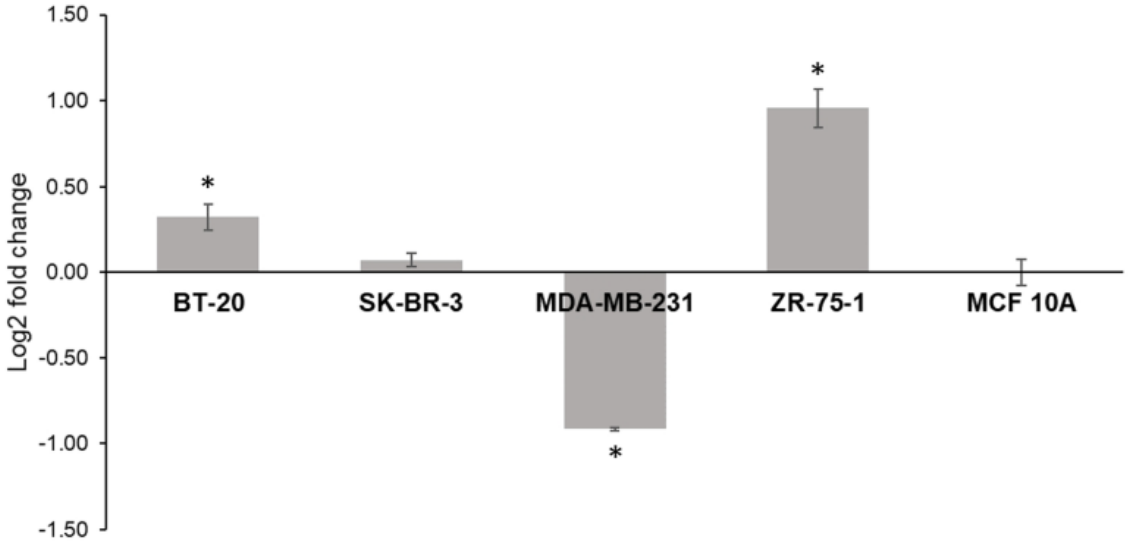

Figure 1. *DNAJC25* expression in breast cancer cell lines relative to MCF 10A (*P< 0.05).

Combined bisulfite restriction analysis (COBRA)

Genomic DNAs were isolated by DNeasy tissue kit (Qiagen, Germany) and treated with bisulfite by using EpiJET Bisulfite Conversion Kit (Cat. No: K146) (Thermo Scientific, MA, USA). The region of CpG island overlapping the DNAJC25 promoter was amplified by a Taq DNA polymerase (Cat. No: LSG-EP0402) (Thermo Scientific, MA, USA). (Forward 5'-GGG GAA GGT GTT TAG TGA TAT AT-3', Reverse 5'-AAT AAA ACC CCT CCA CCA AAA C-3'). PCR amplicons were digested with *Hha*I restriction enzyme (Cat. No: ER1851) (Thermo Scientific, MA, USA) as previously described [11]. *M.Sss*I enzyme (Cat. No: EM0821) (Thermo Scientific, MA, USA) was used to in-vitro methylate the genomic DNA of MCF 10A cell line and used as a positive control.

RESULTS

Differential expression of *DNAJC25* in breast cancer cell lines

DNAJC25 mRNA level is significantly reduced in MDA-MB-231 (claudin-low, triple negative) breast cancer cell line compared to the non-tumorigenic mammary epithelial cell line (MCF 10A). BT-20 (basal, triple negative) and ZR-75-1 (luminal, ER+) cell lines have significantly high levels of *DNAJC25* mRNA (*P*< 0.05) (Figure 1) [12]. No significant difference was observed between SK-BR-3 (HER2+) and MCF 10A cell lines regarding their *DNAJC25* transcript levels.

DNAJC25 mRNA expression is reduced in clinical breast cancer samples

According to the UALCAN in-silico tool [8], *DNAJC25* mRNA expression is reduced in clinical breast cancer samples (TCGA, n= 1097) compare to the normal samples (n= 114) (*P*= 1.47e-02) (Figure 2a). HER2+ (n= 37) and Triple-Negative Breast Cancer (TNBC) (n= 116) clinical tumor samples have significantly low levels of *DNAJC25* mRNA compared to normal samples (n= 114) (P= 7.04e-08 and *P*= 5.044e-12 respectively). Luminal samples (n= 566) have reduced levels of *DNAJC25* transcripts (*P*= 7.19e-01) (Figure 2b).

DNAJC25 expression is favorable for post-progression survival in breast cancer

The Kaplan–Meier plotter tool (http://kmplot.com) [9] shows that high *DNAJC25* expression is favorable for post-progression survival in breast cancer (n= 173, *P*= 0.0035) (Figure 3). But *DNAJC25* expression has no significant survival value for the overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) (Supplementary Information). Breast cancer subtypes (basal, luminal A, luminal B, HER2+) were also analyzed for survival outcomes based on their *DNAJC25* mRNA expression levels (Table 1, Supplementary Information). High *DNAJC25* mRNA expression is favorable for luminal A subtype in DMFS and PPS. *DNAJC25* mRNA expression is also favorable for HER2+ subtype in DMFS. But high *DNAJC25* mRNA expression is unfavorable for luminal B subtype in RFS and DMFS. It is also unfavorable for basal subtype in DMFS (Table 1, Supplementary Information).

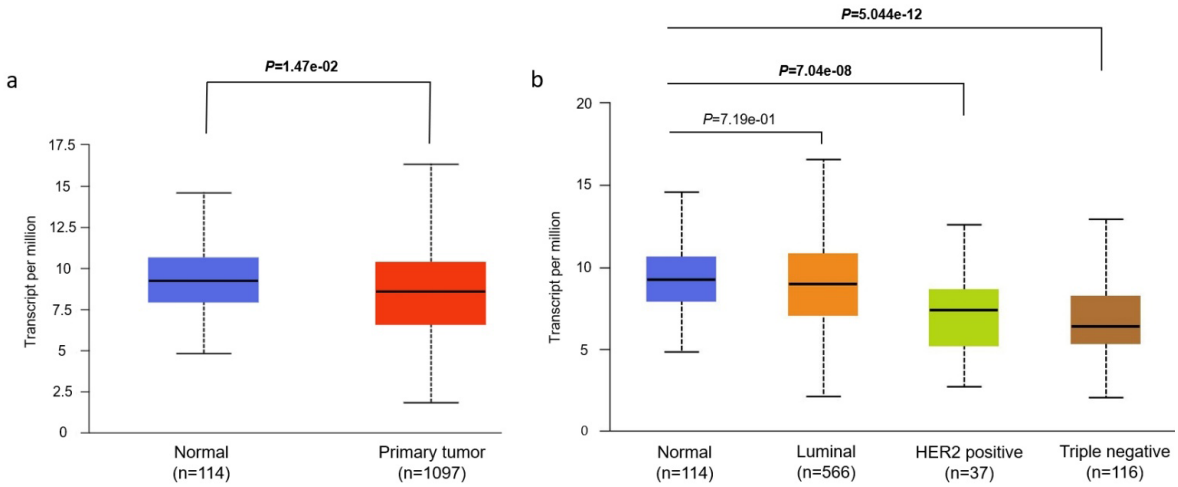

Figure 2. Relative *DNAJC25* transcript levels in clinical tumor samples. Transcript levels of primary tumors (a), and samples of breast cancer subtypes (b) relative to normal samples are shown. Modified screen-view from UALCAN web resource was used. Significant P values are shown as bold.

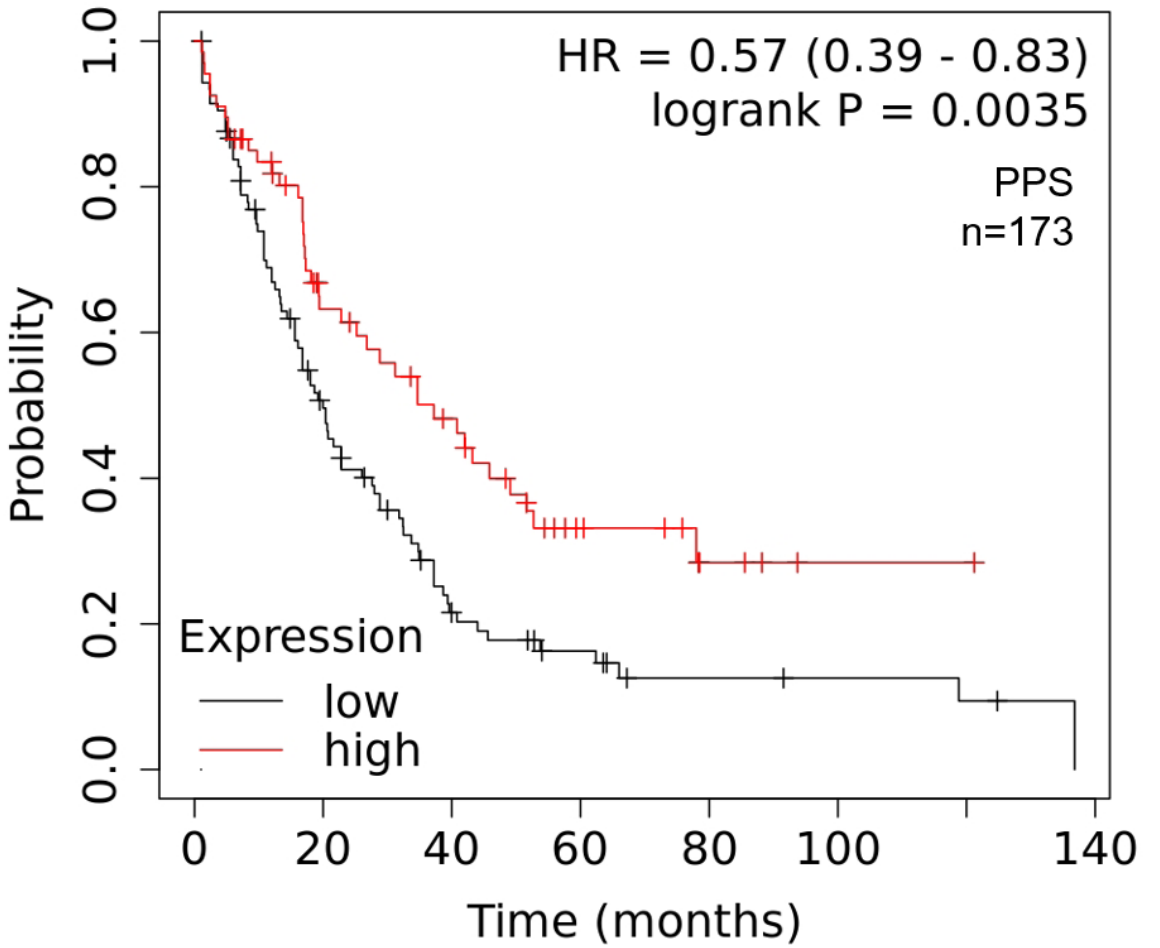

Figure 3. *DNAJC25* expression is favorable for post-progression survival (PPS) in breast cancer (n= 173, P= 0.0035). Kaplan-Meier plot was generated by KM plotter (The probeset Affy ID: 226859_at).

Table 1. Survival outcomes of *DNAJC25* mRNA expression in breast cancer subtypes analyzed by KM-plotter tool.

Breast Cancer Subtypes	Overall Survival	Relapse-Free Survival	Distant Metastasis Free Survival	Post Progression Free Survival
Basal	N.S. P= 0.14, HR= 1.66 n= 153	N.S. P= 0.077, HR= 1.34 n= 360	Unfavorable P= 0.047, HR= 2.08 n= 145	N.S. P= 0.078, HR= 0.46 n= 33
Luminal A	N.S. P=0.16 HR= 0.68 n=271	N.S. P= 0.076, HR= 1.28 n= 841	Favorable P=0.011, HR= 0.44 n= 281	Favorable P=0.045, HR= 0.55 n= 75
Luminal B	N.S. P= 0.13, HR= 1.68 n= 129	Unfavorable P= 0.0058, HR= 1.54 n= 407	Unfavorable P= 0.0065, HR= 2.41 n= 156	N.S. P= 0.17, HR= 0.56 n= 37
HER2+	N.S. P= 0.31, HR= 0.66 n= 73	N.S. P= 0.13, HR= 1.47 n= 156	Favorable P= 0.023, HR= 0.27 n= 82	N.S. P= 0.2, HR= 1.76 n= 28

(N.S.: Not Significant)

Genetic alterations of *DNAJC25* gene are uncommon in breast cancer

Frequency of point mutations and copy number variations (CNV) of *DNAJC25* is very low in breast cancer (Sanger COSMIC database, v92) [10] (Table 2).

DNAJC25 promoter region is hypomethylated in breast cell lines and clinical samples.

DNAJC25 gene has two experimentally validated promoter region, designated as *DNAJC25_1* and *DNAJC25_2* [13]. *DNAJC25_1*, is very active promoter region compared to the *DNAJC25_2* and it is located within the CpG island [13, 14] (Figure 4a). So, primers are designed to amplify the CpG island containing the *DNAJC25_1* promoter region. COBRA results showed that breast cell lines are not methylated. (Figure 4b). According to UALCAN web resource, clinical breast cancer samples (n=793) and normal samples (n=97) are also hypomethylated (beta values <0.25) (Figure 4c) [8]. Besides, no significant difference was observed between normal and tumor samples (*P*=1.01e-01). Samples of breast cancer subtypes are also hypomethylated (data not shown).

Table 2. Genetic alterations of *DNAJC25* in breast cancer.

	Mutated samples/Samples tested (Percentage of samples mutated)
Point Mutations	11/2569 (%0.43)
Copy Number Variations	
Gain	-
Loss	1/1492 (%0.07)

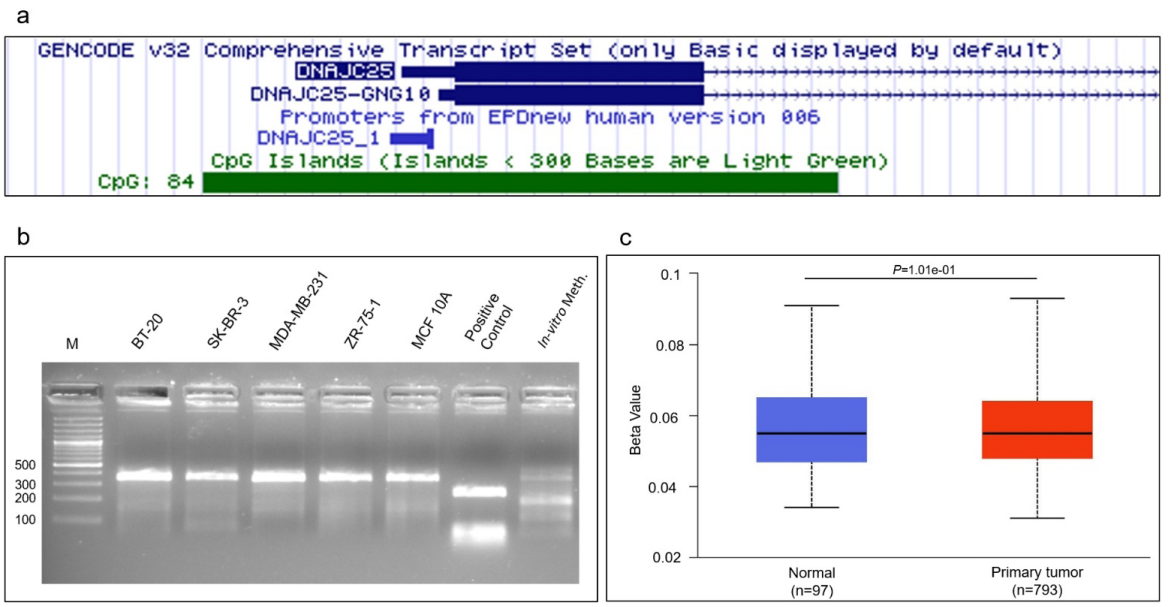

Figure 4. *DNAJC25* promoter region is hypomethylated in breast cell lines and clinical samples. a. Promoter region of *DNAJC25* gene (*DNAJC25*_1) overlapping the CpG island (screen-view of UCSC genome browser, http://genome.ucsc.edu) b. COBRA results of breast cell lines with the expected digestion fragments of 188 bp, 112 bp, 53 bp from a 353 bp PCR amplicon. M: DNA ladder; Positive Control: an amplicon of 597 bp having cutting site of HhaI restriction enzyme with the expected digestion fragments of 239 bp and small fragments (70bp<); In-vitro Meth: In-vitro methylated genomic DNA. c. Modified screen view from UALCAN in-silico tool showing hypomethylation of *DNAJC25* promoter in clinical breast samples (beta values <0.25).

DISCUSSION

Several lines of evidence showed that *DNAJC25* gene act as a tumor suppressor gene in hepatocellular carcinoma [4]. Besides, high *DNAJC25* mRNA is favorable for endometrial cancer (http://www.proteinatlas.org) [5]. The aim of this study is to analyze the expression, genetic/epigenetic regulation, and the prognostic value of the DNAJC25 gene in breast cancer.

Breast cell lines have differential *DNAJC25* mRNA expression; upregulated in BT-20 (triple negative) and ZR-75-1 (luminal, ER+) cell lines, downregulated in MDA-MB-231 cell line (TNBC) relative to the MCF 10A (P< 0.05) (Figure 1) [12]. BT-20 and MDA-MB-231 cell lines are both triple negative (ER-, PR-, HER2-) cell lines, but the expression levels of *DNAJC25* are significantly different (Figure 1). It is important to emphasize that MDA-MB-231 cell line is also regarded as "claudin-low" phenotype as the genes involved in tight junctions and cell-cell adhesion such as claudin-3, claudin-4, claudin-7, and E-cadherin are down-regulated [12, 15]. Moreover, different mutational signatures of those cell lines may also affect the expression level of *DNAJC25* [16].

DNAJC25 mRNA levels are reduced in clinical breast cancer samples compared to the normal samples (P=1.47e-02) (Figure 2). Regarding the prognostic survival values, *DNAJC25* expression is favorable for post-progression survival, but not OS, RFS, and DMFS (Figure 3, Supplementary Information). As we observed differential *DNAJC25* mRNA expression in breast cell lines representative of different subtypes (Figure 1), clinical samples from the breast cancer subtypes (basal, luminal A, luminal B, HER2+) were also analyzed for their *DNAJC25* expression (Figure 2b) and survival outcomes based on their *DNAJC25* expression levels (Table 1, Supplementary Information) by using UALCAN and KM-plotter tools, respectively. *DNAJC25* mRNA expression is reduced in clinical samples representing the breast cancer subtypes compare to the normal samples (Figure 2b). But survival outcomes have some subtype specific differences (Table 1, Supplementary Information). It should be noted that the cohorts used for the expression and survival analyses are not the same. UALCAN tool uses the TCGA datasets, but the KM-plotter uses the data gathered from Gene Expression Omnibus (GEO) and ArrayExpress.

Since, genetic alterations are infrequent, DNA methylation was sought as a possible regulation mechanism for *DNAJC25*. *DNAJC25* promoter is not methylated in breast cell lines (Figure 4b) and clinical breast cancer samples (Figure 4c). High levels of *DNAJC25* mRNA in BT-20 and ZR-75-1 cell lines could be explained by promoter hypomethylation. But this epigenetic event could not explain low levels of *DNAJC25* mRNA in MDA-MB-231 compare to MCF 10A. Some other epigenetic mechanisms, such as histone modifications or miRNAs, may be affecting the cellular *DNAJC25* levels in MDA-MB-231 cell line.

In conclusion, *DNAJC25* is downregulated in breast cancer, and its expression is favorable for post-progression survival. Functional assays are needed to clarify the role and regulation of this gene in breast cancer.

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