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Research Article

DOI:10.2478/rrlm-2022-0035

High *DNAJA4* expression correlates with poor survival outcomes in breast cancer

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Abstract

***Background**: DNAJA4 (PRO1472) is a heat shock protein that has been associated with several types of cancers, including breast cancer. We aimed to reveal the protein expression, clinical outcomes, and regulatory mechanisms of DNAJA4 gene in breast cancer by employing tissue microarrays, transcriptomic datasets, and in-silico tools.* ***Methods**: DNAJA4 protein expression and its clinical implications were evaluated by immunohistochemistry assay (normals = 32; tumors = 121). RNA-seq and DNA microarray datasets were analyzed by using breast cancer gene-expression miner (Bc-GenExMiner v4.8) to estimate the survival probabilities of breast cancer patients. DNAJA4 promoter methylation level was analyzed in clinical samples by UALCAN in-silico tool (normals = 97; tumors = 793).* ***Results**: DNAJA4 protein expression is significantly high in clinical breast cancer samples compared to the normal samples (P = 0.016). High DNAJA4 mRNA expression is correlated with poor overall survival (OS), disease-free survival (DFS), and distant metastasis-free survival (DMFS) in breast cancer patients (P < 0.05). Mutations or copy number variations of DNAJA4 are uncommon in clinical samples. Reduced promoter methylation was observed in clinical breast cancer samples.* ***Conclusion**: We suggest DNAJA4 expression as a new biomarker candidate for breast cancer. Promoter hypomethylation could be an important epigenetic factor in the upregulation of DNAJA4 expression in breast cancer.*

***Keywords**: breast cancer, survival, methylation, expression, DNAJA4*

Received: 6th April 2022; Accepted: 14th September 2022; Published: 23rd September 2022

Introduction

Breast cancer is a common cancer type among women worldwide (24.5%) with the highest mortality rates (15.5%) (1). Breast cancer has multi-subtypes, and this heterogeneous nature makes it particularly important to identify new biomarkers for clinical use (2).

Heat shock proteins (HSPs) are evolutionarily conserved proteins, and their expression was induced by environmental and metabolic stresses. DNAJ (HSP40) proteins constitute the largest HSP family and some of them have already been studied as diagnostic or prognostic biomarkers for many types of cancers. Besides, their expressions are associated with carcinogenesis (3).

In the last decade, *DNAJA4* has been associated with breast cancer and some other types of cancer. An in vitro study showed that *DNAJA4* gene is silenced in c-Myc overexpressing lung cancer cell line (209myc) by promoter hypermethylation (4). *DNAJA4* gene was shown to be hypermethylated in cell lines and clinical samples of rhabdomyosarcoma and Ewing sarcoma (5, 6).

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DNAJA4 acts as a metastasis suppressive factor by elevating the level of ApoE expression and it is downregulated by some miRNAs in melanoma (7). It is interesting to note that *DNAJA4* mRNA levels were found to be raised upon the treatment of HepG2 (human hepatocyte) cell line with the flavokawains, which have anti-cancer properties (8).

Unlike some other cancer types mentioned above, *DNAJA4* is upregulated in breast cancer (9, 10). Besides, *DNAJA4* mRNA expression is not significantly correlated with survival values in breast cancer (9-11). However, the results of the respective studies neither include the DFS, and DMFS (9, 11), nor the subtypes (10, 11).

In our study, new pieces of evidence were obtained regarding the protein expression, subtype-specific prognostic values, genetic alterations, and promoter methylation level of *DNAJA4* gene in breast cancer.

Material and Methods

Tissue Microarrays

Tissue microarrays (Biomax Inc., Rockville, MD, USA) were used in our immunohistochemistry study. They contain normal breast tissue samples (n = 32) and invasive ductal carcinoma samples (n = 121). Clinicopathological features of the samples can be seen in Supplementary material 1.

The statement of informed consent

Tissue microarrays were bought from Biomax Inc. (Rockville, MD, USA) which collects the tissues under HIPPA (Health Insurance Portability and Accountability Act) approved protocols with consents from all donors.

Immunohistochemistry

Novolink™ Min Polymer Detection System Kit (Cat. No: RE7290-K) (Novolink, Inc., CA, USA) was used to analyze DNAJA4 protein on tissue microarrays. Briefly, slides were incubated at 60°C overnight and deparaffinized by incubating them in xylene for 20 min. A series of ethanol was used for the rehydration of tissues sections. Slides were then cooked in citrate buffer (10 mM, pH 6.0) for antigen retrieval.

Unspecific background staining, which may result from endogenous peroxidases, was prevented by using the peroxidase block solution. Slides were incubated at 4°C overnight with the anti-DNAJA4 primary antibody (HPA041790; Atlas Antibodies, Stockholm, Sweden) (dilution 1:50). After the incubation with Novolink™ Polymer (30 min.), peroxidase activity was developed by DAB working solution (5 min.). Then, slides were counterstained with hematoxylin and different degrees of positivity (dark brown staining) were observed.

The qualitative scoring system was used to interpret the antigen expression according to an arbitrary scoring range (0-3) (12). Fisher's exact test was used for statistical analyses by using GraphPad software with a significance level of $P < 0.05$.

In silico Tools

Breast cancer gene-expression miner (Bc-GenExMiner v4.8) was employed to analyze the prognostic values of *DNAJA4* mRNA expression using a univariate Cox proportional hazards model with a significance level of $P < 0.05$ (13). RNA-seq (TCGA and SCAN-B) and DNA Microarray (METABRIC, and Affymetrix) datasets were analyzed in our study. The information of the datasets used in our study can be seen in Supplementary material 1.

The intrinsic molecular subtypes of breast cancer (normal-like, basal-like, luminal A, luminal B, HER2 positive) were classified by PAM50 (14) and analyzed for their survival outcomes.

UALCAN interactive tool was used to reveal the promoter methylation levels of *DNAJA4* gene in clinical breast cancer samples relative to normal

samples (TCGA dataset) (15). DNA methylation array data is used to create the box whisker plots which show average beta values ranging from "0" (unmethylated) to "1" (fully methylated). Statistical significance is calculated by Student's t-test considering unequal variance with a significance level of $P < 0.05$.

Screening of genetic alterations

Point mutations and copy number variations of *DNAJA4* gene were investigated by using Sanger COSMIC (Catalogue of Somatic Mutations in Cancer) database (v96) (16).

Results

DNAJA4 protein expression is upregulated in clinical breast cancer samples

According to our immunohistochemistry results, DNAJA4 protein expression (+1, +2, +3) is significantly more common in invasive ductal carcinoma samples (n = 121) relative to adjacent normal breast samples (n = 32) ($P = 0.016$) (Figure 1, Figure 2).

No significant associations were observed between the DNAJA4 protein expression and clinicopathological features of tumor samples (Supplementary material 2, Supplementary Table 1). The survival value of DNAJA4 protein expression could not be estimated for those clinical samples because the survival information of the patients is not available.

High DNAJA4 mRNA expression is associated with poor survival outcomes in breast cancer

Breast cancer gene-expression miner (Bc-GenExMiner, v4.8) was used to estimate the survival value of *DNAJA4* mRNA expression in clinical samples (13). According to the RNA-Seq and DNA microarray datasets, high *DNAJA4* expression is significantly correlated with poor overall survival (OS) in breast cancer (Figure 3a, 3b). Similar significant results were also obtained for disease-free survival (DFS) and distant metastasis-free survival (DMFS) (Figure 3c, 3d, 3f), except for the DMFS value gathered from RNA-seq datasets (Figure 3e).

Subtype-specific survival analyses were also performed in our study (Table 1). Higher levels of *DNAJA4* transcripts are significantly associated with the lower OS rates in luminal A and

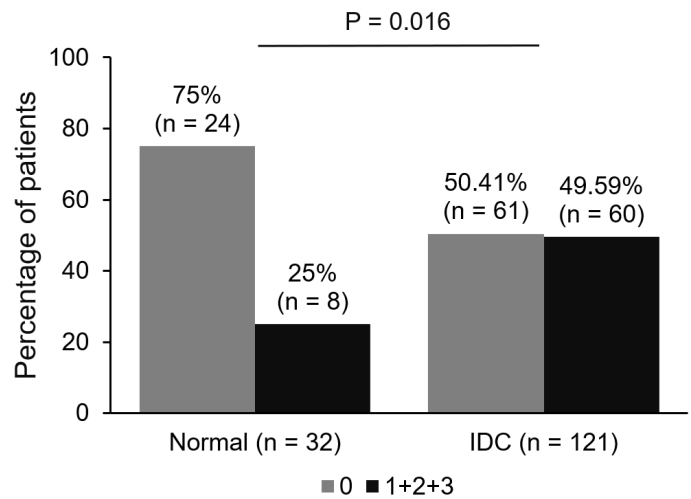

Fig. 1. DNAJA4 protein expression is upregulated in clinical breast cancer samples.

The intensity of immunostaining was graded relatively based on the following levels of staining scores: 0 (Negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The samples were grouped as negative (0) and positive scores (1+2+3). IDC: invasive ductal carcinoma samples.

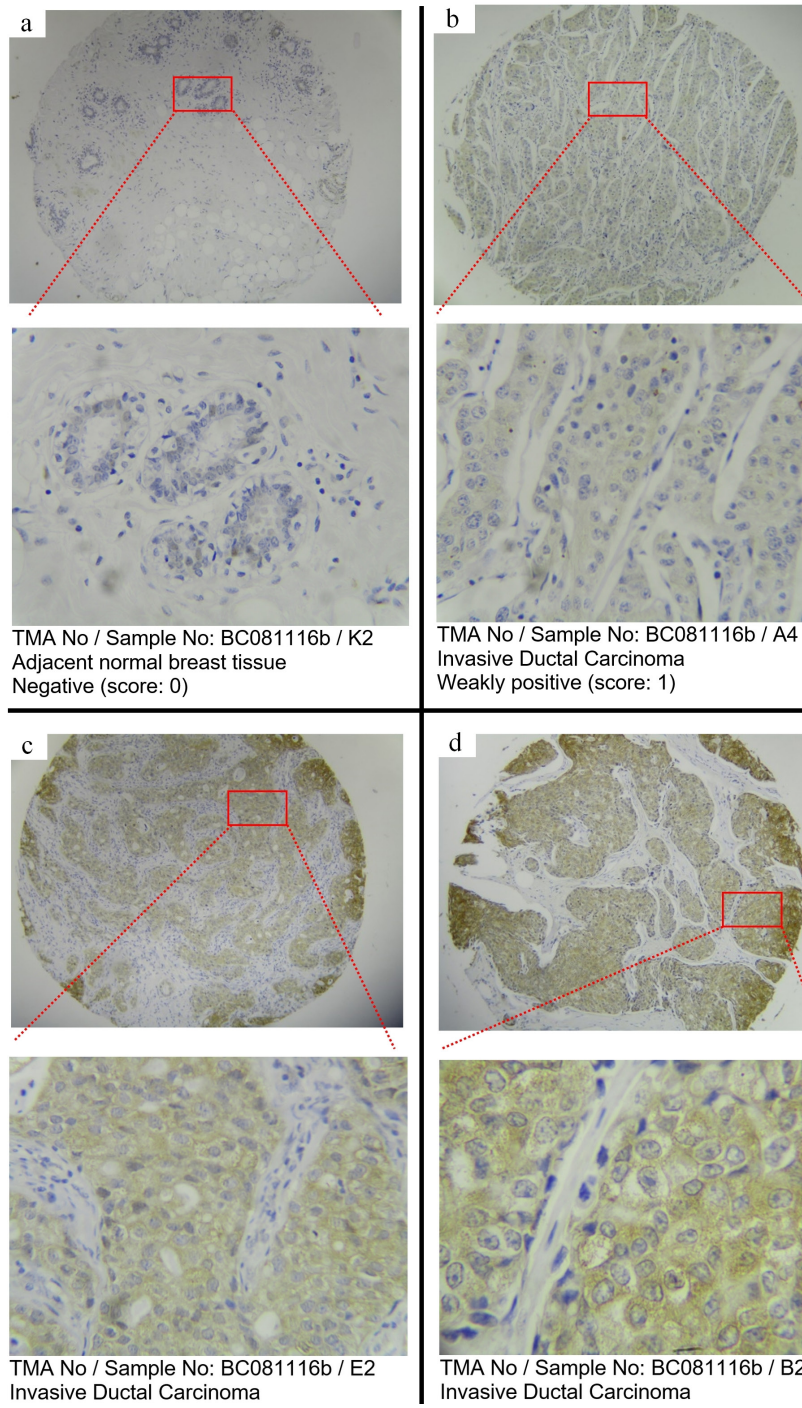

Fig. 2. Representative photographs from immunohistochemistry assay. (a) DNAJA4 protein expression was lost in normal breast tissue. (b, c, d) Invasive ductal carcinoma tissues have uniform and cytoplasmic DNAJA4 protein expression (brown) throughout the tissue. The selected regions (red rectangular) were magnified 400 times.

Table 1. Kaplan-Meier survival analyses of *DNAJA4* mRNA expression in breast cancer subtypes. Bc-GenExMiner (v4.8) in-silico tool was utilized for survival analysis of the breast cancer subtypes which are classified by PAM50.

	Overall Survival		Disease-Free Survival		Distant Metastasis-Free Survival	
	RNA-seq	DNA Microarray	RNA-seq	DNA Microarray	RNA-seq (TCGA)	DNA Microarray
Basal-like	N.S. P = 0.1999 HR = 1.35 n = 712	N.S. P = 0.0650 HR = 1.25 n = 969	N.S. P = 0.2419 HR = 1.30 n = 712	N.S. P = 0.1011 HR = 1.14 n = 1733	N.S. P = 0.0662 HR = 6.87 n = 136	N.S. P = 0.1749 HR = 1.16 n = 1263
Luminal A	N.S. P = 0.3216 HR = 0.80 n = 1205	Unfavorable P = 0.0147 HR = 1.26 n = 1407	N.S. P = 0.4078 HR = 0.82 n = 1205	Unfavorable P = 0.0377 HR = 1.16 n = 2463	Favorable P = 0.0435 HR = 0.23 n = 268	Favorable P = 0.0388 HR = 0.76 n = 1837
Luminal B	N.S. P = 0.0654 HR = 1.46 n = 891	N.S. P = 0.2882 HR = 1.11 n = 955	Unfavorable P = 0.0365 HR = 1.50 n = 891	N.S. P = 0.2909 HR = 1.09 n = 1686	N.S. P = 0.0644 HR = 2.56 n = 281	N.S. P = 0.1734 HR = 1.15 n = 1260
HER2 Positive	N.S. P = 0.1112 HR = 1.45 n = 619	Unfavorable P = 0.0036 HR = 1.45 n = 780	N.S. P = 0.0672 HR = 1.48 n = 619	Unfavorable P = 0.0073 HR = 1.28 n = 1318	N.S. P = 0.0989 HR = 0.13 n = 51	Unfavorable P = 0.0370 HR = 1.30 n = 966
Normal-like	N.S. P = 0.1109 HR = 0.58 n = 558	N.S. P = 0.0560 HR = 1.32 n = 632	N.S. P = 0.0720 HR = 0.55 n = 558	Unfavorable P = 0.0082 HR = 1.34 n = 1105	CSD n = 5	Unfavorable P = 0.0146 HR = 1.52 n = 829

CSD: Completely separated data; HR: Hazard ratio; N.S.: Not Significant; Favorable: High *DNAJA4* expression is associated with high survival probability; Unfavorable: High *DNAJA4* expression is associated with low survival probability

HER2 positive subtypes. DFS analyses showed that *DNAJA4* expression is unfavorable in all subtypes except the basal-like subtype. Breast cancer patients with luminal A subtypes show good DMFS rates with higher *DNAJA4* expression. But *DNAJA4* expression is unfavorable in HER2 positive and normal-like subtypes for DMFS. The basal-like subtype shows no significant association with the *DNAJA4* expression in any survival type.

Genetic alterations of DNAJA4 gene are infrequent in breast cancer

Based on the data obtained from Sanger COSMIC database (v96) (16), mutations and copy number variations of *DNAJA4* are infrequent in breast cancer (Table 2) which suggests that genetic alterations may not be the main contributor to the regulation of *DNAJA4* expression in breast cancer.

Methylation levels of DNAJA4 promoter are reduced in clinical breast cancer samples

CpG methylation is an important epigenetic regulatory mechanism in cancers such that DNA hypomethylation could promote carcinogenesis by activating the oncogenes, and DNA hypermethylation usually results in gene silencing (17). Experimentally validated promoter regions of *DNAJA4* gene, designated as *DNAJA4_1*, *DNAJA4_2*, are overlapped with the CpG island (18, 19) (Figure 4a).

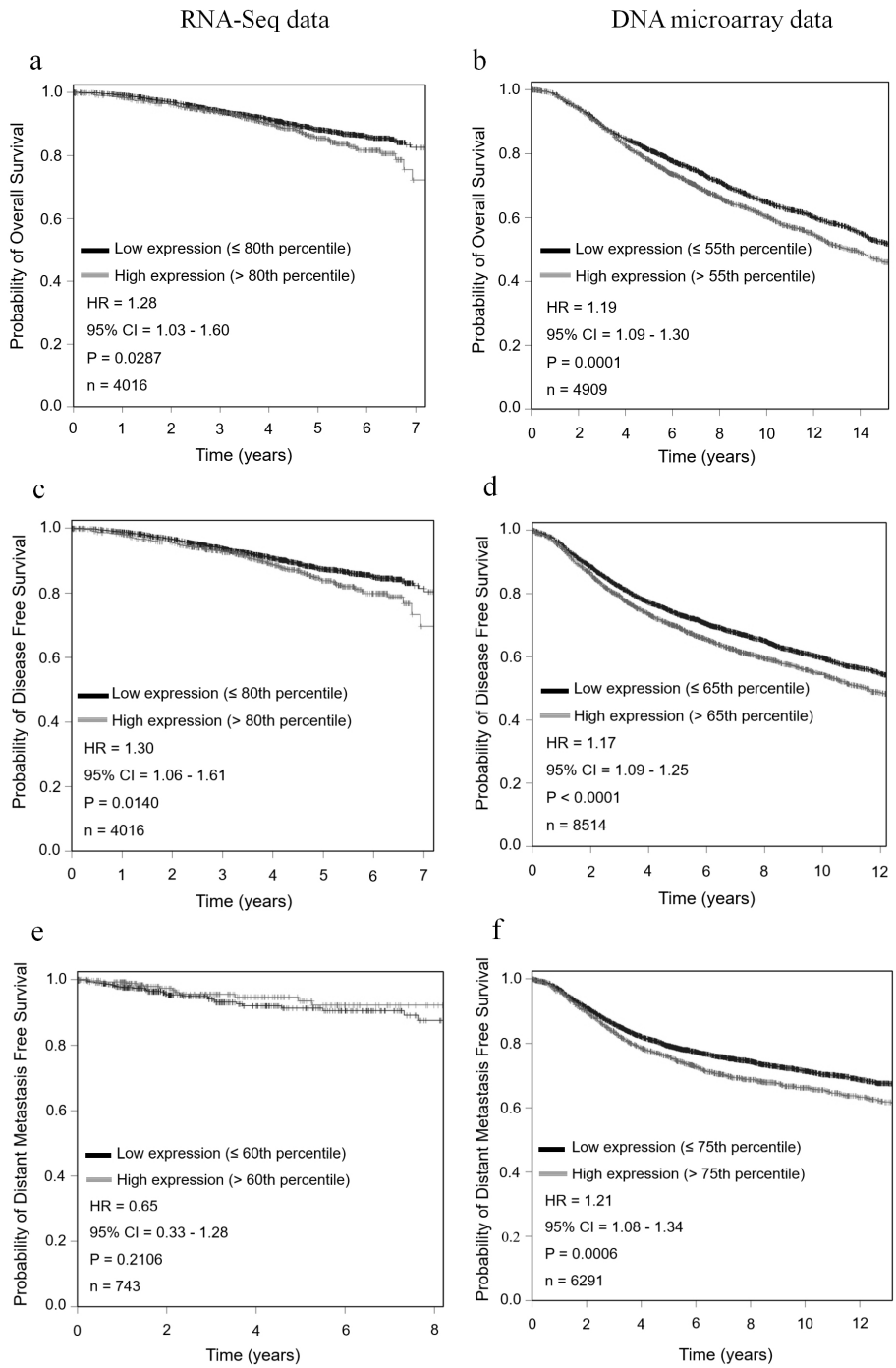

Fig. 3. High *DNAJA4* mRNA expression is correlated with poor survival outcomes in breast cancer. Kaplan-Meier survival analyses of RNA-seq (a, c, e) and DNA microarray (b, d, f) datasets were performed by Bc-GenExMiner (v4.8) in-silico tool. Breast cancer patients were stratified for high and low *DNAJA4* mRNA expression. The splitting criterion is chosen as "optimal" for the discretization and splitting is done according to all percentiles (20th to 80th). HR: Hazard ratio; CI: Confidence interval.

Table 2. Mutations and copy number variations of *DNAJA4* in breast cancer according to Sanger-COSMIC database (v96).

	Mutated samples/Samples tested (Percentage of samples mutated)
Point Mutations	21/2758 (0.76%)
Copy Number Variations	
Gain	6/1492 (0.4%)
Loss	1/1492 (0.07%)

Analysis of the TCGA clinical cohort with UALCAN in silico tool showed that the samples of the luminal and triple-negative subtypes have significantly reduced levels of *DNAJA4* promoter methylation compared to normal samples (Figure 4b, 4c). It should be noted that clinical tumor samples (TCGA) of luminal B and HER2+ subtypes have significantly higher *DNAJA4* mRNA levels compared to normal samples as shown in a previously published study (9). Analysis of the same datasets by using UALCAN in silico tool confirmed the findings (Supplementary material 2, Supplementary Figure 1).

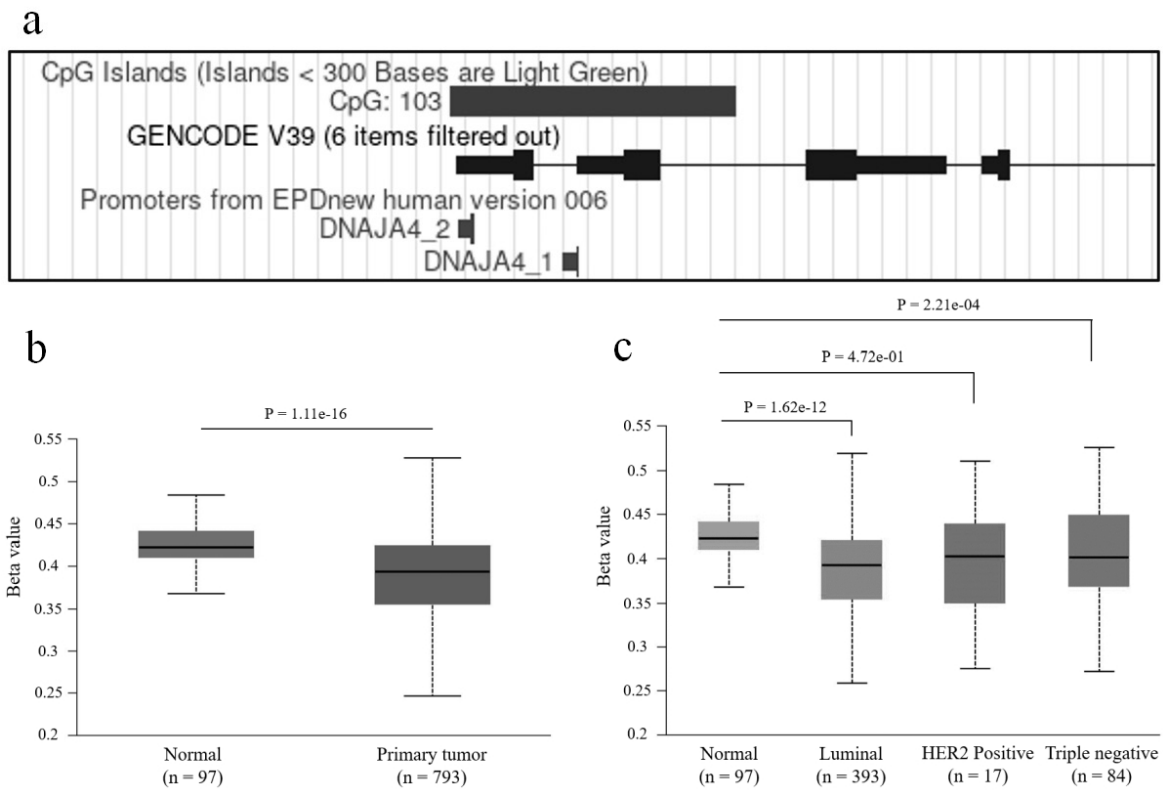

Fig. 4. Methylation level of *DNAJA4* promoter is reduced in clinical breast cancer samples. (a) The screen view from the UCSC genome browser shows promoter regions of *DNAJA4* gene overlapping the CpG island (http://genome.ucsc.edu). (b) The methylation level of *DNAJA4* promoter region in clinical breast cancer samples relative to normals. The box whisker plots show average beta values, ranging from unmethylated (0) to fully methylated (1), generated by UALCAN in-silico tool (http://ualcan.path.uab.edu). (c) Samples of luminal and triple-negative subtypes have reduced methylation levels at the *DNAJA4* promoter region.

Discussion

Our immunohistochemical analysis in a clinical cohort provides an additional layer of data concordant with the previous transcriptomic analysis (9, 10). DNAJA4 protein expression is high in breast cancer samples relative to adjacent normal breast tissues. We did not show any significant correlation between the protein expression and clinicopathological features of tumor samples. It must be mentioned that the number of samples with different clinical characteristics is quite low for statistical analyses (Supplementary material 2, Supplementary Table 1).

According to Zoppino's transcriptomic analysis, *DNAJA4* expression is not significantly associated with overall survival in breast cancer (9). The respective study did not provide information about the DFS, and DMFS (9). The survival outcomes of HSPs, including *DNAJA4*, were also analyzed by two other studies (10, 11). They also did not show any significant association between *DNAJA4* expression and survival. But they neither analyzed the subtypes (10, 11) nor the DFS, and DMFS values (11).

The power of our study comes from the fact that we analyzed larger cohorts from RNA-seq and DNA microarray datasets for three types of survival outcomes: OS, DFS, and DMFS. Moreover, we examined breast cancer subtypes classified by PAM50 (14). Breast cancer is a multi-subtype cancer which complicates the prognosis and selection of the most appropriate treatment for it (2). By analyzing the subtypes for different survival outcomes, we tried to identify a new prognostic biomarker candidate that may help clinicians monitor breast cancer patients.

Our results showed that high *DNAJA4* mRNA expression is unfavorable for OS, DFS, and DMFS in breast cancer. Subtype-specific survival values usually show poor survival rates with increasing *DNAJA4* mRNA expression (Table 1). *DNAJA4* expression is favorable in luminal A subtype for DMFS, which supports the metastasis suppressive role of DNAJA4 in melanoma (7) and emphasizes the need to be studied in breast carcinogenesis.

Since genetic alterations of the *DNAJA4* gene are infrequent in clinical samples, epigenetic mechanisms, such as promoter hypomethylation or deregulated miRNAs could have a role in the upregulation of the *DNAJA4* gene in breast cancer. In support of this concept, there are two experimentally validated promoter regions overlapping with the CpG island (18, 19). We revealed that the methylation status of the *DNAJA4* promoter region is reduced in luminal breast cancer samples which have significantly higher *DNAJA4* mRNA levels compared to normal samples. In alignment with this finding, an in vitro study showed that *DNAJA4* is highly expressed in luminal breast cancer cell lines (MCF7, ZR-75-1, T47D, BT-474) and hypermethylated in basal cell lines (MDA-MB-468, MDA-MB-231, BT-20) (20). So, it is plausible to suggest that promoter hypomethylation could result in high *DNAJA4* expression in luminal breast cancer. *DNAJA4* gene is repressed by c-MYC mediated promoter hypermethylation (4). In this context, it is worth noting that *c-MYC* mRNA expression is significantly reduced in all breast cancer subtypes relative to the normal samples (Supplementary material 2, Supplementary Figure 2). Thus, this knowledge may help to explain the reduced level of promoter methylation of *DNAJA4* in breast cancer. Regulation of *DNAJA4* by miRNAs should also be considered since there are miRNAs previously shown to be targeted *DNAJA4* in melanoma as previously stated (7).

In conclusion, higher DNAJA4 protein expression was detected in clinical breast cancer samples relative to normal samples. High *DNAJA4* expression is unfavorable in breast cancer patients. Good DMFS values are shown in the luminal A subtype which would be related to

the metastasis suppressive role of DNAJA4. Genetic alterations of *DNAJA4* are uncommon but epigenetic factors in the form of promoter hypomethylation could be an important regulatory mechanism for *DNAJA4* expression. Functional assays would be helpful to further decipher the function and the regulation of *DNAJA4* in breast cancer.

Acknowledgment

This work was supported by grants from Zonguldak Bulent Ecevit University (Zonguldak, Turkey) (Grant number 2018-50737594-02) and Scientific and Technological Research Council of Turkey (TUBITAK) (Grant number 217S251).

Authors' contribution

TA: Conceptualization, methodology, formal analysis, resources, writing (original draft preparation), visualization, supervision, project administration and funding acquisition, investigation, validation.
OI: Investigation, validation.

Conflict of interest

None to declare.

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