HLJ1 (*DNAJB4*) Gene Is a Novel Biomarker Candidate in Breast Cancer

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Abstract

Breast cancer is the most common cancer type and cause of cancer-related mortality among women worldwide. New biomarker discovery is crucial for diagnostic innovation and personalized medicine in breast cancer. Heat shock proteins (HSPs) have been increasingly reported as biomarkers and potential drug targets for cancers. *HLJ1* (*DNAJB4*) belongs to the *DNAJ* (*HSP40*) family of HSPs and is regarded as a tumor suppressor gene in lung, colon, and gastric cancers. However, the role of the *HLJ1* gene in breast cancer is currently unknown. We evaluated the role of the *HLJ1* gene in breast cancer progression by analyzing its *in vitro* and *in vivo* expression and its genetic/epigenetic alterations. *HLJ1* expression was found to be reduced or lost in breast cancer cell lines (SK-BR-3, MDA-MB-231, ZR-75-1) compared with the nontumorigenic mammary epithelial cell line (MCF 10A). In a clinical context for breast cancer progression, the *HLJ1* expression was significantly less frequent in invasive breast carcinoma samples ($n=230$) compared with normal breast tissue ($n=100$), benign neoplasia ($n=53$), and ductal carcinoma *in situ* ($n=21$). In methylation analyses by the combined bisulfite restriction analysis assay, the CpG island located in the 5'-flanking region of the *HLJ1* gene was found to be methylated in breast cancer cell lines. *HLJ1* expression was restored in the ZR-75-1 cell line by DNA demethylating agent 5-Aza-2'-deoxycytidine (5-AzaC) and histone deacetylase inhibitor trichostatin A. These new observations support the idea that *HLJ1* is a tumor suppressor candidate and potential biomarker for breast cancer. Epigenomic mechanisms such as CpG methylation and histone deacetylation might contribute to downregulation of *HLJ1* expression. We call for future functional, epigenomic, and clinical studies to ascertain the contribution of *HLJ1* to breast cancer pathogenesis and, importantly, evaluate its potential for biomarker development in support of personalized medicine diagnostic innovation in clinical oncology.

Keywords: breast cancer biomarkers, *DNAJB4*, *HLJ1*, epigenomics, precision medicine

Introduction

DESPITE ADVANCES in prevention, diagnosis, and treatment of breast cancer, it remains as the most common cancer type and cause of cancer-related mortality among women worldwide (Ferlay et al., 2015). New biomarker discovery is much needed for diagnostic innovation and personalized therapeutics in breast cancer, as well as in clinical oncology broadly (Dzobo et al., 2016; Thomford et al., 2016; Toss and Cristofanilli, 2015).

Heat shock proteins (HSPs) have been reported as biomarkers and potential drug targets for cancers (Khalil et al., 2011; Sterrenberg et al., 2011). In this context, *HLJ1* (*DNAJB4*) belongs to the *DNAJ* (*HSP40*) family of HSPs and is regarded as a tumor suppressor gene in lung, colon, and gastric cancers (Liu et al., 2014; Simões-correia et al., 2014; Tsai et al., 2006; Wang et al., 2005). *HLJ1* has also been suggested as a potential biomarker and therapeutic target in lung cancer (Chen et al., 2008; Lai et al., 2013; Tsai et al., 2006; Wang et al., 2007). Moreover, *in vitro* studies showed that induction of

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TABLE 1. THE CLINICOPATHOLOGICAL FEATURES OF THE BREAST CELL LINES USED IN THE STUDY

Cell line	Subtype ^{a,b}	Immunoprofile ^a			Disease ^c	Tumorigenicity ^c
		ER	PR	HER2		
BT-20	Basal A/basal	—	—	—	Carcinoma	Yes
SK-BR-3	Luminal/HER2+	—	—	+	Adenocarcinoma	Yes
MDA-MB-231	Basal B/claudin-low	—	—	—	Adenocarcinoma	Yes
ZR-75-1	Luminal/luminal	+	—	—	Ductal carcinoma	Yes
MCF 10A	Basal B/nonmalignant basal	—	—	—	Fibrocystic disease	No

^aKao et al. (2009).^bHeiser et al. (2012).^cATCC (www.lgcstandards-atcc.org).

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

HLJI inhibits cancer cell invasion in hepatocellular carcinoma cells (Wang et al., 2007). Importantly, the role of *HLJI* remains unclear in breast cancer despite the growing importance of this molecular target in abovementioned cancer types.

There are several lines of evidence that collectively suggest *HLJI* as an antioncogenic factor: *HLJI* inhibits cell cycle progression through controlling the *STAT1/P21^{WAF1}* pathway (Tsai et al., 2006), suppresses invasion by inducing *E-Cadherin* (Wang et al., 2005), and enhances apoptosis by activating *JNK* and *CASPASE-3* (Lin et al., 2010). *HLJI* also suppresses malignancy by inhibiting the catalytic activity of SRC proto-oncogene (Chen et al., 2016).

DNAJ (*HSP40*) HSPs comprise the largest and most diverse subgroup of the HSP family. The *DNAJ* subfamily consists of 49 members and is divided further into three subclasses: *DNAJA* (4 members), *DNAJB* (13 members), and *DNAJC* (32 members). These cochaperones regulate major chaperones (*HSP70*, *HSP90*), which have tumor suppressor or oncoprotein functions. These regulatory actions make the *DNAJ* members more specific and selective drug targets compared with other major chaperones (Sterrenberg et al., 2011). Besides, it has been suggested that *DNAJ* proteins may also possess chaperone-like activities independently of *HSP70* chaperones (Hageman et al., 2010).

To date, many *DNAJ* members have been implicated in cancer development and metastasis of different organs, such as *DNAJA1* (glioblastoma, prostate), *DNAJA3* (osteosarcoma, melanoma, kidney, hematopoietic, breast), *DNAJB6* (breast), *DNAJB11* (KSHV-related tumors, ovarian), *DNAJC9* (cervical), *DNAJC10* (neuroblastoma), and *DNAJC15* (ovarian, brain, breast) (Khalil et al., 2011; Sterrenberg et al., 2011).

In this study, we evaluated the potential role of *HLJI* in breast cancer progression by analyzing its *in vitro* and *in vivo* expression and its genetic/epigenetic alterations. We showed that *HLJI* expression is reduced or lost in breast cancer cell lines and invasive breast carcinoma samples. Moreover, we suggest that epigenetic mechanisms in the form of CpG methylation and histone deacetylation could contribute to downregulation of *HLJI* expression.

Materials and Methods

Cell lines and clinical study samples

Breast cancer cell lines, BT-20 (ATCC[®] HTB-19TM), SK-BR-3 (ATCC[®] HTB-30TM), MDA-MB-231 (ATCC[®] HTB-26TM), and ZR-75-1 (ATCC[®] CRL-1500TM), and nontumorigenic mammary epithelial cell line MCF 10A (ATCC[®] CRL-

10317TM) were used and cultured according to American Type Culture Collection (ATCC) recommendations (ATCC, Manassas, VA, USA) (Table 1).

For the clinical study, we evaluated 404 breast tissue samples (tissue microarray [TMA] sample set) from 245 patients (study cohort) (Tables 2 and 3). In a subset of 118 patients, supplementary samples were collected from the tissue adjacent to the sample of the primary histological diagnosis. These supplementary samples have been used for comparison between histological groups. The normal tissue samples ($n=100$) were obtained from adjacent tissue of samples of the 245 patients with invasive breast carcinoma ($n=203$), ductal carcinoma *in situ* (DCIS; $n=6$), and benign neoplasia ($n=36$). For association and survival analysis, only samples of the primary breast cancer diagnosis were evaluated.

Formalin-fixed and paraffin-embedded (FFPE) tissue samples were collected between 1989 and 1993 and archived in the Institute of Pathology at the University Hospital Schleswig Holstein in Luebeck, Germany. The study was performed in accordance with the approval of the local Ethics Committee at the same institute (#08-012).

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the breast cell lines by using RNeasy Mini Kit (Qiagen, Germany). Isolated RNAs were then used to synthesize cDNA by using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., MA, USA). Relative quantification of *HLJI* transcripts was performed with Power SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) on ABI Prism 7000 Sequence Detection System (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. *HLJI* and *TBP* (TATA box-binding

TABLE 2. HISTOPATHOLOGICAL FEATURES AND NUMBERS OF PATIENTS (STUDY COHORT) AND THE TISSUE MICROARRAY SAMPLES USED IN THE STUDY

Histopathology	Patients	TMA samples
Normal tissue	—	100
Benign neoplasia	36	53
DCIS	6	21
Invasive carcinoma	203	230
TOTAL	245	404

DCIS, ductal carcinoma *in situ*; TMA, tissue microarray.

TABLE 3. CHARACTERISTICS OF THE CLINICAL STUDY COHORT

	Total (n = 245)	Benign neoplasia (n = 36)	DCIS ^a (n = 6)	Invasive carcinoma (n = 203)
Age at diagnosis (years)				
Mean	59.71	45.96	69.60	61.63
Median	58.00	47.00	71.00	60.00
Range	18–93	18–77	59–75	30–93
95% CI	57.46–61.96	39.55–52.38	61.87–77.33	59.32–63.95
Overall survival (years)				
Mean	11.77	16.58	12.55	10.97
Median	12.84	20.45	14.75	9.32
Range	0–22.45	0.56–21.45	2.32–20.45	0–22.45
95% CI	10.69–12.86	14.05–19.12	3.26–21.83	9.79–12.16
Disease-free survival (years)				
Mean	11.37	16.58	12.55	10.50
Median	11.05	20.45	14.75	8.07
Range	0–22.45	0.56–21.45	2.32–20.45	0–22.45
95% CI	10.25–12.49	14.05–19.12	3.26–21.83	9.28–11.71

^aDuctal carcinoma *in situ*.
CI, confidence interval.

protein)-specific primer pairs were used with cDNAs from breast cell lines. The *TBP* gene was used as an internal control (Gur-Dedeoglu et al., 2009). Relative levels of *HLJ1* transcripts were measured by the comparative C_T method ($2^{-\Delta\Delta C_T}$ method) (Livak and Schmittgen, 2001). Statistical analyses were performed using Student's *t*-test.

Western blot

Total cell lysates from breast cell lines were prepared in RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) containing protease inhibitor cocktail (Roche cComplete Mini; Sigma-Aldrich Co. LLC., St. Louis, MO, USA). Insoluble material was removed by centrifugation at 10,000 *g* for 20 min at 4°C and protein contents were measured with bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equalized protein samples were resolved by 4–12% SDS-PAGE (NuPAGE® Novex® Bis-Tris Gels; Thermo Fisher Scientific, Inc.) and then transferred onto polyvinylidene fluoride membrane.

DNAJB4 rabbit polyclonal antibody (Cat No: 13064-1-AP; Proteintech Group, Inc., IL, USA) (dilution, 1:1000) was used as the primary antibody, and HRP-linked anti-rabbit IgG (Cat. No: 7074; Cell Signaling Technology, Inc., MA, USA) was used as the secondary antibody (dilution, 1:2000). Membrane was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). α -Tubulin (11H10) Rabbit mAb (Cat. No: 2125; Cell Signaling Technology, Inc.) was used as the loading control (dilution, 1:2000).

Immunohistochemistry

Clinical specimens were formalin-fixed, paraffin-embedded, and implemented into a TMA. The TMAs were constructed using a semiautomated arrayer (TMArrayer; Pathology Devices, MD, USA) as described previously (Oberländer et al., 2014). Tissue cores with a diameter of 1.5 mm were taken from selected regions of FFPE donor

tissue blocks and transferred into an empty paraffin recipient block. Detailed information of immunohistochemistry (IHC) method was supplied in Supplementary Data.

Combined bisulfite restriction analysis

DNeasy tissue kit (Qiagen, Germany) was used to extract genomic DNA from breast cell lines. Genomic DNAs were treated with bisulfite by using Methylamp™ DNA Modification Kit (EpiGentek, NY, USA) according to the manufacturer's instructions. Seminested primer pairs targeting *HLJ1* CpG islands were used to amplify bisulfite-treated DNAs. To analyze the methylation status, amplicons were restriction digested with *TaqI* and *Hpy188I* (New England BioLabs, Ipswich, MA), as previously described (Xiong and Laird, 1997).

5-Aza-2'-deoxycytidine and trichostatin A treatments

ZR-75-1 cells were plated in 75-cm² plastic tissue culture flasks (T-75) at a density of 8×10^5 cells/flask and grown for 24 h before treatment. ZR-75-1 cells were treated with 10 μ M 5-Aza-2'-deoxycytidine (5-AzaC, Cat No: A3656; Sigma-Aldrich Co. LLC.) or 300 nM trichostatin A (TSA, Cat No: T1952; Sigma-Aldrich Co. LLC.). TSA treatment was performed in the last 24 h. All media were replenished every 12 h over a period of 72 h. Control cultures were left untreated or received a mock treatment with dimethyl sulfoxide (DMSO) (final concentration, 0.1%) since both drugs were dissolved in DMSO (Cat No: D2650; Sigma-Aldrich Co. LLC.). Treatments were performed in triplicates.

Statistical analysis

The semiquantitatively scored *HLJ1* expression and non-binary clinical characteristics were dichotomized according to survival outcomes, group sizes, and median cutoff (e.g., age). For dichotomization, only samples with primary diagnosis were included. Other categorical nonbinary variables were also stratified by either survival outcomes or group sizes.

FIG. 1. *HLJ1* expression in breast cell lines. (A) Real-time qRT-PCR results showing *HLJ1* mRNA expression in breast cancer cell lines (BT-20, SK-BR-3, MDA-MB-231, ZR-75-1) and nontumorigenic mammary epithelial cell line (MCF 10A). *TBP* was used as internal control. Standard deviations of three biological replicates are shown. (B) *HLJ1* expression was also assessed with western blot analysis by using total cell lysates from breast cell lines. α -Tubulin was used as loading control. Twenty micrograms of total protein was loaded per sample.

Associations between *HLJ1* expression and clinical characteristics were examined using the two-sided χ^2 -test or Fisher's exact test. Kaplan–Meier plots and log-rank tests were used to assess the differences between groups in overall survival (OS). Associations with OS were determined using univariate Cox regression, providing a hazard ratio and 95%

confidence interval for each variable. Multivariate Cox regression analysis was used to determine whether *HLJ1* expression was independent of other prognostic factors. A $p \leq 0.05$ was considered as statistically significant. Statistical analysis was performed using SPSS Statistics, version 22 (IBM Corporation, Somers, NY, USA).

FIG. 2. *HLJ1* protein expression in breast samples. Comparison between histological groups regarding *HLJ1* expression in breast samples ($n = 404$). DCIS, ductal carcinoma *in situ*.

Results

HLJ1 expression in breast cancer cell lines and clinical breast samples

We analyzed *HLJ1* mRNA and protein expression levels in breast cell lines by real-time qRT-PCR and western blotting, respectively. *HLJ1* mRNA expression was found to be reduced or very low in MDA-MB-231, SK-BR-3, and ZR-75-1 cell lines compared with MCF 10A cell line, which is a nontumorigenic mammary epithelial cell line (Fig. 1A). Moreover, *HLJ1* protein expression was found to be lost in ZR-75-1 cell line (Fig. 1B). We could not observe a good correlation between mRNA and protein levels of SK-BR-3 and MDA-MB-231 cell lines (Fig. 1A, B).

According to the IHC results, *HLJ1* protein expression (score 1 + 2 + 3) was also significantly less frequent in invasive breast carcinoma samples (47.3%) compared with normal breast samples (78.1%, $p=0.00001$), benign neoplasia samples (74.5%, $p=0.0007$), and DCIS samples (75%, $p=0.039$) (Fig. 2, Supplementary Fig. S1).

In addition, we found significant associations between *HLJ1* expression and clinical characteristics of breast carcinomas: *HLJ1* expression was more frequent in ER+ and PR+ samples compared with ER- and PR- samples ($p=0.0048$ and $p=0.016$, respectively) (Table 4). Furthermore, a significant association with the molecular subtype could be determined: while *HLJ1* was more frequently expressed in the samples of luminal A subtype, it was less frequent in triple negative samples and HER2-overexpressing samples ($p=0.031$, Table 4). However, a significant association between *HLJ1* expression and OS of breast cancer patients could not be observed (Fig. 3).

CpG methylation in the 5'-flanking region of HLJ1

Our *in silico* analysis (Li and Dahiya, 2002) showed that there are CpG islands in the 5'-flanking region and in a region driving basal transcriptional activity of the *HLJ1* gene (Wang et al., 2005). Those regions were designated as region-1 and region-2, respectively (Fig. 4). Genomic DNAs from the cell lines treated with bisulfite were amplified using the seminested primer pairs targeting those two regions. PCR amplicons were restriction digested by *TaqI* and *Hpy188I* restriction enzymes to analyze methylation status of region-1 and region-2, respectively (Fig. 5A, B). We did not detect CpG methylation in a region driving basal transcriptional activity. However, region-1, which contains some sequences similar to heat shock transcription factor 1 (HSF1) binding motifs (Jaeger et al., 2014; Trinklein et al., 2004), was found to be methylated in all cell lines (Fig. 5A), suggesting that methylation of this region could have some regulatory effects.

Restoration of HLJ1 expression in ZR-75-1 cell line

To explore the role of DNA methylation and/or histone deacetylation as mechanisms that might operate on *HLJ1* downregulation in breast cancers, we treated ZR-75-1 cells with DNA methyl transferase inhibitor (5-AzadC) or histone deacetylase inhibitor (TSA). Both drugs successfully restored *HLJ1* expression in ZR-75-1 cells, which implies that epigenetic mechanisms could play a role in the downregulation of *HLJ1* (Fig. 6A, B).

TABLE 4. ASSOCIATIONS BETWEEN *HLJ1* EXPRESSION AND CLINICAL CHARACTERISTICS IN INVASIVE BREAST CARCINOMAS (N=203)

Variable	HLJ1 expression	
	Score 0, n (%)	Score 1 + 2 + 3, n (%)
Age at diagnosis ^a (years)		
<58	47 (53)	42 (47)
>58	56 (53)	49 (47)
<i>P</i>		1.000
Recurrence		
No	87 (51)	84 (49)
Yes	16 (70)	7 (30)
<i>P</i>		0.120
Tumor stage		
pT1	22 (46)	26 (54)
pT2–pT4	66 (56)	52 (44)
<i>P</i>		0.304
Lymph node stage		
pN0	39 (53)	34 (47)
pN1–pN3	40 (50)	40 (50)
<i>P</i>		0.747
Metastasis stage		
pM0	90 (52)	84 (48)
pM1	13 (65)	7 (35)
<i>P</i>		0.345
UICC stage		
Early	50 (48)	54 (52)
Late	38 (61)	24 (39)
<i>P</i>		0.110
Grade		
G1–G2	70 (51)	68 (49)
G3	21 (62)	13 (38)
<i>P</i>		0.258
Ki67 status		
Low (<20%)	89 (51)	81 (49)
High (>20%)	13 (57)	10 (43)
<i>P</i>		0.825
ER		
Negative	50 (66)	26 (34)
Positive	51 (44)	64 (56)
<i>P</i>		0.0048
PR		
Negative	46 (64)	26 (36)
Positive	52 (45)	63 (55)
<i>P</i>		0.016
Her2		
Negative	88 (51)	83 (49)
Positive	15 (68)	7 (32)
<i>P</i>		0.175
Molecular class		
Luminal A	32 (41)	47 (59)
Luminal B Her2–	12 (55)	10 (45)
Luminal B Her2+	5 (50)	5 (50)
HER2 overexpr.	8 (89)	1 (11)
Triple negative	27 (61)	17 (39)
<i>P</i>		0.031

^aMedian age at diagnosis of the full study cohort.

^bStatistically significant values are highlighted in bold.

FIG. 3. Kaplan-Meier overall survival plot of *HLJI* protein expression in invasive breast carcinoma ($n=203$). Log-rank test and univariate Cox regression analysis were used to assess the difference between groups of patients stratified by dichotomized *HLJI* protein expression (Score 0 and Score 1+2+3). The survival rates for patients with *HLJI*-negative breast carcinomas were 62% (95% CI, 52–72%) and for patients with *HLJI*-positive carcinomas 73% (95% CI, 63–84%) at the time point of 5 years. No significant differences were found in the present sample ($p>0.05$). CI, confidence interval.

Discussion

The tumor suppressor role of *HLJI* has been well documented in lung cancer, colorectal cancer, and gastric cancer (Liu et al., 2014; Simões-correia et al., 2014; Tsai et al., 2006;

Wang et al., 2005). Moreover, high expression of *HLJI* was noted as a significant prognostic factor for reduced cancer recurrence and longer OS in NSCLC patients (Tsai et al., 2006). *HLJI* shows its antioncogenic properties through the *STAT1/P21^{WAF1}* pathway (Tsai et al., 2006), *E-Cadherin* (Wang et al., 2005), *JNK* and *CASPASE-3* (Lin et al., 2010), and *SRC* (Chen et al., 2016).

HLJI was also demonstrated to inhibit invasion and metastasis by reducing nucleophosmin (NPM1) oligomerization through direct binding, which results in increased AP-2 α function and decreased matrix metalloproteinase-2 (*MMP2*) and signal transducer and activator of transcription 3 (*STAT3*) activities (Chang et al., 2010). Most of these pathways or genes are important for breast carcinogenesis, which highlighted the tumor suppressive role of *HLJI* for breast cancer (Banerjee and Resat, 2016; Berx et al., 2001; Cellurale et al., 2012; Duffy et al., 2000; Finn, 2008; Pellikainen et al., 2002).

Two herbal chemicals, curcumin (Chen et al., 2008) and andrographolide (Lai et al., 2013), were shown to induce *HLJI* expression and suppress tumorigenesis in lung adenocarcinoma and nonsmall cell lung cancer, respectively. High concentration of DMSO ($\geq 1\%$) was also shown to induce *HLJI* and inhibit malignant properties of lung adenocarcinoma cells (Wang et al., 2012). These results and the above studies strongly suggest that targeted induction of *HLJI* may represent a promising approach for cancer therapy.

Despite the significance of *HLJI* in cancer progression, *HLJI* genetic/epigenetic alterations and functional importance in breast cancer are currently unknown. Breast cancer is the most common cancer type and the most common cause of cancer-related mortality among women worldwide (Ferlay et al., 2015). Therefore, identification of new diagnostic/prognostic biomarkers and drug targets is of high interest for this malignancy, highlighting the significance of evaluating *HLJI* in this context.

We first evaluated *HLJI* expression level among breast cell lines (Fig. 1A, B). The malignant cell lines, MDA-MB-231,

FIG. 4. CpG islands in 5'-region of *HLJI*. *In silico* analysis of *HLJI* locus by MethPrimer program (Li and Dahiya, 2002) showed CpG islands. Location of the primers (F, forward; R, reverse) used in COBRA assay is shown by arrows. Vertical red lines denote CpG sites, numbered boxes denote exons. CpG islands are shown as light blue areas. COBRA, combined bisulfite restriction analysis.

FIG. 5. COBRA. *TaqI* (A) and *Hpy188I* (B) restriction enzymes were used for region-1 and region-2, respectively. (-), No restriction digestion; M, marker. An amplicon having an *Hpy188I* cutting site was used with (+) and without (Uncut) a restriction enzyme.

SK-BR-3, and ZR-75-1, showed reduced or very low levels of *HLJ1* mRNA compared with the nonmalignant breast cell line, MCF 10A. Moreover, *HLJ1* protein was found to be lost in the ZR-75-1 cell line. Immunohistochemical analysis of clinical breast samples confirmed our *in vitro* observations and showed that *HLJ1* was significantly less frequently expressed in invasive breast carcinoma samples than in normal breast samples (Fig. 2, Supplementary Fig. S1).

The *HLJ1* expression was significantly correlated with estrogen receptor (ER) and progesterone receptor (PR) pos-

itivity, as well as with the luminal A subtype, all of which are associated with a better prognosis. Triple negative and HER2-overexpressing samples less frequently expressed *HLJ1*, which are related to worse prognosis (Table 4) (Dai et al., 2015). Although we could not find a significant association between *HLJ1* expression and OS of breast cancer patients (Fig. 3), a study with a larger sample size might be necessary to ascertain the impact of *HLJ1* expression on OS.

According to the COSMIC database, genetic alterations of *HLJ1* in the form of mutations or copy number variations

FIG. 6. Restoration of *HLJ1* expression in ZR-75-1 cell line. Real-time qRT-PCR (A) and western blotting (B) results showing restoration of *HLJ1* expression in ZR-75-1 cells treated with 5-AzadC and TSA. *TBP* and α -tubulin were used as internal controls in real-time qRT-PCR and western blotting, respectively. DMSO was used as mock treatment. Thirty micrograms of total protein was loaded per sample. 5-AzadC, 5-Aza-2'-deoxycytidine; DMSO, dimethyl sulfoxide; TSA, trichostatin A.

(CNVs) are very rare in cancers. In the case of breast cancer, *HLJI* mutation rate is only 0.19%. CNVs are seen at the *HLJI* locus (1p31.1) with a rate of 0.06% and 0.46%, either as loss or gain, respectively (Forbes et al., 2015).

It is known that DNA methylation and alterations of chromatin structure are predominant epigenetic mechanisms that downregulate tumor suppressor genes in cancers (Esteller, 2007). So, epigenetic mechanisms could also be responsible for *HLJI* downregulation in breast cancers. We found CpG islands in the 5'-flanking region and in a region having basal transcriptional activity (Wang et al., 2005) (Fig. 4). According to our combined bisulfite restriction analysis (COBRA) results, the 5'-flanking region having sequences similar to HSF1 binding motifs (heat shock elements [HSEs]) was found to be methylated in all breast cell lines (Jaeger et al., 2014; Trinklein et al., 2004) (Fig. 5A). It is important to note that findings regarding these HSEs are mainly based on our *in silico* observations. HSEs may vary in their consensus sequence, length, and/or orientation (Crinelli et al., 2015; Jaeger et al., 2014).

Moreover, we successfully restored *HLJI* expression in ZR-75-1 cells with 5-AzadC and TSA (Fig. 6A, B). It is therefore plausible to suggest that epigenetic mechanisms in the form of CpG methylation and histone deacetylation may contribute to the downregulation of *HLJI* expression. *HLJI* could also be regulated by miRNAs in breast cancer. A study employing the CellMiner web-based application suggested that some miRNAs could be involved in post-transcriptional regulation of *HLJI* in some breast cell lines (Ozgur et al., 2014; Reinhold et al., 2012).

Conclusions

HLJI was found to be downregulated in breast cancers and epigenomic events such as CpG methylation and histone deacetylation could be responsible for this downregulation. Consistent with previous studies showing tumor suppressor properties of *HLJI*, our findings suggest that *HLJI* is a tumor suppressor candidate and potential biomarker in breast cancer. *HLJI* was previously regarded as a promising therapeutic target in lung cancers. In this regard, we call for future functional, epigenomic, and clinical studies to ascertain the contribution of *HLJI* to breast cancer pathogenesis and, importantly, evaluate its potential for biomarker development in support of personalized medicine diagnostic innovation in clinical oncology.

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Authors' Contributions

All authors met the ICMJE criteria for authorship. T.A. conceived the study hypotheses, designed and implemented all experiments except IHC, interpreted the data, and drafted the first version of the manuscript. N.D., J.K.H., T.G., and C.T. performed the IHC study and interpreted the results.

T.G., J.K.H., E.O., and T.R. contributed to data interpretation and participated in drafting and finalizing the manuscript.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

5-AzadC	= 5-Aza-2'-deoxycytidine
ATCC	= American Type Culture Collection
CNV	= copy number variations
COBRA	= combined bisulfite restriction analysis
DCIS	= ductal carcinoma <i>in situ</i>
DMSO	= dimethyl sulfoxide
FFPE	= formalin-fixed and paraffin-embedded
HSF1	= heat shock transcription factor 1
HSPs	= heat shock proteins
IHC	= immunohistochemistry
OS	= overall survival
TMA	= tissue microarray
TSA	= trichostatin A