The Promoter Methylation Status and mRNA Expression Levels of *CTCF* and *SIRT6* in Sporadic Breast Cancer

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Promoter hypermethylation causes gene silencing and is thought to be an early event in carcinogenesis. This study was to detect promoter methylation status and mRNA expression levels of CCCTC-binding factor (*CTCF*) and sirtuin 6 (*SIRT6*), and to explore the relationship between methylation and mRNA expression in breast cancer patient samples. Promoter methylation analysis and expression profile analysis of two genes were performed by methylation-specific PCR, bisulfite sequencing PCR, and quantitative real-time PCR in cancer lesions and matched normal tissues. The promoter region of *CTCF* has not been hypermethylated in all patient samples. In contrast, methylation of *SIRT6* gene was present in invasive cancers (93.5%) and matched normal tissues (96.8%) from 62 patients. Promoter hypermethylation of *SIRT6* was also observed in ductal carcinoma *in situ* (three of three) and matched normal tissues (two of three). mRNA expression of *CTCF* and *SIRT6* in invasive tumors showed a lower level than that in paired normal tissues ($p=0.008$ and $p=0.030$, respectively). The fold change values of *CTCF* expression were significantly lower in invasive ductal cancer lesions with Ki-67-positive status ($p=0.042$). In conclusion, our data showed that the methylation status of *CTCF* and *SIRT6* promoter regions was not statistically different in cancer lesions compared with matched normal tissues. No significant association between promoter methylation status and expression profiles of *CTCF* and *SIRT6* was found in invasive breast cancers.

Introduction

BREAST CANCER IS THE most frequently diagnosed cancer among women worldwide, with more than 1.3 million cases each year. Breast cancer in Chinese females is the most frequently diagnosed cancer with crude incidence and mortality rates of 43.19/100,000 and 9.31/100,000, respectively (Chen *et al.*, 2012). Biological markers of diagnostic and/or prognostic values are available to predict the progression and development of breast cancer.

Breast cancer progression is due to the accumulation of genomic alterations leading to oncogene overexpression and tumor suppressor loss inducing growth advantage and clonal expansion. Epigenetic changes are considered to be an early event during tumor development and one of the hallmarks of cancer (Jones and Baylin, 2007). Hypermethylation of promoter CpG islands represents an alternative mechanism to inactivate tumor suppressor genes, DNA repair genes, cell cycle regulators, and transcription factors (Jones and Baylin, 2007). Specific patterns of CpG island methylation could result from clonal selection of cells having growth advantages due to silencing of associated tumor suppressor genes. Methylation analysis has shown great potential as a molecular tool for early detection of cancer and risk assessment of precursor lesions, such as ductal carcinoma *in situ* (DCIS) (Brooks *et al.*, 1998; Lamy *et al.*, 2002; Schulmann *et al.*,

2005; Levenson, 2007; Park *et al.*, 2011). DNA methylation markers have also been suggested as markers for treatment response, have been shown to have independent prognostic value, and are possible therapeutic targets in breast cancer, due to the potential reversibility of DNA methylation (Jovanovic *et al.*, 2010). For this reason, identification of methylated genes provides a potential target for molecular detection and therapy of breast carcinoma.

Previous candidate gene studies investigated promoter hypermethylation of breast cancer lesions and identified aberrant methylation at the promoters of *RASSF1A*, *Hin-1*, *RAR-β*, *Cyclin D2*, *twist*, *GSTP1*, *ABCB1*, *FOXCl*, *MGMT*, *MLH1*, *PPP2R2B*, *PTEN*, *BRCA2*, *CDH13*, *MSH6*, *RARRES1*, *SLIT2*, and *WIF1* and others (Fackler *et al.*, 2003; Lee, 2007; Mugerud *et al.*, 2010; Moelans *et al.*, 2011; Peng *et al.*, 2012; Alvarez *et al.*, 2013). Identification of these genes has helped begin to elucidate the molecular pathogenesis of breast carcinoma, as it reveals intracellular pathways that are altered that likely contribute to oncogenesis. CCCTC-binding factor (*CTCF*) and sirtuin 6 (*SIRT6*) have currently been suggested to function as a potential tumor suppressor in breast cancer (Fiorentino and Giordano, 2012; Sebastian *et al.*, 2012) and CpG islands mapped to the promoter regions of *CTCF* and *SIRT6* [chr16:67595310-67644735 and chr19:4182537-4183596, respectively, UCSC assembly: Feb. 2009 (GRCh37/hg19)]. Based on a PubMed search, we found that *CTCF* and

SIRT6 had not been analyzed in breast cancers by other studies previously.

The objective of the current study was to detect promoter methylation status and mRNA expression levels of *CTCF* and *SIRT6* and to explore the relationship between methylation and mRNA expression in breast cancers and matched normal tissues from Chinese females. In addition, the association between the methylation status and mRNA expression changes of these two genes and the key clinicopathological features was investigated. We would like to emphasize that this study was not designed to study the prognosis. For one thing, the patients were treated differently according to tumor characteristics. For another, all tissue samples were collected between 2011 and 2012. This situation impeded our ability to acquire survival time. Hence, no follow-up data can be presented.

Materials and Methods

Patient material

Fresh breast tumors and matched adjacent normal tissues [farther from any malignant lesion (distance > 2 cm)] of 65 patients (female), with the median age of 49 years (range 35–79), whose cancers fulfilled all of the inclusion criteria (no inflammatory features, no previous treatment, and no evidence of any other cancers) were procured randomly from the Department of Pathology of West China Hospital of Sichuan University (SCU), China, between 2011 and 2012. Several types of primary breast cancer lesions procured in this study included pure invasive ductal carcinoma (IDC), pure invasive lobular carcinoma (ILC), mucinous carcinoma, metaplastic carcinoma, other invasive carcinomas, and pure DCIS. Breast cancers procured in our study were roughly divided into two groups: invasive tumor consisting of pure IDC and other several types of invasive carcinomas (ILC, mucinous carcinoma, metaplastic carcinoma, and other invasive carcinomas) and tumor *in situ* that mainly is DCIS. Immediately after procurement, tissues were re-evaluated and macrodissected by our breast pathologists, flash frozen in liquid nitrogen, and stored at -80°C until DNA and RNA extraction. Grading of IDCs only was done according to the usual methods (Elston and Ellis, 1991). IDCs were subdivided into grade I ($n=3$), grade II ($n=22$), and grade III ($n=25$). Grading was done independently by two pathologists and discordances were discussed until consensus was reached. The tumor–node–metastasis staging of invasive tumors was done according to the American Joint Committee on Cancer (AJCC) staging system for breast cancer (Singletary *et al.*, 2003). This study was approved by the ethics committee of the hospital, and consent was obtained from the patients according to the institutional review board of the hospital. Patients' clinical information, collected at the Department of Pathology of West China Hospital of SCU, China, can be found in Table 1.

Clinical endpoints

Immunohistochemical (IHC) staining of paraffin-embedded material was performed for the estrogen receptor (ER), progesterone receptor (PR), and a proliferation marker (Ki-67). A cutoff limit for positive staining was chosen for ER > 10% (ER EP1; Roche, Basel, Switzerland), PR > 10% (PR EP2; Roche), and Ki-67 $\geq 15\%$ (Ki-67 MIB-1; DAKO A/S, Glostrup, Denmark) stained tumor cells, irrespective of the intensity of the staining. Staining of human epidermal

TABLE 1. PATIENTS' CLINICAL INFORMATION

Characteristics	n	%
Age (years) (62)		
≤ 49	34	54.8
> 49	28	45.2
Location (62)		
Left	34	54.8
Right	28	45.2
Tumor size (mm) (62)		
≤ 20	26	41.9
20–50	26	41.9
≥ 50	10	16.1
Histological type (65)		
DCIS	3	4.6
IDC	52	80.0
ILC	2	3.1
MucinousC	3	4.6
MetaplasticC	1	1.5
Others	4	6.2
Grade in IDC (50)		
I	3	6.0
II	22	44.0
III	25	50.0
pN (61)		
Positive	27	44.3
Negative	34	55.7
ER status (62)		
Positive	46	74.2
Negative	16	25.8
PR status (62)		
Positive	42	67.7
Negative	20	32.3
Ki-67 status (61)		
Positive	42	68.9
Negative	19	31.1
HER-2 status (62)		
Positive	10	16.1
Equivocal	18	29.0
Negative	34	54.8
TNM staging (62)		
Stage I	14	22.6
Stage II	34	54.8
Stage III	10	16.1
Stage IV	4	6.5
CK5/6 status (61)		
Positive	7	11.5
Negative	54	88.5
P63 status (40)		
Positive	9	22.5
Negative	31	77.5
E-cadherin status (21)		
Positive	19	90.5
Negative	2	9.5

Numbers in parentheses are numbers of evaluated cases among 65 tumors.

CK5/6, cytokeratin 5/6; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; HER-2, human epidermal growth factor receptor-2; IDC, invasive ductal cancer; ILC, invasive lobular carcinoma; MetaplasticC, metaplastic carcinoma; MucinousC, mucinous carcinoma; Others, other invasive carcinomas; pN, lymph node status; PR, progesterone receptor; TNM, tumor–node–metastasis.

growth factor receptor-2 (HER-2) was subdivided into positive (3+), equivocal (2+), and negative (0/1+) (HER-2, 4BS; Roche) by IHC staining of paraffin-embedded material. IHC data also included status for the following: CK5/6, P63, and E-cadherin (positivity cutoff value of 1%). Staining was performed in an automatic staining machine (Ventana Medical Systems, Tucson, AZ).

DNA and RNA extraction

DNA from normal and abnormal tissues of 65 patients, who underwent surgery, was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction. RNA extraction was performed with the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions from invasive cancer lesions and adjacent normal tissues of 48 patients. DNA and RNA integrity was checked by agarose gel electrophoresis. RNA quality was verified using an Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). The extracted DNA and RNA were quantitated by a UV spectrophotometer and stored at -80°C .

Methylation-specific PCR and bisulfite sequencing PCR

DNA extracted from tissue samples was subjected to bisulfite modification to convert all unmethylated cytosines into uracils leaving methylated cytosines unaltered allowing specific detection of methylated or unmethylated DNA. Bisulfite modification of 500 ng genomic DNA was carried out with the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, CA) following the manufacturer's protocol. We selected the promoter/upstream by 500 bases and 5'

UTR exons region with CpG island(s) for each gene based on UCSC [UCSC assembly: Feb. 2009 (GRCh37/hg19), see Fig. 1], to design two sets of primers for each gene examined. Primers for methylation-specific PCR (MSP) were designed using MethPrimer (Li and Dahiya, 2002). We also designed bisulfite sequencing PCR (BSP) primers using MethPrimer that amplify a fragment of bisulfite-treated genomic DNA for each gene examined containing the corresponding MSP product (Fig. 1). Primers for each gene examined were synthesized by Sangon Biotech (Shanghai, China) and primer sequences and conditions used for MSP and BSP are given in Table 2. Promoter methylation analysis was performed by MSP as previously described (Herman *et al.*, 1996). For the reaction, 1 μL (about 50 ng) sodium bisulfite-treated DNA was added to 19 μL reaction buffer [0.2 μL TaKaRa LA Taq (5 U/ μL), 10 μL 2 $\times$ GC Buffer I, 3.2 μL dNTP mixture, and 4 μL sterile water] (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) containing 0.8 μL each of forward and reverse primers (10 μM) specific to the unmethylated and methylated DNA sequences. Cycling conditions were 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 53.0 – 55.0°C (optimized for each primer pair, see Table 2) for 30 s, and 72°C for 45 s, with a final extension cycle of 72°C for 4 min. No template controls were used for each set of MSP assays. SssI methylase-treated human sperm DNA was used as a positive control and human sperm DNA as a negative control. PCR products were analyzed on 3% agarose gel and the ethidium bromide-stained PCR products were imaged with the Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA). Each PCR analysis was performed twice. Invasive tumor samples and matched normal tissue samples of three patients, which proved to be methylated or unmethylated by MSP, were verified by BSP.

FIG. 1. Promoter region of CCCTC-binding factor (*CTCF*) (a) and sirtuin 6 (*SIRT6*) (b) analyzed by methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP). For each region, CpG sites and islands around the transcription start site (TSS) predicted by MethPrimer are shown, and the location of the primers for the methylated and the unmethylated condition and BSP primers is given in the picture.

TABLE 2. PRIMER SEQUENCES AND CONDITIONS FOR METHYLATION-SPECIFIC PCR AND BISULFITE SEQUENCING PCR

Gene	Primers	Annealing temperature (°C)	Product size (bp)
CTCF M	F: 5'-GGGCGGAGAGATTATTAAGTC-3' R: 5'-CAATAAACTAAAAAACTCCCGAA-3'	54.8	199
CTCF U	F: 5'-GGGTGGAGAGATTATTAAGTTGT-3' R: 5'-CAATAAACTAAAAAACTCCCAA-3'	54.8	199
SIRT6 M	F: 5'-GGGATATGGATAGTTTGTGTTTC-3' R: 5'-CCTCTTTACATACAAATATTTACGAA-3'	53.0	133
SIRT6 U	F: 5'-GGGGATATGGATAGTTTGTGTTTT-3' R: 5'-CCTCTTTACATACAAATATTTACAAA-3'	55.0	134
CTCF	F: 5'-AGGTGGTTTGGGGATATTAAGAG-3' R: 5'-AACCAATAAACTAAAAAACTCCC-3'	56.0–53.0 (TD)	275
SIRT6	F: 5'-GGGGATATGGATAGTTTGTGTTT-3' R: 5'-CAATAAAATTTCCCTTATTAACC-3'	55.0–52.5 (TD)	300

CTCF, CCCTC-binding factor; F, forward; M, methylation; R, reverse; SIRT6, sirtuin 6; TD, touch down; U, unmethylation.

cDNA synthesis and quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) was performed on invasive cancer lesions and matched normal tissues from 48 patients. cDNA was synthesized in a total volume of 20 μ L with 1 μ g total RNA using the PrimeScript[®] RT reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa Biotechnology (Dalian) Co., Ltd.) according to the manufacturer's instruction and used as a template for qPCR analysis. According to the MIQE guidelines for qPCR (Bustin *et al.*, 2009), we refer to reference genes instead of housekeeping genes and Cq (quantification cycle) instead of Ct (threshold cycle). qPCR primers for each gene examined were designed using the Beacon Designer 7.6 (PREMIER Biosoft, Palo Alto, CA). All primers used for qPCR (Table 3) were synthesized by Sangon Biotech and tested for their efficiency (between 90% and 100%) and specificity (melting curve analysis). qPCR was performed on the CFX96 Touch[™] Real-Time PCR Detection System (Bio-Rad). For each qPCR, 2 μ L of diluted cDNA, 5 μ L of SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus; TaKaRa Biotechnology (Dalian) Co., Ltd.), and 0.4 μ L each of forward and reverse primers (10 μ M) were added to obtain a final volume of 10 μ L. Cycle conditions were initially 95°C for 30 s, 40 cycles of 10 s at 95°C, 10 s at 57.0–64.4°C (optimized for each primer pair, see Table 3), 30 s at 72°C, followed by melting curve analysis. Each sample was run in triplicate. No template controls were used for each time of qPCR assays. Interassay variations were <5% (data not shown). Relative gene expression levels were normalized to the reference gene *GAPDH*. Differences in the

expression of each gene were quantified using the Pfaffl approach (Pfaffl, 2001).

Statistical analysis

Descriptive percentage and median statistics were used to summarize patients' data and promoter hypermethylation status, and mRNA expression levels of two genes were analyzed. The chi-square test (χ^2) or Fisher's exact test was applied to evaluate the correlation between promoter hypermethylation and clinicopathological features. Analysis of the alteration of mRNA expression of each gene examined in 48 pairs of invasive cancers and matched normal tissues was determined by the two-sided nonparametric Wilcoxon signed-rank test. Differences in fold change of mRNA expression (invasive cancer compared with matched normal tissue from 48 patients) of each gene analyzed in relation to the clinicopathological parameters were determined by the two-sided nonparametric Mann–Whitney *U* test and Kruskal–Wallis *H* test. Two-sided $p < 0.05$ was used as the threshold for significance. All statistical analyses were performed using the software of IBM SPSS Statistics 19 (IBM SPSS, Inc., Chicago, IL).

Results

Promoter methylation status for two genes examined in breast cancers and adjacent normal tissues

We have found that the promoter region of *CTCF* has not been hypermethylated in invasive cancer lesions and matched normal tissues from 62 patients. In contrast, methylation of

TABLE 3. QUANTITATIVE REAL-TIME PCR PRIMERS FOR TARGET GENE EXAMINED

Primer name	Sequence (5' → 3')	Annealing temperature (°C)	Product size (bp)
CTCF-2F	CATCCAGCATCAGAAGTC	57.0	77
CTCF-2R	TCCTGTCTACAAGCGTAA		
SIRT6-1F	CTCCTCCGCTTCCTGGTC	64.4	123
SIRT6-1R	CGTCTTACACTTGGCACATTCTT		
GAPDH-1F	GAGTCAACGGATTTGGTCTG	60.6	238
GAPDH-1R	TTGATTTTGGAGGGATCTCG		

1 of 1F or 1R, transcript variant 1; 2 of 2F or 2R, transcript variant 2.

SIRT6 gene was present in invasive cancer lesions [93.5% (58 of 62)] and matched normal tissues [96.8% (60 of 62)] from 62 patients. In the IDC cases, promoter hypermethylation of *SIRT6* was detected in IDCs [92.3% (48 of 52)] and matched normal tissues [96.2% (50 of 52)]. In addition, the promoter region of *CTCF* has not been hypermethylated in DCIS and matched normal tissues from three patients, and promoter hypermethylation of *SIRT6* was observed in DCIS (three of three) and matched normal tissues (two of three). Three pairs of invasive tumor and matched normal tissue samples, which proved to be methylated or unmethylated by MSP, were confirmed by BSP (Fig. 2). Figure 2 showed representative samples for methylation for *CTCF* and *SIRT6*, respectively, examined in breast cancers and matched adjacent normal tissues.

mRNA expression of two genes analyzed in 48 pairs of invasive cancers and matched normal tissues

We used Oncomine to analyze previously published microarray data, and the results obtained showed that the ectopic expression of *CTCF* and *SIRT6*, potential tumor suppressor genes, is involved in breast cancer by DNA microarrays. In this study, we investigated the expression of two genes analyzed in 48 pairs of normal and invasive tumor tissues using qPCR. The mean values of *CTCF* and *SIRT6* measured by qPCR were 77.00 and 216.28, respectively, and the medians were 39.50 (range: 3.66–755.30) and 142.92 (range: 0–1907.10), respectively, in adjacent normal tissues. In contrast to the observations in normal tissues, the mean values of *CTCF* and *SIRT6* were 35.64 and 119.94, respectively, and the medians were

20.67 (range: 1–149.94) and 91.73 (range: 1–435.47), respectively, in invasive tumors. We further analyzed the expression of two genes in 40 pairs of IDCs and matched normal tissues, and we found that the mean values of *CTCF* and *SIRT6* measured by qPCR were 80.47 and 235.58, respectively, and the medians were 39.50 (range: 4.46–755.30) and 165.04 (range: 0–1907.10), respectively, in matched normal tissues. In contrast to the observations in matched normal tissues, the mean values of *CTCF* and *SIRT6* were 37.72 and 130.25, respectively, and the medians were 23.55 (range: 1–149.94) and 106.02 (range: 1–435.47), respectively, in IDCs.

Using the nonparametric Wilcoxon signed-rank test, we found that *CTCF* and *SIRT6* displayed a significantly different level of mRNA expression between paired normal and invasive tumor tissues ($p=0.008$ and $p=0.030$, respectively). Their mRNA expression in invasive tumors showed a lower level than that in paired normal tissues (Fig. 3A, B). In the IDC cases, both *CTCF* and *SIRT6* in IDCs showed significantly lower expression values compared with paired normal tissues ($p=0.014$ and $p=0.025$, respectively) (Fig. 3C, D).

Based on the previous report (Charafe-Jauffret *et al.*, 2005), we considered that the significant downregulation or upregulation status for each gene in each paired sample was assigned using a threshold corresponding to twice the expression found in matched normal tissue samples. Samples with a fold change -2 or less (we replace a fold change value that is <1 by the negative of its inverse) were defined as low expression, and 2 or more were defined as high expression. mRNA low expression (equal or more than twofold decrease) of *CTCF* and *SIRT6* was found in invasive tumors [45.8%

FIG. 2. MSP and BSP analysis for *CTCF* and *SIRT6* promoter hypermethylation in tumor (T) and matched normal (N) breast tissues. (Top) U represents amplification of unmethylated allele, and M represents methylated allele. Positive controls are referred as Pos.C. H₂O, water control. L (DNA ladder) represents molecular weight. In BSP circle graph (bottom), black circle represents methylated CpG, and white circle represents unmethylated CpG. For *CTCF*, the 199 bp band amplified by unmethylation primer indicated that *CTCF* is unmethylated, and no methylated CpG sites were found in the sequencing results in tumor and matched normal breast tissues. For *SIRT6*, the MSP electrophoretogram above represented the methylation status of the CpG island, which is about 200 bp apart from *SIRT6*, and the results showed that this island is methylated in tumor and matched normal breast tissues. The circle graph (center) indicated that the CpG island that was detected by MSP is methylated in three pairs of tumor and matched normal breast tissues.

FIG. 3. The expression of two genes analyzed in paired normal and invasive tumor tissues. (A, B) Differences in relative expression levels of *CTCF* and *SIRT6* in paired normal tissue versus invasive tumor, respectively. (C, D) Differences in relative expression levels of *CTCF* and *SIRT6* in paired normal tissue versus invasive ductal cancer (IDC), respectively. Expression of two genes analyzed was measured relative to the expression of the reference gene *GAPDH*. We scaled the normalized expression for each gene by dividing the expression level of each sample by the lowest level of expression in all the samples. The software set the lowest level of expression to a value of 1, and rescaled all the sample expression levels. Black horizontal bars represent median value for each group.

(22/48) and 41.7% (20/48), respectively] and IDCs [both 45.0% (18/40)]. Meanwhile, mRNA high expression (equal or more than twofold increase) of *CTCF* and *SIRT6* was observed in invasive tumors [16.7% (8/48) and 22.9% (11/48), respectively] and IDCs [15% (6/40) and 22.5% (9/40), respectively].

No significant relationship was found between the methylation status and mRNA expression changes of *CTCF* and *SIRT6* in invasive tumors and matched normal tissues from Chinese females.

Correlations between mRNA expression changes and clinicopathological features

A lower *CTCF* expression (less fold change) was significantly related to the Ki-67-positive status of IDC ($p=0.042$, Fig. 4A). The fold change values of *SIRT6* expression were significantly lower in IDCs with left site ($p=0.042$, Fig. 4B). In addition, no significant association was seen between mRNA expression changes and clinicopathological features for these two genes studied in 48 pairs of invasive tumors and matched

FIG. 4. Correlation between mRNA expression changes and clinicopathological features in 40 pairs of IDCs and matched normal tissues. (A) The lower *CTCF* expression (less fold change) was significantly related to Ki-67-positive status of IDC. (B) The fold change values of *SIRT6* expression were significantly lower in IDCs presenting left site. All *p*-values were obtained by using a false discovery rate <5%.

normal tissues, but there were two trends toward the association with clinicopathological features for *CTCF*. The fold change values of *CTCF* expression were higher in ILC versus other types of invasive tumors ($p=0.059$), whereas the fold change values of *CTCF* expression were lower in invasive tumors with the HER-2-positive status ($p=0.089$).

Discussion

Promoter hypermethylation is an important gene silencing mechanism thought to be an early event in carcinogenesis (Baylin and Herman, 2000). Understanding the epigenetic role in breast cancer is important to gain further insight into breast carcinogenesis and for the identification of potential biomarkers for diagnosis and treatment (Laird, 2003; Mulero-Navarro and Esteller, 2008). In the present study, we determined promoter methylation status and mRNA expression levels of *CTCF* and *SIRT6* and explored the relationship between methylation and mRNA expression in breast cancer lesions and adjacent normal tissues from Chinese females. In addition, we investigated the association between the methylation status and mRNA expression changes of two genes studied and the key clinicopathological features.

We have found that the promoter region of *CTCF* has not been hypermethylated in invasive cancer lesions and matched normal tissues from 62 breast cancer patients. In contrast, methylation of *SIRT6* gene was present in invasive cancer lesions (93.5%) and matched normal tissues (96.8%) from 62 patients. In addition, the promoter region of *CTCF* has not been hypermethylated in DCIS and matched normal tissues from three patients, and promoter hypermethylation of *SIRT6* was observed in DCIS (three of three) and matched normal tissues (two of three). Promoter methylation of

CTCF and *SIRT6* was not statistically different in cancer lesions compared with matched normal tissues.

Using the nonparametric Wilcoxon signed-rank test, we found that *CTCF* and *SIRT6* displayed a significantly different level of mRNA expression between paired normal and invasive tumor tissues ($p=0.008$ and $p=0.030$, respectively). Their mRNA expression in invasive tumors showed a lower level than that in paired normal tissues (Fig. 3A, B). In the IDC cases, both *CTCF* and *SIRT6* in IDCs showed significantly lower expression values compared with paired normal tissues ($p=0.014$ and $p=0.025$, respectively) (Fig. 3C, D). mRNA low expression (equal or more than twofold decrease) of *CTCF* and *SIRT6* was found in invasive tumors (45.8% and 41.7%, respectively) and IDCs (both 45.0%). However, mRNA high expression (equal or more than twofold increase) of *CTCF* and *SIRT6* was observed in invasive tumors (16.7% and 22.9%, respectively) and IDCs (15% and 22.5%, respectively). Our data indicate that aberrant expression of the two genes studied could contribute to the progression of invasive breast cancer.

CTCF is an 11-zinc finger protein that is an evolutionary conserved and ubiquitously expressed regulator of fundamental genomic processes, including transcription (Baniahmad *et al.*, 1990; Filippova *et al.*, 1996; Vostrov and Quitschke, 1997), imprinting (Bell and Felsenfeld, 2000; Hark *et al.*, 2000), long-range chromatin interactions, and subnuclear localization (Yusufzai *et al.*, 2004; Splinter *et al.*, 2006; Hou *et al.*, 2008). Among its multiple functions, a growing amount of evidence implicates *CTCF* in the epigenetic regulation of genes responsible for the control of the cell cycle, and its misregulation can lead to aberrant epigenetic silencing of genes involved in cancer development (Recillas-Targa *et al.*, 2011). *CTCF* was also

recognized as a potential tumor suppressor gene in breast cancer (Filippova *et al.*, 1998, 2002; Rasko *et al.*, 2001; Fiorentino and Giordano, 2012), and *CTCF* suppresses breast cancer growth (Tiffen *et al.*, 2013). To our knowledge, all publications currently available mainly describe that loss of *CTCF* binding due to hypermethylation leads to subsequent silencing of tumor suppressor gene loci, including *BRCA1* (Butcher *et al.*, 2004), retinoblastoma (*Rb*) (De la Rosa-Velazquez *et al.*, 2007), *p16(INK4a)* (Witcher and Emerson, 2009), *p53* (Soto-Reyes and Recillas-Targa, 2010), and retinoic acid receptor responder 1 (*RARRES1*) (Peng *et al.*, 2012). In our study, none of the invasive breast cancer and DCIS samples was methylated at the *CTCF* promoters examined, although we have used the commonly studied promoter/upstream by 500 bases and 5' UTR exons region with CpG island(s). Solis *et al.* recently reported that no differences were found in the methylation status between healthy and neoplastic breast tissue for *CTCF* (Soto-Reyes-Solis *et al.*, 2013). Our results obtained by the same method are consistent. mRNA expression of *CTCF* in invasive breast cancers and pure IDCs showed the significantly lower level than that in paired normal tissues ($p=0.008$ and $p=0.014$, respectively) (Fig. 3A, C). mRNA low expression (equal or more than twofold decrease) of *CTCF* was found in invasive tumors (45.8%) and IDCs (45.0%). However, mRNA high expression (equal or more than twofold increase) of *CTCF* was observed in invasive tumors (16.7%) and IDCs (15%). We found low mRNA expression of *CTCF* in invasive tumors similar to previously published data that *CTCF* gene expression was significantly lower in breast cancer compared with normal tissues calculated from Oncomine. In the study by Docquier *et al.* (2005), the levels of *CTCF* in breast cancer cell lines and tumors, however, were found elevated compared with breast cell lines with finite lifespan and normal breast tissues. The discrepancy is most likely related to differences in the populations that were studied and to the number of cases studied. No significant relationship between the methylation status and mRNA expression of *CTCF* gene was found, thus suggesting that methylation may not be the main culprit for its mRNA low expression in invasive breast cancers. Although other mechanisms that include mutation or copy number changes, including deletion, may be the cause of the above observation, we concluded that the influence of mutation and copy number variation on the expression may be insignificant at the mRNA level according to previous reports (Docquier *et al.*, 2005; Van Wezel *et al.*, 2005), and were, therefore, not considered in our study. A study by Rakha *et al.* (2004) showed that there was a significant correlation between expressions of *CTCF* and histological grades; a lower expression was associated with grade III tumors. In our study, *CTCF* showed no significant difference in mRNA expression levels and histological grades. However, the lower *CTCF* expression (less fold change) was significantly related to the Ki-67-positive status of IDC ($p=0.042$, Fig. 4A), and we conclude that the impaired *CTCF* function may promote ductal breast cancer cell proliferation and metastasis.

SIRT6, a member of the sirtuin family, is an NAD⁺-dependent histone H3 lysine 9 (H3K9) deacetylase (Michishita *et al.*, 2008) that is involved in multiple cellular processes, including metabolism (Lerrer and Cohen, 2013), aging (Lerrer and Cohen, 2013), DNA repair, and genomic insta-

bility (Mostoslavsky *et al.*, 2006). Indeed, *SIRT6* has been recently described as a tumor suppressor that regulates cancer metabolism (Sebastian *et al.*, 2012). Mechanistically, *SIRT6* suppresses aerobic glycolysis and Myc-dependent ribosome biosynthesis, and lack of this sirtuin leads to robust metabolic reprogramming, sufficient to promote tumorigenesis. In line with this, *SIRT6* expression is downregulated in human pancreatic and colorectal cancers, and conditional deletion of *SIRT6* promotes intestinal tumorigenesis *in vivo* (Sebastian *et al.*, 2012). Consistent with a tumor-suppressive function, it has recently been shown that overexpression of *SIRT6* induces massive apoptosis in cancer cell lines containing breast tumor cell lines, but not in normal, non-transformed cells (Van Meter *et al.*, 2011). In our work, methylation of *SIRT6* gene was detected in invasive cancer lesions (93.5%) and matched normal tissues (96.8%) from 62 patients. Promoter hypermethylation of *SIRT6* was also observed in DCIS (three of three) and matched normal tissues (two of three). Promoter methylation of *SIRT6* was not significantly different in cancer lesions compared with matched normal tissues. mRNA expression of *SIRT6* in invasive breast cancers and pure IDCs showed the significantly lower level than that in paired normal tissues ($p=0.030$ and $p=0.025$, respectively) (Fig. 3B, D). mRNA low expression (equal or more than twofold decrease) of *SIRT6* was found in invasive tumors (41.7%) and IDCs (45.0%). However, mRNA high expression (equal or more than twofold increase) of *SIRT6* was observed in invasive tumors (22.9%) and IDCs (22.5%). Our finding that mRNA expression of *SIRT6* in invasive breast cancer basically corresponds to published data that *SIRT6* gene expression in breast cancer showed the lower level than that in paired normal tissues calculated from Oncomine. Surprisingly, for a candidate tumor suppressor, the mRNA expression levels of *SIRT6* in partly invasive breast tumors were found upregulated compared with paired normal breast tissues. However, examples of increased levels of mRNA expression with tumor suppressive functions in cancer cells have been previously reported (e.g., *BRCA1* and *BRCA2*) (Zwahlen *et al.*, 2000; Egawa *et al.*, 2001). However, we guess whether upregulation of endogenous *SIRT6* might be linked to promotion of tumorigenesis. The mechanism and physiological relevance of this observation need further investigation. About correlations between mRNA expression changes of *SIRT6* and clinicopathological features, we found that the fold change values of *SIRT6* expression were significantly lower in IDCs with left site ($p=0.042$, Fig. 4B). IHC analysis of 118 breast cancer patient samples revealed that high *SIRT6* nuclear staining is significantly associated with a poorer overall survival ($p=0.018$; Kaplan–Meier analysis) (Khongkow *et al.*, 2013). This study, however, was not designed to study prognosis.

Since this study was not designed to study prognosis due to some limitations, follow-up studies are needed to investigate these biological markers and their potential in predicting prognosis.

In conclusion, our data showed that the methylation status of *CTCF* and *SIRT6* promoter regions was not statistically different in cancer lesions compared with matched normal tissues. No significant association between promoter methylation status and expression profiles of *CTCF* and *SIRT6* genes was seen in invasive breast cancers.

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Disclosure Statement

No competing financial interests exist.

References

- Alvarez, C., Tapia, T., Cornejo, V., Fernandez, W., Munoz, A., Camus, M., Alvarez, M., Devoto, L., and Carvallo, P. (2013). Silencing of tumor suppressor genes RASSF1A, SLIT2, and WIF1 by promoter hypermethylation in hereditary breast cancer. *Mol Carcinog* **52**, 475–487.
- Baniahmad, A., Steiner, C., Kohne, A.C., and Renkawitz, R. (1990). Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell* **61**, 505–514.
- Baylin, S.B., and Herman, J.G. (2000). DNA hypermethylation in tumorigenesis - epigenetics joins genetics. *Trends Genet* **16**, 168–174.
- Bell, A.C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* **405**, 482–485.
- Brooks, J.D., Weinstein, M., Lin, X., Sun, Y., Pin, S.S., Bova, G.S., Epstein, J.I., Isaacs, W.B., and Nelson, W.G. (1998). CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol Biomarkers Prev* **7**, 531–536.
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., *et al.* (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**, 611–622.
- Butcher, D.T., Mancini-DiNardo, D.N., Archer, T.K., and Rodenhiser, D.I. (2004). DNA binding sites for putative methylation boundaries in the unmethylated region of the BRCA1 promoter. *Int J Cancer* **111**, 669–678.
- Charafe-Jauffret, E., Ginestier, C., Monville, F., Fekairi, S., Jacquemier, J., Birnbaum, D., and Bertucci, F. (2005). How to best classify breast cancer: conventional and novel classifications (review). *Int J Oncol* **27**, 1307–1313.
- Chen, W.Q., Zeng, H.M., Zheng, R.S., Zhang, S.W., and He, J. (2012). Cancer incidence and mortality in china, 2007. *Chin J Cancer Res* **24**, 1–8.
- De la Rosa-Velazquez, I.A., Rincon-Arango, H., Benitez-Bribiesca, L., and Recillas-Targa, F. (2007). Epigenetic regulation of the human retinoblastoma tumor suppressor gene promoter by CTCF. *Cancer Res* **67**, 2577–2585.
- Docquier, F., Farrar, D., D'Arcy, V., Chernukhin, I., Robinson, A.F., Loukinov, D., Vatolin, S., Pack, S., Mackay, A., Harris, R.A., *et al.* (2005). Heightened expression of CTCF in breast cancer cells is associated with resistance to apoptosis. *Cancer Res* **65**, 5112–5122.
- Egawa, C., Miyoshi, Y., Taguchi, T., Tamaki, Y., and Noguchi, S. (2001). Quantitative analysis of BRCA1 and BRCA2 mRNA expression in sporadic breast carcinomas and its relationship with clinicopathological characteristics. *Jpn J Cancer Res* **92**, 624–630.
- Elston, C.W., and Ellis, I.O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **19**, 403–410.
- Fackler, M.J., McVeigh, M., Evron, E., Garrett, E., Mehrotra, J., Polyak, K., Sukumar, S., and Argani, P. (2003). DNA methylation of RASSF1A, Hin-1, RAR-beta, cyclin D2 and twist in *in situ* and invasive lobular breast carcinoma. *Int J Cancer* **107**, 970–975.
- Filippova, G.N., Fagerlie, S., Klenova, E.M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P.E., Collins, S.J., and Lobanenko, V.V. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol* **16**, 2802–2813.
- Filippova, G.N., Lindblom, A., Meincke, L.J., Klenova, E.M., Neiman, P.E., Collins, S.J., Doggett, N.A., and Lobanenko, V.V. (1998). A widely expressed transcription factor with multiple DNA sequence specificity, CTCF, is localized at chromosome segment 16q22.1 within one of the smallest regions of overlap for common deletions in breast and prostate cancers. *Genes Chromosomes Cancer* **22**, 26–36.
- Filippova, G.N., Qi, C.F., Ulmer, J.E., Moore, J.M., Ward, M.D., Hu, Y.J., Loukinov, D.I., Pugacheva, E.M., Klenova, E.M., Grundy, P.E., *et al.* (2002). Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter its DNA-binding specificity. *Cancer Res* **62**, 48–52.
- Fiorentino, F.P., and Giordano, A. (2012). The tumor suppressor role of CTCF. *J Cell Physiol* **227**, 479–492.
- Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M., and Tilghman, S.M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405**, 486–489.
- Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., and Baylin, S.B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* **93**, 9821–9826.
- Hou, C., Zhao, H., Tanimoto, K., and Dean, A. (2008). CTCF-dependent enhancer-blocking by alternative chromatin loop formation. *Proc Natl Acad Sci U S A* **105**, 20398–20403.
- Jones, P.A., and Baylin, S.B. (2007). The epigenomics of cancer. *Cell* **128**, 683–692.
- Jovanovic, J., Ronneberg, J.A., Tost, J., and Kristensen, V. (2010). The epigenetics of breast cancer. *Mol Oncol* **4**, 242–254.
- Khongkow, M., Olmos, Y., Gong, C., Gomes, A.R., Monteiro, L.J., Yague, E., Cavaco, T.B., Khongkow, P., Man, E.P.S., Laohasinnarong, S., *et al.* (2013). SIRT6 modulates paclitaxel and epirubicin resistance and survival in breast cancer. *Carcinogenesis* **34**, 1476–1486.
- Laird, P.W. (2003). The power and the promise of DNA methylation markers. *Nat Rev Cancer* **3**, 253–266.
- Lamy, A., Sesboue, R., Bourguignon, J., Dautreux, B., Metayer, J., Frebourg, T., and Thiberville, L. (2002). Aberrant methylation of the CDKN2a/p16INK4a gene promoter region in preinvasive bronchial lesions: a prospective study in high-risk patients without invasive cancer. *Int J Cancer* **100**, 189–193.
- Lee, J.S. (2007). GSTP1 promoter hypermethylation is an early event in breast carcinogenesis. *Virchows Arch* **450**, 637–642.

- Lerrer, B., and Cohen, H.Y. (2013). The guardian: metabolic and tumour-suppressive effects of SIRT6. *EMBO J* **32**, 7–8.
- Levenson, V.V. (2007). Biomarkers for early detection of breast cancer: what, when, and where? *Biochim Biophys Acta* **1770**, 847–856.
- Li, L.C., and Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427–1431.
- Michishita, E., McCord, R.A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., Cheung, P., Kusumoto, R., Kawahara, T.L.A., Barrett, J.C., *et al.* (2008). SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* **452**, 492–U416.
- Moelans, C.B., Verschuur-Maes, A.H.J., and van Diest, P.J. (2011). Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma *in situ* and invasive breast cancer. *J Pathol* **225**, 222–231.
- Mostoslavsky, R., Chua, K.F., Lombard, D.B., Pang, W.W., Fischer, M.R., Gellon, L., Liu, P.F., Mostoslavsky, G., Franco, S., Murphy, M.M., *et al.* (2006). Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* **124**, 315–329.
- Muggerud, A.A., Ronneberg, J.A., Warnberg, F., Botling, J., Busato, F., Jovanovic, J., Solvang, H., Bukholm, I., Borresen-Dale, A.L., Kristensen, V.N., *et al.* (2010). Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma *in situ* and early invasive breast cancer. *Breast Cancer Res* **12**.
- Mulero-Navarro, S., and Esteller, M. (2008). Epigenetic biomarkers for human cancer: The time is now. *Crit Rev Oncol Hematol* **68**, 1–11.
- Park, S.Y., Kwon, H.J., Lee, H.E., Ryu, H.S., Kim, S.W., Kim, J.H., Kim, I.A., Jung, N., Cho, N.Y., and Kang, G.H. (2011). Promoter CpG island hypermethylation during breast cancer progression. *Virchows Arch* **458**, 73–84.
- Peng, Z., Shen, R., Li, Y.W., Teng, K.Y., Shapiro, C.L., and Lin, H.J. (2012). Epigenetic repression of RARRES1 is mediated by methylation of a proximal promoter and a loss of CTCF binding. *PLoS One* **7**, e36891.
- Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.
- Rakha, E.A., Pinder, S.E., Paish, C.E., and Ellis, I.O. (2004). Expression of the transcription factor CTCF in invasive breast cancer: a candidate gene located at 16q22.1. *Br J Cancer* **91**, 1591–1596.
- Rasko, J.E., Klenova, E.M., Leon, J., Filippova, G.N., Loukinov, D.I., Vatolin, S., Robinson, A.F., Hu, Y.J., Ulmer, J., Ward, M.D., *et al.* (2001). Cell growth inhibition by the multifunctional multivalent zinc-finger factor CTCF. *Cancer Res* **61**, 6002–6007.
- Recillas-Targa, F., de la Rosa-Velazquez, I.A., and Soto-Reyes, E. (2011). Insulation of tumor suppressor genes by the nuclear factor CTCF. *Biochem Cell Biol* **89**, 479–488.
- Schulmann, K., Sterian, A., Berki, A., Yin, J., Sato, F., Xu, Y., Olaru, A., Wang, S., Mori, Y., Deacu, E., *et al.* (2005). Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk. *Oncogene* **24**, 4138–4148.
- Sebastian, C., Zwaans, B.M.M., Silberman, D.M., Gymrek, M., Goren, A., Zhong, L., Ram, O., Truelove, J., Guimaraes, A.R., Toiber, D., *et al.* (2012). The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. *Cell* **151**, 1185–1199.
- Singletary, S.E., Allred, C., Ashley, P., Bassett, L.W., Berry, D., Bland, K.I., Borgen, P.L., Clark, G.M., Edge, S.B., Hayes, D.F., *et al.* (2003). Staging system for breast cancer: revisions for the 6th edition of the AJCC Cancer Staging Manual. *Surg Clin North Am* **83**, 803–819.
- Soto-Reyes, E., and Recillas-Targa, F. (2010). Epigenetic regulation of the human p53 gene promoter by the CTCF transcription factor in transformed cell lines. *Oncogene* **29**, 2217–2227.
- Soto-Reyes-Solis, E., Morales-Espinosa, D., Cantu, D., Alvarado-luna, G., Green, D., Hernandez, C.C., Alcalá-Moreno, I., Cisneros-Soberanis, F., Motola, D., Rodríguez-Cid, J., *et al.* (2013). Characterization of DNA methylation of the promoters CTCF and BORIS (CTCF) in breast and ovarian cancer. *J Clin Oncol* **31**(Suppl), e22151 (abstract).
- Splinter, E., Heath, H., Kooren, J., Palstra, R.J., Klous, P., Grosveld, F., Galjart, N., and de Laat, W. (2006). CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. *Genes Dev* **20**, 2349–2354.
- Tiffen, J.C., Bailey, C.G., Marshall, A.D., Metierre, C., Feng, Y., Wang, Q., Watson, S.L., Holst, J., and Rasko, J.E.J. (2013). The cancer-testis antigen BORIS phenocopies the tumor suppressor CTCF in normal and neoplastic cells. *Int J Cancer* **133**, 1603–1613.
- Van Meter, M., Mao, Z.Y., Gorbunova, V., and Seluanov, A. (2011). SIRT6 overexpression induces massive apoptosis in cancer cells but not in normal cells. *Cell Cycle* **10**, 3153–3158.
- Van Wezel, T., Lombaerts, M., van Roon, E.H., Philippo, K., Baelde, H.J., Szuhai, K., Cornelisse, C.J., and Cleton-Jansen, A.M. (2005). Expression analysis of candidate breast tumour suppressor genes on chromosome 16q. *Breast Cancer Res* **7**, R998–R1004.
- Vostrov, A.A., and Quitschke, W.W. (1997). The zinc finger protein CTCF binds to the APBbeta domain of the amyloid beta-protein precursor promoter. Evidence for a role in transcriptional activation. *J Biol Chem* **272**, 33353–33359.
- Witcher, M., and Emerson, B.M. (2009). Epigenetic silencing of the p16(INK4a) tumor suppressor is associated with loss of CTCF binding and a chromatin boundary. *Mol Cell* **34**, 271–284.
- Yusufzai, T.M., Tagami, H., Nakatani, Y., and Felsenfeld, G. (2004). CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol Cell* **13**, 291–298.
- Zwahlen, D., Tschann, M.P., Grob, T.J., Peters, U.R., Fink, D., Haenggi, W., Altermatt, H.J., Cajot, J.F., Tobler, A., Fey, M.F., and Aebi, S. (2000). Differential expression of p73 splice variants and protein in benign and malignant ovarian tumours. *Int J Cancer* **88**, 66–70.

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