

Original Article

Functional genetic variants within the SIRT2 gene promoter in breast cancer

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Received March 5, 2018; Accepted October 12, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Breast cancer (BC) is the most common cancer in women worldwide. However, BC etiology remains unclear. Approximately 5%-10% of BC cases are inheritable while the rest are sporadic. Large scale genomic studies have reported the molecular landscape and driver genes of BC, explaining the occurrence and development of only a small portion of BC cases. Low frequency and rare genetic variants with large effects may account for missing heritability in BC cases. SIRT2 is a member of sirtuin family of NAD (+)-dependent class III deacetylases. SIRT2 is involved in mitotic progression, oxidative stress, metabolism, microtubule dynamics, cell migration, apoptosis, and differentiation. Recent evidence has suggested that SIRT2 is involved in tumorigenesis. In this study, SIRT2 gene promoter was analyzed in groups of female BC patients (n = 200) and ethnic-matched controls (n = 184). Two novel heterozygous DNA sequence variants (DSVs) (g.38900839G>C and g.38900478G>A) were identified in two patients with luminal B subtype, but not in the controls. These DSVs significantly decreased the transcriptional activity of the SIRT2 gene promoter (P < 0.01) in BC cell lines (MCF-7 and BT-474 cells). The SNP, g.38900291C>G (rs2053071), which was found in both BC patients and controls with similar frequencies, did not significantly alter the transcriptional activity of the SIRT2 gene promoter. Therefore, the SIRT2 gene promoter DSVs may alter transcriptional activity of SIRT2 gene promoter and change SIRT2 levels, contributing to breast cancer development as a rare risk factor.

Keywords: Breast cancer, genetics, SIRT2, promoter, DNA sequence variants

Introduction

Breast cancer (BC) is the most common cancer in women, worldwide. The etiology of BC, however, remains unclear. Approximately 5%-10% of BC cases are inheritable. Up to 90% are due to mutations in BRCA1 and BRCA2 [1]. Most BC cases are sporadic with different morphologies and behaviors [2-4]. Over the past decade, large scale genomic studies have reported the molecular landscape and driver genes of BC [5, 6]. Genetic diagnosis and targeted therapy have been progressively adopted, obtaining good effects in specific BC patients [7, 8]. However, these data only explain the occurrence and development of a small portion of BC cases. The prognosis of some subtypes (such as bas-

al-like breast cancer) is still poor [9, 10]. It has been proposed that low frequency and rare genetic variants with large effects may account for the missing heritability for BC [11]. Emerging evidence has suggested that epigenetic factors also contribute to BC development [12-14].

Sirtuins are a highly conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent protein lysine modifying enzymes that play multiple roles in many cellular processes, such as metabolism, transcription, and DNA repair. Sirtuins have been implicated in the development of cancer and act as either a tumor suppressor or promoter, depending on the cellular context and tumor type [15-17]. SIRT2 is mainly localized to the cytoplasm and can move to the

nucleus during mitosis [18]. SIRT2 maintains cellular homeostasis through the deacetylation of its substrates, such as histone H4K16, H3K56, α -tubulin, PR-Set7, phosphoenolpyruvate carboxykinase 1, NF- κ B subunit p65, RIP1 (receptor-interacting protein 1), Foxo1, Foxo3, p53, Cdh1, and Cdc20 [19-27]. SIRT2 also regulates mitotic progression [28], oxidative stress response [25], metabolism [29], microtubule dynamics [30], cell migration [31], apoptosis [32], neurotoxicity [33], and the inhibition of differentiation [24, 34]. Increasing evidence has suggested that SIRT2 is involved in tumorigenesis [35-38].

It has been reported that SIRT2 has two opposing roles, either as a tumor suppressor or an oncogene [20, 39]. Previous studies have indicated that SIRT2 functions as a tumor suppressor by maintaining mitotic integrity in a cell culture system [40-42]. Mice lacking SIRT2 develop liver, gastrointestinal, and mammary tumors, as well as pancreatic ductal adenocarcinoma and lung adenocarcinoma [27, 43]. A deficiency of SIRT2 in a murine model causes the development of gender-specific tumorigenesis, with the development of mammary tumors in females and hepatocellular carcinoma (HCC) in males [27]. Levels of SIRT2 are more frequently decreased in human breast tumors [27]. Expression of SIRT2 was shown to be downregulated in several human cancers, including gliomas [40, 41], head and neck squamous cell carcinoma (HNSCC) [42], and esophageal adenocarcinoma (EAC) [44]. Regions of the SIRT2 gene are also frequently deleted in human gliomas [40].

SIRT2 may also have tumor-promoter qualities [36, 45]. SIRT2 knockdown has led to both necrotic and apoptotic cell death in C6 glioma cells [46] and cervical carcinoma HeLa cells [32]. SIRT2 has been shown to be upregulated and involved in the aberrant proliferation and survival of leukemic cells [47]. SIRT2 inhibits the transcription of developmentally downregulated protein 4 (NEDD4), an E3 ubiquitin ligase that targets Myc proteins for degradation through the deacetylation of acetyl histone H4-K16, resulting in the stabilization of N-myc and C-myc proteins, ultimately promoting neuroblastoma and pancreatic cancer [48]. SIRT2, together with HDAC6, targets K-Ras in many cancers through the deacetylation of lysine 104 site, dramatically promoting the tumorigenesis of cancer cell lines [49]. SIRT2 contributes to cell motility and invasiveness of hepato-

cellular carcinoma (HCC). Upregulation of SIRT2 in primary HCC tumors has been significantly correlated with the presence of microscopic vascular invasion, more advanced tumor stage, and shorter overall survival [50]. SIRT2 promotes bladder cancer cell migration and invasion by targeting cortactin together with HDAC6 [38]. In addition, expression of SIRT1 and SIRT4 are significantly downregulated in BC tissues and SKBR3 BC cells. In contrast, SIRT2, SIRT3, and SIRT5 genes are upregulated [51]. Opposing data suggests that the roles of SIRT2 in carcinogenesis depend on the features of diverse tumors. It was speculated that DNA sequence variants (DSVs) in the SIRT2 gene promoter may influence expression of SIRT2 genes and be involved in the tumorigenesis of breasts. Therefore, occurrence of DSVs in the SIRT2 gene promoter was genetically and functionally investigated in large cohorts of sporadic BC patients and healthy controls.

Materials and methods

BC patients and healthy controls

All female BC patients (n = 200, ages ranging from 26 to 81 years, median age 50.00 years) were recruited from April 2014 to June 2017, from the Division of Breast Surgery, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong, China. BC patients were diagnosed by two senior pathologists, including different subtypes, 42 luminal A, 93 luminal B, 33 HER2-Enriched, 26 triple negative, and 6 ductal carcinomas *in situ* (DCIS). Ethnically-matched healthy female controls (n = 184, ages ranging from 23 to 82 years, median age 52.00 years) were recruited from the same hospital during the same period. All BC patients had no family history of BC. Controls with a family history of breast disease were excluded from this study. This study was carried out according to principles of the Declaration of Helsinki and was approved by the Human Ethics Committee of Affiliated Hospital of Jining Medical University. Written informed consent was obtained from all participants.

Direct DNA sequencing

Leukocytes were isolated from venous blood and genomic DNAs were extracted. Two overlapped DNA fragments, 764 bp (-1292 bp~ -521 bp) and 678 bp (-598 bp~ +154 bp), covering the SIRT2 gene promoter region (1446

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Table 1. PCR primers for the SIRT2 gene promoter

PCR primers	Sequences	Location	Position	Products
Sequencing				
SIRT2-F1	5'-GGCATACAGCAGTAAACACAAC-3'	38901154	-1292	772 bp
SIRT2-R1	5'-CTAGCTATGATCCTAACCCAAG-3'	38900383	-521	
SIRT2-F2	5'-ACAATGTGGATTCCAGGAGC-3'	38900460	-598	752 bp
SIRT2-R2	5'-TTTGGTACAACACCCAGAGC-3'	38899709	+154	
Functioning				
SIRT2-F	5'-(KpnI)-GGCATACAGCAGTAAACACAAC-3'	38901154		1446 bp
SIRT2-R	5'-(HindIII)-TTTGGTACAACACCCAGAGC-3'	38899709	+154	

PCR primers are designed based on the genomic DNA sequence of the SIRT2 gene (NC_000019.10). Transcription start site (TSS) is at the position of 38899862 (+1).

bp, from -1292 bp to +154 bp to the transcription start site) were generated by PCR and directly sequenced. PCR primers were designed based on the genomic sequence of human SIRT2 gene (NCBI, NC_000019.10) (Table 1). Sequencing was performed with an Applied Biosystems 3500XL genetic analyzer. Sequences were aligned and compared with the wild type SIRT2 gene promoter.

Functional analysis with dual-luciferase reporter assay

DNA fragments of wild type and variant SIRT2 gene promoters (1446 bp, from -1292 bp to +154 bp to the transcription start site) were generated by PCR and inserted into the KpnI and Hind III sites of pGL3-basic to generate expression vectors. PCR primers are shown in Table 1. Designated expression vectors were transiently transfected into human embryonic kidney cells (HEK-293), MCF-7, or BT-474 cells. Forty-eight hours post-transfection, the cells were collected and luciferase activities were measured using a dual-luciferase reporter assay system on a Promega Glomax 20/20 luminometer. Vector pRL-TK expressing renilla luciferase was used as an internal control for transfection. Empty vector pGL3-basic was used as a negative control. Transcriptional activities of the SIRT2 gene promoters were represented as ratios of luciferase activities over renilla luciferase activities. All experiments were repeated three times, independently, in triplicate. Transcriptional activities of the wild type SIRT2 gene promoter were designed as 100%.

Statistical analysis

Two-tailed Student's t-test was employed to analyze expression levels of the reporter gene. Two-way ANOVA was applied to analyze fre-

quencies of DSV and SNPs. All analyses were performed using SPSS v13.0. A threshold of $P < 0.05$ is defined as statistically significant.

Results

DSVs identified in BC patients and controls

The present study analyzed the occurrence of DSVs in BC patients and controls. Eight DSVs, including three single-nucleotide polymorphisms (SNP), were identified in this study. Locations and frequencies of the DSVs and SNPs are depicted in Figure 1A and summarized in Table 2. Two heterozygous DSVs (g.38900839G>C and g.38900478G>A) were identified in two BC patients (41 and 50 years old, respectively), but not in the controls. Both BC patients were luminal B subtype. DNA sequencing chromatograms of these novel DSVs are shown in Figure 1B. Three heterozygous DSVs, g.38900413A>C, g.38900030G>A, and g.38899852C>T, were only found in controls. In addition, three SNPs, g.38901007delT (rs10713585), g.38900291C>G (rs2053071), and g.38900145C>T (rs116900177), were found in both BC patients and controls. The SNP, g.38900145C>T (rs116900177), was seen more in controls than BC patients ($P < 0.05$). Sequencing chromatograms of these DSVs and SNPs are not shown.

Putative binding sites for transcription factors affected by DSVs

To determine whether DSVs affect putative binding sites for transcription factors, the SIRT2 gene promoter was analyzed with the JASPAR program (<http://jaspar.genereg.net/>). DSVs identified in BC patients may abolish, create, or modify the putative binding sites for transcription factors. The DSV g.38900839G>C may

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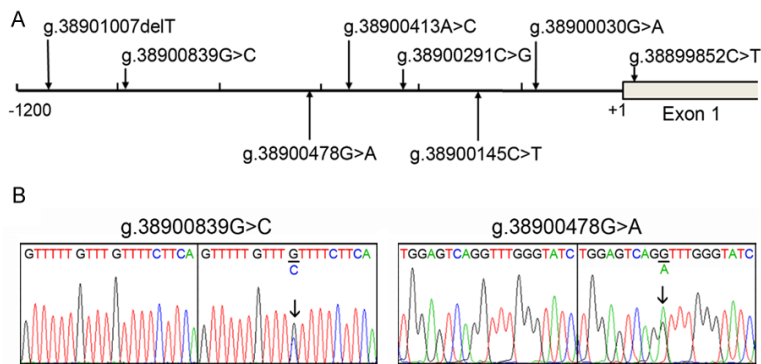


Figure 1. Locations and sequencing chromatograms of DSVs in the SIRT2 gene promoter. A. Locations of the DSVs in the SIRT2 gene promoter in BC patients and controls. The numbers represent the genomic DNA sequences of the human SIRT2 gene (Genbank accession number NC_000019.10). The transcription start site is at the position of 38899862 in the first exon. B. Sequencing chromatograms of the novel DSVs in the SIRT2 gene promoter identified in BC patients. Sequence orientations of the DSVs are forward. Left panels show wild type and right panels heterozygous DSVs, which are marked with arrows.

modify the binding sites for forkhead box (FOX) transcription factors, including FOXA1, FOXC2, FOXD2, FOXI1, FOXL1, FOXO4, FOXO6, FOXP2, and FOXP3. The DSV g.38900478G>A may abolish a binding site for SNAI2, a zinc finger factor that controls the undifferentiated state of human epidermal progenitor cells.

Functional analysis of the DSVs by dual-luciferase reporter assay

Wild type and variant SIRT2 gene promoters were cloned into luciferase reporter vector (pGL3-basic) to generate expression vectors, including empty pGL3-basic (negative control), pGL3-WT (wild type SIRT2 gene promoter), pGL3-38900839C, pGL3-38900478A, and pGL3-38900291G. After transfecting into cultured cell lines, human embryonic-kidney cells (HEK-293), MCF-7 (human breast adenocarcinoma cell line), and BT-474 (invasive ductal carcinoma of the breast cells), the cells were collected. Dual-luciferase activities were assayed and relative transcriptional activities of the SIRT2 gene promoters were calculated. Transcriptional activities of the wild type SIRT2 gene promoter were set as 100%.

In HEK-293 cells, DSVs (g.38900839G>C and g.38900478G>A) that were identified only in BC patients significantly decreased the activity of SIRT2 gene promoter ($P < 0.01$ for both DSVs). The SNP, g.38900291C>G (rs20530-

71), found in both BC patients and controls with similar frequencies, did not alter the activity of SIRT2 gene promoter ($P > 0.05$) (Figure 2). To further investigate the effects of DSVs in BC cells, this study examined the transcriptional activity of DSVs found in breast BC patients (g.38900839G>C and g.38900478G>A) in MCF-7 and BT-474 cells. Both DSVs significantly decreased the activity of the SIRT2 gene promoter in MCF-7 and BT-474 cells ($P < 0.01$). Similarly, the SNPs, g.38900291C>G (rs205307-1), did not significantly alter the activity of the SIRT2 gene promoter ($P > 0.05$) (Figure 2).

Taken together, DSVs identified in BC patients altered the activity of the SIRT2 gene promoter in HEK-293 cells, MCF-7, and BT-474 cells, indicating that effects on the SIRT2 gene promoter were not tissue-specific.

Discussion

Genetic variants in the SIRT2 gene have been associated with human diseases, including myocardial infarction and type 2 diabetes [52, 53]. SNP rs10410544 T allele in SIRT2 has been associated with Alzheimer's disease in the APOE ϵ 4-negative Caucasian population, as a minor genetic risk factor [54]. The intronic SNP (rs10410544) in the SIRT2 gene significantly increases risk of Alzheimer's disease [55]. The SIRT2 gene SNP (rs45592833), located in the 3'-untranslated regions (3'UTR), has been significantly associated with human longevity [56]. A variant in SIRT2 gene 3'-UTR has been associated with susceptibility to colorectal cancer [57]. In this study, two heterozygous DSVs (g.38900839G>C and g.38900478G>A) were identified in two BC patients, but not in the controls. These DSVs significantly altered the transcriptional activity of the SIRT2 gene promoter in both HEK-293 and BC cell lines (MCF-7 and BT-474 cells). Therefore, these SIRT2 gene promoter DSVs may change SIRT2 levels, contributing to breast cancer development. Although occurrence of DSVs in SIRT2 gene promoter is rare in BC patients, the pres-

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Table 2. DSVs within SIRT2 gene promoters in BC patients and controls

DSVs	Genotypes	Location ¹	Controls (n = 184)	BC (n = 200)	P value
g.38901007delT (rs10713585)	T/T	-1145 bp	0	0	0.907
	T/-		6	6	
	-/-		178	194	
g.38900839G>C	G/C	-977 bp	0	1	-
g.38900478G>A	G/A	-616 bp	0	1	-
g.38900413A>C	AC	-551 bp	1	0	-
g.38900291C>G (rs2053071)	CC	-429 bp	29	46	0.084
	GC		84	95	
	GG		71	59	
g.38900145C>T (rs116900177)	CT	-283 bp	13	4	0.015
g.38900030G>A	GA	-168 bp	1	0	-
g.38899852C>T	CT	+11 bp	1	0	-

¹, DSVs are located upstream (-) to the transcription start site of SIRT2 gene at 38899862 of NC_000019.10.

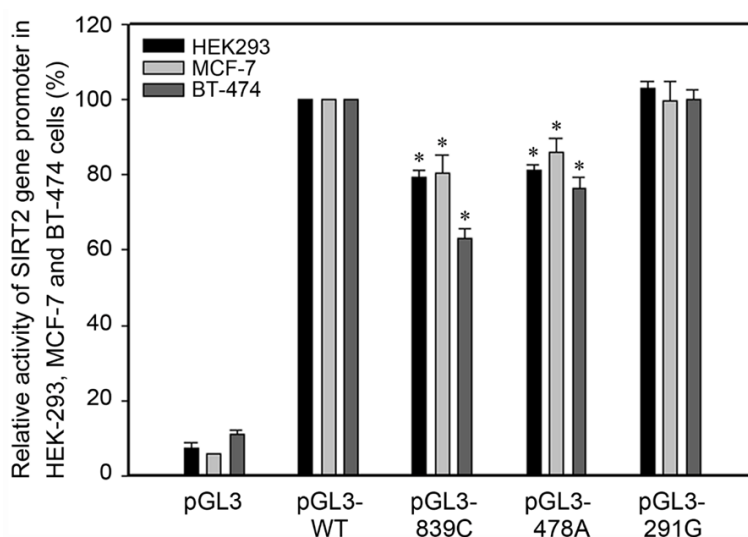


Figure 2. Relative transcriptional activities of wild type and variant SIRT2 gene promoters in HEK-293, MCF-7, and BT-474 cells. Wild type and variant SIRT2 gene promoters were cloned into reporter gene vector pGL3 and transfected into HEK-293, MCF-7 and BT-474 cells. The transfected cells were collected and dual-luciferase activities were assayed. Empty vector pGL3-basic is used as a negative control. Transcriptional activity of the wild type SIRT2 gene promoter was designed as 100%. Relative activities of SIRT2 gene promoters were calculated and shown in bar graphs. Set 1, pGL3-empty; 2, pGL3-WT; 3, pGL3-38900839C; 4, pGL3-38900478A; 5, pGL3-38900291G. WT, wild type. *, P < 0.01.

ent study highlights the mechanisms of SIRT2 in the tumorigenesis of breasts. Present data provides supportive evidence that SIRT2 may function as a tumor repressor for BC.

The human SIRT2 gene has been localized to chromosome 19q13.1, which has 16 exons and spans a region of 20,960 bp. The SIRT2 gene promoter is a TATA- and CCAAT-box less

promoter, containing a 670 bp CpG island and a number of NF- κ B and GATA transcription factor binding sites [58]. In human cells, SIRT2 genes are directly regulated by P53 [59]. In addition, the SIRT2 gene is a direct target of microRNA-7 [60]. Expression of SIRT2 mRNAs was significantly reduced in tumor tissue samples from basal cell carcinoma patients, compared with non-tumor tissues [61]. In this study, two functioning DSVs in the SIRT2 gene promoter were identified in patients with luminal B subtype of breast cancer. Further investigations to find transcription factors binding to these DSVs will provide new information characterizing the human SIRT2 gene promoter.

Many studies have demonstrated that SIRT2 is implicated in diverse types of BC. SIRT2 deacetylase stabilizes

Slug to control the malignancy of basal-like breast cancer (BLBC). Genetic depletion and pharmacological inactivation of SIRT2 in BLBC cells reverse Slug stabilization, cause the loss of clinically relevant pathological features of BLBC, and inhibit tumor growth [62]. SIRT2 dependent epigenetic silencing of arrestin domain-containing 3 (ARRDC3), a tumor suppressor, may contribute to the aggressive nature of

BLBC cells [63]. As a tumor suppressor, SIRT2 regulates cell cycle progression and genome stability by modulating the mitotic deposition of histone H4 lysine 20 (H4K20) methylation [18, 64]. SIRT2 has also been found to deacetylate histone H4 lysine 16 (H4K16) mediating chromatin condensation during G2/M transition [63, 66]. SIRT2 stabilizes N-MYC and c-MYC proteins by downregulating ubiquitin-protein ligase NEDD4 expression [48]. SIRT2 modulates H4K20 me 1-3 depositions, which are important for cell cycle regulation, through the deacetylation of H4K16 [18]. Several SIRT2 inhibitors have also been reported to have anticancer effects [35, 45, 67, 68]. Specific inhibitors combined targeting of both SIRT1 and SIRT2 induced p53 acetylation and cell death in MCF-7 cells [68]. Therefore, genetic variants in the SIRT2 gene promoter may change SIRT2 levels, affecting cell proliferation of breast cancer cells. Human SIRT2 gene expression may be manipulated with genetic approaches or pharmaceutical agents for targeted purposes.

Previous studies have shown that SIRT2 gene expression was altered in diverse types of tumors [35-38]. SIRT2 levels are more frequently decreased in human breast tumors [27]. To date, molecular mechanisms related to SIRT2 gene expression remain unclear. Present results suggest that genetic variants exist in the human SIRT2 gene promoter in BC patients. The novel DSVs identified in BC patients significantly altered the transcriptional activity of the SIRT2 gene promoter in cultured breast cancer cell lines. Therefore, DSVs in SIRT2 gene promoter may change SIRT2 levels, contributing to breast cancer development as a rare risk factor. Present findings may provide a genetic basis for translational and targeted therapeutic studies for BC patients.

Acknowledgements

This study was supported by the Natural Science Foundation of Shandong Province (ZR20-14HM115) and Scientific Research Project of Shandong University (12721742).

Disclosure of conflict of interest

None.

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