

# Quantitative detection of methylation of *FHIT* and *BRCA1* promoters in the serum of ductal breast cancer patients

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**Abstract.** The development of carcinoma has been found to be associated with epigenetic modifications. The aim of this study was to estimate the methylation levels of *FHIT* and *BRCA1* promoters using the bisulphite sequencing method (BSP) and high-resolution melting curve analysis (HRM) in the serum of patients with ductal breast carcinoma as a biomarker for the possible application of early diagnosis of breast cancer. The results showed that the methylation levels of both *BRCA1* and *FHIT* promoters were higher in the serum of the breast ductal carcinoma group (BDC group) than those of the breast fibroadenoma group (BFA group), and the healthy individuals group (HI group). However, the methylation levels of the *BRCA1* promoters were very low in all three groups compared to the levels of *FHIT*. The advanced quantitative detection of the samples with HRM showed that the *FHIT* promoter methylation level of the cfDNA in each serum was also very high in the BDC group compared to the HI group. The methylation level of *FHIT* was found to be significantly associated with breast cancer ( $p < 0.05$ ). In conclusion, the methylation quantitative detection of *FHIT* promoter in serum may be useful for the early diagnosis of ductal breast carcinoma.

Keywords: *FHIT*, *BRCA1*, methylation, breast cancer, cfDNA

## 1. Introduction

Invasive ductal carcinoma is the most common type of breast cancer. However, in early diagnosis it is difficult to distinguish from fibroadenoma, which is the most common benign tumor of the breast. According to the data, the development of carcinoma is a complicated process that involves gene mutation and epigenetic changes. For example, individuals possessing the breast cancer susceptibility gene 1 (*BRCA1*) mutation have a higher risk of breast and ovarian cancers [1]. Some data shows epigenetic alterations may result in the silencing of the tumor suppressor gene [2]. The methylation of gene promoters is the main mechanism of epigenetic alterations. Wei M, et al. reported that *BRCA1* promoter methylation in sporadic breast cancer was associated with a reduced number of *BRCA1* copies [3]. Therefore, the detection of promoter methylation of the tumor suppressor gene may be a

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useful method for the early diagnosis of tumors, but it may be difficult in application for its invasion.

Cell free DNA (cfDNA) is DNA isolated from serum or plasma. The cfDNA is set off to the circulatory system by the cells, including tumor cell. It is found in low concentrations in healthy individuals [4], but are at elevated levels in trauma, stroke, and cancer patients in which necrosis, apoptosis, and especially metastasis has occurred [5-7]. Furthermore, methylation patterns in cfDNA are similar to those in cancer tissues in the same patient [8], which suggests that methylation patterns in cfDNA may be useful for early diagnosis and monitoring of cancer.

In this paper, considering the low concentration of cfDNA in serum, the levels of methylation of the two tumor suppression genes, *BRCA1* and the fragile histidine triad gene (*FHIT*), of the cfDNA in the pool of patients' serum from each group were investigated using the Bisulphite sequencing method (BSP). Subsequently, the methylation levels of *FHIT* in the patients' samples were evaluated with high-resolution melting curve analysis (HRM), which may prove to be a suitable method for the early diagnosis of breast cancer.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich Chemical Company, unless otherwise stated.

### 2.2. Clinical samples

The studied subjects were obtained from 36 patients with invasive breast ductal carcinoma (BDC group), 30 patients with breast fibroadenoma (BFA group), and 30 healthy individuals (HI group) at Jilin Central hospital from January 2014 through August 2014. All of the serum samples for analysis were extracted and stored at  $-80^{\circ}\text{C}$  within 2-3 hours after blood draw. This study was approved by the institutional review board of the Jilin Central hospital in Jilin Province, PRC, and informed consent was obtained from all participants.

### 2.3. DNA isolation and bisulfate treatment

The cfDNA for BSP was extracted from the mixture of serum samples of each group with cfDNA extraction kits (Omega) and then concentrated with DNA Concentrator (Genevac Ltd). The cfDNA for MS-HRM was extracted from each of the serum samples with cfDNA extraction kits (Omega). All of the cfDNA was bisulfate modified with EZ DNA Methylation Gold<sup>TM</sup> Kits (Zymo Research, USA) according to the manufacturer's protocol using <500 ng of each DNA sample.

### 2.4. Bisulfite sequencing PCR

The primers of *FHIT* and *BRCA1* were designed with methyl\_primer\_express\_software v1.0 and synthesized by BGI Company, all of which are listed in Table 1. The amplifications were performed in 50  $\mu\text{L}$  reaction mixtures containing 2  $\mu\text{L}$  bisulfite-modified genomic DNA, 25  $\mu\text{L}$  Hot start ExTaq premix, and 1  $\mu\text{L}$  primers (10  $\mu\text{M}$ ). The touchdown PCR was performed according to the following protocol:  $95^{\circ}\text{C}$  denaturation for 5 min, followed by 15 cycles of  $98^{\circ}\text{C}$  for 10 sec, annealing for 30 sec arranging from  $68^{\circ}\text{C}$  to  $53^{\circ}\text{C}$  for *FHIT* and  $63.5^{\circ}\text{C}$  to  $48.5^{\circ}\text{C}$  for *BRCA1* (decreasing  $1^{\circ}\text{C}$  per cycle),

Table 1  
Sequence of specific primers used for BSP and MS-HRM

Gene	Primer sequences	Length (bp)	GenBank accession no.
<i>BRCA1</i>	F: ATTTTTTGTGGTAAAGGT	317	NG_005905.2
	R: AACATAATATCCCCCTCAAAC		
<i>FHIT</i>	F: TTTGTAATTTAGGATTTGGG	289	NG_007551.1
	R: CTAATTCCAAACCTCAACATCTT		

extension at 72°C for 30 sec; followed by another 25 cycles of 98°C for 10 sec, annealing temperatures of 53°C (for *FHIT*) or 48.5°C (for *BRCA1*) for 30 sec and 30 sec elongation at 72°C, and final incubation at 72°C for 10 min. The amplified fragments were sub-cloned. At least twenty clones of each sample were selected for sequencing. The sequencing data of ten sub-clones were selected and analyzed by the BiQ Analyzer. The CpG islands were screened for black spot methylation sites. The percentage of methylation for each sample was calculated as the number of methylated CpG dinucleotides/(10×12 or 19) × 100% for *BRCA1* and *FHIT*, respectively.

### 2.5. HRM

Methylation standards were constructed by diluting 100% methylated bisulfite-modified control DNA and 100% unmethylated bisulfite-modified control DNA (EpiTect PCR Control DNA Set, Qiagen,) in a pool of normal bisulfite-modified DNA at ratios of 25%, 50%, and 75%. HRM was performed in a total volume of 25 µL containing: 1 µL of modified template DNA, 12.5 µL 2×HRM Analysis PreMix (HRM Analysis Kit, Tiangen), 1 µL of each primer (10 µM), and 9.5 µL PCR grade water. The cycling conditions were: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, annealing for 30 sec at 60°C, extension at 72°C for 30 sec; followed by an HRM step of 95°C for 1 min, and 40°C for 1 min, 65°C for 15 sec, and continuous acquisition to 95°C at one acquisition per 0.02°C, and then detected using the PikoREAL. HRM data were analyzed using the dedicated HRM software (PikoREAL, Thermo Scientific). All experiments were performed in triplicate.

### 2.6. Statistical analyses

SPSS 16.0 software was used for statistical analyses.  $\chi$  tests were used to test the differences of *FHIT* methylation level in the groups. *p* values below 0.05 were considered statistically significant.

## 3. Results

### 3.1. The methylation level of *FHIT* and *BRCA1* in BSP

The results of BSP showed that although the level of *BRCA1* promoter methylation was higher in the BDC group (10%) than those of the BFA group (2.5%) and HI group (1.7%), all three groups were very low. Conversely, the levels of *FHIT* promoter methylation were very high, and higher in the BDC group (64.2%) than in the BFA group (36.8%) and HI group (35.3%), as shown in Figures 1 and 2.

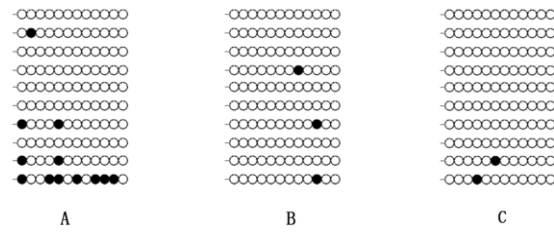


Fig. 1. Methylation of *BRCA1* promoter in serum. A) BDC group (10.0%); B) BFA group (2.5%); C) HI group (1.7%).

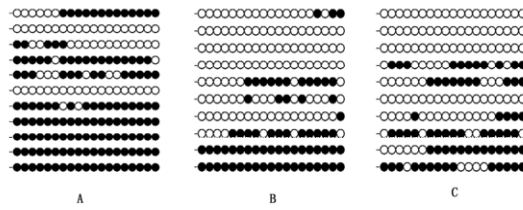


Fig. 2. Methylation of *FHIT* promoter in serum. A) BDC group (64.2%); B) BFA group (36.8%); C) HI group (35.3%).

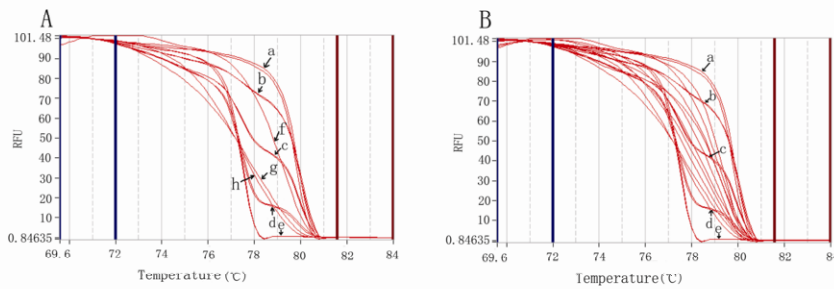


Fig. 3. Level of *FHIT* promoter methylation in MS-HRM. A) Methylation of *FHIT* promoter in serum of three groups: g = BDC group; f = BFA group; h = HI group; a-e = Standard curve of 100%, 75%, 50%, 25%, and 0% methylation, respectively; B) Methylation of *FHIT* promoter in serum of some patients.

3.2. The methylation level of *FHIT* with HRM

The results of MS-HRM of the groups' samples were similar to those of BSP. *FHIT* promoter methylation levels in the serums of the BDC group were higher (>50%) than those of the other two groups (>25% and <50%), as shown in Figure 3A. The advanced detection of HRM of each sample showed that 83.3% of the BDC group had higher (>50%) methylation levels of *FHIT* promoter than those of the two other groups (see Table 2). These results showed that the methylation of *FHIT* promoters was significantly associated with ductal breast cancer ( $p < 0.05$ ).

Table 2

Groups	Methylation level of <i>FHIT</i> with MS-HRM		
	Level of Methylation (ratio)		
	25-50%	50-75%	75-100%
HI (n=30)	15 of 30 (50.0%)	12 of 30 (40.0%)	3 of 30 (10.0%)
BFA (n=30)	17 of 30 (56.7%)	11 of 30 (36.7%)	2 of 30 (6.6%)
BDC (n=36)	6 of 36 (16.7%)	18 of 36 (50%)	12 of 36 (33.3%)

Note:  $\chi^2 = 16.602$   $P = 0.002 (<0.05)$ .

#### 4. Discussion

*BRCA1* is a tumor suppressor gene associated with many female carcinomas [9]. Its functions include controlling chromatin remodeling and transcription, cell cycle regulation, and DNA repair processes in many organs [10]. Previous studies have reported that low expressions of *BRCA1* mRNA result from DNA methylation [11], which may be the mechanism by which *BRCA1* transcription is silenced. This paper evaluated the quantitative methylation of *BRCA1* in the serum of the BDC, NP, and BFA groups using BSP; although the levels of methylation in the BDC group were higher than those of the other groups, the levels of methylation of the *BRCA1* promoter in all three groups were very low. This was different from the research of Katarzyna Jaworska-Bieniek's [12], which may be due to different methods, different samples, or different kinds of breast tumors. Cameron Snell, et al. detected the methylation of *BRCA1* in peripheral blood. The methylation levels were found to be very low: below 10% in breast cancer patients and only 0.1% in healthy individuals [13]. These results were consistent with the results of this study. Considering the low levels of methylation and the difficulty of quantitative detection with HRM, the methylation of another tumor suppressor gene, *FHIT*, was estimated.

*FHIT* was first recognized as a putative tumor suppressor in humans in 1996 [14]. Its protein is essential for protecting DNA from damage-induced cancer initiation and progression by modulating genome stability, oxidative stress, and levels of accumulating DNA damage [15]. It is reduced or has no expression in many types of cancer, including breast cancer, and especially in malignant breast cancer [16, 17]. Mohammad Raish, et al. discovered that the methylation frequency in *FHIT* genes is significantly higher in poorly- and moderately-differentiated tumors in postmenopausal patients. Loss of the protein expression of *FHIT* is partially associated with the methylation status in premenopausal women [18]. In this study, the quantitative methylation levels of *FHIT* promoters of cfDNA in the BDC, BFA, and NP groups were detected with BSP. The results showed the levels of methylation of cfDNA in the BDC group were approximately twice those of the other two groups. Also, when the HRM of each patient's sample was carried out, the results showed the methylation level was significantly associated with the malignancy of the breast cancer for the first time in cfDNA.

Cell-free DNA was first studied over fifty years ago [19]. Elevated levels of cfDNA in breast cancer patients were discovered in 2007 [20], and were not influenced by the female menstrual cycle [21]. Following the development of techniques, more characteristics of cfDNA were found. It is thought circulating tumour DNA (ctDNA) has a greater correlation with changes in tumour burden than two other circulating biomarkers (CA 15-3 and circulating tumor cell counts) in individuals with metastatic breast cancer [22]. Vera Kloten, et al. also found promoter hypermethylation of tumor suppressor genes *ITIH5*, *DKK3*, and *RASSF1A* in cfDNA with methylation-specific polymerase chain reaction (MSP) and believed it to be a novel biomarker for blood-based breast cancer screening [23]. In this paper, the methylation levels of *BRCA1* and *FHIT* were quantitatively detected with BSP, the gold standard method, but the patient samples were pooled by group before BSP because of the low concentration of cfDNA, the possible bias may occur, so the methylation levels of *FHIT* of each sample were estimated with HRM, which is a new quantitative method suitable for a number of sample measurements.

In summary, the level of methylation of *FHIT* promoters in cfDNA was found to be significantly associated with ductal breast carcinoma. The quantitative detection of *FHIT* promoter methylation with HRM in cfDNA may be useful for the early diagnosis of ductal breast cancer.

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