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Research paper

Methylation of NBPF1 as a novel marker for the detection of plasma cell-free DNA of breast cancer patients

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ABSTRACT

Background: Recent studies revealed that tumor-specific gene methylation can be detected in the circulating cell-free DNA (cfDNA) of cancer patients; therefore, methylated cfDNA is considered a promising biomarker. Human neuroblastoma breakpoint family member 1 (NBPF1) was originally identified in a neuroblastoma (NB) patient. The present study is the first to evaluate the presence of NBPF1 gene methylation in cfDNA in plasma of breast cancer patients.

Methods: Differentially methylated cfDNA was screened using bisulfite sequencing with a next-generation sequencer (BS-seq) among 25 breast cancer patients, 25 patients with a benign breast disease and 25 healthy female volunteers. Then, five specific methylation sites in the NBPF1 gene were verified in 11 breast cancer samples, and two further sites in the NBPF1 promoter were detected in 52 breast cancer patients (stages I-III), 31 patients with benign breast disease and 30 healthy controls by using methylation-specific PCR (MSP). Furthermore, the association between the methylation statuses of NBPF1 and the clinicopathological characteristics of breast cancer patients was analyzed.

Results: BS-seq demonstrated that the NBPF1 methylation levels in breast cancer patients were higher than those in patients with benign breast disease and healthy controls. The MSP results showed that the methylation rates of two sites in the NBPF1 promoter were 67.1% and 61.4% in breast cancer patients, 48.2% and 59.6% in patients with benign breast disease, and 40.9% and 48.1% in healthy controls, respectively. The methylation rates of one site were significantly different among the three groups ($p < .05$), with the highest rate in breast cancer patients. Moreover, there was no statistically significant correlation between the NBPF1 promoter methylation and the major clinicopathological features of the patients.

Conclusions: These results indicate that hypermethylation of the NBPF1 promoter occurs in a significant proportion of breast tumors and that NBPF1-methylated cfDNA may serve as a potential tumor marker for breast cancer.

1. Introduction

Breast cancer (BC) is by far the most frequently occurring cancer in women. Every year, 522,000 women die from BC [1]. In breast cancer, ultrasound, puncture cytological examination and mammography are used as screening tools for early diagnosis but have limitations because they are less sensitive or specific when identifying cancer in the early stages [2,3].It has also been clearly shown that DNA methylation is one of the most frequently occurring epigenetic events in the mammalian genome and that alterations in DNA methylation are very common in cancer cells [4]. In 1999, cell-free DNA (cfDNA) circulating in plasma of breast cancer patients was suggested as a very promising tumor biomarker for early detection and prognosis [5].

The methylation of tumor suppressor genes is one of the most common events in carcinogenesis and has been detected in various malignant diseases, including breast cancer [6–9]. Recent studies have also revealed that tumor-specific gene methylation can be detected in the cfDNA of cancer patients and that methylated cfDNA can be a promising biomarker [10–12]. Several studies have shown that cfDNA is present in the plasma or serum of cancer patients, and its methylation patterns have been associated with the tumor burden and malignant progression [13,14].

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Human neuroblastoma breakpoint family member 1 (NBPF1) was originally identified in a neuroblastoma (NB) patient based on its disruption in a de novo, constitutional translocation between chromosomes 1p36.2 and17q11.2 [15,16]. NBPF1 is a member of the NBPF family of proteins, which consists of dozens of recently duplicated genes that are primarily located in segmental duplications on human chromosome 1. Accumulating evidence has indicated that altered expression of NBPF1 is associated with several types of cancer pathogenesis, such as gastric cancer and neuroblastoma [17,18]. Moreover, a recent article reported that NBPF1 acts as a tumor suppressor in neuroblastoma by inducing the G1 cell-cycle arrest [19]. Although NBPF1 has been implicated in several types of diseases, there has been no report on the methylation of NFBP1gene as a novel marker for the detection of plasma cfDNA in breast cancer patients.

The bisulfite conversion of DNA followed by BS-seq opens the possibility of discovery studies to quantify DNA methylation patterns at the base-pair resolution. The rapidly developing BS-seq technologies may provide a quantitative methylation ratio within a broad CpG area. We can quantitatively detect the methylation levels of genes, and methylated cfDNA of NBPF1 gene in plasma can be analyzed by using methylation-specific PCR (MSP).MSP can rapidly detect the methylation status of any group of CpG sites within a CpG island and does not require methylation-sensitive restriction enzymes. It also requires minute amounts of DNA and is very sensitive, as it can detect < 0.1% of methylated alleles in a specific locus [20,21].Given the accessibility of methodology, a few studies have extended methylation analysis to cfDNA by using the BS-seq and MSP in multiple tumors, but such studies are still scarce in breast cancer [22].

In this study, we used BS-seq to explore differentially methylated cfDNA in plasma of breast cancer patients and, as a first step, validated NBPF1 as a differentially methylated gene, to further suggest that methylated cfDNA of NBPF1 gene may serve as a potential tumor marker for breast cancer.

2. Materials and methods

2.1. In the training study

2.1.1. Patients and healthy controls

Peripheral blood samples from 25 consecutive breast cancer patients without treatment before surgery and 25 patients with breast fibroadenomas, included as a separate benign tumor group, were collected at the Peking Union Medical College Hospital from 2016 to 2017. For every patient enrolled, a complete diagnostic examination was performed to evaluate the presence or the absence of distant metastasis, consisting of chest X-rays, mammography, ultrasound of the liver and a whole-body bone scan. Computed tomography scans and/or magnetic resonance imaging studies were performed, if clinically indicated. Twenty-five healthy female volunteers were enrolled as a control group. The blood specimens were centrifuged, and the plasma was stored at −80 °C until the assays were performed.

The breast cancer cases were at different stages (I–III) and reviewed by experienced pathologists. The breast cancer molecular subtypes were characterized based on the guideline of the St. Gallen International Expert Consensus [23].All control individuals were healthy when donating their blood, and the health condition was certified by physicians at the Peking Union Medical College Hospital. The average ages in the benign tumor group and healthy controls matched those of the cancerous cases.

Informed consent was obtained from each patient included in this study. The study, along with the procedures used, was approved by the Institutional Research Ethics Committee of PUMCH.

2.1.2. cfDNA extraction and sodium bisulfite treatment

A total of 2 mL of peripheral whole blood was collected from studied participants in EDTA-K2 tubes. Within 2 h of blood collection, plasma was isolated from the whole blood samples.

There were 3 groups with 25 samples in each. Aliquots (200 μL) from each sample in each group were mixed thoroughly and processed for cfDNA extraction with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Cat. No. 55114) according to the manufacturer's instructions. Extracted DNA was modified with sodium bisulfite (SB) using the EZ DNA Methylation-Gold Kit (Cat. No. D5005), to convert all unmethylated cytosines to uracil, leaving methylated cytosines intact. The converted DNA was stored at −70 °C until use.

2.1.3. Specific gene screening using bisulfite sequencing (BS-seq)

The BS-seq methylation assay was performed with an Illumina Hiseq X10high-throughput sequencer according to the manufacturer's instructions. The data alignment and methylation calling were analyzed using the Bismarck software (Roche Diagnostics). The methylation level for each gene was assigned by averaging the methylation levels of all CpG sites. The methylation index (MI) was calculated by dividing the number of cytosines by the total read number at each CpG site.

2.1.4. Isolation of cfDNA circulating in plasma

Cell-free DNA was isolated from plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, Cat. No. 51183) according to the manufacturer's instructions. Aliquots (800 μL) of plasma were mixed with 80μL of proteinase K (18 mg/mL) and 800 μL of Buffer AL, and incubated for 10 min at 56 °C. Further DNA isolation was done as described in the manufacturer's protocol. DNA concentration was determined in the Multiskan GO spectrophotometer (Thermo Scientific, USA).

2.1.5. Sodium bisulfite conversion

Extracted DNA was modified with sodium bisulfite (SB) to convert all non-methylated cytosines to uracil, while methylated cytosines were not converted. The bisulfite conversion was carried out in 50 μL of denatured DNA using the EZ DNA Methylation Gold Kit according to the manufacturer's instructions. The converted DNA was stored at −20 °C until use.

2.1.6. MSP amplification system and the product electrophoresis

The methylation status of five sites for the DMR (NBPF1) in circulating cfDNA was detected by MSP. Each MSP reaction was performed in a total volume of 20μL containing 10μL of the KAPA 2G Robust Mix (KAPA 2G Robust PCR Kit, KK5701), 7 μL of ddH2O, 2 μL of template DNA, 0.5 μL of forward primer and 0.5 μL of reverse primer. SB-treated DNA was amplified in two separate MSP reactions, one with primers specific for methylated and one with primers specific for unmethylated DNA. We used the T100™ Thermal Cycler (BIORAD, USA) for amplification by the following PCR profile: 1 cycle at 94 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension cycle of 72 °C for 5 min.MSP products for five methylated and unmethylated sites of NBPF1 were resolved on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8.0) and visualized by ethidium bromide staining.

2.2. Statistical analysis

The Chi-square test was used to determine the differences in methylation levels of plasma cfDNA between breast cancer patients and patients with benign breast disease and healthy controls.

The association of the NBPF1 methylation rate in cfDNA with clinicopathological features for the group of enrolled breast cancer patients was analyzed by the Chi-square test and the Fisher's exact test; a P value < .05 was considered statistically significant. We also use the Bonferroni post hoc test for multiple comparisons. The SPSS 16.0 software (SPSS Inc., Chicago, IL) was used for all statistical analyses.