Biomedicine & Pharmacotherapy 68 (2014) 699–707

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Original article

Differential DNA methylation status between breast carcinomatous and normal tissues

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ARTICLE INFO

Article history:
Received 8 June 2014
Accepted 8 July 2014

Keywords:
Breast cancer
DNA methylation
Microarray

ABSTRACT

Breast cancer has been considered to be a multifactorial disease with a wide array of well-characterized gene mutations and chromosomal abnormalities. However, it is becoming evident that the onset or development of breast cancer also depends on epigenetic factors, although the mechanisms have not been fully elucidated. We performed a genome-wide analysis of DNA methylation of breast carcinomatous tissues and paired normal tissues to examine the differences in methylation between them. Methylation-specific polymerase chain reaction (MSP) was used to validate the hypermethylated genes screened out by DNA methylation microarray. We found that hypomethylation and hypermethylation occurred in 2753 and 1795 genes, respectively, in breast carcinomatous tissues. Meanwhile, gene ontology analysis and ingenuity pathway analysis revealed the function and pathway of several genes whose methylation status was altered in breast carcinomatous tissues. In addition, we investigated the promoter methylation status of four genes in breast carcinomatous tissue and paired normal tissues ($n = 30$) by MSP. Promoter hypermethylation of *CRABP1*, *HOXB13*, *IFNGR2*, and *PIK3C3* was found in 37% (11/30), 23% (7/30), 17% (5/30), and 2% (2/30) of the carcinomas, respectively. Mutation of these four important genes was critical to many types of cancer. Our results suggest that DNA methylation mechanisms may be involved in regulating the occurrence and development of breast cancer.

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1. Introduction

Breast cancer is one of the most frequently occurring malignancies worldwide. Its incidence has increased in recent years, especially in developing countries [1]. Detection of breast cancer at an early stage is the key to successful treatment and a favorable outcome [2]. To date, no efficient methods to identify breast cancer in its preclinical or presymptomatic stages have been established. The development of strategies capable of detecting breast cancer in its preclinical or presymptomatic stages is desirable. The search for a sensitive and specific biomarker for diagnosis of breast cancer is ongoing.

Breast cancer has been considered to be a multifactorial disease with a wide array of well-characterized gene mutations and chromosomal abnormalities. In the past 20 years, studies on cancer epigenetics have revealed that a given gene can exhibit multiple aberrant epigenetic changes resulting in numerous possible genetic alterations [3]. Cancer epigenetics has recently become a full-fledged field focusing on the different mechanisms involved in epigenetic regulation, including DNA methylation, histone modification, and nucleosomal remodeling. DNA methylation is an epigenetic marker that is erased in the early embryonic period, and then re-established in each individual [4]. It has been speculated that DNA methylation plays an important role in the onset or development and progression of cancers.

Although many studies on aberrant DNA methylation in breast cancer have been performed, the understanding of methylation patterns as a diagnostic tool for breast cancer remains limited. Numerous data have shown that promoter hypermethylation that induces inactivation of multiple tumor suppressor genes is an

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http://dx.doi.org/10.1016/j.biopha.2014.07.014
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important mechanism in the multistep process of genetic carcinogenesis [5–7]. Generally, breast cancer is characterized by global hypomethylation and promoter hypermethylation of tumor suppressor genes [8–10]. Global hypomethylation has contributed to the overexpression of oncogenes, while promoter hypermethylation leads to epigenetic silencing of the target genes [11]. DNA hypermethylation is a common early event in carcinogenesis and can thus serve as a potential predictor in breast cancer diagnosis. Identification of DNA hypermethylation signatures in human breast carcinomatous tissues and paired normal tissues is likely to screen out diagnostic and predictive biomarkers in breast cancer.

In this study, we used a comprehensive methylation profiling technique termed "methylated CpG island recovery assay" or MIRA [12,13]. This assay was used in conjunction with CpG island and promoter microarrays (MIRA-chip) to characterize the CpG island methylome in human breast carcinomatous and paired normal tissues. Analysis of functional gene pathways and construction of a signaling network from the aberrant methylated genes were performed. The significant biological functions and pathways of the methylated genes associated with breast cancer were then discussed. We also selected several genes from the microarray to validate the promoter hypermethylation status using methylation-specific PCR (MSP) in 30 cases of breast carcinomatous tissue specimens and their paired autologous normal tissues. This approach may provide valuable insights into methylation patterns and changes therein and offers a possibility of correlating them with gene expression levels.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethical review committee of Nanjing Maternity and Child Health Care Hospital affiliated to Nanjing Medical University. The specimens were collected for a clinical study of breast cancer, and signed informed consent was obtained from all study participants for sample collection and analysis.

2.2. Patients and samples

All samples, including 30 fresh breast carcinomatous tissues and paired normal tissues, were obtained at surgery from women with breast cancer who underwent breast surgery at Nanjing Maternity and Child Health Care Hospital from 2009 to 2011. None of the patients had undergone previous chemotherapy or radiotherapy. Cancer staging (pTNM) was defined according to the 7th edition of the American Joint Committee on Cancer staging manual [14]. All pathological types of breast cancer were infiltrating ductal carcinomas, and all patients with breast cancer were at stage I–III (pT1N0M0–pT2N3M0). Paired normal tissues were also obtained from a quadrant other than that harboring the tumor. Fresh specimens were snap-frozen in liquid nitrogen and subsequently stored at −70 °C until used.

2.3. DNA extraction, MIRA, and microarray hybridization

Total DNA was extracted from fresh tissues using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total DNA were fragmented by sonication to an average size of approximately 500 bp as determined on agarose gels. Enrichment of the methylated double-stranded DNA fraction by MIRA was performed as previously described [12,13]. Labeling of amplicons, microarray hybridization, and scanning were performed according to the NimbleGen protocol. NimbleGen tiling arrays were used for hybridization (385K Human CpG Island plus Promoter arrays). The single-array design covered all 28,226 University of California, Santa Cruz (UCSC) Genome Browser-annotated CpG islands and the promoter regions for all RefSeq genes. The promoter region covered was 1 Kb (−800 to +200 relative to the transcription start sites). For all samples, the MIRA-enriched DNA was compared with the input DNA.

2.4. Analysis of aberrantly methylated genes

Log2 ratio data of microarray results were converted into P value scores using the Kolmogorov–Smirnov test with a 750-bp window through the NimbleScan software. Probes were selected as positive if their P value scores were > 2 ($P < 0.01$). For our analysis, we defined a methylated region of interest (methylation peak) as a region with at least four consecutive positive probes (i.e., no gaps) covering a minimum length of 400 bp. This stringent definition gave few false-positive results. Identified methylation peaks were mapped relative to known transcripts defined in the UCSC Genome Browser HG18 RefSeq database.

2.5. Gene ontology analysis

The results of the promoter microarrays of patients with breast cancer were analyzed using gene ontology (GO) terms. Testing of over-represented GO terms was performed using the GO stats package [15]. The overlapping probabilities of differentially methylated region datasets were calculated using a hypergeometric test [16,17]. We analyzed the main functions of the differentially expressed genes according to the GO database, which classifies the key functions of the National Center for Biotechnology Information (NCBI) [18,19]. In general, Fisher's exact test and the Chi^2 test were used to classify the GO categories, and the false-discovery rate (FDR) [20] was calculated to correct the P value; the smaller the FDR, the smaller the error in judging the P value. The FDR was defined as $FDR = 1 - N_k/T$, where N_k indicates the number of Fisher's test P values less than the Chi^2 test P values. We calculated P values for the GO terms of all differential genes. Enrichment was used to measure the significance of the function: if the enrichment increases, the corresponding function is more specific, which helps us to find more concrete functions of those GOs in the experiment. Within the significant category, the enrichment Re was given by: $Re = (n_f/n)/(N_f/N)$, where n_f is the number of differential genes within the particular category, n is the total number of genes within the same category, N_f is the number of differential genes in the entire microarray, and N is the total number of genes in the microarray [21].

2.6. Ingenuity pathway analysis

Gene networks and canonical pathways representing key genes were identified using the curated ingenuity pathway analysis (IPA) database according to KEGG, Biocarta, and Reatome, as previously described. We again turned used Fisher's exact test and the Chi^2 test to select the significant pathway, and the threshold of significance was defined by the P value and FDR. The enrichment Re was calculated as in the above equation [22,23].

2.7. Signal-net

The networks of genes were built according to the relationships among the genes, proteins, and compounds in the KEGG database [24–28]. Signal-net deconstructs the KEGG database, breaking the KEGG pathway database restricted to a particular interaction between genes. Therefore, signal-net allows for examination of a protein's relationship to the upstream or downstream within the

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