Isolation and bioinformatics analysis of differentially methylated genomic fragments in human gastric cancer

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Abstract

AIM: To isolate and analyze the DNA sequences which are methylated differentially between gastric cancer and normal gastric mucosa.

METHODS: The differentially methylated DNA sequences between gastric cancer and normal gastric mucosa were isolated by methylation-sensitive representational difference analysis (MS-RDA). Similarities between the separated fragments and the human genomic DNA were analyzed with Basic Local Alignment Search Tool (BLAST).

RESULTS: Three differentially methylated DNA sequences were obtained, two of which have been accepted by GenBank. The accession numbers are AY887106 and AY887107. AY887107 was highly similar to the 11th exon of LOC440683 (98%), 3’ end of LOC440887 (99%), and promoter and exon regions of DR5 (94%). AY887106 was consistent (98%) with a CpG island in ribosomal RNA isolated from colorectal cancer by Minoru Toyota in 1999.

CONCLUSION: The methylation degree is different between gastric cancer and normal gastric mucosa. The differentially methylated DNA sequences can be isolated effectively by MS-RDA.

Key words: Gastric cancer; DNA methylation; Differential sequences; Methylation-sensitive representational difference analysis

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INTRODUCTION

Epigenetic inactivation of tumor suppressor genes (TSG) is frequently associated with tumor pathogenesis[1,2]. The major mechanism of this epigenetic inactivation is through hypermethylation of promoter CpG islands (CGIs). In general, DNA hypermethylation of gene-associated CpG islands results in either down-regulation or complete abrogation of gene expression, indicating that aberrant DNA methylation could execute a similar function to genetic abnormalities, such as inactivating mutations or deletions in the disease state[1,3,4]. Numerous studies have indicated that several gene classes such as adhesion molecules, inhibitors of angiogenesis, DNA repair, cell cycle regulators, metastasis suppressors, etc, are frequently hypermethylated in human primary tumors[5,6]. Gastric cancer is the second most common cause of cancer death in the world[7]. Compared with other malignant tumors, the incidence and mortality rate of gastric cancer rank first in China[8,9]. In the present study, DNA methylation is characterized as an important mode of epigenetic modification in the development and progression of gastric cancer by affecting gene transcription, increasing the frequency of gene mutation, and enhancing genomic instability[5]. However, the entire picture of methylation-silenced genes in gastric cancers is still unclear, and further searching for methylation-silenced genes is necessary.

Because analysis of methylation of known genes has limitations, genome-wide screening for CGIs methylated in gastric cancer is needed. In this study, methylation-sensitive representational difference analysis (MS-RDA)[14,15], an advanced biotechnique, was used to detect the methylation
status of two genomes and elucidate the role of methylation in carcinogenesis. Moreover, with this strategy, novel genes related to gastric cancer can be obtained and new targets can be offered for gastric cancer research.

MATERIALS AND METHODS

Materials

Tissues from three gastric adenocarcinomas and adjacent normal tissue samples (> 5 cm away from the center of cancer) were collected from gastric cancer patients at a hospital affiliated with Nanhua University. Tumor and adjacent normal tissues were isolated and stored in liquid nitrogen. The histological types were confirmed by histological examination. The study protocol was approved by the Ethics Committee of Nanhua University.

DNA extraction and HpaII digestion

Genomic DNAs of three gastric adenocarcinomas and adjacent normal mucosa samples were prepared by serial extraction with phenol and chloroform, followed by ethanol precipitation. A 10 μg sample of genomic DNA was digested with 10 μL methylation-sensitive four-base recognition restriction enzymes HpaII (Fermentas Co., 10 U/μL), and was incubated for 20 h at 37℃.

Amplicon preparation

Adaptors RHpa24 and RHpa11 (Table 1) were ligated to the mixture of digestion products of three gastric adenocarcinomas and adjacent normal mucosa samples, annealed by gradual cooling from 50℃ to 10℃ for 40 min, and then ligated to DNA fragments by overnight incubation with T4 DNA ligase at 16℃[17]. The ligation product was amplified for 26 cycles (each cycle including 1 min incubation at 95℃ and 3 min at 72℃, with the last cycle followed by an extension at 72℃ for 10 min) with RHpa24 oligonucleotide as a primer as reported. Three gastric adenocarcinoma samples served as the testers, and three adjacent normal mucosa samples served as the drivers. The DNA fragments with RHpa adaptor were amplified effectively and testers and driver amplicons were obtained[14]. Amplicons were detected by 1.0% agarose gel electrophoresis.

Subtractive hybridizations

The RHpa adaptor in the tester and driver amplicons were removed by digestion with the isoschizomerase MspI (Fermentas Co.10 U/μg) and separated with a DNA fragment purification and filtration kit (Takara Co., Japan). The JHpa II 24/11 adaptor was then ligated to the tester amplicon with T4 DNA ligase. The subtractive hybridizations with gastric cancer amplicons serving as the tester and normal gastric mucosa amplicons serving as the driver were presumed to be the positive hybridizations, and the converse was presumed to be the reverse hybridizations. The tester DNA with J adaptor at its ends was mixed with the driver DNA at a ratio of 1:40. The DNA mixture was purified by phenol extraction and ethanol precipitation, dissolved in 4 μL of 3 × EE buffer (3 mmol/L EDTA/3 mmol/L N-[2-hydroxyethyl] pipedacine-N’-[3-propanssulfonic acid, pH 8.0], denatured at 96℃ for 10 min, and reannealed at 67℃ for 24 h. One-tenth of the reannealed product was amplified by PCR with JHpa24 oligonucleotide as the primer for 13 cycles. Tester/tester and tester/driver double-stranded DNA fragments had J adaptors on either both ends or only one end, respectively, and could be amplified exponentially or linearly. DNA fragments linearly amplified, existing as single-stranded DNA, were digested with mung-bean nuclease (Takara Co., Japan), and the remaining double-stranded DNA was again amplified by PCR for 26 cycles with JHpa24[18].

For the second and third cycles of subtractive hybridization to a new adaptor (N/S adaptor for the second cycle; J for the third cycle) (Table 1), the ratio of the tester DNA to the diver DNA was 1:400 and 1:4000, respectively[18], and the differentially methylated sequence between gastric cancer and normal gastric mucosa could be obtained. The experiment fluidogram is displayed in Figure 1.

Cloning, sequencing and BLAST

After the final subtractive hybridization, products were digested with 10 μL methylation-sensitive four-base recognition restriction enzymes HpaII (Fermentas Co., 10 U/μL), and was incubated for 20 h at 37℃.

Table 1 Adaptors and sequences in MS-RDA

| Primer | Sequences |
|--------|-----------|
| RHpa II a | 5'-AGC ACT CTC CAG CCT CTC ACC GAC-3' |
| RHpa II b | 5'-CGG TCG GTG AG-3' |
| JHpa II a | 5'-ACC GAC GTC GAC TAT CCA TGA AAC-3' |
| JHpa II b | 5'-CGG TTT CAT GG-3' |
| NHpa II a | 5'-AGG CAA CTG TGC TAT CCG AGG GAC-3' |
| NHpa II b | 5'-CGG TCC TCT CGG-3' |
| SHpa II a | 5'-ACT TCT ACG GCT GAA TTC CGC CAC-3' |
| SHpa II b | 5'-CGG TCT CGG AAT-3' |

MS-RDA: Methylation-sensitive representational difference analysis.
The target fragments were subsequently cloned into a pGEM-T vector (Promega Co., USA). Plasmid DNA was transformed into *E. coli* strain JM109. Bacteria were incubated in LB medium for 1 h at 37°C, then plated onto agar plates containing ampicillin (100 μg/mL), X-gal (20 μg/mL) and IPTG (12.1 μg/mL) and incubated for 14 h at 37°C. Positive (white) colonies were picked out and identified by PCR. The primer was the JHpa2 oligonucleotide. After the positive colonies were sequenced, nucleic acid sequence homology searches were performed using the BLAST server at the National Center for Biotechnology Information.

**RESULTS**

**Amplicons of gastric cancer and normal gastric mucosa**

The digestion products of gastric cancer and normal gastric mucosa genomic DNAs were ligated with the RHpa II adaptor using PCR, and the amplicons were enriched (Figure 2). As shown in the figure, amplicons appeared as bright smears between 200-1000 bp.

**Differentially methylated DNA fragments**

After three cycles of the positive hybridizations, a differentially methylated fragment of about 400 bp could be seen (Figure 3A). After two cycles of the reverse hybridizations, two differentially methylated fragments could be obtained (Figure 3B), one at approximately 400 bp and the other located between 200-300 bp.

**Cloning, sequencing and BLAST**

Three differentially methylated DNA fragments were cloned into pGEM-T, and the positive colonies were identified by PCR (Figure 4) and sequenced (Figure 5). The differentially methylated DNA fragment from the positive hybridizations was problematic because of its complex secondary structure. Two differentially methylated DNA fragments from the reverse hybridizations were accepted by GenBank. The accession numbers were AY887106 and AY887107, respectively. The two differentially methylated DNA fragments were: AY887106:

```plaintext
GGTTGACGCGCATATCGAATCCGACTGTTTAAT
- TAAAACAAAGCATCGCGAAGGCCCGCGC-
- GGGTTGGACGCGCATATCGAATCCGACTGTTTAAT
- TAAAACAAAGCATCGCGAAGGCCCGCGC-
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AY887107:

```plaintext
CTCGTTGAAGTGAAATTCATGAGGAGAGAT-
- GCCGTGGGGGGCTCGGCGGGGGCACCGC-
- CACTGGGGCCCGTGCAGGTGGTCACCGCT-
- GCCTGCTGACCCTACTCGTCATCTGGACCTT
- GCTGGGCAACGTGCTGGTGTCCGCAGCCATC-
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BLAST indicated that AY887107 was highly similar to the 11th exon of gene LOC440683 located on chromosome 1q21.1, while the 3' end of gene LOC440887 located on chromosome 2p11.1 included the promoter and 1st exon region of gene DRD5 located on chromosome 4p16.1, respectively. Their similarity rates were 98%, 99% and 94% respectively. AY887106 located on ribosomal RNA was linked with a CpG island isolated from colorectal cancer by Toyota et al [19].

The characteristics of differentially methylated DNA fragments are shown in Table 2.

**DISCUSSION**

Given the role of aberrant DNA methylation in cancer initiation and progression, a distinct effort has been put towards the development of strategies that could facilitate early cancer detection. It is clear that aberrant DNA methylation is an early event in tumor development, as indicated by reports where aberrantly hypermethylated sites could be detected in seemingly normal epithelia from a...
patients, years before the overt development of cancer. Thus, utilizing DNA methylation as a biomarker might prove to be a useful tool not only for early diagnosis but also for the detection and assessment of high-risk individuals. This strategy is reported to be successful in various bodily fluids, biopsy materials, lymph nodes obtained at surgery, and serum.

Although Southern Blotting and Restriction Landmark Genomic Scanning have been used for detection of abnormal DNA methylation, it is limited to the detection of known genes and is a complicated and difficult procedure. Lisitsyn et al. established a representational difference analysis (RDA) based upon subtractive hybridization in 1993. For this, two genomic DNAs were obtained at surgery, and serum.

In this study, MS-RDA was performed using a mixture of DNAs from mucosa of three gastric adenocarcinomas serving as the tester and adjacent normal mucosa serving as the driver. This strategy might have neglected infrequent methylations that occurred in only one gastric adenocarcinoma or that were induced by individual differences. Our data indicate that gene LOC440887 and LOC440683 were highly homologous to the sequences AY887107. Using GenBank, we found that gene LOC440887 and gene LOC440683 are novel genes that are seldom studied. LOC440887 is probably linked to the etiology of myeloid/lymphoid or mixed-lineage leukemia and closely related to the MLL3 gene, which is located on chromosome region 7q36 and encodes a protein homologous to ALR (acute lymphoblastic leukemia-1 related). A previous study by Tan et al. demonstrated that MLL3 does map to 7q36, a chromosome region that is frequently deleted in myeloid leukemia and may be involved in leukemogenesis. Ruault et al. found partial duplications of the MLL3 gene in the juxtacentromeric region of chromosomes 1, 2, 13, and 21. The LOC440887 gene may be a duplicated region containing a fragment of the MLL3 gene on chromosome 2. Digital Northern analysis was applied to the MLL3 gene using the NCBI Web Station. The outcome indicated that the human stomach tissue could express the MLL3 gene and that there were expression differences between the gastric cancer and the normal stomach tissue. We are currently investigating the relative differences in expression level between the tissue samples. Moreover, BLAST indicated that AY887107 was highly similar to the promoter and 1st exon region of gene DRD5 located on chromosome 4p16.1, with a similarity rate of 94%. The DRD5 gene encodes the D5 receptor.

Table 2 Characteristics of differentially methylated DNA fragments by MS-RDA

| Accession number | Screening time | Size (bp) | GC (%) | GC/ GC | Location | S | E | Similarity rate (%) |
|------------------|----------------|-----------|--------|--------|----------|---|---|---------------------|
| AY887106         | 2              | 395       | 50.6   | 0.9286 | 8q13.3   | 745 | 0 | 98                  |
| AY887107         | 2              | 264       | 66.3   | 0.7000 | 2p11.1   | 521 | e-145 | 99                  |
|                  |                |           |        |        | 1q21.1   | 498 | e-138 | 98                  |
|                  |                |           |        |        | 4p16.1   | 435 | e-119 | 94                  |
subtype of the dopamine receptor. The D5 subtype is a G-protein coupled receptor which stimulates adenyl cyclase. In both gastric and duodenal mucosa, Mezey et al. demonstrated the presence of significant amounts of the D5 receptor that could serve as a target for locally produced dopamine. Dopamine is a protective agent in the gastrointestinal (GI) tract in both rats and humans. The relationship between DRD5 gene methylation and gastric cancer still needs further studies. The sequence AV887106, which maps to ribosome RNA, has 98% homology to a differentially methylated CpG island genomic sequence obtained from human colon carcinomas by Toyota et al.

Our research also suggested that this location is likely to be methylated in gastrointestinal carcinomas. In view of the above-mentioned analysis, we will carry out a relative study of three differentially methylated DNA fragments so that novel genes related to gastric cancer can be obtained and new targets can be offered for gastric cancer research.

Various methylation-based strategies, including MS-RDA, restriction landmark genome scanning, arbitrarily primed PCR, and CpG island microarray, have been developed and proven to be useful for identifying hypermethylated sequences. MS-RDA was previously established to detect differences in the methylation status of two genomes. As for the specificity of MS-RDA, it was shown that DNA fragments, that are unmethylated in the tester and almost completely methylated in the driver, are efficiently isolated. This indicated that genes that are in a biallelic methylation state or in monoallelic methylation with loss of the other allele are efficiently isolated. Takai et al. first reported the silencing of HTR1B and reduced expression of EDN1 in human lung cancers using MS-RDA. Subsequently, Miyamoto et al. found methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung, and pancreatic cancers. Then in 2004, Hagihara et al. documented that MS-RDA was used for isolation of 111 DNA fragments derived from CpG islands (CGIs), and 35 of them were from CGIs in the 5’ regions of known genes in human pancreatic cancers. MS-RDA is effective in identifying silenced genes in various cancers. MS-RDA also has some disadvantages such as complexity of the procedure, requiring high homology between the “tester and driver”, inefficiency with amplicons less than 1 kb. In a word, MS-RDA is a promising method for the study of DNA methylation in gastric cancer. It may also be used for isolating novel tumor suppression genes.

COMMENTS

Background
Epigenetic inactivation of tumor suppressor genes (TSG) is frequently associated with tumor pathogenesis. The purpose of this study was to isolate the differentially methylated DNA sequences between gastric cancer and normal gastric mucosa.

Research frontiers
Genome-wide screening for CGIs differentially methylated between gastric cancer and normal gastric mucosa.

Innovations and breakthroughs
Because analysis of methylation of known genes has limitations, genome-wide screening for CGIs methylated in gastric cancer is needed. In this study, methylation-sensitive representational difference analysis (MS-RDA), an advanced biotechnique, was used to detect the methylation status of gastric cancer and normal gastric mucosa.

Applications
This study found that methylation status is different between gastric cancers and normal gastric mucosa. It elucidated the role of methylation in carcinogenesis, and moreover, new targets were offered for gastric cancer research.

Terminology
Methylation is a term used in chemical sciences to denote the attachment or substitution of a methyl group on various substrates. DNA methylation profiling is gaining momentum as an epigenetic approach to understanding the effects of aberrant methylation (either hyper- or hypomethylation) both in basic research and in clinical applications.

Peer review
The manuscript by Liao et al describes a method to screen differences in methylation status of DNA between gastric cancer samples and normal gastric mucosa. The authors found 3 DNA regions that have changed methylation status between the normal and neoplastic tissue. The data presented is novel and interesting.

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