Online Submissions: wjg.wjgnet.com
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2008 March 7; 14(9): 1333-1338
World Journal of Gastroenterology ISSN 1007-9327

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

Ai-Jun Liao, Qi Su, Xun Wang, Bin Zeng, Wei Shi

Ai-Jun Liao, Qi Su, Cancer Research Institute, Nanhua University, The First Affiliated Hospital, University of South China, Hengyang 421001, Hunan Province, China
Xun Wang, Bin Zeng, Wei Shi, Department of Gastroenterology, The First Affiliated Hospital, University of South China, Hengyang 421001, Hunan Province, China
Author contributions: Liao AJ and Su Q contributed equally to this work; Liao AJ, Su Q, Wang X, Zeng B and Shi W designed the research; Liao AJ and Wang X performed the research; Liao AJ, Su Q, Wang X and Zeng B analyzed the data; and Liao AJ, Su Q, Wang X and Zeng B wrote the paper.
Supported by The Key Project Fund of the Science and Technology Program of Hunan Province, No. 04SK1004; the Scientific Research Fund of Educational Department, Hunan Province, No. 99C195
Correspondence to: Dr. Qi Su, Cancer Research Institute, University of South China, Hengyang 421001, Hunan Province, China. suqi1@hotmail.com
Telephone: +86-734-8279347 Fax: +86-734-8281547
Received: August 3, 2007 Revised: January 2, 2008

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 DRD5 (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

Peer reviewer: Sharon DeMorrow, Assistant Professor, Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Liao AJ, Su Q, Wang X, Zeng B, Shi W. Isolation and bioinformatics analysis of differentially methylated genomic fragments in human gastric cancer. *World J Gastroenterol* 2008; 14(9): 1333-1338 Available from: URL: http://www.wjgnet.com/1007-9327/14/1333.asp DOI: http://dx.doi.org/10.3748/wjg.14.1333

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[5-9]. Gastric cancer is the second most common cause of cancer death in the world[10]. Compared with other malignant tumors, the incidence and mortality rate of gastric cancer rank first in China[11,12]. 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[13]. 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)[14-16], 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 oligonucleotides 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 JHpaⅡ$_{24/11}$ 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] pipecazine-N'-[3-propansulfonic 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 JHpa$_{24}$ 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 JHpa$_{24}$[14].

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.

Table 1 Adaptors and sequences in MS-RDA

Primer	Sequences
RHpaⅡ$_{24}$	5'-AGC ACT CTC CAG CCT CTC ACC GAC-3'
RHpaⅡ$_{11}$	5'-CGG TCG GTG AG-3'
JHpaⅡ$_{24}$	5'-ACC GAC GTC GAC TAT CCA TGA AAC-3'
JHpaⅡ$_{11}$	5'-CGG TTT CAT GG-3'
NHpaⅡ$_{24}$	5'-AGG CAA CTG TGC TAT CCG AGG GAC-3'
NHpaⅡ$_{11}$	5'-CGG TCC CTC GG-3'
SHpaⅡ$_{24}$	5'-ACT TCT ACG GCT GAA TTC CGC CAC-3'
SHpaⅡ$_{11}$	5'-CGG TGT CGG AAT-3'

MS-RDA: Methylation-sensitive representational difference analysis.

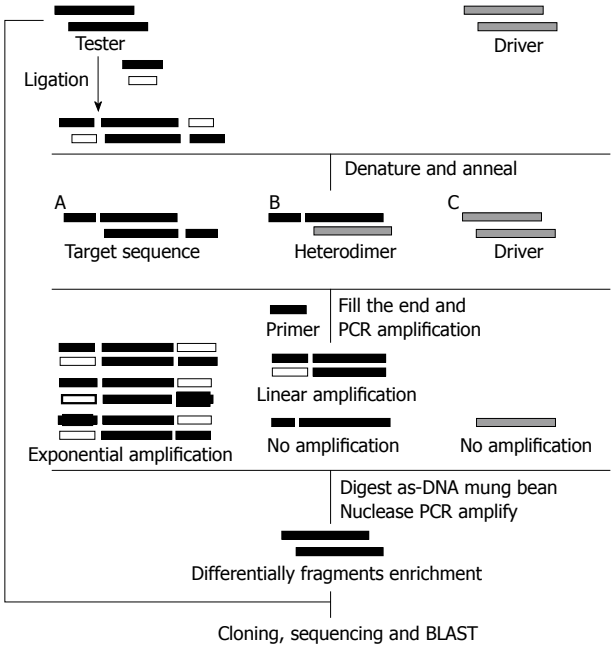

Figure 1 Schematic diagram of MS-RDA experimental strategy.

Cloning, sequencing and BLAST

After the final subtractive hybridization, products were electrophoresed through 1.0% agarose gel, and the target bands were recovered with an agarose gel recovery kit

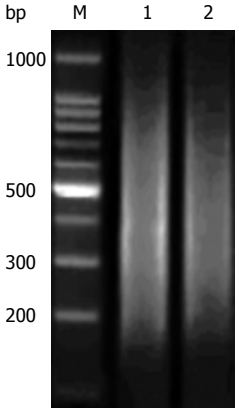

Figure 2 Amplicons of gastric cancer and normal gastric mucosa. M: DNA marker; 1: Gastric cancer amplicon; 2: Normal gastric mucosa amplicon.

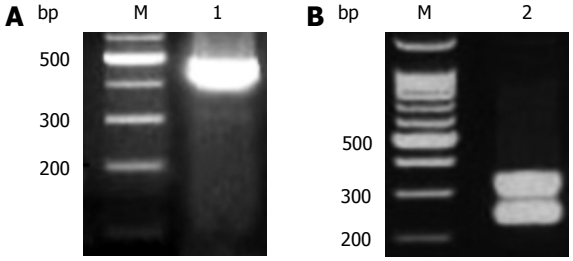

Figure 3 **A**: Results after the third cycle of competitive hybridization. **B:** After a second cycle of competitive hybridization. M: DNA marker; 1: differentially methylated fragment of approximately 400 bp; 2: Two differentially methylated fragments, one is approximately 400 bp and the other lies between 200-300 bp.

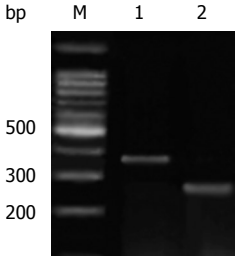

Figure 4 PCR results. M: DNA marker; 1: Differentially methylated fragment of approximately 400 bp; 2: Differentially methylated fragment of approximately 200-300 bp.

(Takara Co., Japan). 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℃, then plated onto agar plates containing ampicillin (100 μg/mL), X-gal (20 μg/cm^2) and IPTG (12.1 μg/cm^2) and incubated for 14 h at 37℃. Positive (white) colonies were picked out and identified by PCR. The primer was the JHpa24 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: CCGGCGCCTAGCAGCCGACTTANAACTG-GTGCGGACCAGGGGAATCCGACTGTTTAAT-TAAAACAAAGCATCGCGAAGGCCCGCGGC-GGGTGTTGACGCGATGTGATTTCTGCCCAGT-GCTCTGGATGTCAAAGTGAAGAAATTCAAT-GAAGCGCGGGTAAACGGCGGGAGTAACCAT-GACTCTCTTAAGGTAGCCAAATGCCTCGTCATC-TAATTAGTGACGCGCATGAATGGATGAACGAGA-TTCCCCCTGTCCCTACCTACTATCCAGCGAAAC-CACAGCCAAGGGAACGGGCTTGGCGGAAT-CAGCGGGGAAAGAAGACCCTGTTGAGCTT-GACTCTAGTCTGGCACGGTGAAGAGACAT-GAGAGGTGTAGAATAAGTGGGAGGCTCCCGG; AY887107: ATACCAGCAGCTGGCGCAGGGGAAT-GCCGTGGGGGGCTCGGCGGGGGCACCGC-CACTGGGGCCCGTGCAGGTGGTCACCGCCT-GCCTGCTGACCCTACTCGTCATCTGGACCTT-GCTGGGCAACGTGCTGGTGTCCGCAGCCATC-GTGTGGAGCCGCCACCTGCGCGCCAAGAT-GACCAACGTCTTCATCGTGTCTCTACCTGTGT-CAGACCTCTTCGTGGCGCTGCTGGTCATGTCCT-GGAAGGCAGTCGCCGAGGTGGCCGG.

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

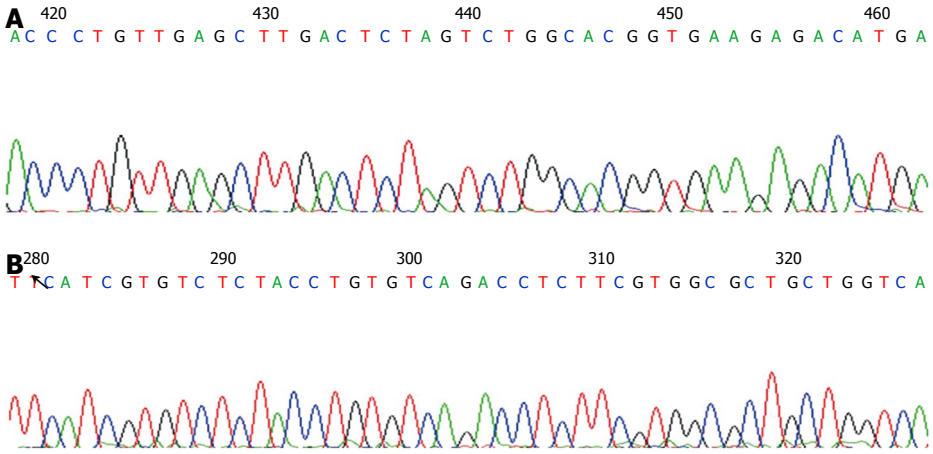

Figure 5 AY887106 (**A**) and AY887107 (**B**) sequencing.

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

Accession number	Screening time	Size (bp)	GC (%)	CG/GC	Similarity			
					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

patients, years before the overt development of cancer[20]. 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[21-23]. 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[24]. Lisitsyn *et al*[17] established a representational difference analysis (RDA) based upon subtractive hybridization in 1993. For this, two genomic DNAs were digested with sensitive restriction enzyme in order to reduce the structural complexity of genomic DNA. After several cycles of subtractive hybridizations and PCR, the target fragments differentially isolated from two genome specimens were obtained. Subsequently, Ushijima *et al*[14] developed MS-RDA technology and successfully isolated the differentially methylated genomic fragments in mouse liver tumor. MS-RDA uses the methylation-sensitive four-base recognition restriction enzyme HpaII to digest two genomic DNAs in order to reduce the methylation site complexity of the DNA. The digestion product was ligated to the RHpa II $_{24/11}$ adaptor, and PCR was used to effectively amplify the shorter fragments, while longer fragment amplification was difficult by PCR. Accordingly, the difference between "driver" and "tester" can be transformed to the distinction of different digestion sites[17]. After several cycles of subtractive hybridizations and PCR, the differentially methylated fragments form homopolymers and are amplified exponentially, while the identical sequences between "driver" and "tester" form heterodimers and are amplified linearly. Thus, the identical sequences will be eliminated, while the target fragments, existing in the "tester" but lacking in the "driver", have been amplified effectively. As for the specificity of MS-RDA, it was shown that the contrast between "tester" and "driver" is not a difference in digestion fragment size but rather a difference in the methylation status of two genomes. MS-RDA is an effective method for identifying the silenced genes in various cancers and marker genes linked with diseases.

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*[25] 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*[26] 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

subtype of the dopamine receptor. The D5 subtype is a G-protein coupled receptor which stimulates adenylyl cyclase. In both gastric and duodenal mucosa, Mezey *et al*[27] 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 AY887106, 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*[19]. 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[24,28], arbitrarily primed PCR[29], and CpG island microarray[30], 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[31]. Takai *et al*[32] first reported the silencing of HTR1B and reduced expression of EDN1 in human lung cancers using MS-RDA. Subsequently, Miyamoto *et al*[33] 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*[34] 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[35]. 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.

REFERENCES

1 **Jones PA**, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415-428
2 **Feinberg AP**. Cancer epigenetics takes center stage. *Proc Natl Acad Sci USA* 2001; **98**: 392-394
3 **Liao AJ**, Ling QH, Liu GX, Wei S. The methylation status of Exon 1 of p16 gene in human gastric cancer. *Shiyong Aizheng Zazhi* 2001; **16**: 284-287
4 **Herman JG**, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003; **349**: 2042-2054
5 **Han SY**, Iliopoulos D, Druck T, Guler G, Grubbs CJ, Pereira M, Zhang Z, You M, Lubet RA, Fong LY, Huebner K. CpG methylation in the Fhit regulatory region: relation to Fhit expression in murine tumors. *Oncogene* 2004; **23**: 3990-3998
6 **Kim H**, Kwon YM, Kim JS, Lee H, Park JH, Shim YM, Han J, Park J, Kim DH. Tumor-specific methylation in bronchial lavage for the early detection of non-small-cell lung cancer. *J Clin Oncol* 2004; **22**: 2363-2370
7 **Kim JS**, Lee H, Kim H, Shim YM, Han J, Park J, Kim DH. Promoter methylation of retinoic acid receptor beta 2 and the development of second primary lung cancers in non-small-cell lung cancer. *J Clin Oncol* 2004; **22**: 3443-3450
8 **Maruyama R**, Sugio K, Yoshino I, Maehara Y, Gazdar AF. Hypermethylation of FHIT as a prognostic marker in nonsmall cell lung carcinoma. *Cancer* 2004; **100**: 1472-1477
9 **Sathyanarayana UG**, Padar A, Huang CX, Suzuki M, Shigematsu H, Bekele BN, Gazdar AF. Aberrant promoter methylation and silencing of laminin-5-encoding genes in breast carcinoma. *Clin Cancer Res* 2003; **9**: 6389-6394
10 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108
11 **Xie HL**, Su Q, He XS, Liang XQ, Zhou JG, Song Y, Li YQ. Expression of p21(WAF1) and p53 and polymorphism of p21(WAF1) gene in gastric carcinoma. *World J Gastroenterol* 2004; **10**: 1125-1131
12 **Sun XD**, Mu R, Zhou YS, Dai XD, Zhang SW, Huangfu XM, Sun J, Li LD, Lu FZ, Qiao YL. Analysis of mortality rate of stomach cancer and its trend in twenty years in China. *Zhonghua Zhongliu Zazhi* 2004; **26**: 4-9
13 **Ushijima T**, Sasako M. Focus on gastric cancer. *Cancer Cell* 2004; **5**: 121-125
14 **Ushijima T**, Morimura K, Hosoya Y, Okonogi H, Tatematsu M, Sugimura T, Nagao M. Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc Natl Acad Sci USA* 1997; **94**: 2284-2289

15 **Kaneda A**, Takai D, Kaminishi M, Okochi E, Ushijima T. Methylation-sensitive representational difference analysis and its application to cancer research. *Ann N Y Acad Sci* 2003; **983**: 131-141
16 **Ushijima T**. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer* 2005; **5**: 223-231
17 **Lisitsyn N**, Lisitsyn N, Wigler M. Cloning the differences between two complex genomes. *Science* 1993; **259**: 946-951
18 **Hubank M**, Schatz DG. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* 1994; **22**: 5640-5648
19 **Toyota M**, Ho C, Ahuja N, Jair KW, Li Q, Ohe-Toyota M, Baylin SB, Issa JP. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res* 1999; **59**: 2307-2312
20 **Issa JP**, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001; **61**: 3573-3577
21 **Belinsky SA**. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 2004; **4**: 707-717
22 **Laird PW**. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003; **3**: 253-266
23 **Miyamoto K**, Ushijima T. Diagnostic and therapeutic applications of epigenetics. *Jpn J Clin Oncol* 2005; **35**: 293-301
24 **Parkes V**, Modha N, Ulrich JM, Jones T, Francis GE. DNA-protein interaction sites in differentiating cells. II. A subset of alphoid repetitive sequences with retinoic acid induced protein attachment and an unusual purine-pyrimidine 'signature'. *Exp Hematol* 1996; **24**: 568-579
25 **Tan YC**, Chow VT. Novel human HALR (MLL3) gene encodes a protein homologous to ALR and to ALL-1 involved in leukemia, and maps to chromosome 7q36 associated with leukemia and developmental defects. *Cancer Detect Prev* 2001; **25**: 454-469
26 **Ruault M**, Brun ME, Ventura M, Roizes G, De Sario A. MLL3, a new human member of the TRX/MLL gene family, maps to 7q36, a chromosome region frequently deleted in myeloid leukaemia. *Gene* 2002; **284**: 73-81
27 **Mezey E**, Eisenhofer G, Hansson S, Harta G, Hoffman BJ, Gallatz K, Palkovits M, Hunyady B. Non-neuronal dopamine in the gastrointestinal system. *Clin Exp Pharmacol Physiol Suppl* 1999; **26**: S14-S22
28 **Plass C**, Shibata H, Kalcheva I, Mullins L, Kotelevtseva N, Mullins J, Kato R, Sasaki H, Hirotsune S, Okazaki Y, Held WA, Hayashizaki Y, Chapman VM. Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLGS-M. *Nat Genet* 1996; **14**: 106-109
29 **Liang G**, Gonzalgo ML, Salem C, Jones PA. Identification of DNA methylation differences during tumorigenesis by methylation-sensitive arbitrarily primed polymerase chain reaction. *Methods* 2002; **27**: 150-155
30 **Yan PS**, Chen CM, Shi H, Rahmatpanah F, Wei SH, Caldwell CW, Huang TH. Dissecting complex epigenetic alterations in breast cancer using CpG island microarrays. *Cancer Res* 2001; **61**: 8375-8380
31 **Kaneda A**, Takai D, Kaminishi M, Okochi E, Ushijima T. Methylation-sensitive representational difference analysis and its application to cancer research. *Ann N Y Acad Sci* 2003; **983**: 131-141
32 **Takai D**, Yagi Y, Wakazono K, Ohishi N, Morita Y, Sugimura T, Ushijima T. Silencing of HTR1B and reduced expression of EDN1 in human lung cancers, revealed by methylation-sensitive representational difference analysis. *Oncogene* 2001; **20**: 7505-7513
33 **Miyamoto K**, Asada K, Fukutomi T, Okochi E, Yagi Y, Hasegawa T, Asahara T, Sugimura T, Ushijima T. Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. *Oncogene* 2003; **22**: 274-280
34 **Hagihara A**, Miyamoto K, Furuta J, Hiraoka N, Wakazono K, Seki S, Fukushima S, Tsao MS, Sugimura T, Ushijima T. Identification of 27 5′ CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. *Oncogene* 2004; **23**: 8705-8710
35 **Furuta J**, Nobeyama Y, Umebayashi Y, Otsuka F, Kikuchi K, Ushijima T. Silencing of Peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. *Cancer Res* 2006; **66**: 6080-6086

S- Editor Zhu LH **L- Editor** Ma JY **E- Editor** Ma WH