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Significance of MUC2 gene methylation detection in pancreatic cancer diagnosis

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ABSTRACT

Purpose: This study was conducted to explore the diagnostic value of MUC2 gene methylation in pancreatic cancer.
Methods: Methylation restriction enzyme digestion (Msp I/Hap II) and polymerase chain reaction (PCR) were performed to detect methylation of the MUC2 gene in fecal and blood specimens from seven study subjects with pancreatic cancer (PC), chronic pancreatitis (CP), or normal controls (CON). Simultaneously, blood CA 19-9 levels were detected as a positive indicator of PC.
Results: MUC2 methylation was detected in 50% of PC cell lines. In fecal samples, the MUC2 methylation rate in PC ($n = 30$) was 43.3%, which was significantly higher than those in CP ($n = 8$, 0%, $P < 0.05$) and CON ($n = 20$, 5.0%, $P < 0.05$). In blood samples, the MUC2 methylation rate in PC ($n = 40$) was 52.5%, which was significantly higher than those in CP ($n = 15$, 0%, $P < 0.01$) and CON ($n = 25$, 4.0%, $P < 0.01$). For PC diagnosis, MUC2 gene methylation in blood samples showed higher specificity and positive predictive value than CA 19-9. The combined detection in the feces and blood showed a 60% MUC2 methylation rate in PC ($n = 10$), which was higher than those in the CP ($n = 5$, 0%, $P < 0.01$) and CON ($n = 12$, 0%, $P < 0.01$).
Conclusions: The study can clearly indicate that combined detection of MUC2 gene methylation in the peripheral blood and feces could be used as a new screening and early diagnosis method for pancreatic cancer.

Introduction

Pancreatic cancer (PC) has high degree of malignancy and is the eighth leading cause of cancer deaths worldwide with early metastasis and a very poor prognosis. The overall 5-year survival rate is less than 9% [1]. Currently, the early diagnosis of PC remains difficult. Serum carbohydrate antigen (CA 19-9) is the most common tumor marker assessed in pancreatic cancer patients; At 95% specificity, CA 19-9 (>37 U/mL) had a sensitivity of 68% up to 1 year, and 53% up to 2 years before diagnosis [2]. Additional biological markers for the early diagnosis of PC are needed.

In recent years, epigenetics has developed rapidly, and a model of DNA methylation maintenance at CpG sites has been widely examined. DNA methylation can be described by a dynamic stochastic model, in which DNA methylation at each site is determined by the local activity of DNA methyltransferases (Dnmts), DNA demethylases, and the DNA replication rate. Through the targeting and regulation of these enzymes, DNA methylation is controlled by the network of chromatin marks [3]. DNA methylation plays an important role in the process of carcinogenesis. Inactivation of tumor suppressor genes by local hypermethylation of the 5′-terminal CpG island is an important step in the malignant transformation of cells [4,5].

Mucin (MUC) is a high-molecular weight glycoprotein secreted by various epithelial cells in the body and is often abnormally expressed in malignant tumors and precancerous lesions. Among which, MUC2 was found to have tumor suppressive effects [6]. The tumor suppressive effects of MUC2 gene are related with hypermethylation of its promoter region in the CpG island [6,7]. Studies have also reported that abnormal expression of MUC2 is closely

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related to PC incidence [8–10]. Therefore, this study aimed to establish a simple, feasible, and inexpensive clinical test method to detect methylation of the MUC2 gene in PC. This could provide a specific molecular diagnostic marker for the clinical diagnosis of PC, which may lead to immediate treatment and improved outcomes.

Materials and methods

Cell line and culture

AD293 human embryonic kidney cells were purchased from Shanghai Science and BioTech Co., Ltd. (Shanghai, China). The SW1990 PC cell line was purchased from ATCC (Manassas, VA, USA). ASPC, BxPC3, CfPAC, PANC1, and the PaTu8988 s PC cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All PC cell lines were incubated in DMEM containing 10% fetal bovine serum and 10 U/mL of penicillin and streptomycin at 37 °C, 5% CO_2, and saturated humidity using the wall-adherence culture method.

Specimen collection

All specimens were sourced from our People's Hospital. The blood specimens were sampled from 40 patients with pathologically confirmed PC, 15 patients with chronic pancreatitis (CP) [11], and 25 healthy controls (CON, all excluded as having other tumor diseases) who visited our hospital from 2015 to 2018. The fecal specimens were sampled from 30 patients with pathologically confirmed PC, 8 patients with CP and 20 healthy controls who visited our hospital from 2015 to 2018.

Analysis of DNA methylation

A restriction endonuclease (Hind III, Shanghai Huamei Bioengineering Co., Ltd., Shanghai, China), which did not contain the restriction site in the target polymerase chain reaction (PCR) amplification fragments, together with one methylation restriction endonuclease (Msp I/Hap II: Dalian Baosheng Bioengineering Co., Ltd., Dalian, China) were used for the enzymatic digestion of the genomic DNA. After PCR amplification, the appearance of one 259-base pair (bp) band indicated methylation of the MUC2 gene; the absence of this band was considered to indicate an unmethylated state. For PCR amplification, the following primers were used: MUC2 upstream: 5′-TGTGTTGGCATTCAGGCTAC-3′; downstream: 5′-GCAGGGGCGGTGTGGGTT-3′ 259 bp (Shanghai Bioengineering Co. Ltd).

Because the two primers contained the Hap II recognition sequence CCGG, the DNA dual strands were not cut when C was methylated, and the desired DNA fragment was amplified, indicating positive DNA methylation. However, Msp I is a methylation-insensitive endonuclease and can cut both DNA strands regardless of C methylation, preventing amplification of the desired DNA fragment.

Statistical analysis

SPSS17.0 software (SPSS, Inc., Chicago, IL, USA) was used for analysis, and the Fisher's exact test was used to calculate the P value. Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were calculated according to the following formulas: sensitivity = true positive number/(true positive number+false negative number); specificity = true negative number/(true negative number+false positive number); positive predictive value = true positive number/total positive number; negative predictive value = true negative number/total negative number; and diagnostic accuracy=(true positive number+true negative number)/total number of cases.

Results

Extraction of genomic DNA

Genomic DNA was successfully extracted from all strains of PC cells (Fig. 1A), blood samples (Fig. 1B), and fecal samples (Fig. 1C).

Methylation of PC cell lines

All samples amplified the 259 bp fragment prior to the restriction enzyme digestion by Msp I/Hap II. After digestion using the methylation-insensitive Msp I enzyme, the specific PCR product disappeared. Additionally, the specific PCR product disappeared from PANC1, BXPC3, and Patu8988 after digestion using the methylation-sensitive Hap II enzyme, indicating that the enzyme digestion was complete. The three PC cell strains did not show methylation of the MUC2 gene. However, digestion of ASPC, CFPAC, SW1990, and AD293 by the methylation-sensitive Hap II enzyme still showed the 256-bp fragment, suggesting that the MUC2 gene was methylated in the PC cell lines (Fig. 2A).

Methylation in peripheral blood samples

The results of methylation in the peripheral blood samples are shown in Fig. 2B. The methylation rate of PC was 40.0% (16/40), CP was 0% (0/15), and CON was 4% (1/25, Table 1). There were

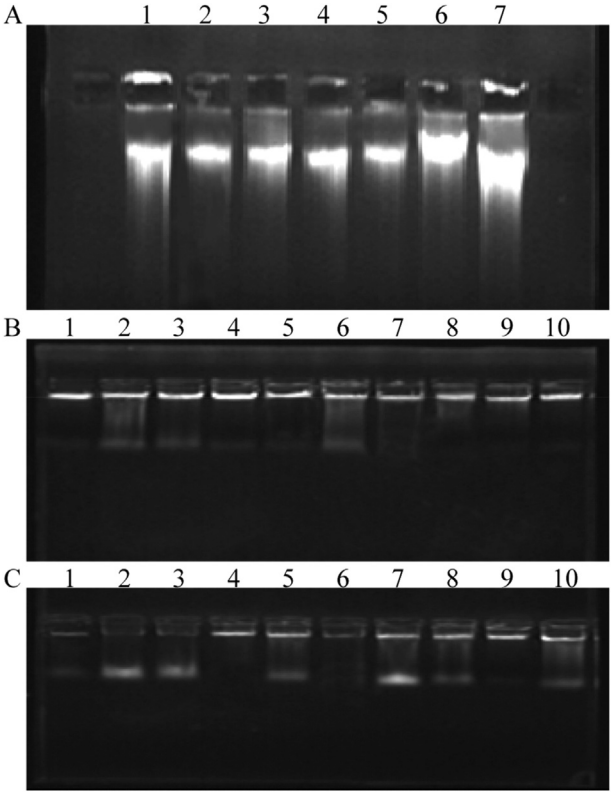

Fig. 1. A: The extraction of DNA of pancreatic cancer cell lines. Lane 1-7: AD293, ASPC, BXPC3, CFPAC, PANC1, Patu8988, and SW1990, respectively; B: The extraction of DNA in peripheral blood. Lane 1-4: N1, N2, N3, and N4 in Group CON; Lane 5-8: 17CA, 25CA, 27CA, and 28CA in Group PC; Lane 9-10: 10CP and 12CP in Group CP; C: DNA extraction from feces samples. Lane 1-5: 11CA, 14CA, 22CA, and 28CA in group PC; Lane 6-7: 5CP and 7CP in group CP; Lane 8-10: N3, N6, and N7 in group CON

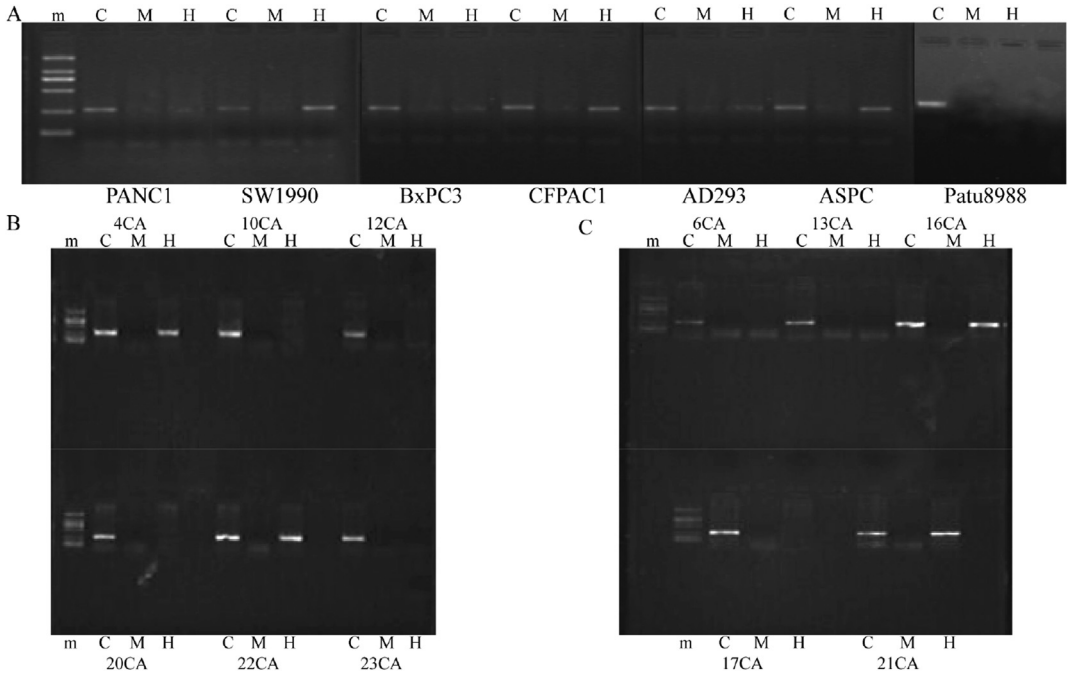

Fig. 2. Methylation-restricted enzymatic digestion+PCR (m: marker; C: amplification bands of DNA templates after non-restricted enzymatic digestion; M: amplification bands of DNA templates after Msp I digestion; H: amplification bands of DNA templates after Hap II digestion). A: DNA from PC; B: DNA from the whole blood; C: DNA from feces.

Table 1
Methylation status of MUC2 in the peripheral blood of pancreatic cancer patients, chronic pancreatitis patients and health controls.

Disease	Number	Methylation status of MUC2	Methylation rate
PC	40	16	40%
CP	15	0	0%
CON	25	1	4.0%

Table 2
The comparison between methylation status of MUC2 and CA19-9 in the diagnosis value of pancreatic cancer.

Diagnosis method	(+)	(-)
Methylation status of MUC2	21 (52.5%)	19 (47.5%)
CA 19-9 (>37 μ/ml)	23 (57.5%)	17 (42.5%)

significant differences among the groups ($P < 0.01$). However, there were no significant differences in PC diagnosis between the MUC2 methylation and CA 19-9 assays ($P > 0.05$, Table 2). The sensitivity, specificity, diagnostic accuracy, a positive predictive value, a negative predictive value was respectively 40%, 97.5%, 68.8%, 94.1% and 61.9% for PC detection through CpG island methylation of the MUC2 gene in the peripheral blood. The values of the above indices were 65.0%, 85%, 75%, 81.3%, and 70.8% for the CA 19-9 assay, respectively (Table 3). Compared to the CA 19-9 assay, diagnostic specificity and positive predictive value against PC were improved in the methylation detection of the MUC2 gene in the peripheral blood, but there were no significant differences in sensitivity and diagnostic accuracy ($P > 0.05$).

Table 4
Methylation status of MUC2 in the feces of pancreatic cancer patients, chronic pancreatitis patients and health controls.

Disease	Number	Methylation status of MUC2	Methylation rate
PC	30	13	43.3%
CP	8	0	0%
CON	20	1	5.0%

Methylation in fecal samples

As shown in Fig. 2C, the methylation rate of PC was 43.3% (13/30), CP was 0% (0/8), and CON was 5.0% (1/20) (Table 4). There were significant differences among the groups ($P < 0.05$). The sensitivity, specificity, diagnostic accuracy, positive predictive value, and negative predictive value were 36.7%, 96.4%, 65.5%, 91.7%, and 58.7% for PC detection through CpG island methylation of the MUC2 gene in feces, respectively (Table 5).

Joint detection of methylation in peripheral blood and fecal samples

Simultaneous detection revealed that the methylation rate of PC was 60% (6/10), while CP (0%, 0/5) and CON (0%, 0/12) did not show methylation (Table 6). This difference was significant ($P < 0.01$). The sensitivity, specificity, diagnostic accuracy, positive predictive

Table 3
Evaluation of methylation status of MUC2 in the pancreatic cancer patients' peripheral blood for diagnosis of pancreatic cancer.

Diagnosis method	Sensitivity	Specificity	Diagnostic accuracy	Positive predictive value	Negative predictive value
Methylation status of MUC2	40%	97.5%	68.8%	94.1%	61.9%
CA 19-9 (>37 μ/ml)	65.0%	85.0%	75%	81.3%	70.8%

Table 5
Evaluation of methylation status of MUC2 in the pancreatic cancer patients' feces for diagnosis of pancreatic cancer.

Specimen	Sensitivity	Specificity	Diagnostic accuracy	Positive predictive value	Negative predictive value
Feces	36.7%	96.4%	65.5%	91.7%	58.7%

Table 6
Methylation status of MUC2 in the peripheral blood and feces of pancreatic cancer patients, chronic pancreatitis patients and health controls.

Disease	Number	Methylation status of MUC2	Methylation rate
PC	10	6	60.0%
CP	5	0	0%
CON	12	0	0%

value, and negative predictive value were 60.0%, 100%, 85.2%, 100%, and 81.0% for PC detection through simultaneous detection, respectively (Table 7).

Discussion

Pancreatic cancer (PC) is one of the most aggressive and lethal malignancies and ranks as the eighth most common cause of death from cancer worldwide [1,12]. The development of new screening methods, comprehensive analysis of high-risk populations, and improvements in the early diagnosis of PC are key for successful prognosis.

DNA methylation is a type of gene modification [13]. That tumor occurrence may be related to the inactivity of tumor suppressor genes caused by methylation of the CpG island [14]. Because local hypermethylation of the CpG island precedes the malignant proliferation of cells, detecting methylation can be useful as an early predictor of tumorigenesis [15].

In this study, DNA fragments were digested simultaneously with Hind III and Hap II. Following purification of the DNA fragments (which retained the methylated regions), PCR amplification, and analysis were conducted. This approach reduced the issue of false-positive results caused by methylation-sensitive restriction enzyme (MS-RE)-induced incomplete digestion. Therefore, the specificity and sensitivity of the results can be confirmed through simple and rapid testing.

None of the available biomarkers possess a sufficiently high accuracy to be implemented for screening. Endoscopic ultrasound has high resolution and detects lesions as small as 1–2 mm. Potentially EUS-guided fine-needle aspiration (FNA) biopsy can distinguish benign from malignant disease when focal lesions are present [16]. KRAS mutations are commonly detected in the pancreatic juice samples of patients undergoing pancreatic screening, even subjects without any detectable evidence of pancreatic neoplasia by pancreatic imaging, (most of whom probably have PanIN-1 lesions), demonstrating that pancreatic juice sample analysis can provide information about the presence of pancreatic neoplasia that is complementary to the information provided by pancreatic imaging tests [17]. However, the invasive examinations and technical difficulties during specimen collection greatly impacted the assay results. Sidransky first conducted fecal DNA analysis to help identify benign and malignant colorectal diseases because many colorectal tumor cells are shed into the intestinal lumen [18]. However, any intestinal-related tumors shed cells into this organ; as such, analysis of fecal genes is possible because the tumor cells may be more tolerant to degradation than normal cells [19].

In this study, the methylation rate of the fecal specimens from 30 PC patients was 43.3%, 0% in the 8 CP patients, and 5.0% in the 20 normal controls, showing a significant difference between PC and CP/CON ($P < 0.05$). In addition, the sensitivity, specificity, diagnostic accuracy, positive predictive value, and negative predictive value were 43.3%, 96.4%, 58.8%, 92.9%, and 61.4% for PC detection by detecting CpG island methylation of the MUC2 gene in feces, respectively.

The detection results for the peripheral blood specimens revealed that methylation was detected in 21 of the 40 PC specimens (52.5%), was not detected in 15 CP cases, and was detected in 1 of the 25 normal controls (4.0%). The difference between PC and CP/CON was significant ($P < 0.01$). The sensitivity, specificity, diagnostic accuracy, positive predictive value, and negative predictive value were 52.5%, 97.5%, 75%, 95.5%, and 67.2% for PC detection by detecting CpG island methylation of the MUC2 gene in the peripheral blood, respectively. Compared to the CA 19-9 assay, diagnostic specificity and positive predictive value were improved by the detection of the MUC2 gene methylation in the peripheral blood samples for PC diagnosis. The results of the joint detection using peripheral blood and fecal specimens revealed hypermethylation in 6 of the 10 PC patients (60%), but no cases of methylation were found in the 5 CP patients and 12 normal controls. The difference between PC and CP/CON was significant ($P < 0.01$). In the combined detection of peripheral blood and fecal specimens, methylation diagnosis of PC sensitivity, specificity, diagnostic accuracy, positive predictive value, and negative predictive value were 60.0%, 100%, 85.2%, 100%, and 81.0% for PC detection through the joint detection, respectively. Therefore, detection of MUC2 methylation may become an important auxiliary method to diagnose and screen PC in the clinic.

In this study, a dual-enzyme digestion (Hind III + Msp I/Hap II) and PCR method were used to detect methylation of the MUC2 gene in PC, which improved the traditional single enzyme digestion method and avoided false-positive results due to incomplete digestion. Because of this enzymatic digestion, PCR showed greatly improved sensitivity for DNA methylation detection. DNA as low as approximately 10 ng is required. Furthermore, this method does not require methylation-specific primers, involves simple operations, and is inexpensive. As such, it is a feasible and convenient method for detecting DNA methylation. Therefore, joint detection, using methylation restriction enzyme digestion and PCR, against hypermethylation of the MUC2 promoter region in the peripheral blood and feces may become a new diagnostic method for PC. However, its exact clinical value requires further investigation.

Table 7
Evaluation of methylation status of MUC2 both in the pancreatic cancer patients' peripheral blood and feces for diagnosis of pancreatic cancer.

Specimen	Sensitivity	Specificity	Diagnostic accuracy	Positive predictive value	Negative predictive value
Peripheral blood & feces	60.0%	100.0%	85.2%	100.0%	81.0%

Declaration of competing interest

The authors declare that they have no competing interests.

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