Disease Markers 32 (2012) 195–202
DOI 10.3233/DMA-2011-0877
IOS Press

Hypermethylated *FAM5C* and *MYLK* in serum as diagnosis and pre-warning markers for gastric cancer

Lu Chen[1], Liping Su[1], Jianfang Li, Yanan Zheng, Beiqin Yu, Yingyan Yu, Min Yan, Qinlong Gu, Zhenggang Zhu and Bingya Liu*
[a]*Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China*
[b]*Department of Surgery, Shanghai Jiao Tong University School of Medicine, Shanghai, China*

Abstract. Most cases of gastric cancer (GC) are not diagnosed at early stage which can be curable, so it is necessary to identify effective biomarkers for its diagnosis and pre-warning. We have used methylated DNA immunoprecipitation (MeDIP) to identify genes that are frequently methylated in gastric cancer cell lines. Promoter regions hypermethylation of candidate genes were tested by methylation-specific polymerase chain reaction (MSP) in serum samples, including GC ($n = 58$), gastric precancerous lesions (GPL, $n = 46$), and normal controls (NC, $n = 30$). Eighty two hypermethylated genes were acquired by array analysis and 5 genes (*BCAS4, CHRM2, FAM5C, PRAC* and *MYLK*) were selected as the candidate genes. Three genes (*CHRM2, FAM5C* and *MYLK*) were further confirmed to show methylation rates increased with progression from NC to GPL, then to GC. There was obvious decrease in detection of *FAM5C* and *MYLK* hypermethylation, but not *CHRM2*, from preoperative to postoperative evaluation ($P < 0.001$). Combined detection of *FAM5C* and *MYLK* hypermethylation had a higher sensitivity in GC diagnosis (77.6%,45/58) and pre-warning (30.4%,14/46) than one single gene detection and also had a high specificity of 90%. The combined hypermethylated status of *FAM5C* and *MYLK* correlated with tumor size ($P < 0.001$), tumor invasion depth ($P = 0.001$) and tumor-node-metastasis (TNM) stage ($P = 0.003$). Hypermethylated *FAM5C* and *MYLK* can be used as potential biomarkers for diagnosis and pre-warning of GC.

Keywords: Gastric cancer, hypermethylated DNA, biomarker, diagnosis, pre-warning, FAM5C and MYLK

1. Introduction

Gastric cancer (GC) is the third most common type of cancer and the second leading cause of cancer-related death in the world [1]. Despite advances in the management of this disease, the prognosis remains dismal. GC patients have a high mortality rate, with a 5-year survival rate of approximately 20% [2]. One of the main factors limiting the survival rate is that most cases are asymptomatic until advanced stages. GC, in most instances, represents a sequential process, i.e., progressing from inflammation to intestinal metaplasia, then to gastric epithelial dysplasia, and finally, to GC [3]. Gastric precancerous lesions (GPL) which confer a high risk for GC were including chronic atrophic gastritis (CAG), intestinal metaplasia (IM) and gastric epithelial dysplasia (GED). Therefore, it is crucial to explore effective biomarkers for diagnosis and pre-warning of GC.

Epigenetic perturbation of gene regulation is pathologically important to tumorigenesis [4], and methylation of CpG island (CGIs) is among the crucial epigenetic pathways regulating gene transcription. It had

[1]Co-first authors.

*Corresponding author: Bingya Liu, M.D., PhD., Shanghai Key Laboratory of Gastric Neoplasms, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University, Shanghai 200025, China. Tel.: +86 21 64674654; Fax: +86 21 64373909; E-mail: byliu@sjtu.edu.cn.

ISSN 0278-0240/12/$27.50

identified important correlation between methylation frequency and gastric cancers [5]. Most of the recent studies investigating methylation status of specific genes were restricted to tissues only [6–8]. Thus far, little data are available on hypermethylated DNA analyses in the sera of GC and GPL patients for diagnosis and pre-warning.

In this study, we determined the early diagnostic and pre-warning potential of DNA hypermethylation in the serum of gastric cancer patients. Five candidate genes were selected according to the results of the methylated DNA immunoprecipitation (MeDIP) assay and then were measured in the sera from patients with GC, GPL and NC. Our study showed that hypermethylated *FAM5C* and *MYLK* might be potential biomarkers for diagnosis and pre-warning of GC.

2. Materials and methods

2.1. Clinical samples and cell lines

Nine GC cell lines (SGC7901, MKN-45, and MKN-28 were obtained from Cell Bank of Chinese Academy of Sciences, AGS, NCI-N87, NCI-SNU-1, NCI-SNU-16, HTB-103, and BGC823 were obtained from ATCC) and 6 samples of normal gastric mucosa were prepared for methylated DNA immunoprecipitation (MeDIP) assay analysis. These 9 GC cell lines were conserved by Shanghai Institute of Digestive Surgery and authenticated by flow cytometry three months before experiment. Serum samples of 58 GC patients with average age of 62.0 years were diagnosed according to the WHO criteria [9]. The 46 GPL patients (3 GEDs, 20 IMs, and 23 CAGs) with average age of 60.9 years were diagnosed according to the gastroscopy examination. NC with average age of 55.2 years were taken from 30 healthy volunteers. GC and GPL patients and NC were detected without Helicobacter pylori infection. Local research ethics committees approved the collection of samples and informed consent was obtained from each patient.

2.2. Methylated DNA immunoprecipitation (MeDIP) and CpG island microarray assay

RNA-free genomic DNA extracted from 9 GC cell lines and 6 normal gastric mucosa samples were enriched with the methyl-CpG binding domain (MBD) protein by capturing mCG. MBD-enriched immunoprecipitation (IP) samples were labeled with Cy5, whereas the remaining non-enriched samples were labeled with Cy3 as control (INPUT) DNA. Then, two samples were hybridized on NimbleGen human CpG island microarray containing 300,000 probes. The two-color array was scanned and data were analyzed by the NimbleScan software. Each feature on the array had a corresponding scaled $\log_2$ratio, which was the ratio of the INPUT and IP signals. From the scaled $\log_2$ratio data, one-sided Kolmogorov-Smirnov (KS) test was applied to determine whether the probes were drawn from a significantly more positive distribution than those in the rest of the array. The resulting score for each probe is the -$\log_{10}$ p-value which was called Peak. Peak data $\geqslant 2$ was considered to indicate statistical difference between INPUT and IP samples.

2.3. DNA isolation and bisulphite modification

DNA was extracted from serum samples by using QIAamp Circulating Nucleic Acid Kit (QIAGEN). The concentration of DNA was measured by spectroscopy at a wavelength of 260 nm. Sulfurization modification, which converted unmethylated cytosine to uracil and left methylated cytosine unaltered, was done with less than 2 μg DNA. The samples were purified by use of EpiTect Bisulfite Kit (QIAGEN). Modified DNA was stored at -80°C until used.

2.4. Methylation-specific polymerase chain reaction (MSP)

Bisulfite-modified DNA was amplified using primers specific for either methylated or unmethylated promoter sequences of the target genes. The information of primers and their products is shown in Table 1. Polymerase chain reaction (PCR) was performed in 25-μl reaction volumes containing 2.5 μl of 10×PCR buffer (100S Tris-HCl, pH 8.3, 500 mmol/L KCl, 15 mmol/L MgCl2), 2 μl deoxynucleotide triphosphates (200 μmol/L each, final concentration), 5 pmol each primer, 1 μl modified DNA (10 ng), and 0.5U Taq polymerase (Hot Start Version, TaKaRa). Touch-down PCR was used for amplification; its condition was 3 min at 95°C, followed by 8 cycles of denaturation at 95°C for 20 s, annealing at the specific temperature for 25 s (decreased by 0.5°C at each cycle), and extension at 72°C for 30 s. This reaction was followed by 35 cycles of the above-mentioned program in which the annealing temperature has been decreased by 4°C and a final extension step at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gels. Each exper-

Table 1
Primers for methylation-specific polymerase chain reaction (MSP)

Primers	Regions	Sequences	Products length(bp)
BCAS4-M-F	chr20:48844271-480	GGGTTTATTTAGGTCGGTTTTC	128
BCAS4-M-R		TAACGATCCTCGTCTTCCTC	
BCAS4-U-F		TAGGTTTGGAATTGTTTGTT	128
BCAS4-U-R		CCAAAACTAACACACTTTACTC	
CHRM2-M-F	chr7:136204314-622	TAGATTAGTTAAGTGGTTGAGACGA	133
CHRM2-M-R		ATAAAAATAAAACGAAAAAAACGAA	
CHRM2-U-F		TAGATTAGTTAAGTGGTTGAGATGA	132
CHRM2-U-R		TAAAAATAAAACAAAAAAAACAAA	
FAM5C-M-F	chr1:188714132-341	TTTTTTTAGGTTTTATTTTTTTGGC	101
FAM5C-M-R		TAACTTCTAATACAAAACGTTCGAA	
FAM5C-U-F		AGTTTTTTTAGGTTTTATTTTTTTGGTG	110
FAM5C-U-R		AAAACTCTAACTTCTAATACAAAACATTCA	
PRAC-M-F	chr17:44157439-737	AATCGAGGATTTTAAGTTGGATTAC	126
PRAC-M-R		ACCAAAAAATATAAATACATACGAA	
PRAC-U-F		TTGAGGATTTTAAGTTGGATTATGG	126
PRAC-U-R		AAACCAAAAAATATAAATACATACAAA	
MYLK-M-F	chr3:125085114-300	TAATGGGATTTTAGTTAACGC	134
MYLK-M-R		GACCCGCGAATACCAAAC	
MYLK-U-F		TAATAATGGGATTTTAGTTAATGT	134
MYLK-U-R		ACAACCCACAAATACCAAACC	

iment was repeated at least three times and we have removed the false results. The EpiTect PCR control DNA set (QIAGEN) was used as positive control of methylated and unmethylated DNA.

2.5. *Statistical analyses*

The methylation frequencies in serum samples were compared using the $\chi 2$ test. ROC curve analysis was used to assess methylation detection significance for GC diagnosis. The association between clinicopathological characteristics and combined DNA hypermethylation was compared using $\chi 2$ tests. For all tests, probability values less than 0.05 were regarded as statistically significant. All datas were analyzed with SPSS 13.0 for Windows (SPSS, Chicago, IL).

3. Results

3.1. *Identification of candidate hypermethylated genes*

DNA samples from 9 gastric cancer cell lines and 6 normal gastric mucosae were prepared by MeDIP, and the resulting methylated and unmethylated fractions were hybridized to Nimblegen human CpG island CHIP. Eighty two hypermethylated genes in cancer cells compared with normal mucosae were acquired (Fig. 1). As our aim was to identify novel high penetrance markers for the GC diagnosis and pre-warning, many loci previously reported as methylated in GC were not included in this study. Instead, 5 genes, *BCAS4* (breast carcinoma amplified sequence 4), *CHRM2* (cholinergic receptor muscarinic 2), *FAM5C* (family with sequence similarity 5, member C), *PRAC* (prostate

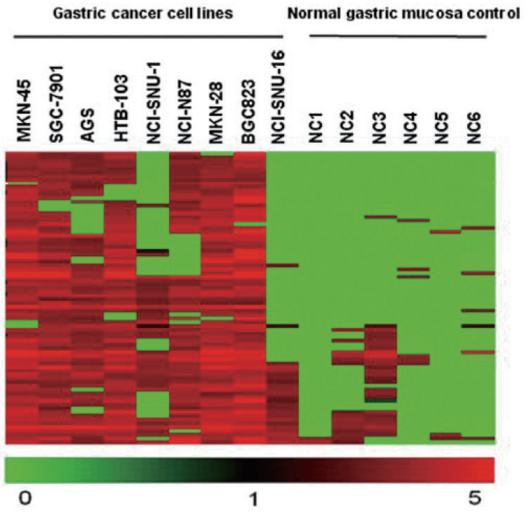

Fig. 1. Methylated DNA immunoprecipitation (MeDIP) CpG island microarray assay analysis. DNA was extracted from 9 GC cell lines and 6 normal gastric mucosa and methylated DNA was enriched by MeDIP. Methylated DNA and un-enriched DNA were hybridized on NimbleGen human CpG island microarray. The two-color array was scanned and data were analyzed by the NimbleScan software. Red color represents high levels of DNA methylation. Green color represents low levels of DNA methylation.

Table 2
Methylated ratio peak in CpG island microarray for five selected genes

	BCAS4	CHRM2	FAM5C	PRAC	MYLK
MKN-45	7.03	6.03	6.26	< 2.00	4.04
SGC-7901	5.85	4.32	4.36	3.45	3.6
AGS	3.15	2.99	< 2.00	4.47	3.42
HTB-103	8.04	4.49	3.65	4.87	3.23
MKN-28	9.12	3.9	4.72	4.87	3.38
BGC823	7.16	6.48	8.66	5.39	3.22
NCI-N87	7.45	< 2.00	< 2.00	3.62	2.62
NCI-SNU-1	4.12	2.47	< 2.00	< 2.00	2.22
NCI-SNU-16	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
NC1	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
NC2	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
NC3	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
NC4	2.04	< 2.00	< 2.00	< 2.00	< 2.00
NC5	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
NC6	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00

NC, normal gastric mucosa.

cancer susceptibility candidate) and *MYLK* (myosin light chain kinase) that were not previously reported as methylated were selected for the subsequent analysis. Besides, Peak data of four genes were lower than 2 in normal controls except *BCAS4*. The exact peak data obtained by CHIP analysis for these 5 loci were shown in Table 2.

3.2. Identification of hypermethylated genes in a set of serum samples

To evaluate the significance of the DNA promoter region methylation for diagnosis and pre-warning of GC according to MeDIP assay results, five genes were further examined in the serum samples collected from patients with GC, GPL and NC. Methylation rates of three genes (*CHRM2, FAM5C* and *MYLK*) were confirmed to show methylation rates increased with progression from NC to GPL, then to GC (*CHRM2* (6.7%,15.2%,31.05%), *FAM5C* (3.3%,6.5%,31.0%) and *MYLK* (6.7%,28.3%,70.7%)). However, methylation of *BCAS4* and *PRAC* were shown to be frequently detected in NC and there was no correlation with the histological progression. The DNA methylation rates of these five genes were shown in Fig. 2A and representative examples of methylation analysis of *CHRM2*, *FAM5C* and *MYLK* were shown in Fig. 2B.

3.3. Alteration of DNA methylation rates in pre- and postoperative serum samples of GC patients

We next compared the DNA methylation rates of *CHRM2*, *FAM5C* and *MYLK* in serum collected from 58 GC patients before operation or at the 3rd day post operation. The methylation rate of *FAM5C* was 31.0% before operation and decreased to 3.5% post operation. Meanwhile, the methylation rate of *MYLK* decreased from 70.7% (preoperative) to 20.7% (postoperative). Our results showed significant decreases in the hypermethylation status of *FAM5C* and *MYLK* from the pre- to postoperative period ($P < 0.001$). However, no notable differences were detected in the hypermethylated *CHRM2* between pre- and postoperative sera of GC patients. The DNA methylation rates of these three genes detected in the pre- and postoperative sera are shown in Fig. 3A, and representative examples of the methylation analysis are shown in Fig. 3B.

3.4. Combined detection analysis of FAM5C and MYLK hypermethylation

One of FAM5C and MYLK hypermethylation detected in serum samples as positive for combine detection. ROC curve analysis was used to assess DNA methylation detection significance for GC diagnosis. Area under the ROC curve of *FAM5C* was 0.639, *MYLK* was 0.820, combined *FAM5C* and *MYLK* was 0.838 (Fig. 4A). Combined detection of *FAM5C* and *MYLK* hypermethylation for GC diagnosis was more significant than one single gene. Positive rate of the combined detection in GC sera was 77.6% (45/58), which had a statistical difference in contrast to normal control (10.0%, 3/30) ($P < 0.001$) (Fig. 4B). Combined dection in GPL (30.4%, 14/46) was also had a statistical difference in contrast to normal control ($P = 0.037$) (Fig. 4B). Moreover, there was a significant decrease in the combined detection of *FAM5C* and *MYLK* hypermethylation from the pre- (77.6%, 45/58) to postoperative period (24.1%, 14/58) ($P < 0.001$)(Fig. 4C).

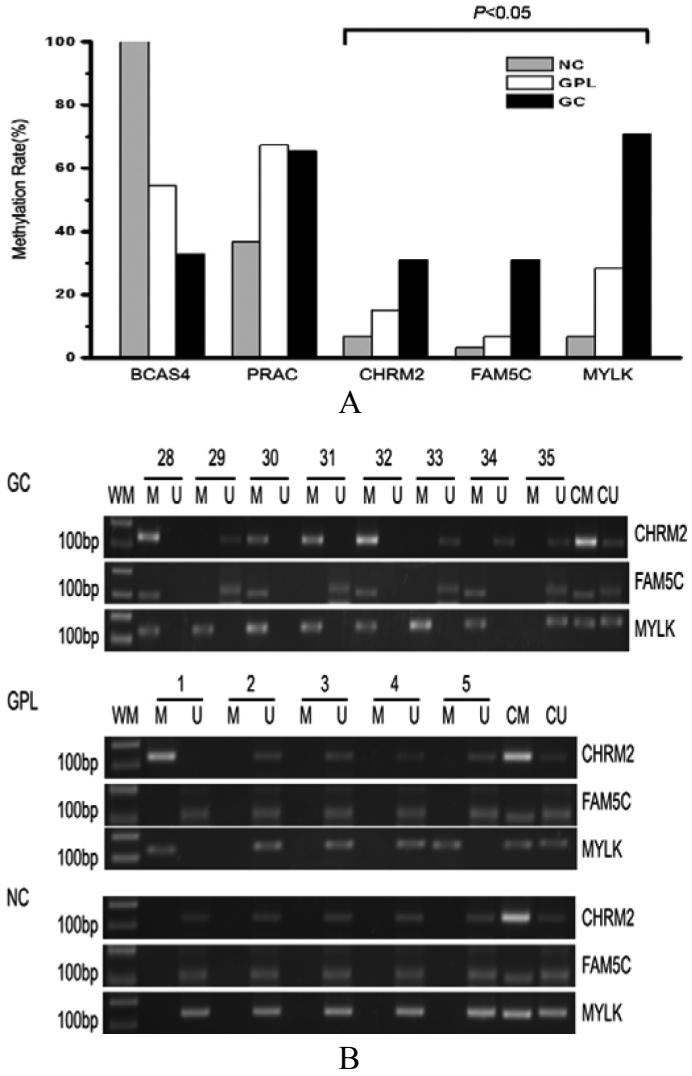

Fig. 2. Methylation-specific polymerase chain reaction (MSP) analysis of serum samples. Methylation status of five candidate genes (*BCAS4, CHRM2, FAM5C, PRAC* and *MYLK*) in serum samples including GC ($n = 58$), GPL ($n = 46$) and NC ($n = 30$) were detected by MSP. A Histogram representing methylation rates of five candidate genes in serum samples is shown. Three genes (*CHRM2*, *FAM5C* and *MYLK*) were confirmed to show methylation rates increased with progression from NC to GPL, then to GC. B MSP results for three genes (*CHRM2*, *FAM5C* and *MYLK*) in representative samples are shown. GC: gastric cancer, GPL: gastric precancerous lesions, NC: normal controls. WM, weight marker; M, PCR product with primers specific for methylated DNA; U, PCR product with primers specific for unmethylated DNA; CM, methylated DNA control; CU, unmethylated DNA control.

3.5. Association between clinicopathological features and combined FAM5C and MYLK methylated pattern in GC sera

The combined hypermethylated status of *FAM5C* and *MYLK* in the pretherapeutic sera of the 58 GC patients was analyzed for its association with clinicopathological parameters. No correlation between the methylated status of these two genes and age, differentiation grade and lymph node metastasis was found. However, the combined hypermethylated status of *FAM5C* and *MYLK* was shown to correlate with tumor size ($P < 0.001$), tumor invasion depth ($P = 0.001$) and TNM stage ($P = 0.003$) (Table 3).

4. Discussion

In this study, we evaluated the DNA promoter region methylation of five novel genes (*BCAS4*, *CHRM2*,

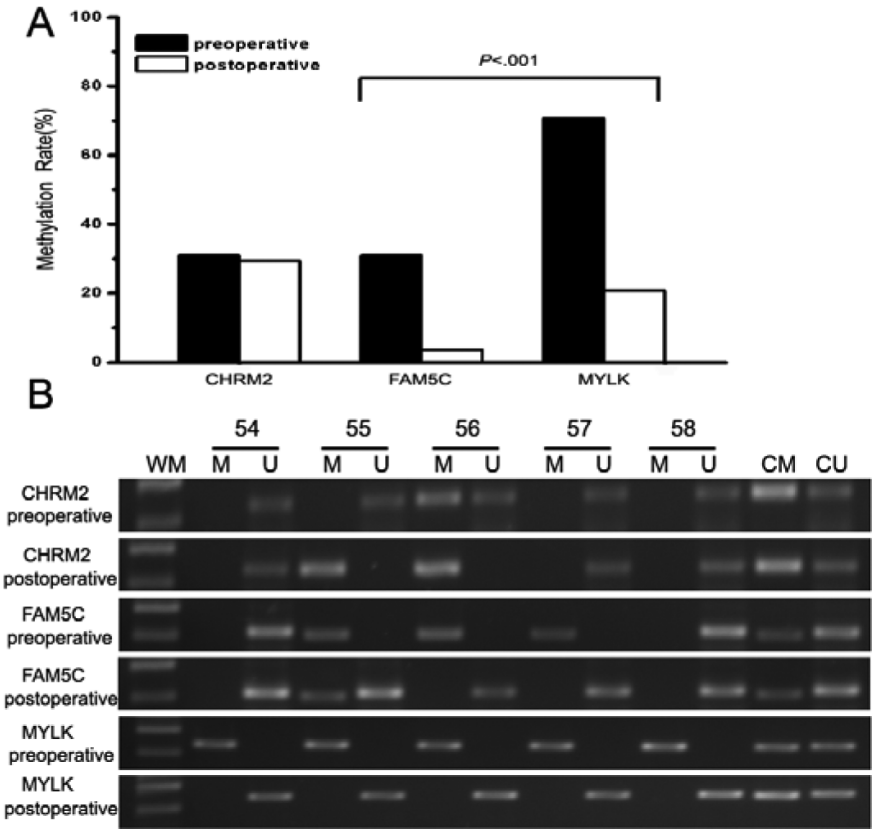

Fig. 3. Alteration of DNA methylation rates of *CHRM2, FAM5C* and*MYLK* in pre- and postoperative serum samples. Methylation status of *CHRM2, FAM5C* and*MYLK* in pre- and postoperative GC serum samples were detected by MSP. A Histogram representing methylation rates of three genes in pre- and postoperative serum samples is shown. There were significant decreases in the hypermethylation status of *FAM5C* and *MYLK* from the pre- to postoperative period ($P < 0.001$). B MSP results for three genes (*CHRM2*, *FAM5C* and *MYLK*) in representative samples is shown. WM, weight marker; M, PCR product with primers specific for methylated DNA; U, PCR product with primers specific for unmethylated DNA; CM, methylated DNA control; CU, unmethylated DNA control.

FAM5C, *PRAC*, and *MYLK*) that were selected on the basis of the MeDIP assay results in NC, GPL (CAG, IM, and GED), and GC patients.

Several studies have addressed the diagnostic utility of epigenetic biomarkers in the detection of human cancer [10]. It is now necessary to develop noninvasive and affordable methods of "serologic biopsy" for the asymptomatic population. With this goal in mind, we evaluated five candidate genes (*BCAS4*, *CHRM2*, *FAM5C*, *PRAC*, and *MYLK*) in serum samples from patients with GC, GPL, and NC. Detecting aberrant DNA methylation events in patients' sera is preferred for several reasons. First, compared with conventional serum tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, DNA methylation markers are associated with higher levels of sensitivity and specificity [11,12]. MSP assays can detect alterations of DNA methylation based on low levels of circulating tumor DNA [13]. Although Pyrosequencing results are the "golden standard" of DNA methylation, this method is very expensive and can just be fit for detection of the small samples. Second, compared with mRNA and protein, DNA is a more stable molecule. Third, in contrast with mutations, the incidence of aberrant DNA methylation is higher, with smaller specific regions of the genome effected [14], thereby making it is easier to detect methylated targets. But MSP assays results were showed by agarose electrophoresis, which was not convenient to clinical application. So we can use the Methylight method [15] to detect DNA methylation aberrant in serum samples and apply it to clinical practice.

Most previous studies focused on single gene rather than multiple genes. We found that the combined detection of hypermethylated *FAM5C* and *MYLK* achieved a more significant diagnosis for GC than one single gene.

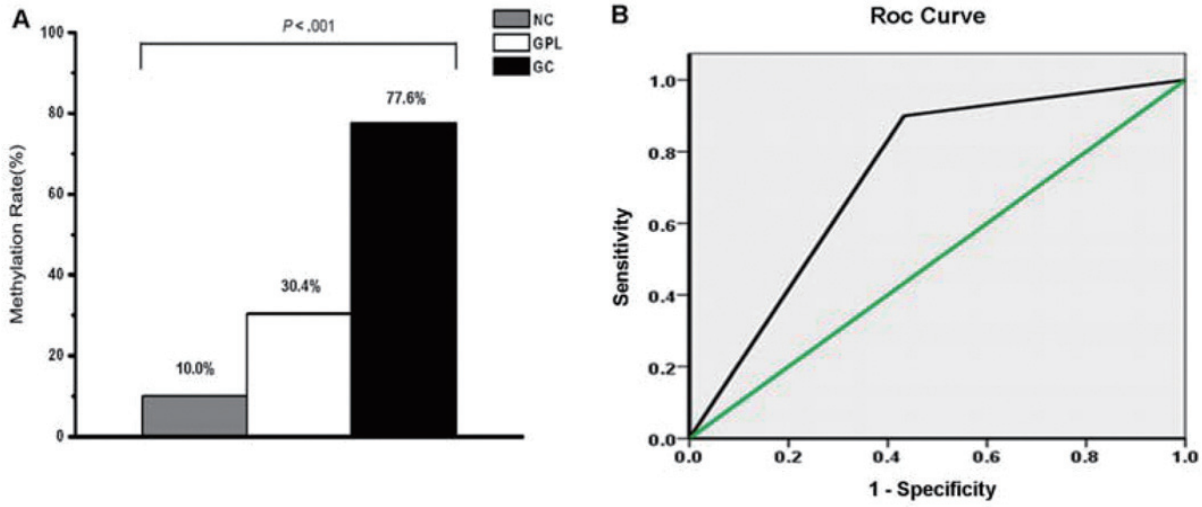

Fig. 4. Combined detection of *FAM5C* and *MYLK* hypermethylation in GC, GPL and NC serum specimens. A ROC curve which evaluate combined detection significance for GC diagnosis. Combined detection of *FAM5C* and *MYLK* hypermethylation for GC diagnosis was more significant than one single gene. B Histogram representing the combined detection rates in serum samples of GC, GPL and NC. Combined detection of *FAM5C* and *MYLK* in GC ($P < 0.001$) and GPL ($P = 0.037$) sera were higher than in NC. C, histogram representing the combined detection rates in pre- and postoperative serum samples. There was a significant decrease in the combined detection from the pre- to postoperative period.

Table 3
Clinicopathological parameters and combined detection of *FAM5C* and *MYLK* hypermethylation in GC serum

Characteristics	M[a]	U[b]	χ^2	P
Tumor size			14.099	< 0.001
$>$ 5 cm	30	1		
$\leqslant$ 5 cm	15	12		
Differentiation Grade			1.180	0.352
I–II	20	8		
III–IV	25	5		
Tumor invasion depth			12.026	0.001
T1-2	11	10		
T3-4	34	3		
Lymph node status			2.057	0.212
N0	21	9		
N1-3	24	4		
TNM Stage			9.757	0.003
I–II	16	11		
III–IV	29	2		

a: M means one gene of *FAM5C* and *MYLK* detected as methylation; b: U means both genes of *FAM5C* and *MYLK* detected as unmethylation.

Combined detection had a higher sensitivity in GC diagnosis and pre-warning than general methods [16,17]. Furthermore, the obvious decrease of combined detection of hypermethylated *FAM5C* and *MYLK* in sera of postoperative GC patients in contrast to in preoperative samples and the combined detection of *FAM5C* and *MYLK* methylation status correlates with tumor size, which can effectively reflect tumor burden status and also can help to evaluate the efficiency of operation and follow-up for tumor recurrence. The DNA methylation level can be used to discriminate tumor stages in GC [18]. Besides, we found that combined detection of *FAM5C* and *MYLK* methylation status correlates with tumor invasion depth and TNM stage. This correlation can be used for evaluation of disease condition and prognosis.

BCAS4 and *PRAC* play a negative regulatory role in the development of breast and prostate cancer, respectively [19,20]. Therefore, our findings might explain the high methylation rates in NC. There are currently no reports on the relationship of *CHRM2* and cancer. *FAM5C* is identified as a novel tumor suppressor gene in tongue squamous cell carcinoma [21] and might has similar biological functions in GC. It has been found *MYLK* inhibitor ML-7 and ML-9 can inhibit invasion of mammary, prostate, and pancreatic cancer cells *in vitro* [22–24]. *MYLK* mRNA expression level increases obviously in non-small cell lung cancer cells and is related with recurrence and metastasis [25]. These findings suggest that *MYLK* may be an oncogene. It seemed to be complicated that *MYLK* shows DNA hypermethylation in GC. Actually, our study found that some GC highly expressed *MYLK*, which restricted in the stromal cells (unpublished). Up-regulated expression of *MYLK* in stroma cells may regulate GC cells proliferation, apoptosis, invasion and migration.

In summary, our findings suggested that combined detection of hypermethylated *FAM5C* and *MYLK* might

be a potential method for GC diagnosis and pre-warning, monitoring recurrence and auxiliary judgment of TNM stage. However, it is necessary to enlarge clinical samples and validate the potential significance for the clinical application of these findings.

Acknowledgements

This study was supported by grants from National Natural Science Foundation of China (No. 30872476, No. 30900670), National High Technology Research and Development Program of China (863 Program, No. 2006AA02A301 and No. 2007AA02Z179), Science and Technology Commission of Shanghai Municipality (No. 10jc1411100, 09DZ1950100, and 09DZ2260200), Shanghai Key Discipline (S30204), and Key Projects in the National Science & Technology Pillar Program of China (No. 2008BA152B03).

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward and D. Forman, Global cancer statistics, *CA Cancer J Clin* **61** (2011), 69–90.

[2] S. Nomura and M. Kaminishi, Surgical treatment of early gastric cancer, *Dig Surg* **24** (2007), 96–100.

[3] H. Tomita, A. Hirata and Y. Yamada, Suppressive effect of global DNA hypomethylation on gastric carcinogenesis, *Carcinogenesis* **31** (2010), 1627–1633.

[4] M. Vauhkonen, H. Vauhkonen and P. Sipponen, Pathology and molecular biology of gastric cancer, *Best Pract Res Clin Gastroenterol* **20** (2006), 651–674.

[5] C.D. Jee, M.A. Kim, E.J. Jung, J. Kim and W.H. Kim, Identification of genes epigenetically silenced by CpG methylation in human gastric carcinoma, *Eur J Cancer* **45** (2009), 1282–1293.

[6] W. Guo, Z. Dong, Y. Guo, G. Kuang, Z. Yang and Z. Chen, Detection of promoter hypermethylation of the CpG island of E-cadherin in gastric cardiac adenocarcinoma, *Eur J Med Res* **14** (2009), 453–458.

[7] Y.C. Wang, Z.H. Yu, C. Liu, L.Z. Xu, W. Yu and J. Lu, Detection of RASSF1A promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients, *World J Gastroenterol* **14** (2008), 3074–3080.

[8] F. Ksiaa, S. Ziadi, K. Amara, S. Korbi and M. Trimeche, Biological significance of promoter hypermethylation of tumor-related genes in patients with gastric carcinoma, *Clin Chim Acta* **404** (2009), 128–133.

[9] J. Harder and O.G. Opitz, Gastric cancer–risk factors and medical therapy, *Praxis* (*Bern 1994*) **95** (2006), 1021–1028.

[10] T. Ushijima, Detection and interpretation of altered methylation patterns in cancer cells, *Nat Rev Cancer* **5** (2005), 223–231.

[11] H. Koike, D. Ichikawa, H. Ikoma, E. Otsuji, K. Kitamura and H. Yamagishi, Comparison of methylation-specific polymerase chain reaction (MSP) with reverse transcriptase-polymerase chain reaction (RT-PCR) in peripheral blood of gastric cancer patients, *J Surg Oncol* **87** (2004), 182–186.

[12] H. Koike, D. Ichikawa, H. Ikoma, N. Tani, D. Ikoma and E. Otsuji, Comparison of serum aberrant methylation and conventional tumor markers in gastric cancer patients, *Hepatogastroenterol* **52** (2005), 1293–1296.

[13] J.G. Herman, J.R. Graff, S. Myöhänen, B.D. Nelkin and S.B. Baylin, Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands, *Proc Natl Acad Sci USA* **93** (1996), 9821–9826.

[14] H.H. Zhou, S.Y. Yan, X.Y. Zhou, X. Du, T.M. Zhang and X. Cai, MLH1 promoter germline-methylation in selected probands of Chinese hereditary non-polyposis colorectal cancer families, *World J Gastroenterol* **14** (2008), 7329–7334.

[15] C.A. Eads, K.D. Danenberg, K. Kawakami, L.B. Saltz, C. Blake and D. Shibata, MethyLight: a high-throughput assay to measure DNA methylation, *Nucleic Acids Res* **28** (2000), E32.

[16] Y. Hao, Y. Yu, L. Wang, M. Yan, J. Ji and Y. Qu, IPO-38 is identified as a novel serum biomarker of gastric cancer based on clinical proteomics technology, *J Proteome Res* **7** (2008), 3668–3677.

[17] M. Carpelan-Holmström, J. Louhimo, U.H. Stenman, H. Alfthan and C. Haglund, CEA, CA 19-9 and CA 72-4 improve the diagnostic accuracy in gastrointestinal cancers, *Anticancer Res* **22** (2002), 2311–2316.

[18] N. Oue, Y. Mitani, J. Motoshita, S. Matsumura, K. Yoshida and H. Kuniyasu, Accumulation of DNA methylation is associated with tumor stage in gastric cancer, *Cancer* **106** (2006), 1250–1259.

[19] M. Løvf, G.O. Thomassen and A.C. Bakken, Fusion gene microarray reveals cancer type-specificity among fusion genes, *Genes Chromosomes Cancer* **50** (2011), 348–357.

[20] S. Edwards, C. Campbell and P. Flohr, Expression analysis onto microarrays of randomly selected cDNA clones highlights HOXB13 as a marker of human prostate cancer, *Br J Cancer* **92** (2005), 376–381.

[21] T. Kuroiwa, N. Yamamoto, T. Onda and T. Shibahara, Expression of the FAM5C in tongue squamous cell carcinoma, *Oncol Rep* **22** (2009), 1005–1011.

[22] L.Z. Gu, W.Y. Hu, N. Antic, R. Mehta, J.R. Turner and P. de Lanerolle, Inhibiting myosin light chain kinase retards the growth of mammary and prostate cancer cells, *Eur J Cancer* **42**, (2006), 948–957.

[23] K. Kaneko, K. Satoh, A. Masamune, A. Satoh and T. Shimosegawa, Myosin light chain kinase inhibitors can block invasion and adhesion of human pancreatic cancer cell lines, *Pancreas* **24** (2002), 34–41.

[24] X. Zhou, Y. Liu, J. You, H. Zhang, X. Zhang and L. Ye, Myosin light-chain kinase contributes to the proliferation and migration of breast cancer cells through cross-talk with activated ERK1/2, *Cancer Lett* **270** (2008), 312–327.

[25] Y. Minamiya, T. Nakagawa, H. Saito, I. Matsuzaki, K. Taguchi and M. Ito, Increased expression of myosin light chain kinase mRNA is related to metastasis in non-small cell lung cancer, *Tumour Biol* **26** (2005), 153–157.