

***p16* promoter hypermethylation: A useful serum marker for early detection of gastric cancer**

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Abstract

AIM: To determine *p16* promoter hypermethylation in gastric tumoral tissue and serum samples, its impact on p16-protein expression, and correlation with clinical and histological features.

METHODS: Samples were obtained from 52 histologically confirmed cases of gastric adenocarcinoma. Gastric tissue and serum of 50 age- and sex-matched individuals with normal gastroscopy and biopsy were obtained as control samples. Methylation-specific polymerase chain reaction (MSP) was used to evaluate

methylation status of *p16* promoter. p16-protein expression was analyzed by immunohistochemical staining on paraffin-embedded sections.

RESULTS: Methylation was detected in 44.2% (23/52) of tumoral tissues. 60.9% of them were also methylated in serum, i.e., 26.9% of all patients (14/52). Methylation was not detected in tissue and sera of control samples. p16-protein expression was decreased in 61.5% of cases (32/52), and was significantly associated with promoter hypermethylation ($P < 0.001$). Methylation was significantly more frequent in higher pathological grades ($P < 0.05$). Methylation was not associated with other clinicopathological features and environmental factors including *H pylori* infection and smoking.

CONCLUSION: *p16* promoter hypermethylation is an important event in gastric carcinogenesis. It is the principle mechanism of *p16* gene silencing. It is related to malignant tumor behavior. Detection of DNA methylation in serum may be a biomarker for early detection of gastric cancer.

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Key words: Gastric cancer; *p16*; Hypermethylation; Methylation specific PCR

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INTRODUCTION

Gastric cancer is one of the most widespread cancers and the second leading cause of cancer-related death worldwide^[1]. It is estimated that gastric cancer is the most common cancer in Iran (Age Standardized Rate = 26.1 per 10⁵), and the incidence rate is higher than the world

average^[2]. Several genetic and epigenetic alterations have been suggested to play important roles in the carcinogenesis pathway, affecting oncogenes, tumor suppressor genes, apoptosis-regulating or mismatch repair genes^[3]. Repression of genes by CpG island methylation in the promoter region, which is normally unmethylated, is the most frequent epigenetic alteration, in which the DNA structure is affected while the genetic code remains intact^[4]. Thus, an increasing number of genes, methylated at the promoter region, are targeted as possible tumor markers for different purposes such as early detection, classification and tumor prognosis, therapeutic strategies and patient follow up^[5-9].

Detection of circulating tumoral DNA was first reported about three decades ago^[10]. Free DNA is thought to originate from apoptotic and necrotic tumoral cells^[11]. More recently, detection of promoter hypermethylation in serum of patients has been reported in some malignancies such as colorectal or esophageal cancer^[12,13]. Correlation of clinicopathological features with methylation patterns, which help to predict patient outcome, has been indicated in several studies^[14-18].

p16 is a cell-cycle regulator that induces G1-phase arrest by inhibition of cyclin D-dependent protein kinase 4 (CDK4) and 6 (CDK6), thus interfering with phosphorylation of the retinoblastoma protein (pRb) and further inhibition of transcription of proteins that promote passage of the cell through the restriction point of the G1 stage^[19]. *p16* inactivation breaks down the regulatory mechanism of the cell cycle. As a tumor suppressor gene, being silenced by any mechanism will promote carcinogenesis. This study was conducted to assess the methylation of *p16* promoter in gastric tumoral tissue and serum samples and its impact on gene expression, and correlation with clinical and histological features for the first time in the Iranian population.

MATERIALS AND METHODS

Sample collection and DNA preparation

Tumoral tissue and corresponding serum samples were obtained from 52 consecutive histologically confirmed gastric adenocarcinoma patients. Patients undergoing any therapeutic intervention were excluded. Tumoral tissues were obtained by gastrectomy or endoscopy in unresectable metastatic cases, formalin-fixed and paraffin-embedded. Tumors were histologically verified as gastric adenocarcinoma and subtyped into intestinal, diffuse, or mixed type, as suggested by Lauren^[20]. Grading was also determined and staging was performed using tumor, node and metastasis (TNM) classification, sixth edition. Fifty age- and sex-matched individuals, with normal gastroscopy and biopsy, were included as a control group. Paraffin-embedded tissue and corresponding serum samples were taken as well. All patients and control individuals gave informed consent according to institutional guidelines, and the study was approved by the research ethics committee of Mashhad University of Medical Sciences. Paraffin-embedded tissues were retrieved by using xylene and alcohol, digested by proteinase K, extracted with phenol/chloroform/isoamyl alcohol, and precipitated in ethanol. Serum samples were isolated by DNA extraction

Table 1 Primer sets used in MSP

Primer sets	Sense primer: 5'-3'	Antisense primer: 5'-3'	Size (bp)
<i>p16-W</i>	CAGAGGGTGGGG CGGACCGC	CGGGCCCGGGCCGTGG	140
<i>p16-M</i>	TTATTAGAGGGTG GGGCGGATCGC	GACCCCGAACC GCGAC CGTAA	150
<i>p16-U</i>	TTATTAGAGGGTG GGGTGGATTGT	CAACCCCAAACCACAA CCATAA	151

W: Unmodified or wild-type primers; M: Methylated-specific primers; U: Unmethylated-specific primers.

kit (QIAamp DNA mini kit, QIAGEN, Canada). *H pylori* infection was detected and double checked in serum with an ELISA (Trinity *H pylori* kit, Ireland). None of the patients had a history of *H pylori* eradication.

DNA modification (bisulfite treatment)

DNA modification with sodium bisulfite causes unmethylated cytosine bases to convert to uracil, while methylated cytosine is resistant and remains unchanged. After treatment, methylated alleles have a different sequence as compared with unmethylated alleles, which can be used to design allele-specific PCR primers, and methylation-specific PCR (MSP) takes advantage of this fact^[21]. Two micrograms of genomic DNA was first denatured by heating (97°C for 10 min followed by chilling on ice at 0°C for 5 min), and incubated for 20 min at 48°C after adding 3 mol/L NaOH (2 µL). Bisulfite solution (2.5 mol/L sodium metabisulfite and 125 mmol/L hydroquinone) was added and incubated for 12 h at 48°C in the dark, for modification. Modified DNA was purified by using Wizard DNA purification resin (DNA Cleanup Kit; Promega, Madison, WI, USA) according to the manufacturer's instructions. Modified DNA was treated with 3 mol/L NaOH (5 µL) in 37°C for 10 min and precipitated with ammonium acetate 5 mol/L (75 µL), 2.5 volumes 100% ethanol and 2 µL glycogen (20 mg/mL; Fermentase; UAB, Lithuania) and dissolved in 20 µL 5 mmol/L Tris (pH 8.0).

MSP

Specific primer sets for unmethylated (*p16U*) and methylated (*p16M*) DNAs, described by Herman *et al*^[21], were utilized (Table 1). The PCR mixture contained 1 × buffer (Finzymes OY, Finland) with 2 mmol/L MgCl₂, 500 nmol/L each primer, 0.2 mmol/L dNTPs, 1 U Hot Start Taq polymerase (Finzymes OY, Finland). The PCR amplification of the modified DNA samples consisted of one cycle of 95°C for 10 min, 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min; then one cycle of 72°C for 10 min. DNA from L132 (embryonic lung cell line) cells was used as a positive control for unmethylated DNA. In order to make a positive control for methylated DNA, normal lymphocyte DNA was treated with M.Sss1 CpG methyltransferase (New England BioLabs, USA) before bisulfite treatment. Six microliters of amplified PCR products were loaded onto 2.5% agarose gels and non-denaturing 8% polyacrylamide gels, stained with ethidium bromide, and directly visualized under UV illumination.

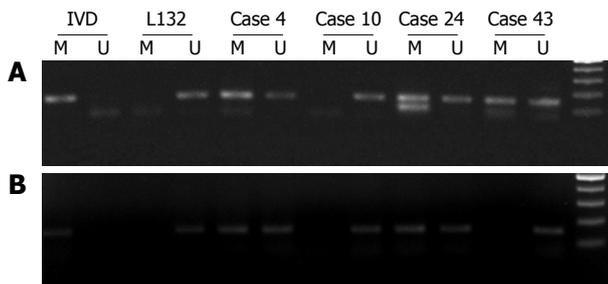


Figure 1 Analysis of p16 promoter hypermethylation in tissue and corresponding serum of patients. (A) MSP analysis. IVD served as a positive control for hypermethylated DNA and L132 as a positive control for unmethylated DNA. Patients 4, 24 and 43 were hypermethylated, which revealed 150 bp bands with hypermethylated primers. Patient 10 was not methylated. (B) MSP analysis in corresponding sera of samples depicted in A. Patients 4 and 24 were hypermethylated in serum as well. Patients 43 and 10 were not methylated.

Table 2 Results of methylation in tumoral tissue and corresponding serum

	Tissue (+)	Tissue (-)
Serum (+)	14	0
Serum (-)	9	29

(+): Methylated; (-): Unmethylated.

Immunohistochemical staining

Immunohistochemical staining was performed using the CINtec p16^{INK4A} Histology Kit, clone E6H4 (Dako, Carpinteria, CA, USA) and the DakoCytomation Autostainer Instrument, according to the manufacturer’s instructions. Briefly, 4-µm-thick formalin-fixed, paraffin-embedded sections were dewaxed, rehydrated and boiled in Target Retrieval Solution of Dako in a microwave oven for 40 min. After endogenous peroxidase blocking, the slides were incubated with primary p16^{INK4A} antibody (clone E6H4) at 1:25 dilutions for 30 min. The antigen-antibody reaction was visualized by 3,3'-diaminobenzidine (DAB) chromogen for 10 min, followed by acidified hematoxyline counterstaining for 1 min. p16-positive cervical squamous cell carcinoma was used as an external positive control, and non-neoplastic stromal cells served as internal positive controls for p16 in every tumor section.

Statistical analysis

Statistical analysis was performed using SPSS software (ver. 11.5). The correlation between two variables was evaluated using Pearson’s χ^2 and Fisher’s exact test. Statistical significance was defined as $P < 0.05$.

RESULTS

The studied population consisted of 38 men and 14 women. The median age was 64.5 and the average was 63.7 years (range, 38-81). Hypermethylation of p16 promoter was detected in 44.2% of tumoral gastric tissue (23/52), while normal gastric samples were all unmethylated. MSP analysis of the p16 promoter in gastric cancer is shown in Figure 1. Corresponding serum samples were also examined. Among the patients with methylated gastric

Table 3 Clinicopathological features of p16 promoter hypermethylation

Variable	n (%)	Methylated	Unmethylated	P value
Total		23	29	
Gender				
Male	38 (73.1)	17	21	0.904
Female	14 (26.9)	6	8	
Age (yr)				
< 64	26 (50.0)	8	18	0.051
> 64	26 (50.0)	15	11	
Pathological grade				
1	20 (39.2)	5	15	< 0.05
2	11 (21.6)	7	4	
3	20 (39.2)	10	10	(1 vs 2&3)
Pathological type				
Intestinal	27 (62.7)	13	14	0.754
Diffuse	14 (27.5)	5	9	(Intestinal vs Diffuse)
Mixed	5 (9.8)	4	1	0.161
				(Intestinal vs Mix)
				0.140
				(Diffuse vs Mix)
Anatomical site				
Cardia	22 (44.0)	7	15	0.124
Body	15 (30.0)	9	6	
Pylorus	13 (26.0)	6	7	(Cardia vs Noncardia)
Distant metastasis				
Absent	39 (75.0)	20	19	0.259
Present	13 (25.0)	9	4	
Smoking				
Yes	9 (17.6)	4	5	1.000
No	42 (82.4)	19	23	
H pylori infection				
Positive	30 (60.0)	11	19	0.201
Negative	20 (40.0)	11	9	

tissue, 60.9% were also methylated in their corresponding serum, i.e., 26.9% of all cases (14/52). All normal individuals in the control group were unmethylated in their sera (Table 2).

Clinicopathological features of p16 promoter hypermethylation are tabulated in Table 3. There was no association between gender and promoter hypermethylation. Methylation had higher frequency in older patients ($P = 0.051$). Methylation was significantly lower in well-differentiated tumors as compared with higher pathological grades ($P < 0.05$). There was no significant difference in methylation status between intestinal and diffuse types. However, it was observed that 4/5 mixed type tumors were methylated, and all five were negative for p16 protein expression. Methylation was more frequent in non-cardiac type of tumor as compared with cardiac type (68.2% vs 31.8%), without statistical significance. p16 promoter methylation had no correlation with distant metastasis. No association was observed between methylated circulating DNA and lymph node metastasis.

Among environmental factors, we assessed H pylori infection and smoking. Sixty percent of patients (30/50) were infected with H pylori and 17.6% (9/51) were smokers. p16 promoter methylation had no correlation with smoking and H pylori infection.

Immunohistochemical staining of p16

Nuclear p16 immunostaining was positive for protein

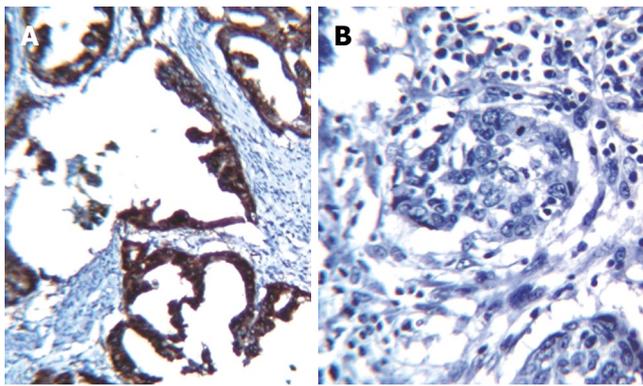


Figure 2 Immunohistochemical staining with monoclonal anti-p16 protein. (A) Nuclear reactivity showed expression of p16 protein (case 48). (B) p16-negative tumor (case 15) failed to stain due to decreased expression of p16 protein.

expression. Positive tumors varied in intensity of nuclear staining, with the proportion of cells ranging from 10% to 95%. In a few positive tumors, we observed marked heterogeneity in different areas with respect to p16 reactivity. Negative staining was observed in 61.5% of patients (32/52). Immunohistochemical staining is depicted in Figure 2. We assessed the correlation between immunohistochemical staining and methylation status (Table 4). Among the p16-negative tumors, 62.5% were methylated, which showed strong correlation between negative immunostaining and promoter region hypermethylation ($P < 0.001$). There was significant correlation with pathological grade and mixed subtype, as well as methylation pattern, but no association was observed with other clinicopathological and demographic features and environmental factors.

DISCUSSION

Several genetic and epigenetic alterations play an important role in gastric carcinogenesis. Tumor suppressor and other tumor-related genes are the main targets. Aberrant alterations of *p16*, as a tumor suppressor gene, are important events in several tumors, including gastric cancer, and hypermethylation of CpG islands in the promoter region is responsible for a great proportion of tumors^[22-25]. In this study, we demonstrated that 44.2% of tissues from gastric adenocarcinoma were methylated. The result was consistent with previous studies by Shim *et al*^[26] and Ding *et al*^[27] who reported 42% and 45% methylation of *p16* promoter in gastric cancer.

Different mechanisms are suggested for decreased p16 protein expression, including homozygote deletion, point mutation and promoter methylation^[28]. The first two mechanisms occur in $< 10\%$ of tumors^[29,30]. We report that 61.5% of gastric adenocarcinomas were negative for p16 protein expression. Among p16-negative tumors, 62.5% were methylated in their promoter region, which was strongly correlated with decreased protein expression. We conclude that the principle mechanism for decreased p16-protein expression in gastric cancer is hypermethylation of the promoter region. There were three patients with normal protein expression despite promoter hypermethylation. All of these exhibited tumor heterogeneity, although

Table 4 Results of methylation analysis in p16-positive and negative cases

Immunohistochemistry	Methylated	Unmethylated
p16-positive	3	17
p16-negative	20	12

partial methylation in one or both alleles might have been another possible reason. There were also twelve p16-negative patients with normal methylation status. Alternative mechanisms such as point mutation or homozygote deletion might be responsible for decreased protein expression.

Promoter hypermethylation was detected from sera of 60.9% of patients with tumor methylation, which accounted for 26.9% of all cases. This was similar to the study performed by Koike *et al*^[31] who reported 27% methylated sera in gastric cancer patients. There are some interesting features that introduce *p16* hypermethylation as a good serum marker for early detection of gastric cancer. As compared with other methods, serum markers are easier to use, less expensive and less invasive. Among serum markers, hypermethylation has some distinct features. It is a frequent event in cancer, while rare or absent in normal tissue, which leads to high specificity for the purpose of tumor diagnosis. Small specific regions of genome are affected, which make it easily detectable, in contrast with mutations. MSP is a sensitive technique that detects free circulating DNA even in small amounts^[32]. Among many genes altered by methylation in gastric cancer, *p16* promoter methylation is an early event in carcinogenesis^[33]. We conclude that *p16* is a good serum marker for early detection of gastric cancer.

In this study we showed that hypermethylation is less frequent in well-differentiated tumors. We conclude that *p16* promoter hypermethylation is associated with tumor malignant behavior. There were no significant differences between intestinal and diffuse types in our population, but mixed subtype was strongly associated with silenced expression. However, there were only five cases with mixed type among our patients, and additional studies with a larger number of cases are needed to confirm the finding. In the population studied, 86.5% were at stage 3 and 4, which showed that gastric cancer was diagnosed in advanced stages, which necessitates early detection. We did not statistically compare patients at different stages, because of unbalanced distribution and the consequent bias. Methylation status in cardiac tumors was also compared with that of non-cardiac tumors. We know that cardiac tumors have different behavior from non-cardiac tumors. Methylation tends to occur more in distal tumors (more than two-fold higher), without statistical significance. We did not find any association between *H pylori* and methylation in our population, despite a few previous studies that suggested *H pylori* induces methylation^[34,35]. Smoking, the other studied environmental factor, had no correlation with promoter hypermethylation. However, our patients consisted of only 17.6% (9/51) smokers, which was not sufficient to draw any strong conclusion. Promoter hypermethylation was not affected by gender, although it tended to occur more often in older patients. Precise

matching and absence of promoter hypermethylation in the control individuals decreased the possibility of methylation induction by aging, unrelated to cancer. No association was observed between circulating methylated DNA and lymph node metastasis. This indicates that the origin of circulating methylated DNA is not from lymph node metastasis. Hypermethylation status in serum was not associated with other clinicopathological features either.

In conclusion, *p16* promoter hypermethylation is an important event in gastric carcinogenesis. It is the principle mechanism of *p16* gene silencing. It is associated with tumor malignant behavior. Detection of *p16* hypermethylation in serum may be a useful biomarker for early detection of gastric cancer. Assessment of other serum markers may increase the sensitivity of screening in future studies. Moreover, promoter hypermethylation will regress after treatment. Further studies will determine whether promoter hypermethylation is a good surveillance marker for patient follow-up after treatment.

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COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide. This is because most cases are diagnosed at an advanced stage, and there is no standard method for early detection. Several genetic and epigenetic alterations play important roles in gastric carcinogenesis. Methylation, the most important epigenetic alteration, influences gene repression without affecting genetic coding. Several recent studies have been conducted to assess the clinical implications of epigenetics in cancer. Free circulating, tumor-derived DNA is a good target for early detection as a serum marker. *p16* is a tumor suppressor gene, which acts as a cell-cycle regulator. It is silenced in early stages of gastric carcinogenesis.

Research frontiers

p16 promoter hypermethylation related to clinicopathological features and early detection of gastric cancer.

Innovations and breakthroughs

A thorough analysis of *p16* promoter hypermethylation, correlations with demographic and clinicopathological features and environmental factors, and implications as an early diagnostic marker were performed for the first time in the Iranian population.

Applications

Although *p16* promoter hypermethylation is very specific for early detection of gastric cancer, further assessment of other genes will be useful to raise the sensitivity and provide a panel of serum markers for the purpose.

Peer review

This was a very interesting study. It examined *p16* promoter hypermethylation in gastric adenocarcinoma and free DNA. Methylation was detected in 44% of tumors, and in 26.9% of serum. *p16* promoter hypermethylation in serum was closely correlated with that in gastric tissue. Methylation was significantly associated with pathological grade.

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