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Promoter hypermethylation of p16 and APC in gastrointestinal cancer patients

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Original Article

ABSTRACT

Background/Aims: Cancer is a consequence of the disruption of cellular regulation. Epigenetic is one of the reasons of this disruption. Epigenetic factors play a role in the carcinogenesis by affecting proto-oncogenes and tumor suppressor genes and it is one of the most popular research areas in recent years. DNA methylation, which is an epigenetic mechanism, occurs in the early stages of tumorigenesis. Promoter methylation which causes the silence of tumor suppressor genes have been studied extensively in various tumor types. The aim of this study was to investigate promoter methylation of certain tumor suppressor genes, *Cyclin-dependent kinase inhibitor 2A (p16)* and *Adenomatous polyposis coli (APC)*, which take part in gastrointestinal tumorigenesis.

Materials and Methods: To detect the promoter methylation of p16 and APC genes, tissue samples from 20 gastrointestinal cancer patients and peripheral blood samples from 15 healthy individuals were collected for Methylation-Specific Polymerase Chain Reaction (MSP) analysis.

Results: According to the statistical analysis, in tumor tissue, positive methylation ratio of *p16* and *APC* genes was found respectively 30% (6/20) and 50% (10/20). The difference of promoter methylation of these genes between tumor tissues and control group was significantly observed (p=0.02 and 0.001, respectively). An alteration of promoter methylation of *APC* gene according to tumor localization was found (p=0.007), but there was no significant difference observed in *p16*.

Conclusion: In our study, promoter methylation which was considered to be occurred as an early event in gastrointestinal carcinogenesis was observed in *p16* and *APC* genes.

Keywords: Promoter hypermethylation, tumor suppressor gene, MSP, gastrointestinal cancer

INTRODUCTION

Tumorigenesis is a multistep process which consists of genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (1). One of these steps is an epigenetic modification which is a cellular process that takes part in tumorigenesis. In addition to the epigenetic modification, global changes in the epigenetic landscape are also hallmarks of cancer.

Epigenetic is heritable changes in gene expression that occur independent of changes in the primary DNA sequence (2-4). DNA methylation, covalent histone modifications, and nucleosome positioning and histone variants are epigenetic mechanisms that have an effect on the gene expression (4). Genetic studies showed that the DNA methylation is required for embryonic development, genomic imprinting and X-chromosome inactivation, and alterations in this epigenetic mark also cause many human diseases, including cancer (5). In a cancer cell, the DNA methylation pattern is a combination of an overall decrease in the level of 5-methylcytosine (hypomethylation) and regional hypermethylation particularly CpG islands is generally disrupted (6). The hypermethylation of DNA is a post-replication modification and it is predominantly found in cytosines of the dinucleotide CpG that is presented throughout the genome at small regions named CpG islands (6). CpG is-

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lands are generally found at the upstream region of genes that is called promoter region and any changes in this area might affect the gene expression. In other words, hypermethylation of genes in a cell may silence the function of related gene (7). It was reported that there is a strong correlation between gene expression and methylation; however, only methylation in the promoter region is associated with gene silencing (8). Aberrant DNA hypermethylation of promoter regions occurs before a bulk of tumor is seen through the proliferation of a single cell (9). Thus, identification of promoter hypermethlaytion may be useful to diagnose the disease at early stages.

Gastrointestinal cancer is one of the most common form of cancer and one of the most important causes of cancer-related death in our nation like the Western world. There is a myriad of reasons that cause gastrointestinal cancer. Moreover, gastrointestinal cancer is a complicated and heterogeneous disease in which genomic instability and DNA promoter methylation play important roles (10). The hypermethylation of CpG islands within the promoter and/or upstream exon regions is an important epigenetic mechanism underlying the inactivation of tumor suppressor genes in this type of cancer (11). It was reported that many tumor suppressor genes, such as *Ras association domain family 1A* (*RASSF1A*), *Cyclin-dependent kinase inhibitor 2A* (*p16)*, *Adenomatous polyposis coli* (*APC)* are epigenetically silenced by aberrant promoter hypermethylation in gastric and colorectal cancer (11-15).

Adenomatous polyposis coli plays an important role in many cellular processes which determine whether a cell may develop into a tumor and *p16* takes part in regulating the cell cycle. Therefore, mutations in these two genes may result in cancer.

In this study, the methylation status of the *p16* and *APC* genes were examined in 20 tumor tissues derived from patients with gastrointestinal cancer and 15 healthy individuals by using Methylation-Specific Polymerase Chain Reaction (MSP), and the correlation between the methylation status and the clinicopathological findings was evaluated.

MATERIALS AND METHODS

Peripheral blood samples and gastrointestinal biopsy specimens of 20 gastrointestinal cancer patients and only peripheral blood samples of 15 healthy individuals were collected from the Department of Gastroenterology, Gülhane Military Medical Academy between 2008 and 2010. All tissue and peripheral blood samples were carried on ice while they were brought to the laboratory and all of them were processed immediately. It was found that all of those gastrointestinal cancer patients have no evidence of other diseases. Clinicopathologic data were available for most of the 20 gastrointestinal cancer patients; for some patients we were missing information on age at surgery (n=1) and tumor stage (n=1). The localizations of tumors are as follows: nine gastric, ten colorectal, and one esophagus tumors. Tumor stages and pathologic features of primary tumors were defined according to the criteria of the American Joint Committee of Cancer. The distribution of tumor stages were as follows: two cases were stage II, nine cases were stage III, and eight cases were stage IV. The study protocol was in adherence to the tenets of the Declaration of Helsinki and the procedure was done according to the ethics committee approval. Informed consent was obtained from all patients and healthy individuals after giving the explanation on the nature and possible consequences of the study.

Genomic DNA was isolated from peripheral blood and biopsy specimens using the phenol-chloroform extraction method and was stored at -20°C. DNA samples were measured at 260 nm with spectrophotometry (Quawell UV-Vis Spectrophotometer Q5000; Quawell Technology, California, USA) and bisulfite modification reaction was conducted by using 1 µg genomic DNA. All of the DNA samples were modified with the reagents provided in the Epigentek BisulFlash™ DNA Modification Kit (Epigentek, New York, USA) and the modification reaction lasted for approximately 25 minutes. After the bisulfite treatment, converted DNA samples were stored at -20°Cand could be used up to four weeks. The modified DNA samples were then subjected to MSP using with methylation-specific primers of the *p16* and *APC* genes. We used methylated primers (M) to amplify methylated regions and unmethylated primers (U) to amplify unmethylated regions. In addition, we also used CpGenome™ Universal Methylated DNA (Millipore, California, USA) as a positive control. Each PCR reaction mixture contained 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM TrisHCl pH 8.8 , 0.1% Tween-20, 2,5 mM $MgCl_2$), 0.1 mM dNTP, 0.4 pmol sense and antisense primers for target genes and 1u Taq DNA polymerase (Bioron, Ludwigshafen, Germany) in a total volume of 25 µl. The primer sequences, PCR conditions, and product sizes are given in Table 1. After PCR amplification, the products were electrophoresed on 3% agarose gel (Sigma-Aldrich, Steinheim, Germany) and visualized under ultraviolet light after ethidium bromide staining.

Statistical analyses were done using SPSS 15.0 for Windows Evaluation Version (SPSS Inc.; Chicago, IL, USA). MSP results were compared between the subject and control group by the χ^2 test, $p<0.05$ was considered as statistically significant.

RESULTS

Tumor tissues of 20 patients who were referred to the Clinic of the Department of Gastroenterology, Gulhane Military Medical Academy and 15 healthy individuals were examined for promoter methylation of *p16* and *APC* by using MSP.

Aberrant methylation of *p16* and *APC* were detected in 6 out of 20 (30%) patients and 10 out of 20 (50%) patients, respectively (Figure 1).

Among the patients and healthy individuals, the difference between the tumor stage and methylation of *p16* and *APC* genes were found insignificant.

Table 1. Primer sequences, PCR conditions and size of the PCR products

Primer	Sequence of 5'-3'	PCR conditions	PCR products
p16-U-F p16-U-R	TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	94°C 25s/64°C 25s/72°C 25s/30 cycle	151 bp
p16-M-F p16-M-R	TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA	94°C 25s/60°C 25s/72°C 25s/30 cycle	150 bp
APC-U-F APC-U-R	GTGTTTTATTGTGGAGTGTGGGTT CCAATCAACAAACTCCCAACAA	94°C 25s/62°C 25s/72°C 25s/30 cycle	108 bp
APC-M-F APC-M-R	TATTGCGGAGTGCGGGTC TCGACGAACTCCCGACGA	94°C 25s/58°C 25s/72°C 25s/30 cycle	104 bp

p16-U-F: The forward primer sequence of the unmethylated sequence of p16 gene; p16-U-R: p16-U-F: The forward primer sequence of the unmethylated sequence of p16 gene; p16-U-R: The reverse primer sequence of the unmethylated sequence of the p16 gene; p16-M-F: The forward primer sequence of the methylated sequence of p16 gene; p16-M-R: The reverse primer sequence of the methylated sequence of p16 gene; APC-U-F: The forward primer sequence of the unmethylated sequence of APC gene; APC-U-R: The reverse primer sequence of the unmethylated sequence of APC gene; APC-M-F: The forward primer sequence of the methylated sequence of APC gene; APC-M-R: The reverse primer sequence of the methylated sequence of APC gene; bp: Base pair

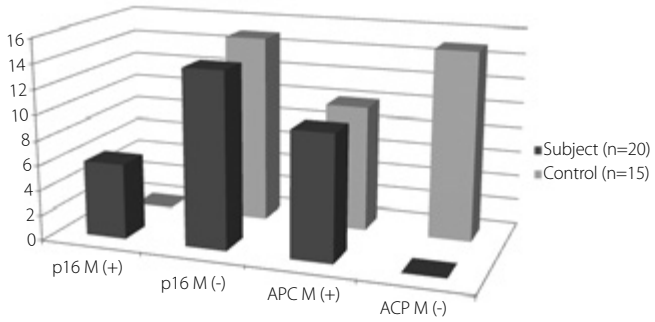

Figure 1. Promoter hypermethylation of p16 and APC genes in gastrointestinal cancer patients and healthy individuals.

We also found that the relation of *p16* methylation and ages of patients was significant (p=0.025); on the other hand, no significance was found for the *APC* gene (Table 2). Methylation of *p16* was seen more in patients who were 60 years and below than patients who were above 60 years.

Methylated and unmethylated status of *p16*, *APC*, and *p* values in different tumor types and stages and also in different age groups are seen in Table 2.

DISCUSSION

Gastrointestinal cancer, which is seen in most countries with a high incidence, is one of the most fatal type of cancer; therefore, it may be important to determine the genetic alterations, such as epigenetic modifications before the surgery as a new parameter to estimate the malignancy of the cancer (15).

In this study, we aimed to show that promoter hypermethylation of tumor suppressor genes correlates with the carcinogenesis by using MSP technique. In our technique, we used Epigentek BisulFlash™ DNA Modification Kit instead of manual method. Before the invention of these new modification kits, bisulfite modification was lasting for nearly more than 16 hours. Both the long reaction time and the labor of this modification make this process hard. Thus, we used kit for modification and there are many studies which use these modification kits for their studies (16-28).

We used gastrointestinal cancer patients as a subject group and we examined the tumor suppressor genes, such as *p16* and *APC*. Among the subject group, we found that 30% of them have methylated *p16* and 50% of them have methylated *APC*. In the control group, methylated *p16* or *APC* were not seen. We can say that methylation of *p16* and *APC* correlates with tumorigenesis (p=0.02 and 0.001, respectively). In the present study, we were supposed to find out if most of the gastrointestinal cancer patients have methylated *p16* promoter because there are many researches which support this idea and our findings also correlate with other researches. Kim et al. (29) suggested that methylation of *p16* causes low expression level of *p16* and that situation may contribute to tumor enlargement. According to the clinicopathological analysis, *p16* metyhlation in serum of the patients with colorectal cancer was related with later Dukes' stage (17). In another study, it is claimed that *p16* gene silencing contributes to the changes in the epigenetic landscape which are widespread in neoplasia (30). In addition, the rate of hypermethylation of *APC* is found to be high in many studies. Cho et al. (31) observed that while 52.5% of breast cancer patients have hypermethylated *APC* gene, there is no *APC* hypermethylation in the control group. It was found that the rate of methylated *APC* gene was 82.5% (33/40) in a study which was about gastric cancer patients (32). Hypermethylation of *APC* promoter, like the mutations in the coding region of *APC*, appears with the same frequency in colon neoplasia (33). Our results which resemble other studies indicate that *p16* or *APC* hypermethylation is an important epigenetic modification which contributes to the tumor enlargement.

Moreover, we studied the unmethylated promoter regions for these genes and we observed that 90% of the patients have unmethylated *p16* and 30% of them have unmethylated *APC*. Similar results were also obtained by the study of

Table 2. Promoter hypermethylation in gastrointestinal tumors and healthy subjects

	p16 M				APC M			
	+	-	Total	p	+	-	Total	p
Subject (n=20)	6 (30.0%)	14 (70.0%)	20 (100.0%)		10 (50.0%)	10 (50.0%)	20 (100.0%)	
Control (n=15)	0 (0.0%)	15 (100.0%)	15 (100.0%)	0.02	0 (0.0%)	15 (100.0%)	15 (100.0%)	0.001
Location of Tumor								
gastric (n=9)	3 (33.3%)	6 (66.7%)	9 (100.0%)		8 (88.9%)	1 (11.1%)	9 (100.0%)	
colorectal (n=10)	3 (30.3%)	7 (70.0%)	10 (100.0%)		2 (20.0%)	8 (80.0%)	10 (100.0%)	
esophagus (n=1)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0.788	0 (0.0%)	1 (100.0%)	1 (100.0%)	0.007
Total	6 (30.0%)	14 (70.0%)	20 (100.0%)		10 (50.0%)	10 (50.0%)	20 (100.0%)	
Tumor Stage								
II and III	4 (36.4%)	7 (63.6%)	11 (100.0%)		6 (54.5%)	5 (45.5%)	11 (100.0%)	
IV	2 (25.0%)	6 (75.0%)	8 (100.0%)	0.599	4 (50.0%)	4 (50.0%)	8 (100.0%)	0.845
Total	6 (31.6%)	13 (68.4%)	19 (100.0%)		10 (52.6%)	9 (47.4%)	19 (100.0%)	
Age								
>60	2 (15.4%)	11 (84.6%)	13 (100.0%)		6 (46.2%)	7 (53.8%)	13 (100.0%)	
≤60	4 (66.7%)	2 (33.3%)	6 (100.0%)	0.025	4 (66.7%)	2 (33.3%)	6 (100.0%)	0.405
Total	6 (31.6%)	13 (68.4%)	19 (100.0%)		10 (52.6%)	9 (47.4%)	19 (100.0%)	

APC: adenomatous polyposis coli

Virmani et al. (34). They claimed that non-malignant cells are always found in tumor specimens. That is why unmethylated sequences are seen in methylated tumor samples (34). Tumor tissue is a complicated structure and has many cells which have different genotype. In other words, through the tumorigenesis, many different genetic alterations affect tumor cells in a tumor tissue and this tissue is a complex of many different cells. Therefore, in a tumor tissue, while some cells may have methylated *p16*, some of them may have unmethylated *p16* because of the complicated structure of tumor which is the sum of genetically different cells. Thus, it can be seen in both methylated and unmethylated types of genes in a tumor tissue (35).

Hypermethylation of tumor suppressor genes causes to silence the expression of the related gene and this process increases with higher stages of tumor. During the tumorigenesis, both the hypermethylation of genes and tumor enlargement develop by affecting each other. Methylation status of tumor suppressor genes has a significant correlation with tumor stage (36). However, we observed that there was no significant relationship between the tumor stage and methylation status of *p16* and *APC* genes. Thus, we think that low number of our subject and control group may affect our results in a negative way.

The relationship between the localization of tumor and the hypermethylation of *APC* gene was found significant (p=0.007) but we did not found any significance for p16 methylation. These genes are important tumor suppressors which take part in the cell cycle; therefore, any changes which occurs in these genes, affects the cell's normal route. Among these two genes, there are many studies which showed that *APC* is methylated more frequently in gastric carcinomas than other carcinomas (37,38). According to our results; methylation of *APC* is seen in gastric tumors than in colon tumors. The results of the study of Esteller et al. (39) also observed that the frequency of promoter hypermethylation of *APC* gene was 18% in colon tumors and 34% in stomach tumors.

DNA methylation is an important mechanism which regulates gene expression and it is affected by aging (40). Recent genome-wide studies showed that DNA methylation in aging and cancer has a strong relationship (41). According to the study of Waki et al. (42), DNA methylation is an age-related phenomenon and Loyo et al. (43) showed that methylation of *APC* increased with age. The probable cause of this is not only the genetic background of people but also the accumulation of cancer inducers in the cells (44-46). It was previously reported that *RASSF1A* methylation increases the breast cancer risk and methylation of this gene increased between ages 32 and 55 (47). In our study, although there was no significant relationship between the age of patients and *APC* methylation, we found a significant relationship between age and *p16* methylation. However, our results contradict with the idea that cancer increases with age. According to our findings, patients whose age were 60 and below shown methylated *p16* more than patients whose age were above 60. We think that our results might be affected by the low number of subjects.

In conclusion, the aim of this study was to investigate the relationship between the gastrointestinal cancer and promoter methylation of *p16* and *APC* genes. We analyzed methylation of *p16* and *APC* with MSP and in the light of our results we can say that gastrointestinal tumor tissue has a significant relationship with the methylation of these genes. Of course, there are many reasons for the cause of gastrointestinal cancer, and methylation of the tumor suppressor genes is one of them. However, during the last decade, researches have attached more importance to this issue and early diagnosis of prospective cancer cells via the methylation profile of tumor suppressor genes may be effective in treatment. Therefore, identifying the relationship between the methylation and cancer will be important for the future treatment strategies and our results showed that promoter methylation of *p16* and *APC* has a significant correlation with gastrointestinal cancer tissues and these findings will enlighten our future studies.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gülhane Military Medical Academy.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.M.; B.E.; S.B.; Design - H.M.; Supervision - H.M.; Resource - H.M.; Materials - Z.P.; Data Collection&/or Processing - B.E.; S.K.; Analysis&/or Interpretation - B.E.; S.K.; H.M.; Literature Search - B.E.; S.K.; E.S.; Writing - B.E.; S.K.; E.S.; Critical Reviews - H.M.

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