BRIEF ARTICLE

Aberrant promoter methylation of *p16* in colorectal adenocarcinoma in North Indian patients

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Abstract

AIM: To investigate *p16* gene methylation and its expression in 30 patients with sporadic colorectal adenocarcinoma in a North Indian population.

METHODS: Methylation specific polymerase chain reaction was used to detect *p16* gene methylation and immunohistochemistry was used to study the p16 expression in 30 sporadic colorectal tumors as well as adjoining and normal tissue specimens.

RESULTS: Aberrant promoter methylation of *p16* gene was detected in 12 (40%) tumor specimens, whereas no promoter methylation was observed in adjoining and normal tissue. Immunohistochemistry showed expression of p16 protein in 26 (86.6%) colorectal tumors whereas complete loss of expression was seen in 4 (13.3%) and reduced expression was observed in 12 (40%) tumors. In the adjoining mucosa, expression of p16 was in 11 (36.6%) whereas no clear positivity for p16 protein was seen in normal tissue. There was a significant difference in the expression of p16 protein in tumor tissue and adjoining mucosa ($P < 0.001$). The methylation of the *p16* gene had a significant effect on the expression of p16 protein ($P = 0.021$). There was a significant association of methylation of *p16* gene with the tumor size ($P = 0.015$) and of the loss/reduced expression of p16 protein with the proximal site of the tumor ($P = 0.047$). Promoter methylation and expression of p16 had no relation with the survival of the patients ($P > 0.05$).

CONCLUSION: Our study demonstrated that promoter hypermethylation of the *p16* gene results in loss/reduced expression of p16 protein and this loss/reduced expression may contribute to tumor enlargement.

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the Western world[1]. The incidence of CRC is very dynamic worldwide and many Asian countries have experienced a two- to four-fold increase in the incidence of CRC during the past few decades[2]. Adopting a Western-style diet high in fat and meat protein and low in fiber, vegetables, and fruit and a more sedentary lifestyle are believed to be the reasons underlying the increase. However, the interaction between these factors and the genetic characteristics of Asian populations might also have a pivotal role. CRC is one of the best-studied systems of multistage human carcinogenesis. The well-described model of colorectal carcinogenesis envisions a stepwise accumulation of early and late genetic alterations in the progression of adenoma to carcinoma[3]. It is becoming increasingly apparent that cancer arises through a series of not only genetic but also epigenetic alterations. The epigenetic mechanisms play a prominent role in CRC. Aberrant DNA methylation has long been noted in colorectal carcinogenesis; imbalances in methylation are thought to occur early in the process and are characterized by genome-wide hypomethylation and regional and locus-specific hypermethylation[4]. Among the common targets for aberrant DNA methylation is the *p16* gene[5,6]. *p16* is a gene which seems to play a major role in colorectal carcinogenesis. It is a tumor suppressor gene which is also known as *MTS-1, INK4a, CDKN2A*, and is composed of three exons, which encode 156 amino acids[7]. This gene is located on chromosome 9p21 and encodes a G1 cyclin-dependent kinase (CDK) inhibitor that can interrupt the tumor cycle by acting as a tumor suppressor gene and induces G1 cell cycle arrest[8,9]. Inactivation of the *p16* gene is now recognized as the second most common molecular defect in human cancer preferentially through *de novo* methylation of its 5'-promoter associated CpG Island[10,11].

In the present study, we looked the occurrence of *p16* gene promoter methylation and expression. We also explored the effect of *p16* gene methylation on protein expression in colorectal adenocarcinoma patients from India, in the cancer tissue, adjoining tissue and normal mucosa. The relationship of *p16* gene methylation and expression with clinicopathological parameters was also studied. To the best of our knowledge this is the first study in Indian population. The prevalence of adenoma is quite low in Indians and this raises a question whether the Indian population follows the same pattern of genetic alterations as in the west. Epigenetic alterations that suppress gene expression are a reversible phenomenon. So a detailed understanding of these alterations will provide insight into possible preventive strategies for colorectal carcinogenesis.

MATERIALS AND METHODS

Sample collection

Between July 2004 and July 2006, 30 patients meeting the selection criteria and diagnosed with colorectal adenocarcinoma undergoing surgery at the Postgraduate Institute of Medical Education and Research, India were prospectively included in this study designed to examine the promoter methylation and expression of the *p16* gene in normal colonic mucosa, adjoining and tumor tissues. Only patients undergoing resectional surgery were included. Patients who had history of prior chemotherapy or radiotherapy, with inoperable tumors, family history of colorectal adenocarcinoma, or those with mucinous/signet cell carcinoma were excluded from the study. Follow-up end point was September, 2007. A written informed consent was obtained from each patient for inclusion in this study, which was carried out after obtaining a formal approval from the Institute Ethics Committee. Apart from the description of the gross features of the tumor at the time of surgery, the rest of the colon was examined for any synchronous polyp or tumor. Fresh samples from tumor, adjoining (2-5 cm from the tumor) tissue and distant mucosa (5-10 cm from the main tumor mass) were taken from the resected colorectal specimen.

For histopathological analysis, freshly removed tissue samples were immediately fixed in 10% buffered formalin for 24 h, embedded in paraffin and histopathological assessment was carried out to determine the tumor grade and invasion. Fresh tissues were snap frozen within 10-15 min of surgical removal and stored at -80°C until further use. Each tissue for the molecular analysis was also assessed histologically by making a crushed smear to verify the presence of tumors and only those samples, which contained > 90% of tumor cells were included for the final analysis. Similarly, the presence of adjoining and normal colorectal mucosa was also confirmed histologically before subjecting the tissue for further analysis. The normal mucosa was used as a control in each case.

Methylation analysis of the p16 gene promoter

All the specimens obtained during surgery procedure were treated with proteinase K and RNase. Each specimen was then subjected to DNA extraction using standard phenol-chloroform procedures[12]. The methylation status of 5' CpG islands of *p16* gene was assessed by bisulfite modification of DNA and methylation specific polymerase chain reaction (MSP) according to the method of Herman *et al*[13].

Bisulfite modification

Briefly, DNA (1 μg) in a volume of 50 μL was denatured by 0.2 mol/L NaOH for 10' at 37°C, then 30 μL of 10 mmol/L hydroquinone (Sigma, St. Louis, USA) & 520 μL of 3 mol/L Na bisulfite, pH 5.0 (Sigma, St. Louis, USA), both freshly prepared, were added to each sample. These were then incubated at 50°C for 16 h. Modified DNA was purified using the Wizard DNA Purification Kit (Promega, Madison, WI, USA). Modification was completed by 0.3 mol/L NaOH treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in distilled water and stored at -20°C.

Methylation-specific polymerase chain reaction

The bisulfite modified DNA was polymerase chain reaction (PCR) amplified by using primers specific for methylated

CpG and unmethylated regions of the *p16* gene promoter. The PCR mixture contained 1 × PCR buffer (16.6 mmol/L ammonium sulphate / 67 mmol/L Tris, pH 8.8 / 6.7 mmol/L $MgCl_2$ / 10 mmol/L 2-mercaptoethanol), dNTPs (each at 2 mmol/L), 10 pmol of each primer (*p16* unmethylated sense: 5'GGTAGTTAGGAAGGTTGTATTGT3', *p16* unmethylated antisense: 5'TCCCTACTCCCAAC-CACA3', *p16* methylated sense: 5'TTGGTAGTTAG-GAAGGTTGTATCGC3', *p16* methylated antisense: 5' TCCCTACTCCCAACCGCG3') and bisulfite treated DNA (approximately 50 ng) or unmodified DNA (50-100 ng) in a final volume of 25 μL. PCR specific for unmodified DNA also included 5% DMSO. Reactions were hot started at 95℃ for 5 min before the addition of 1.5 units of Taq DNA polymerase (Roche, GmbH, Germany). PCR amplification of the modified DNA samples consisted of 1 cycle of 95℃ for 5 min; 40 cycles of 95℃ for 30 s, 64℃ for 1 min, and 72℃ for 1 min; and 1 cycle of 72℃ for 5 min. PCR products amplified by unmethylated and methylated primers were 124 bp and 126 bp respectively.

Each PCR product was loaded onto a 2% Agarose gel, stained with ethidium bromide and visualized under UV illumination. DNA treated *in vitro* with CpG methylase MSssI (Sss1 methyltransferase, New England Biolabs, Ipswich, MA, USA) was used as a positive control for methylated alleles of this gene. Controls without DNA were performed for each of PCR. Each MSP was repeated at least three times.

Immunohistochemistry

Immunohistochemical analysis was performed according to the avidin/biotin complex method using the Novocastra concentrated peroxidase detection system (Novocastra, Newcastle, UK). The specimens were fixed with 10% formalin, embedded in paraffin, cut into sections 5-μm in thickness and mounted on slides coated with poly-L-lysine. The sections were deparaffinized by heating at 60℃ for 30 min, treated with xylenes, and dehydrated in alcohol. Endogenous peroxidase was blocked with 0.03% H_2O_2 in methanol for 20 min. The antigenic sites were unmasked by means of pressure cooker treatment for 15 min in 10 mmol/L Citrate buffer (pH 6.0). The sections were then incubated with p16 primary antibody (BD Biosciences, CA, USA) at 1:50 dilution for 3 h at room temperature. Sections were further incubated with biotinylated universal secondary antibody (1:40 dilution) for 20 min followed by incubation with strept avidin horseradish peroxidase (1:40 dilution) for 20 min at room temperature. The slides were developed using 3-3' diaminobenzidine as the chromogen and counterstained with hematoxylin followed by mounting with DPX. The negative control in each case was served by omission of primary antibody. Inflammatory cells and reactive stromal cells served as positive internal controls for p16 staining

The immunohistochemistry results were scored by taking percentage positivity and intensity of staining into account. An intensity score of 0: no staining, 1: weak positivity, 2: moderate positivity and 3: strong positivity was given.

Statistical analysis

Data were analyzed using statistical analysis software. Pearson's χ^2 was performed to analyze the relationship between *p16* methylation status and expression and with each of the clinicopathological parameters. Mann-Whitney *U* test was performed to determine the relationship between the expression of p16 and each of the clinicopathological parameters. The overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan-Meier method and the Log rank test was used to evaluate the difference between survival of the patients with and without methylation and expression of the *p16* gene.

RESULTS

Clinicopathological features

There were 30 patients (20 males) with age range from 24-90 years (median age 56 years). All the patients were symptomatic at the time of diagnosis. Presentation include abdominal pain (*n* = 18), change in bowel habits (*n* = 17), rectal bleeding (*n* = 15), and loss of appetite (*n* = 12). The other signs and symptoms were subjective weight loss (*n* = 14), abdominal mass (*n* = 6), vomiting or abdominal distention (*n* = 4), anemia (*n* = 5).

The distribution of tumor was 10% (*n* = 3) in cecum, 16.6% (*n* = 5) in ascending colon, 3.3% (*n* = 1) in transverse colon, 3.3% (*n* = 1) in hepatic flexure, 6.6% (*n* = 2) in splenic flexure, 10% (*n* = 3) in descending colon, 33.3% (*n* = 10) in sigmoid colon, and 16.6% (*n* = 5) in the rectum. Thus 18 (60%) patients had the tumor in the distal colon, and 12 (40%) in the proximal colon. None of the patients had a synchronous adenoma or carcinoma. The median length of the tumors was 5 cm (range 2-10 cm). According to the classification of International Union Against Cancer[14], there were 4 patients (13.3%) in stage Ⅰ, 18 (60%) in stage Ⅱ, 6 (20%) in stage Ⅲ and 2 (6.6%) in stage Ⅳ disease. Metastasis was found in 8 patients with distant metastasis in liver (*n* = 2) and lymph nodes (*n* = 6). None of the patients had a family history of CRC or any other kind of malignancy. All tumors were adenocarcinomas and on histological examination 6 were well differentiated, 21 moderately differentiated and 3 poorly differentiated adenocarcinomas.

Methylation analysis of p16 promoter in surgical specimens

Methylation status of the *p16* gene promoter was evaluated in colorectal tumors, adjoining and normal mucosa using MSP. All the surgical specimens showed the presence of a band of unmethylated DNA that could be derived from unmethylated DNA of normal, adjoining mucosal cells and tumor cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts and inflammatory cells. A band of methylated DNA was found in 12 (40%) tumors whereas no methylation was observed in adjoining and normal mucosa (Figure 1).

Expression of p16 protein in surgical specimens

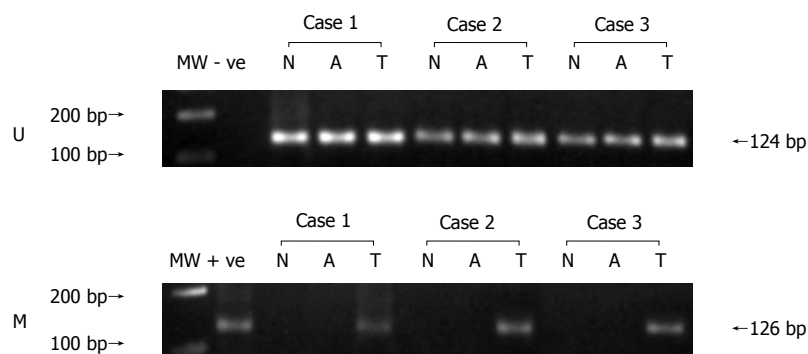Immunohistochemistry was performed to evaluate pos-

Figure 1 Methylation specific polymerase chain reaction for *p16* gene in colorectal tumor tissue, adjoining and normal mucosa. U: Polymerase chain reaction (PCR) with primers for unmethylated *p16*; M: PCR with primers for methylated *p16*; MW: Molecular weight marker; - ve: negative control (without template); + ve: Normal lymphocyte DNA completely methylated with CpG methylase (M.sss1) used as a positive control for methylation; N: Normal mucosa; A: Adjoining mucosa; T: tumor tissue.

sible differences in the expression of p16 protein in tumor tissues, adjoining and normal mucosa. No clear positivity of p16 protein was seen in majority of normal mucosa samples except for a few samples (13.3%), which showed surface nuclear positivity for p16 protein. In contrast, p16 was abundantly expressed in the majority of adjoining mucosa and CRC tissues and was localized both in the nuclei and cytoplasm. In adjoining mucosa 11 (36.6%) samples showed expression of p16 protein. The p16 expression was weak in 6 (20%) and moderate in 5 (16.6%) samples. In the case of tumors, p16 was expressed in 26 (86.6%) samples whereas complete loss of expression was observed in 4 (13.3%). The expression was reduced or weak in 12 (40%), moderate in 10 (33.3%) and strong in 4 (13.3%) tumors (Figure 2A-D). There was a significant difference between the expression of p16 in tumor and adjoining mucosa (131.12 ± 91.55 *vs* 42.80 ± 57.21, $P = 0.001$).

Effect of gene methylation on protein expression
The *p16* gene methylation was compared to the p16 protein expression and the results are summarized in Table 1, showing actual staining intensities of each specimen. The staining intensities were categorized into 3 groups 0-100, 100-200 and 200-300 intensity score. Out of the 26 colorectal adenocarcinoma samples positive for p16 protein expression, 8 had methylation in the promoter region. In the 8 methylated samples, 6 had reduced expression and 2 had moderate expression. However, 4 tumors totally negative for p16 expression also had methylation in their promoter region. There was a significant correlation of methylation of *p16* gene with the loss/reduced expression (the samples with staining intensity between 0-100) of p16 protein ($P = 0.021$, Table 2).

Relationship of p16 methylation and expression with clinicopathological parameters
The relationship between the methylation and level of staining of p16 in all the surgical specimens and several clinicopathological parameters was examined. These parameters included gender, tumor size, tumor site, histological grade, lymph node metastasis, distant metastasis, TNM

staging, age, pre and post-operative serum CEA levels *etc*. A significant correlation was observed between methylation of *p16* gene and the size of the tumor ($P = 0.015$) and of the nuclear positivity of p16 protein with the proximal site of the tumors ($P = 0.047$). There was no correlation between *p16* gene methylation and protein expression with other clinicopathological parameters.

Survival analysis
Follow up data were available for 29 patients. At last follow-up (September, 2007), four patients (13.3%) had died, 4 patients (13.3%) showed the recurrence of disease with distant metastasis in 2 patients (6.6%) and 21 patients (70%) were alive without disease. The association of methylation and expression of p16 with DFS and OS was analyzed. DFS was defined as the time from the date of surgical resection of the tumor to the date of recurrence of the disease and OS was defined as the time from the date of diagnosis of CRC to the date of last follow up. The median follow-up period was 27 mo with a range of 8-39 mo (mean=25.5 ± 8.14 mo). By Kaplan-Meier log-rank survival analysis, survival of the patients with colorectal adenocarcinomas was not associated with the methylation and expression of p16 ($P > 0.05$; Figure 3).

DISCUSSION
CRC is a major cause of cancer death worldwide. The usual treatment is surgery and subsequent chemotherapy and radiotherapy. Many Asian countries have experienced two- to four-fold increase in the incidence of CRC during the past few decades. However, there are no such data from India. So it is important to determine genetic alterations in this cancer as an approach to predicting the malignancy of disease in the Indian population. Development of CRC is a multistep process which includes the involvement of various oncogenes and tumor suppressor genes. Several tumor suppressor genes contain CpG islands in their promoters, a fact which has prompted many workers to investigate the role of methylation in silencing these genes. DNA is methylated only at cytosine located 5' to guanosine in the
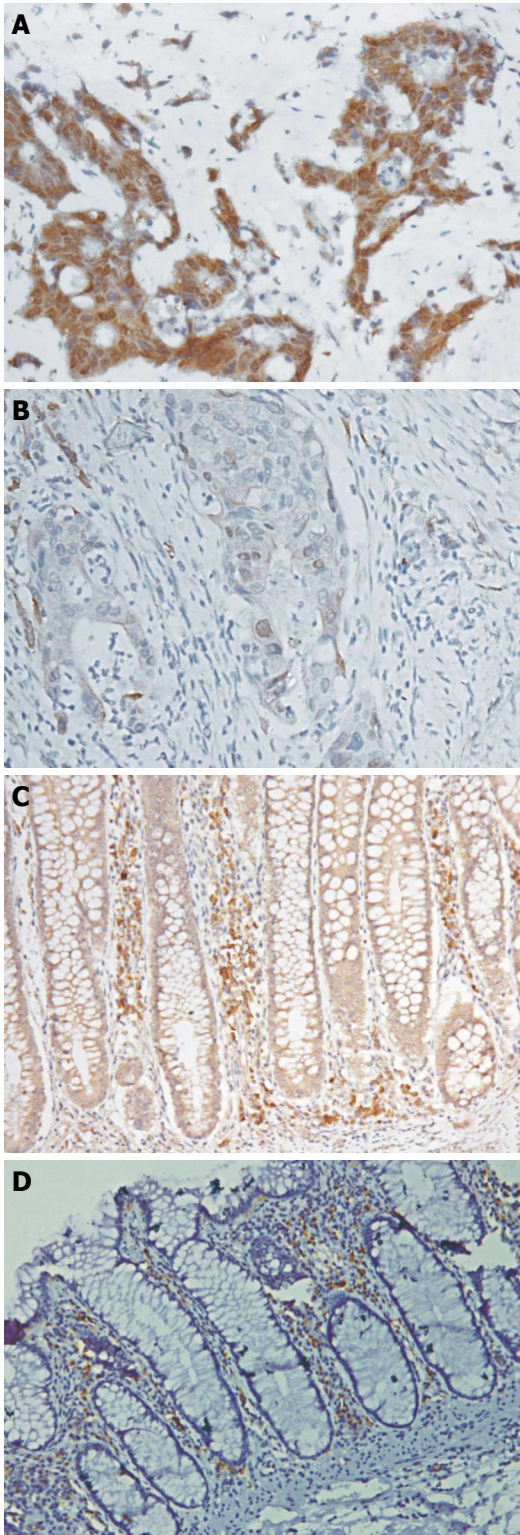
Figure 2 Immunohistochemical staining showing expression of p16 protein.
A: Tumor showing strong nuclear and cytoplasmic positivity of p16; B: Tumor showing reduced cytoplasmic positivity for p16; C: Adjoining mucosa showing moderate cytoplasmic positivity for p16; D: Normal colorectal mucosa showing no positivity for p16 (SABP immunostaining; × 450).

Figure 3 Survival curves of patients with colorectal adenocarcinoma showing correlation. A: *p16* methylation status with probability of disease-free survival (DFS); B: *p16* methylation status with probability of overall survival (OS); C: p16 protein expression with probability of DFS; D: p16 protein expression with probability of OS.

CpG dinucleotide. This modification has important regulatory effects on gene expression *via* blocking transcriptional activation[15]. *p16* is a tumor suppressor gene which plays an important role in the transition of cells through G1 to S phase of the cell cycle by binding to CDK 4 and inhibiting its binding to cyclin D1[16-18]. Methylation of cytosine residues at CpG sites in the *p16* gene promoter, resulting in silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas of varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma[19-32]. The rates of homozygous dele-

Table 1 Comparison of p16 expression to the *p16* gene methylation

Sample No.	Tumor		Adjoining		Normal	
	p16 protein expression	*p16* methylation	p16 protein expression	*p16* methylation	p16 protein expression	*p16* methylation
S-1	90	+	50	-	0	-
S-2	0	+	0	-	0	-
S-3	235	-	0	-	0	-
S-4	100	+	0	-	0	-
S-5	50	+	0	-	0	-
S-6	200	+	100	-	0	-
S-7	150	-	0	-	60	-
S-8	100	-	0	-	0	-
S-9	180	+	0	-	0	-
S-10	225	-	120	-	0	-
S-11	200	-	0	-	0	-
S-12	80	-	0	-	0	-
S-13	70	+	0	-	0	-
S-14	300	-	100	-	0	-
S-15	115	-	-	-	0	-
S-16	90	-	40	-	0	-
S-17	50	+	40	-	0	-
S-18	0	+	0	-	0	-
S-19	0	+	0	-	0	-
S-20	50	+	0	-	30	-
S-21	200	-	0	-	0	-
S-22	40	-	0	-	50	-
S-23	110	-	0	-	0	-
S-24	200	-	120	-	0	-
S-25	40	-	0	-	0	-
S-26	0	+	0	-	0	-
S-27	60	-	30	-	0	-
S-28	180	-	100	-	0	-
S-29	300	-	180	-	70	-
S-30	120	-	100	-	0	-

tion and intragenic mutation of the *p16* gene in CRCs are very low. Therefore, in the present study, methylation of 5' CpG islands of the *p16* gene in tumors, adjoining and normal colonic mucosa was examined by using the PCR-based methylation assay. The prevalence of *p16* hypermethylation in colorectal tumors, reported in different studies, varies according to the technique used. In the present study MSP was used. The percentage of hypermethylation of the *p16* gene in our study was 40% whereas, it ranged from 32%-37% in European[23,33],19%-36% in US[34-36] and 29%-42% in Asian population[37,38]. The percentage of *p16* methylation in CRC observed in our study fell within the range of those quoted in the literature. A band of unmethylated DNA was visible in each specimen. Similar results were also reported by other investigators[34]. This genetic aberration was found to be significantly correlated with the size of the tumor but not with the other clinicopathological parameters examined. Tumors > 5 cm exhibited *p16* gene methylation more than tumors ≤ 5 cm suggesting that *p16* methylation may contribute to tumor enlargement in CRC.

p16 has been shown to be expressed in colorectal adenocarcinoma in some studies[39-41]. The reported percentage of expression has varied, with a majority of studies showing p16 expression in more than three quarters of CRC. In our study, p16 expression was observed in 86.6% of tumors whereas complete loss of expression was observed in 13.3% and reduced expression in 40% of tumors. These observa-

tions are in agreement with the study of Kim *et al*[42] who have shown that the majority of CRCs (96.4%) over expressed p16 protein. Similarly, Lam *et al*[43] reported p16 expression in 78% of the tumors. A study by Palmqvist *et al*[44] reported that only 18% of CRCs were classified as p16 negative, whereas the majority (82%) exhibited p16 positivity. The expression of p16 was more frequently noted in proximally located tumors ($P = 0.047$). The difference between proximal and distal colorectum in p16 expression could account for their different clinical behavior. The high frequency of p16 expression in tumors and the absence of p16 in non tumor epithelium implies that the *p16* aberration is an important step in the pathogenesis of CRC.

There was a significant correlation between methylation and loss or reduced expression of p16 in this study ($P = 0.021$). *p16* methylated tumors showed either loss of expression or reduced expression. Most studies have suggested that detectable *p16* gene methylation is necessarily linked to the inactivation of p16 protein or transcriptional silencing of *p16* gene[10,45-48]. Coexistence of *p16* gene methylation and p16 expression in the same specimen has also been frequently described[39,48,49] and this might reflect the cell heterogeneity, in which some cells contained showed *p16* gene methylation and loss of p16 expression whereas others expressed or even overexpressed p16 protein. Ohhara *et al*[50] have proposed that activation but not inactivation of the *p16* gene was associated with primary CRC. In the present study, we found that the methylation

Table 2 Comparison of p16 protein expression with *p16* gene methylation

| | | | p16 expression (IHC score) | | | Total |
			0-100	100-200	200-300	
p16 Methylation	- ve	Count	6	8	4	18
		% within p16 expression	33.3%	44.4%	22.2%	100.0%
		Count	10	2	0	12
Total	+ ve[1]	% within p16 expression	83.3%	16.6%	0.0%	100.0%
		Count	16	10	4	30
		% within p16 expression	53.3%	33.3%	13.3%	100.0%

[1]$P = 0.021$, Pearson's χ^2 test.

of the *p16* gene results in loss or reduced expression of p16 protein, but the overall percentage of expression of p16 was high, whereas majority of the samples showed reduced p16 expression. The reduced expression of p16 in some tumors lacking methylation suggested that not only the methylation but some other genetic alterations are responsible. This other genetic alteration could possibility be mutation in the coding region of *p16* gene which could also reduce the expression of p16 protein in tumors lacking methylation. Preliminary observation on the same CRC specimens indicate that 20% of the samples of CRC showed mutation in exons 2 and 3 of the coding region of *p16* gene, although more comprehensive analysis of the site and nature of mutation is required. The overexpression of p16 protein in some of the tumors lacking methylation indicated that the activation, but not the inactivation, of the gene was associated with the overexpression and tumor progression. Taken together these results suggest that *p16* hypermethylation may, at least in part, contribute to reduced expression of p16. The elucidation of the relationship between p16 expression and *p16* gene methylation in primary tumors requires further studies on a large number of samples and this may certainly help us to better understand the role of methylation of tumor suppressor genes in carcinogenesis.

There are not many reports in the literature evaluating the prognostic role of *p16* hypermethylation and expression. Some investigators have shown that hypermethylation of the *p16* gene was associated with advanced tumor stage and shorter survival[37,38]. In our patients no correlation could be established between the methylation and expression of p16 and survival.

All reported studies have assumed the adenoma→carcinoma sequence and reported the presence of genetic alterations in adenomas, a precursor lesion that finally develops to carcinoma. However, only a very small proportion of the Asian patient population has develop adenomas. The present study, therefore included adjoining mucosa in order to determine the initial changes in CRC. No methylation was observed in the adjoining mucosa, suggesting that there were no changes near the tumor region and that the process of tumorigenesis is restricted to a limited area. This is the first study in which we analyzed both the adjoining and normal mucosa to determine the initial changes in CRC.

This is the first study in the Indian population in which *p16* gene has been analyzed at both genetic and expression level in CRC in relation to clinicopathological features and prognosis. The frequency of alterations of this gene in this cohort is similar to others. This shows that despite the rare occurrence of synchronous adenomas in the population studied, the frequency of alterations in this gene is almost same as observed in other studies, suggesting that the process of tumorigenesis is similar overall although there is a difference at the initiation stage. This study is the first one in an Indian population. It was limited to by the size of the cohort and confined to North India. We need more data from the other parts of the country to validate our findings

In conclusion, our study demonstrated that majority of CRC tissues expressed p16 protein and that the low or reduced expression of p16 among CRC tissues, probably caused by hypermethylation, may contribute to tumor enlargement.

ACKNOWLEDGMENTS

COMMENTS

Background

Colorectal cancer (CRC) is a worldwide health problem. The incidence of CRC is very dynamic worldwide and during the past few decades there has been a dramatic increase in the incidence of CRC in Asian countries although the exact cause of this increase is not known. Therefore, this study was planned to investigate the molecular genetic alterations responsible for the development of CRC in the Indian population. *p16* is a cell cycle regulator and inactivation of this gene leads to uncontrolled cell proliferation and growth. The inactivation of this gene is mainly associated with aberrant promoter methylation. Therefore in the present study the authors analyzed the promoter methylation and expression of the *p16* gene in colorectal adenocarcinoma in Indian patients.

Research frontiers

p16 is a major genetic marker in the development of colorectal carcinogenesis. It is well studied in various populations but there are no previous data from Indian population.

Innovations and breakthroughs

p16 gene hypermethylation may at least in part contribute to reduced/loss of expression of p16 protein. This is the first study in an Indian population and for the first time *p16* has been analyzed at both genetic and expression levels in CRC in relation to clinicopathological features and prognosis.

Applications

p16 plays a very important role in the development of colorectal carcinogenesis. Inactivation of the gene due to promoter methylation leads to lost or reduced

gene function. Hypermethylation is a reversible phenomenon. The identification of these genetic markers are implicated in colorectal carcinogenesis at its early stages can be very helpful for treating the patients with CRC.

Peer review

This study examines *p16* promoter methylation using methylation-specific PCR in resected tumors from a cohort of 30 colon cancer patients in North India. *p16* methylation in matched adjacent tumor and normal tissue is also examined. p16 expression was determined by IHC score. This paper is appropriate and well written.

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