The blues of P(16)INK(4a): Aberrant promoter methylation and association with colorectal cancer in the Kashmir valley

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Abstract. Hypermethylation of the promoter region of the *p16INK4a* (*p16*) gene plays a significant role in the development and progression of colorectal cancer (CRC). The aim of the present study was to establish the role of the methylation status of the *p16* gene in 114 CRC cases and to correlate it with the various clinicopathological parameters. Analysis of *p16* promoter methylation was performed by methylation-specific PCR. Forty-eight (42.1%) of the CRC cases were found to be methylated for the *p16* gene in our population. The methylation status was found to be associated with the gender, lymph node status, tumour stage, smoking status and tumour grade of the CRC patients. *p16* plays a pivotal role in tumour development and progression to advanced stages.

Introduction

Colorectal cancer (CRC) is a major cause of mortality and morbidity, and the third most common malignancy in the world (1). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. CRC is the third most common type of cancer in men and the second most common cancer in women worldwide (2). Kashmir has been reported as being a high-incidence area of gastrointestinal (GIT) cancers (3,4). In the Kashmir valley, CRC represents the third most common type of GIT cancer, following esophageal and gastric cancer (5,6).

The tumour-suppressor gene *p16INK4a* codes for a cyclin-dependent kinase inhibitor *p16INK4a*, which acts as a negative regulator of cell growth and proliferation in the G1 phase of the cell cycle (7,8). Functionally, *p16INK4a* has been identified as an inhibitor of cyclin-dependent kinase 4 and 6. As a consequence, *p16INK4a* plays a significant role in the proliferation process of many types of tumour cell growth and apoptosis, and correlates closely with the expression of the proliferating cell nuclear antigen ki67, which is usually viewed as the proliferation marker in intestinal tumours (9,10). Most human colorectal carcinomas show genetic alterations in the *p16INK4a*-cyclin D-pRb pathway (11). The *p16INK4a* gene of the cyclin D/pRb pathway has been found to be inactivated in human malignancies with a frequency second only to p53 (12,13). Mutation, homozygous deletions and hypermethylation of the promoter are major mechanisms of *p16INK4a* inactivation (8,14,15).

Hypermethylation of non-mutated promoter regions is one of the common mechanisms for inactivating tumour-suppressor genes, which leads to stable allele-specific loss of transcription function (16). Such methylations tend to occur at the sites of CpG dinucleotides, which are clustered as so-called CpG islands and are frequently found in promoters of *p16INK4a* and other tumour-suppressor genes (13). In fact, the major mechanism of *p16INK4a* gene inactivation has been found to be promoter methylation (11). A number of studies have been carried out on *p16INK4a* in different populations, implicating the role of hypermethylation in the development of cancers (17-23). *p16INK4a* gene promoter methylation has been observed in colorectal dysplasia, adenomas, malignant tumours and normal mucosa adjacent to tumours (18,19,24).

Two significant investigations were previously carried out in the Kashmir valley in order to establish the role of *p16INK4a* mutations and promoter hypermethylation in gastric and esophageal squamous cell carcinoma, respectively (13,25).

Based on the hypothesis that CRC carcinogenesis is a multi-step and multi-gene event, we designed this study to elucidate the role of *p16INK4a* promoter hypermethylation in the development and progression of CRC in the Kashmiri population and to correlate it with the clinicopathological parameters of CRC cases.

Materials and methods

Colorectal cancer cases and controls. This study included 114 CRC cases recruited from the Department of Surgery, Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, India. Tumour and adjacent normal tissue samples from the cases were resected in the General Surgery Department

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Table I. Primer sequences used in the hypermethylation analysis of the promoter region of the *p16* gene.

Gene	Primer sequence	Amplicon size (bp)	Annealing temperature (˚C)
p16 unmethylation reaction	p16-U1 F: 5'-TTATTAGAGGGTGGGGTGGATTGT-3' p16-U1 R: 5'-CAACCCCAAACCACAACCATAA-3'	151	70
	p16-U2 R: 5'-CCACCTAAATCAACCTCCAACCA-3'	234	66
p16 methylation reaction	p16-M1 F: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' p16-M1 R: 5'-GACCCCGAACCGCGACCGTAA-3'	150	70
	p16-M2 R: 5'-CCACCTAAATCGACCTCCGACCG-3'	234	66

(SKIMS) and were collected for this study. Data on all CRC cases were obtained from personal interviews with patients and/or guardians and medical records. All patients and/or guardians were informed of the study and their will to participate in this study was noted in a pre-designed questionnaire (available on request). The mean age of the patients was 52 years.

DNA extraction. Samples were snap-frozen immediately after collection and stored at -70˚C until further analysis. DNA was then isolated from both tissue and blood samples using the ammonium acetate method succeeding proteinase-K digestion. The tissue for DNA extraction from the tumour sample was selected by an experienced pathologist and was ascertained to comprise >90% tumour cells.

Methylation-specific polymerase chain reaction (MS-PCR) of the p16INK4a promoter. Both normal and tumour DNAs were subjected to sodium bisulphite modification using the EZ DNA Methylation kit (Zymo Research, USA). Approximately 10 μl of DNA from each sample was modified as described in the protocol. Previously reported primer sets were used for the amplification of the *p16* promoter (11,26). The unmethylated primer pair *p16*-U1F/U1R produced an amplicon of 151 bp, while *p16*-U1F/U2R produced an amplicon 234 bp in length. Similarly, the methylated primer pair *p16*-M1F/M1R produced an amplicon of 150 bp, while *p16*-M1F/M2R produced an amplicon of 234 bp in length. The presence of unmethylation and/or methylation of the *p16* promoter was ascertained by the presence of both amplicons.

PCR for both unmethylation as well as methylation detection was performed in a 50-μl total volume reaction mixture containing 10 ng of modified genomic DNA, 100 μM of each dNTP, 100 ng of each of the three primers (U1F, U1R and U2R in the case of unmethylation detection; M1F, M1R and M2R in the case of methylation detection), 1.5 mM $MgCl_2$, 5% dimethyl sulphoxide (DMSO), 10X *Taq* buffer and 2 units *Taq* DNA polymerase (Fermentas, MD, USA). The conditions of PCR were as follows: initial denaturation at 95˚C for 7 min, 40 cycles of denaturation at 95˚C for 30 sec, annealing at designated temperatures (˚C) (see Table I) for 45 sec and extension at 72˚C for 45 sec, and final extension at 72˚C for 10 min in a Bio-Rad iCycler. The PCR amplicons were electrophoresed on 2.5% agarose gels and visualised after staining with ethidium bromide.

Universal methylated human DNA (Zymo Research) was used as a positive control for methylated alleles whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. Water was used as a negative PCR control in both reactions.

Statistical analysis. All statistical analyses were performed using PASW software, version 18 (IBM, NY, USA). Pearson's Chi-square two-proportion test was used to evaluate the hypothesis of equal distribution of molecular alterations with different clinicopathological variables. A Fisher's two-tailed test (p-values) of ≤0.05 was considered statistically significant.

Results

A total of 114 CRC cases were included in this study. The patients comprised 67 males and 47 females (male/female ratio of 1.42). The demographic and clinical characteristics of the CRC cases are shown in Table II. Out of 114 confirmed cases of CRC, 109 cases were sporadic, 4 were familial adenomatous polyposis (FAP) and 1 was Lynch syndrome. All but 1 case were adenocarcinoma and only 1 was squamous cell carcinoma (SCC) of basal cell type; 79 came from a rural and 35 from an urban environment; 49 cases had carcinoma in the colon and 65 in the rectum; 74 were smokers and 40 non-smokers; and 89 cases had well-differentiated adenocarcinoma.

MS-PCR analysis revealed a high methylation status of the *p16INK4a* promoter in CRC cases. Forty-eight (42.1%) of the tumours were found to be hypermethylated at the promoter region of *p16INK4a*, while 8 (7%) of the tumours were partially hypermethylated and the remaining 58 (50.9%) of the tumours were not methylated at all (Table II).

Among the 48 patients with tumours which were methylated for the *p16INK4a* gene promoter, 26 were female, 36 had a higher tumour stage (C+D), 38 were smokers and 31 were of a well-differentiated (WD) grade (Table II).

Statistical analysis between the *p16INK4a* hypermethylation status and clinicopathological parameters of the CRC cases revealed a significant association ($p<0.05$) with gender (females), lymph node status (present), tumour stage (C+D), smoking status (smokers) and tumour grade (WD) of the CRC patients (Table II).

Discussion

This is the first study to report on the association of *p16* gene promoter hypermethylation with the risk of development of CRC in the Kashmiri population. The Kashmir valley, located

Table II. Comparison between promoter hypermethylation of the *p16* gene and clinicopathological variables.

Variable	Total (n=114)	Methylation (n=48; 42.1%)	Non-methylation (n=66; 57.9%)	p-value[a]
Age group (years)				0.330
≤50	45	16	29	
>50	69	32	37	
Gender				**0.020**
Male	67	22	45	
Female	47	26	21	
Dwelling				1.000
Urban	35	15	20	
Rural	79	33	46	
Tumour location				0.560
Colon	49	19	30	
Rectum	65	29	36	
Nodal status				**0.006**
Involved	68	36	32	
Not involved	46	12	34	
Tumour grade				**0.006**
A+B	46	12	34	
C+D	68	36	32	
Tumour grade				**0.010**
WD	89	31	58	
MD+PD	25	17	8	
Smoking status				**0.009**
Positive	40	10	30	
Negative	74	38	36	
Pesticide exposure				0.070
Positive	38	11	27	
Negative	76	37	39	

[a]Fischer's exact two-tailed test. Bold figures indicate statistical significance. WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

in the northern part of India and walled by the Himalayas has a unique ethnic population living in discrete temperate environmental conditions and having unique eating habits, which, along with genetic factors, play a large role in the development of GIT cancers (4-6,13). As previously reported, the etiology and incidence of various GIT cancers in this population has been attributed to a probable exposure to nitroso compounds, amines and nitrates reported to be present in local foodstuffs, such as Hoakhe Suen (sun-dried vegetables), Pharei and Hoggade (sundried and/or smoked fish and meat), Hakh (a leafy vegetable of the Brassica family), hot noon chai (salted tea), dried and pickled vegetables and red chilli, and also through smoking Hukka (a water pipe) (3,4,27).

CpG island hypermethylation is one of the essential mechanisms of gene inactivation. Cancer cell lines have in general demonstrated an increased frequency of hypermethylation in comparison to primary tumours (28). The inactivation of tumour-suppressor genes by promoter hypermethylation has been recognised to be as common as gene disruption by mutation in tumourigenesis (29-31). A number of studies on CRC around the globe have demonstrated the role of promoter hypermethylation of a number of different genes in the development and progression of CRC (32-34). Promoter hypermethylation of *p16INK4a*, similarly to that of other genes, plays a pivotal role in the inactivation of *p16INK4a*, which in turn enhances tumour development (11,18,23).

In the present study, we observed hypermethylation of the *p16INK4a* gene promoter in 42.1% (48/114) of CRC cases, which is higher than reported by other major studies in the world (11,20,21). However, our observation is consistent with other studies (22,23) which have reported a similar frequency of *p16INK4a* hypermethylation in CRC tumours in the Japanese population. This may be due to the fact that this population is exposed to a special set of environmental challenges, including extreme temperature, high altitude and special food habits, as well as exposure to agricultural by-products, such as pesticides and nitrosamines (6,13). Liang *et al*, Urosevic *et al* and Krtolica *et al* have already proposed that geographical differences or other unknown factors supplementary to *p16* methylation may increase tumour aggressiveness (35-37).

We found a significant association of the *p16INK4a* methylation status with higher Dukes' stage (C+D). These results are in concordance with those of other studies (20,21,23), which have reported the same observations of a higher methylation status of *p16INK4a* gene promoter with higher Dukes' stage (C+D). Duke's staging has been considered a most significant prognostic determinant in cancers (20). Dukes' staging, proposed by Dr Cuthbert E. Duke in 1932, is a clinical classification for CRC based on the tumour size, local extent and metastatic status, i.e., lymph node involvement. Hence, the present study supports the observation of Yi *et al* (20) in suggesting that *p16INK4a* methylation may be involved in the malignant transformation of CRC. Furthermore, a significant association was found between *p16INK4a* methylation status and lymph node metastasis in our population, as was also reported by Goto *et al* in their study (23).

We also found that females were more likely than males to have *p16INK4a* methylated CRC tumours in our population; this supports the study of Wiencke *et al* (38). They also reported *p16INK4a* methylation to be associated with poorly differentiated tumours. However, contrary to this observation, we found the methylation status of the *p16INK4a* gene to be associated with a well-differentiated tumour grade. Jie *et al* previously demonstrated the association of *p16INK4a* methylation status with the proliferative activity of CRC (39). They reported the loss of proliferation and invasion in CRC in tumours with re-expression of *p16INK4a* protein. Thus, epigenetic silencing of *p16INK4a* may have a role in driving CRC tumours to higher differentiation and consequently to invasiveness.

Another unique observation in our study was the association of the methylation status with smoking status. Tumours of the CRC patients who had a history of smoking (cigarettes and/or Hookah) were found to have methylated *p16INK4a* gene promoter ($p<0.05$).

In conclusion, in the Kashmir valley population, the high level of epigenetic silencing of *p16INK4a* plays a pivotal role in the initial tumourigenesis and also enhances the chances of tumour development and progression to advanced stages.

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