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

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Received July 29, 2011; Accepted October 24, 2011

DOI: 10.3892/mmr.2012.740

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.
(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 µl 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 MgCl₂, 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.

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. 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

| Gene | Primer sequence | Amplicon size (bp) | Annealing temperature (°C) |
|------|-----------------|--------------------|---------------------------|
| p16 unmethylation reaction | p16-U1 F: 5'-TTATTAGAGGGTGTTGATTGT-3' | 151 | 70 |
| | p16-U1 R: 5'-CAACCCCCAACCACAACCATAA-3' | 151 | 70 |
| | p16-U2 R: 5'-CACCTAATTCAACTCTCAACCA-3' | 234 | 66 |
| | p16-M2 R: 5’-CCACCTAAATCGACCTCCGACC-3' | 234 | 66 |

Table I. Primer sequences used in the hypermethylation analysis of the promoter region of the p16 gene.
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 \( p16^{INK4a} \), similarly to that of other genes, plays a pivotal role in the inactivation of \( p16^{INK4a} \), which in turn enhances tumour development (11,18,23).

In the present study, we observed hypermethylation of the \( p16^{INK4a} \) 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 \( p16^{INK4a} \) 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).

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* |
|----------------------|--------------|---------------------------|-----------------------------|----------|
| 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                          |          |

*Fischer's exact two-tailed test. Bold figures indicate statistical significance. WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.
We found a significant association of the \(p16^{INK4a}\) methylation status with higher Duke's 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 \(p16^{INK4a}\) gene promoter with higher Duke's stage (C+D). Duke's staging has been considered a most significant prognostic determinant in cancers (20). Duke's 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 \(p16^{INK4a}\) methylation may be involved in the malignant transformation of CRC. Furthermore, a significant association was found between \(p16^{INK4a}\) 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 \(p16^{INK4a}\) methylated CRC tumours in our population; this supports the study of Wiencke et al (38). They also reported \(p16^{INK4a}\) methylation to be associated with poorly differentiated tumours. However, contrary to this observation, we found the methylation status of the \(p16^{INK4a}\) gene to be associated with a well-differentiated tumour grade. Jie et al previously demonstrated the association of \(p16^{INK4a}\) 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 \(p16^{INK4a}\) protein. Thus, epigenetic silencing of \(p16^{INK4a}\) 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 \(p16^{INK4a}\) gene promoter (p<0.05).

In conclusion, in the Kashmir valley population, the high level of epigenetic silencing of \(p16^{INK4a}\) plays a pivotal role in the initial tumourigenesis and also enhances the chances of tumour development and progression to advanced stages.

Acknowledgements

The authors wish to thank the CRC patients who took part in this study for their cooperation during the interview and sample collection. The authors gratefully acknowledge the financial support provided by the Indian Council of Medical Research (ICMR), New Delhi (5/13/100/2009-NCD-III), for this study and for supporting the fellowship of Dr A. Syed Sameer as Senior Research Fellow. Thanks are also due to the Head and Technical Staff of the surgical theater of the Department of General Surgery who helped in the tissue procurement, and to the anonymous pathologists of the Department of Pathology for the histopathological assessment of the tumour tissues.

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