Indian Journal of Surgical Oncology (September2021) 12(3):454 –459
https://doi.org/10.1007/s13193-021-01325-5

ORIGINAL ARTICLE

Aberrant Promoter Hypermethylation of *p16* and *RASSF1a* Genes in Colorectal Cancer – Significance in Young Patients

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Received: 6 October 2020 / Accepted: 15 April 2021 / Published online: 24 April 2021

Abstract

Objective The clinical profile of colorectal cancers (CRC) in India is different from that described in western countries. Microsatellite instability and APC mutation explain the molecular biology of up to 50% of colorectal cancers. Global genome hypermethylation may be the cause in at least 20% of cases. Few studies from India have examined the epigenetic profile of colorectal cancers. This study was designed to study aberrant promoter hypermethylation of two select tumour suppressor genes (*p16, RASSF1a*) in patients with colorectal cancer and their association with clinicopathologic features.

Methods A total of 41 samples including controls were collected from colorectal cancer patients. DNA was isolated from tumour tissue, and methylation-specific PCR was performed for the 2 genes.

Results *p16* and *RASSF1a* promoter hypermethylation was found in 26% and 48% of CRC cases, respectively. *RASSF1a* promoter hypermethylation was more often seen in young CRC patients aged 40 years or less, and this was found to be statistically significant (p value = 0.037).

Conclusion *RASSF1a* hypermethylation is peculiar to rectal cancers and left-sided colonic tumours in young patients. Large-scale population-based studies with extensive genetic and epigenetic characterization are required for a better understanding and further validation of our findings. For individuals diagnosed with sporadic CRC, these studies on specimen might help predict prognosis and response to therapy.

Keywords Colorectal cancer · Promoter hypermethylation · p16/ CDKN2A · RASSF1a

Introduction

The clinical profile of colorectal cancers (CRC) in India is different from that described in western countries. Demographic studies of CRC from India report a higher incidence of rectal cancer in the young with a higher proportion of signet ring carcinomas, and higher presentation at an advanced stage [1–4]. Most studies have looked into demography and clinicopathologic features; few have examined the genetic profile of colorectal cancers. Genetic instability forms the hallmark of all neoplasia. It is the diversity of this genetic instability that makes each tumour unique and difficult to treat. Diversity also exists within an individual's tumour [5]. This genetic diversity is seen clearly in colorectal cancer which develops through multiple genetic and epigenetic pathways.

On the basis of molecular features, three main pathways for the development of colorectal cancer have been defined: the chromosomal instability(CIN) pathway, the pure microsatellite instability (MSI) pathway, and the CpG island methylator phenotype pathway [6]. Each of these pathways is characterized by specific pathological features and mechanisms of carcinogenesis. These findings indicate the need for tailored treatment of colon cancer, meaning each tumour

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will have to undergo molecular profiling before starting therapy. The molecular aspects of the first two pathways, i.e., CIN and MSI, have been used clinically in the diagnosis and management of patients with colorectal cancer. The epigenetic alterations in colorectal cancer too have the potential to be used as clinical biomarkers for diagnosis and treatment [7]. To use this in clinical practice, technical measurements and characterization of the various epigenetic alterations need to be done in different populations.

Short sequences rich in CpG dinucleotide, usually underrepresented in the genome, can be found in the 5′ region of 50% of all human genes. CpG islands within the promoters are unmethylated in normal cells except for those on the inactive X chromosome. Hypermethylation of promoter CpG islands eventually transforms the chromatin into a closed structure and results in gene inactivation. Inactivation of tumour suppressor genes through this mechanism leads to tumour formation [8]. The *p16 (p16INK4A/ CDKN2A)* and *RASSF1a* are tumour suppressor genes involved in cell cycle regulation. Hypermethylation of both these genes has been observed in colorectal cancers [9].

Methodology

This was a prospective feasibility study designed to study the aberrant promoter hypermethylation of *p16* and *RASSF1a* genes in patients with colorectal cancer and its association with clinicopathologic features. We used methylated CpG island amplification using polymerase chain reaction (PCR) to study the methylation status of *p16* and *RASSF1a.* Informed consent was obtained from all patients. This study was approved by the Institutional Scientific Review Board and the Medical Ethics Committee.

Sample Collection

Tumour sample was collected from patients undergoing upfront surgery for colon and rectum. The tumour specimen was cut open and washed in normal saline. Representative tumour tissue ~1 to 2 g was collected in autoclaved vacutainers and stored immediately at −20 °C. To serve as a negative control, corresponding normal mucosa (cut margins of specimen >5 to 10 cm from tumour) of colorectum was also collected in 10 patients undergoing surgery and stored at −20 °C.

Universally Methylated HeLa Genomic DNA (New England Biolabs Inc., England) served as a positive control.

DNA Isolation

Genomic DNA was extracted from 25 mg of tissue specimen using QIAamp DNA mini kit (Qiagen, CA, USA) following the manufacturer's instructions. The quality of the extracted DNA was examined by electrophoresis, and the yield was measured using the Eppendorf Biospectrophotometer kinetics ™ before proceeding for bisulphite modification.

Bisulphite Modification of Isolated DNA

Using the EZ DNA Methylation-Lightning Kit (Zymo Research, CA, USA), 1 μg of genomic DNA was subjected to bisulphite modification, following the manufacturer's instructions. Bisulphite treatment of DNA converted all the unmethylated cytosine to uracil, leaving the methylated cytosines unaffected. The modified DNA was used as a template for nested and methylation-specific PCR.

Methylation-Specific PCR (MSP)

In the first step, we used a primer set that recognized the bisulphite-modified DNA template, but did not discriminate between methylated and unmethylated alleles. The primers and annealing conditions used are summarized in Tables 1 and 2. The nested PCR product was subjected to second step PCR specific for methylated and unmethylated alleles, respectively. The PCR products were loaded onto 2% agarose gel and visualized by ethidium bromide staining (Figs.1, 2).

Data Analysis

Frequencies of promoter methylation of specific genes, frequencies, and mean of other clinicopathologic variables were computed. To test the association of promoter methylation with other variables, Fisher's exact test was used. A p value of 0.05 was considered to be statistically significant. All tests of statistical significance were two-sided. Data analysis was done using statistical computing software "R" version 4.0.2 (R core team 2020, Vienna, Austria).

Results

A total of 41 samples including 10 controls were collected and analysed. The age of the CRC patients ranged from 30 to 74 years. A total of 7 patients aged 40 or less and 24 aged more than 40. Rectal cancers constituted 48.3% of the cases. The rest were right colon (22.5%) and left colon (29%) cancers. Of all CRC, 52% were moderately differentiated, 32% were poorly differentiated, and 16% were well-differentiated tumours. A majority of the cases (48.3% or 15 out of 31) belong to TNM stage III. Preoperative elevated CEA levels were seen in 42% (13 out of 31) cases.

p16 and *RASSF1a* promoter hypermethylation was found in 26% (8 of 31) and 48% (15 of 31) of cases, respectively. Hypermethylation of both *p16* and *RASSF1a* genes was found in 16% (5 of 31) tumour samples. Of all tumour samples, 42%

Table 1 Primer sequences used

Gene	Primer	Sequence	Amplicon size (bp)	Annealing temperature (°C)
p16 (nested)	FORWARD REVERSE	5′-GAAGAAAGAGGAGGGGTTGG-3′ 5’-CTACAAACCCTCTACCCACC-3	280	56
Methylated *p16*	FORWARD REVERSE	5’-TATTAGAGGGTGGGGCGGATCGC-3′ 5’-GACCCCGAACCGCGACCGTAA-3′	150	58
Unmethylated *p16*	FORWARD REVERSE	5′-TTATTAGAGGGTGGGGTGGATTGT-3’ 5’-CAACCCCAAACCACAACCATAA-3′	150	59
RASSF1a (nested)	FORWARD REVERSE	5′-GGAGGGAAGGAAGGGTAAGG-3’ 5′- CAACTCAATAAACTCAAACTCCC-3’	260	56
Methylated *RASSF1a*	FORWARD REVERSE	5′- GGGTTTTGCGAGAGCGCGT-3′ 5′- GCTAACAAACGCGAACCG-3′	169	60
Unmethylated *RASSF1a*	FORWARD REVERSE	5′-GGTTTTGTGAGAGTGTGTTTAGT-3′ 5’-CACTAACAAACACAAACCAAACA −3′	169	58

Bp, base pair

(13 of 31) showed methylation for either *p16* or *RASSF1a*. *p16* hypermethylation was absent in normal control tissue; however, *RASSF1a* hypermethylation was seen in 60% of normal control tissues. *RASSF1a* promoter hypermethylation was more often seen in young CRC patients aged 40 years or less, and this was found to be statistically significant (p value = 0.037). No significant association of promoter hypermethylation was seen with tumour stage, grade, tumour location, and CEA levels (Table 3). *p16* methylation was more often seen in stage I and II CRC. Although this was not statistically significant (p value = 0.206), this finding suggests that p16 promoter hypermethylation might be an early event in multistep carcinogenesis. RASSF1a promoter methylation was more often seen with left-sided colonic tumours and rectal cancers compared to right colonic tumours, though this finding was not statistically significant (p value = 0.137).

Discussion

Literature review of studies using fresh frozen CRC tissue as in our’s using MSP method show *p16* hypermethylation in the range of 18 to 53% [10–15]. Agnese et al. showed a 21% hypermethylation status of the *p16INK4A* gene promoter [15]. They did not find a significant association between *p16* hypermethylation and clinicopathological variables. Yi et al. studied 62 CRC specimens and found *p16* methylation was more likely to occur in Dukes’ C and D patients [13]. In contrast, we found that 62.5% of *p16* methylation was seen in TNM stage I and II CRC. Xing et al. did a pooled analysis including studies with patients from Japan, China, the USA, Spain, and few others. Their meta-analysis showed that p16 hypermethylation had an unfavourable impact on the overall survival of colorectal cancer [16].

We found *RASSF1a* promoter hypermethylation in 48% of CRC samples. Engeland et al. observed 20% *RASSF1a* methylation in CRC [17]. They analysed 6 controls obtained from noncancer patients and found no *RASSF1a* methylation. On the other hand, Oliveira et al. in their analysis of 31 MSI CRC found 52% *RASSF1a* methylation [18]. This finding was associated with poorly differentiated tumours. A study from Assam, India, reports 41.5% *RASSF1a* methylation [19]. Another study on the North Indian population of 62 CRC patients found 47% promoter hypermethylation of *RASSF1a* [20]. They reported a higher frequency of hypermethylated *RASSF1a* in those with risk factors of smoking and alcohol

Table 2 Methylation-specific reaction conditions

Gene	Initial denaturation	Cycling stage × 40			Final extension
		Denaturation	Annealing	Extension	
P16	95°	95°	56°(N)/58°(M)/59°(UM)	72°	72°
	7 min	30 s	30 s	30 s	5 min
RASSF1A	95°	95°	56°(N)/60°(M)/58° (UM)	72°	72°
	7 min	30 s	30 s	30 s	7 min

N, nested; *M*, methylated; *UM*, unmethylated

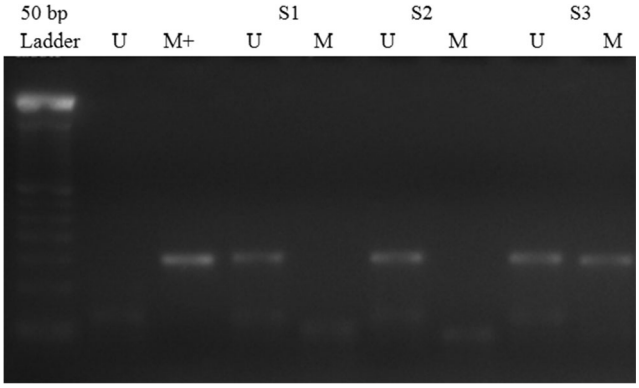

Fig. 1 Gel electrophoresis image *p16* methylation. (*S*, sample; *U*, unmethylated; *M*, methylated; *M+*, positive control)

consumption. This is the first such study from South India on CRC, to the best of our knowledge. Large numbers of patients at our centre come from the Bengal region which probably explains the similar rates of *RASSF1a* methylation found in the studied Indian populations. *RASSF1a* methylation is associated with metastatic CRC and poor prognosis [21]. In our study, although we did not find an association with stage, 60% of *RASSF1a* methylation was seen in stage III and IV CRC.

Multiple studies from India report a higher incidence of colorectal cancer in the young [2, 22, 23]. Aetiology in the young is not clear. Most of these patients do not have a well-documented family history. This might not be related to familial adenomatous polyposis (FAP) as most cases do not have adenomatous polyposis coli [22]. MSI or HNPCC might play a role in young; however, these are often associated with right-sided tumours. We attempted to see if any relation exists between young age and *p16* or *RASSF1a* methylation. A significant proportion of *RASSF1a* hypermethylation was seen in young CRC patients. We also noted a trend for a higher frequency of *RASSF1a* hypermethylation in rectal and left-sided tumours. Similar to our findings, Laskar et al. reported a higher frequency of *RASSF1a* methylation in young patients with rectal cancer, which is associated with poor prognosis [19].

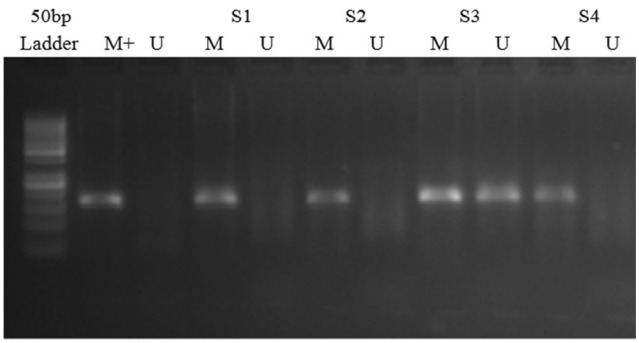

Fig. 2 Gel electrophoresis image of *RASSF1a* methylation. (*S*, sample; *U*, unmethylated; *M*, methylated; *M+*, positive control)

We found that *p16* methylation was absent in normal tissue and *RASSF1a* methylation was seen in 60% of normal colorectal mucosa. Ahuja et al. studied age-related methylation of multiple genes in normal colonic mucosa [24]. They concluded that age-related methylation was gene-specific and that *p16* methylation was not affected by age. Methylation is not only tissue specific but also cell specific; i.e. methylation varies between epithelial and stromal components. Thus precise sampling of tissue is important, so that epithelial cells are targeted. We have used a technique described by Sugai et al. to accurately isolate mucosal glands [25]. Sinha et al. reported 13% *RASSF1a* methylation in adjacent colonic mucosa. They explain it as an early change in the adjacent colonic mucosa, which subsequently leads to carcinogenesis. The normal controls in our study were taken from non-adjacent normal colonic mucosa of patients suffering from CRC – more than 5 cm from tumour, at cut margins of specimen, pathologically verified to be free of tumour. In a more recent study, Sugai et al. compared DNA methylation levels of many genes including *RASSF1a* in cancerous crypts and normal mucosa. They reported that a field effect may be present in CRC, affecting both adjacent and non-adjacent normal mucosa [26]. Sun et al. studied *RASSF1a* methylation using isolated DNA from blood samples and found 48.44% methylation in CRC versus 5.13% in healthy volunteers [25]. However, the estimate of methylation in blood is lesser than in fresh frozen CRC specimens [11, 12]. Age-related methylation is also related to carcinogen exposure, folate, and alcohol intake [27]. Therefore, methylation of genes is dependent on multiple factors. This probably explains the variations in methylation status and its association seen across studies. It is preferable to take negative controls from age-matched, non-cancer individuals and collect information on diet/habits/risk factors of patients and controls for a complete analysis.

Methylation studies might explain relapses in optimally treated early-stage individuals. Nakayama et al. studied tumour DNA in blood samples of patients with recurrent CRC and found p16 methylation in 69% of cases. They proposed that this can be a useful method to detect recurrences [11]. For individuals diagnosed with sporadic CRC, these studies on specimen might help predict prognosis and response to therapy.

Conclusion

With so much heterogeneity in normal and tumour aberrant methylation, translating these tests into clinically useful diagnostic/prognostic platforms still remains a challenge. RASSF1a hypermethylation is peculiar to rectal cancers and left-sided colonic tumours in young patients. Can this be a field change leading to cancer in a subset of the Indian

Table 3 Gene methylation and clinicopathologic characteristics

Variables	Groups	N	Unmethylated p16 (No. /%)	Methylated p16 (No. /%)	p value fisher's	Unmethylated RASSf1a (No. /%)	Methylated RASSF1a (No. /%)	*p* value Fisher's
Age (years)	<=40	7	6(85.7)	1 (14.3)	0.642	1 (14.3)	6 (85.7)	**0.037**
	>40	24	17(70.8)	7 (29.2)		15 (62.5)	9 (37.5)	
Gender	F	14	10(71.4)	4 (28.6)	1	5 (35.7)	9 (64.3)	0.156
	M	17	13(76.5)	4 (23.5)		11 (64.7)	6 (35.3)	
Tumour location	Left colon	9	7(77.8)	2 (22.2)	0.173	2 (22.2)	7 (77.8)	0.137
	Right colon	7	7(100)	0 (0)		5 (71.4)	2 (28.6)	
	Rectum	15	9(60)	6 (40)		9 (60)	6 (40)	
Grade	1	5	3(60)	2 (40)	0.407	2 (40)	3 (60)	0.546
	2	16	11(68.8)	5 (31.2)		10 (62.5)	6 (37.5)	
	3	10	9(90)	1 (10)		4 (40)	6 (60)	
TNM Stage	Stage 1 and 2	12	7(58.3)	5 (41.7)	0.206	6 (50)	6 (50)	1
	Stage 3 and 4	19	16(84.2)	3 (15.8)		10 (52.6)	9 (47.4)	
Pre-op CEA levels (ng/ml)	CEA<=5	18	14(77.8)	4 (22.2)	0.689	7 (38.9)	11 (61.1)	0.149
	CEA>5	13	9(69.2)	4 (30.8)		9 (69.2)	4 (30.8)	

population? If so, is this amenable to therapy? Large-scale population-based studies with extensive genetic and epigenetic characterization are required for a better understanding and further validation of our findings. Close collaboration between clinicians and research scientists is a must to understand the clinical relevance of these findings.

Acknowledgements The authors acknowledge the contribution to statistical work made by Dr. Santu Ghosh, Assistant professor, Department of Biostatistics, St. John's Medical College, Bangalore.

Availability of Data and Material available.

Author Contribution Dr. Medha Sugara conceptualized the work. All authors contributed to the study design and methodology. Dr. Medha Sugara performed the data curation and formal analysis. The investigation was performed by Dr. Medha Sugara and Ms. Shalini N Swamy and Mr. Sandeep Kumar S. Resources were provided by Prof. Ramachandra C and Prof. Ramesh Gawari. The supervision of work was done by Prof. KV Veerendra Kumar. The original draft was prepared by Dr. Medha Sugara. All authors reviewed and approved the final manuscript.

Declarations

Ethics Approval The study was approved by the institutional scientific review board and medical ethics committee of Kidwai Memorial Institute of Oncology, Bangalore (No:KMIO/MEC/007/30.April.2016).

Consent to Participate Informed consent was obtained from all individuals participating in the study.

Consent for Publication The individual participants provided informed consent to the publication of findings of this study.

Conflicts of Interests The authors declare no competing interests.

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