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RESEARCH

High Frequency of Genes' Promoter Methylation, but Lack of *BRAF* V600E Mutation among Iranian Colorectal Cancer Patients

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Abstract Gene silencing due to DNA hypermethylation is a major mechanism for loss of tumor suppressor genes function in colorectal cancer. Activating V600E mutation in *BRAF* gene has been linked with widespread methylation of CpG islands in sporadic colorectal cancers. The aim of the present study was to evaluate the methylation status of three cancer-related genes, *APC2*, *p14ARF*, and *ECAD* in colorectal carcinogenesis and their association with the mutational status of *BRAF* and *KRAS* among Iranian colorectal cancer patients. DNA from 110 unselected series of sporadic colorectal cancer patients was examined for *BRAF* V600E mutation by PCR-RFLP. Promoter methylation of genes in tumors was determined by methylation specific PCR. The frequency of *APC2*, *E-CAD*, and *p14* methylation was 92.6%, 40.4% and 16.7%, respectively. But, no V600E mutation was identified in the *BRAF* gene in any sample. No association was found in cases showing epigenetic *APC*, *ECAD*, and *p14* abnormality with the clinicopathological parameters under study. The association between *KRAS* mutations and the so called methylator phenotype was previously reported. Therefore, we also analyzed the association between the hot spot *KRAS* gene mutations in codons of 12 and 13 with genes' promoter hypermethylation in a subset of this group of patients. Out of 86 tumors, *KRAS* was mutated in 24 (28%) of tumors, the majority occurring in codon 12. *KRAS* mutations were not associated with genes' methylation in this tumor series. These findings suggest a distinct molecular pathway for methylation of *APC2*, *p14*, and *ECAD* genes from those previously described for colorectal cancers with *BRAF* or *KRAS* mutations.

Keywords Colon cancer · *BRAF* · *KRAS* · Methylation

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Introduction

Colorectal cancer (CRC) is the third most common cause of cancer death worldwide. A significant increase in CRC incidence with a predominant distal localization has also been reported in Iran over the last decade [1, 2].

There is increasing evidence suggesting that colorectal cancers develop through different molecular pathways [3]. One of the pathways by which CRC can progress involves transcriptional silencing by hypermethylation of CpG islands, including those present in the promoter regions of tumor suppressor genes, a feature referred as methylator phenotype (CIMP+) [4].

BRAF is a cytoplasmic serine—thereonine kinase that mediates responses to EGF-mediated growth signals through RAS/RAF/MAPK pathway [5]. Activating mutations in the *BRAF* gene have been identified in 5–15% of

sporadic CRCs, more than 80% of which are in the hot spot of exon 15, resulting in V600E substitution [6–8].

The V600E mutation introduces a negative charge in the BRAF that mimics the activating phosphorylation events at nearby threonine and serine residues. Several studies reported a strong association between DNA hypermethylation and *BRAF* mutation in sporadic CRCs [9–11]. The alteration of *KRAS* may also contribute to the methylator phenotype in CRC [3]. *BRAF* and *KRAS* mutations are important early events in CRC development, and have been strongly correlated with the level of methylation in multiple genes' promoter in CRC [12]. It has been reported that CRCs with the *KRAS* mutation were consistent with a methylation frequency between those with a *BRAF* mutation and those with neither mutation [13].

Aberrant methylation of promoter CpG islands might silence genes that are important for normal cellular homeostasis. Tumor suppressor genes act in several pathways controlling cell proliferation such as cell cycle regulators (*p14* and *p16*), cell adhesion (*E-Cadherin*), and WNT signaling (*APC* and *APC2*) [14, 15].

APC2 is one of the candidate cancer genes (CAN gens) that its epigenetic alterations have been recently identified in CRC by analysis of the DNA hypermethylome in human colorectal cancer [15]. APC2 is a homologue of APC tumor suppressor involved in Wnt signaling pathway. Like APC, APC2 also can inhibit β-catenin signaling and has been suggested to function as a tumor suppressor gene and therefore, important in cancer development [16]. Hypermethylation of *APC2* gene has been recently reported in 90% of African-American CRC patients [16, 17]. ECAD is a Ca^{+}-dependent adhesion molecule that mediates intercellular contacts. It plays critical roles in the maintenance of morphogenesis and tissue structure. Loss of *ECAD* expression is associated with the invasion and metastasis [16, 18]. *p14ARF* is another tumor suppressor gene that regulates cell cycle and its expression has been shown to enhance stability of p53 through MDM2 suppression. Transcriptional silencing by methylation of CpG islands within the 5′-flanking region and exon 1β of *p14* gene has been reported in human CRC [19].

In the present study, we investigated the relationship between *APC2*, *p14*, and *ECAD* promoters' methylation and *BRAF V600E* mutation among a group of sporadic CRC patients from south of Iran.

Materials and Methods

Study Population, and Tumor Samples

A total of 110 unselected CRC patients from two referral hospitals in Shiraz, south of Iran were included in this study. Ethics approval for the project was obtained from the Institutional Ethics Committee. Tumor samples were snap frozen immediately after surgical resection and stored at −80°C. All samples were evaluated and subjected to histological diagnosis by expert pathologists and clinical data was obtained. Family history of cancer was analyzed to exclude those pedigrees that met either the Amsterdam I or Amsterdam II criteria. The splenic flexure was used as the anatomical boundary to define proximal and distal CRC.

Methylation-Specific PCR (MS-PCR)

The status of promoter methylation of the *APC2*, *p14,* and *ECAD* genes were determined by MS-PCR. The sequences of primers used for amplification of the promoter regions of genes are listed in Table 1. The MS-PCR reactions were performed as previously described [20]. PCR reactions were "hot started" at 94°C and carried out using 100 ng of bisulfite treated DNA with the following conditions: The PCR reactions were performed in a 50 μL reaction volume containing 25 pmol of each of sense and antisense primers, 0.2 mmol/L dNTPs in 1× PCR buffer provided by the enzyme supplier. The reaction mixture was denatured at 95°C for 5 min, after which 1.5 U Taq polymerase was added; then amplified by 40 cycles, each cycle consisting of 45 s denaturation at 95°C, 45 s annealing at the specified temperatures (Table 1), and 45 s polymerization at 72°C, followed by a single 10-min final extension at 72°C. DNA from peripheral blood lymphocytes was used as negative control in every bisulfite conversion. PCR products were analyzed on 2% agarose gel.

BRAF and *KRAS* Mutation Analysis

The *BRAF* genotyping was performed by PCR—RFLP. Genomic DNA from the samples was used as a template in PCR reactions using two *BRAF* primers encompassing *BRAF* exon 15 where the V600E substitution is known to take place.

Two nucleotides at the 5′-side of the forward primer were changed (Table 1) to create, in cases of a mutant 1799A nucleotide in the DNA template, an MboII recognition site GAAGA(N8)↓. The wild-type allele with 1799T does not contain the MboII recognition sequence at the corresponding position. MboII digestion of the 97 bp PCR product resulted in 63 bp and 34 bp fragments for the mutant (1799A) allele. Amplified fragments were digested overnight with 10U Mbo II restriction enzyme (MBI Fermentas, Vilnius, Lithuania) at 37°C. The PCR products subjected to enzyme digestion were visualized on 2% agarose gel stained with ethidium bromide. The method was validated by direct sequencing of 50 PCR products.

Table 1 Primers' sequence and the annealing temperature used for the related PCR reactions

Primer name	Forward	Reverse	Annealing temperature (°C)
APC2-M	5′-GTCGTTTGTTTAGGTTCGGATC-3′	5′- GACCCGAAATAACCTCGAAACG-3′	60°C
APC2-U	5′-TGGTAGTGTTGTTTGTTTAGGTTTGGATTG-3′	5′-ACCAAAAATCCCAACCCAAAATAACCTCAAAAC-3′	57°C
P14-M	5′- GTGTTAAAGGGCGGCGTAGC-3′	5′- AAAACCCTCACTCGCGACGA-3′	60°C
P14-U	5′- TTTTTGGTGTTAAAGGGTGGTGTAGT-3′	5′- CACAAAAACCCTCACTCACAACAA-3′	58°C
ECAD-M	5′- TTAGGTTAGAGGGTTATCGCGT-3′	5′- TAACTAAAAATTCACCTACCGAC-3′	60°C
ECAD-U	5′- TAATTTTAGGTTAGAGGGTTATTGT-3′	5′- CACAACCAATCAACAACACA-3′	50°C
BRAF 1799	[a]5′- ATAGGTGATTTTGGTCTAGCT**GA**AG-3′	5′- CAATTCTTACCATCCACAAA-3′	51°C
KRAS codons 12/13	5′-CTGCTGAAAATGACTGAATA-3′	5′-ATGG TCCTGCACCAGTAATA-3′	48°C

[a] We used an altered forward PCR primer, which creates, in cases of a mutant 1799A nucleotide in the DNA template an artificial MboII site (see the text). Altered bases are in bold font and underlined

For the mutational analysis of *KRAS*, PCR/non-isotopic single strand conformational polymorphism (SSCP) analysis was employed as described by Servomaa et al. [21]. Briefly, codons 12/13 of the *KRAS* gene were amplified by PCR, as a single fragment, using the primer pair flanking the codons of interest (Table 1). PCR products (2 μl) were denatured for 10 min at 96°C with 2 μl of formamide denaturing dye mixture, cooled on ice, and then applied (4 μl/lane) on 10% nondenaturing polyacrylamide gels. Gels were silver stained and photographed. DNA showing an altered mobility, distinct from that of a normal band in SSCP analysis, was analyzed further by direct sequencing.

Statistical Analysis

Statistical analysis was performed using the SPSS version 11.5 software package (Chicago, IL). Associations between methylation of loci and clinical biological features were evaluated using Chi square and Fisher's exact test as appropriate.

Results

Distribution of the Selected Characteristics of Cases

In this study, the relationship between promoter methylation of 3 tumor suppressor genes involved in CRC carcinogenesis and *BRAF* V600E mutation was investigated. One hundred and ten patients entered the study. Selected characteristics of the study population are presented in Table 2. Cases were more likely to be males, smokers and older than 60 years. The incidence of distal tumors was higher than proximal tumors (data not shown). Twenty seven percent (30) of patients had tumors in the proximal colon and 73% (80) in distal parts. No statistically significant differences were found between proximal and distal cancer cases with respect to sex, age, and smoking status. The majority of distal tumors was found to be well/moderately differentiated and was stage II or higher.

APC2, *p14* and *ECAD* Gene Methylation Profiles

Illustrative examples of MSP reactions for promoter methylation analysis are shown in Fig. 1. The majority of cases showing positive PCR bands for methylated DNA sequences also exhibited them for unmethylated DNA sequences, which were related to tumor heterogeneity or to the occurrence of intermingled normal cells. The epigenetic profile of *APC2*, *p14*, and *ECAD* in CRC is shown in Table 2. We found methylation of *APC2* and *p14* genes in 104 (95.5%) and 18 (16.4%) of the 110 CRC cases, respectively. The *ECAD* gene promoter's methylation was detected in 20 (40.8%) out of 49 CRCs investigated.

We analyzed the correlation between the methylation statuses of the genes' promoter with clinicopathological features. The characteristics of tumors with either one or two (*ECAD* and *p14*) methylated genes are summarized in Table 2. When methylation of individual sites was analyzed, no age or sex differences were detected. We also found no significant difference in the methylation status of either gene by tumor location, smoking status, or stage of tumors (Table 2). Coincidental methylation of *ECAD* and *p14* was documented in five out of 49 CRC patients, all being <60 years old (Table 2). A small trend for a higher risk of coincidental alterations of *ECAD* and *p14* was observed in stage I tumors compared with the higher stages.

BRAF and *KRAS* Mutation Analysis

Samples were analyzed for the presence of a point mutation that frequently occurs in the *BRAF* oncogene leading to a

Table 2 Associations between genes promoter methylation and clinicopathological features of CRC patients

Variables (n)	Methylation positive n (%)							
	APC2	[a]*p*	*p14*	[a]*p*	[b]*ECAD*	[a]*p*	[b]*ECAD* and *p14*	[a]*p*
Total (110)	104 (95.5)		18 (16.4)		20 (40.8)		5 (10.2)	
Age								
<60 (42)	41 (97.6)	0.3	8 (26.1)	0.4	8 (38.1)	0.7	5 (23.8)	0.01
≥60 (68)	63 (92.5)		10 (14.7)		12 (42.9)		0 (0)	
Sex								
Male (72)	68 (95.8)	0.4	11 (15.3)	0.4	11 (39.3)	0.8	3 (14.3)	0.06
Female (38)	35 (92.1)		7 (18.5)		9 (42.9)		2 (7.1)	
Smoking								
Smoker (56)	53 (96.4)	0.4	10 (17.9)	0.7	7 (28)	0.6	1 (4)	0.2
Non-smoker (54)	50 (92.6)		8 (14.8)		13 (54.1)		4 (16.7)	
Location								
Distal (80)	73 (92.4)	0.1	11 (13.8)	0.2	15 (42.9)	0.6	2 (5.7)	0.1
Proximal (30)	30 (100)		7 (23.4)		5 (35.8)		3 (21.4)	
Tumor stage								
I (14)	11 (84.6)	0.2	3 (21.4)	0.2	5 (45.5)	0.9	3 (27.3)	0.08
II (57)	55 (96.5)		8 (14)		10 (38.5)		2 (7.7)	
III (24)	23 (95.8)		2 (8.3)		5 (41.7)		0 (0)	
IV (4)	3 (75)		2 (50)		0 (0)		0 (0)	
Differentiation								
Grade								
Well (5)	4 (80)	0.6	0 (0)	0.6	1 (50)	0.9	0 (0)	0.4
Moderate (44)	44 (100)		7 (16)		7 (43.8)		3 (18.8)	
Poor (48)	42 (89.4)		8 (15.7)		12 (38.8)		2 (6.5)	

[a] *P*-value from Fisher's exact test

[b] The methylation status was investigated in 49 CRC specimens

change of valine to glutamic acid at position 600 of the BRAF protein. The *BRAF* gene had no detectable mutation in all 110 samples studied using the PCR—RFLP assay. These results suggest that *APC2, ECAD*, and *p14* promoters' methylation is a frequent event in CRC tumorigenesis and could occur in a different pathway of CRC development from that involving *BRAF* mutation.

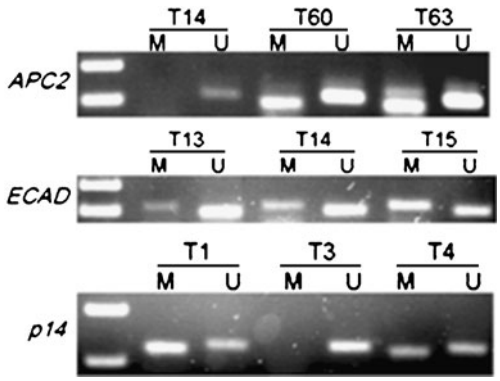

Fig. 1 Representative examples of MSP reactions for promoter methylation analysis of *APC2*, *ECAD*, and *p14* genes in primary CRC tumors. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of a product in those lanes marked M indicates the presence of methylated genes. Lane 1 indicates the 100 bp DNA size marker

It has been proposed that a subset of CRCs with *KRAS* mutations may also accumulate promoter methylation in several genes [22]. Therefore, we analyzed the hot spot *KRAS* gene mutations in codons of 12/13 in a subset of 86 cases of this group of patients (Table 3). We identified oncogenic mutations in 24 out of 86 (28%) tumors, the majority occurring in codon 12 and a few in codon 13. A significant difference in the distribution of *KRAS* mutation was observed between patients with proximal colon tumor

Table 3 *KRAS* codons 12/13 mutation frequencies in colorectal cancers

Variables (n)	*KRAS* mutated n (%)	[a]*p* Value
All patients (86)	24 (27.9)	
Sex		
Female (31)	6 (19.4)	0.2
Male (55)	18 (32.7)	
Age		
<60 (36)	8 (22.2)	0.3
≥60 (50)	16 (32)	
Location		
Left (62)	23 (37.1)	0.002
Right (24)	1 (4.2)	

[a] *P*-value from Fisher's exact test

and those with distal CRC, as verified by the Fisher's exact test (p=0.002). *KRAS* mutations were not associated with genes' methylation in this tumor series (Table 4). Together, these results suggest for the existence of alternate CIMP pathways of CRC development to those involving *BRAF* and *KRAS* mutations.

Discussion

Our data indicates extensive methylation of the *APC2* and *ECAD* genes in CRC patients. We observed a high frequency of 95.5% methylation in the *APC2* promoter. Gene's promoter methylation was not specifically associated with any of the clinicopathological parameters considered in this study. In a recent population-based study, a similar frequency of 96% methylation of the *APC2* gene in African- Americans and 94% in Iranian sporadic CRC patients were reported [23]. *APC2* is a homologue of *APC1* tumor suppressor involved in Wnt signaling pathway. In our previous study in this group of patients, the frequency of *APC1* methylation was 11.8% (unpublished). Therefore, it seems that the *APC2* hypermethylation is a more frequent event than the *APC1* hypermethylation in colorectal carcinogenesis.

ECAD was hypermethylated in 40.8% of primary CRCs, a finding consistent with previous reports. Miranda et al. reported a frequency of 44% for *ECAD* methylation [24]. In another study, half of all CRC samples were found to be hypermethylated in the *ECAD* promoter, although the sample size was relatively small [25]. Mutations of the *ECAD* gene have been only rarely found in most types of sporadic cancers [26]. It seems that promoter methylation is the major oncogenic event for the *ECAD* gene inactivation in CRC.

In the previous studies, hypermethylation of the promoter region in the *p14ARF* gene was reported in 18–33% of human colon cancers [19, 27]. We detected *p14* methylation in 16% of the cases. MSI has been classified into two categories, high-level MSI (MSI-H) and low-level MSI (MSI-L). In a recent study of 234 CRCs, promoter methylation of *p14* was significantly associated with MSI-low (MSI-L) CRC and with *KRAS* mutation [13]. The authors concluded that promoter methylation of *p14* could be an alteration leading to a CRC with MSI-L. The CRC series investigated in this study have also been characterized previously for MSI + and the methylation status of *hMLH1*, and *p16* promoters [20, 28]. Therefore, this allowed us to examine the association between *KRAS* mutation with hypermethylation and MSI status in the present study. MSI analysis was performed using the reference panel of five markers recommended by the "Bethesda guidelines" (D2S123, D5S346, D17S250, BAT25, and BAT26). Tumors were classified as MSI positive if two or more markers showed instability [28]. We found MSI+ tumors in 23.8% of this CRC series and *KRAS* mutations in 28% of tumors (Table 3). We did not find an association between promoter methylation of the *p14* gene and MSI tumors with *KRAS* mutations in these CRC tumors (Table 4). *BRAF* mutation was also reported to positively associate with MSI+status and *hMLH1* methylation [11, 29]. We verified the relationship between the *BRAF* gene mutation and the MSI and the hypermethylation of *hMLH1* gene in this group of patients. As described above, while there was no *BRAF* mutation in 110 CRCs investigated, 23.8% of the patients were MSI+ and 13.2% had methylated *hMLH1* promoter.

We also analyzed the differences in the methylation status of these three tumor-related genes in CRC patients according to their clinicopathological features. We found no significant differences of methylation status of *APC2*, *p14*, and *E-CAD* genes between these subgroups (Table 2). The frequency of coincidental methylation of *ECAD* and *p14* genes was higher in younger patients (<60 years) than that in the old ones. A small trend for a higher risk of coincidental methylation of *p14* and *ECAD* was observed in stage I tumors (Table 2).

In our previous study on this group of cases, we identified 13.2% methylation of *hMLH1*, 2.6% methylation of *hMSH2*, and 19.9% methylation of *p16* genes [20]. About half of the promoters of human genes have CpG islands, and are

Table 4 DNA methylation according to *KRAS* mutation and MSI status in tumor tissue and normal mucosa from 86 CRC patients

Methylated Promoter (N)	No *KRAS* mutation N (%)	*KRAS*/[b]*MSS* N (%)	*KRAS*/[b]MSI-high N (%)	*KRAS*/[b]MSI-low N (%)	[a]*p*-value
p14ARF (12)	7 (58.3)	5 (41.7)	0 (0)	0 (0)	NS
APC2 (80)	58 (72.5)	19 (23.8)	1 (1.3)	2 (2.5)	NS
ECAD (20)	17 (85)	3 (15)	0 (0)	0 (0)	NS
hMLH1 (14)	14 (100)	0 (0)	0 (0)	0 (0)	NS
p16INK4a (12)	11 (91.7)	1 (8.3)	0 (0)	0 (0)	NS

[a] Fisher's exact test for difference in methylation frequency between tumor subgroups; *NS* Not significant (P>0.05)

[b] *MSS* microsatellite stable; *MSI-low* tumors with only one MSI-positive marker; *MSI-high* tumors with two or more MSI-positive makers (see text)

susceptible to hypermethylation. But, it is unclear why certain genes are more frequently affected than others.

There is not yet a consensus on what markers and criteria should be used for classification of CIMP in CRC. A panel of five markers including *RUNX3*, *CACNA1G*, *IGF2*, *NEROG1*, and *SOCS1* were recently proposed for defining CIMP+ tumors [10, 30]. Using above markers, the frequency of CIMP+ was estimated at approximately 15–18% of CRC patients.

It has been previously reported that *BRAF* and *KRAS* mutations are associated with different levels of DNA hypermethylation [12]. CRCs with *BRAF* mutations are associated with a high-level of promoter methylation in multiple loci, but tumors with *KRAS* mutations are associated with a low-level of promoter methylation. CRCs with neither *KRAS* nor *BRAF* mutations have very little methylation. *BRAF* somatic mutations were reported in ~15% of sporadic CRCs [7, 8]. We found no *BRAF* mutations in this study confirming recent findings of Brim et al. [31] reporting the lowest mutation rate of this gene among Iranian (2%) in comparison to Omanis (19%), and African American (10%) patients. Brim et al. used direct sequencing method and we employed PCR-RFLP to analyze the presence of *BRAF* mutation. The difference is unlikely to be due to a failure of our mutation detection methodology because we also verified PCR-RFLP results by direct sequencing in a subset of our samples (see "Materials and Methods" section). We assume that the genetic or environmental factors may underlie the lack of *BRAF* mutation among Iranian CRC patients.

We detected a substantial promoter methylation in genes investigated in 110 CRC specimens. Our results showed the lack of association of the methylation status with either *BRAF* or *KRAS* mutations in this group of CRC patients. Despite the size limitations, our preliminary data suggest possibly a distinct molecular pathway for methylation of *APC2*, p14, and *E-CAD* genes from those previously described for CRCs with *BRAF* or *KRAS* mutations. Further studies will be necessary to substantiate this issue.

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