Research Article

Cancer Epidemiology, Biomarkers & Prevention

Methylation of the Polycomb Group Target Genes Is a Possible Biomarker for Favorable Prognosis in Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) is the second most common cancer in the Kingdom of Saudi Arabia with ever increasing incidence rates. DNA methylation is a common event in CRC where it is now considered an important phenomenon in CRC carcinogenesis and useful for the classification and prognosis of CRC.

Methods: To gain insight into the molecular mechanisms underpinning CRC in Saudi Arabian patients, we profiled the DNA methylation frequency of key genes (*MLH1*, *MSH2*, *RASSF1A*, *SLIT2*, *HIC1*, *MGMT*, *SFRP1*, *MYOD1*, *APC*, *CDKN2A*, as well as five CIMP markers) in 120 sporadic CRC cases. CRC tumors originating from the rectum, left, and right colons are represented in this cohort of formalin-fixed paraffin-embedded tissues.

Results: The most common methylation frequency was detected in the polycomb group target genes (PCGT) including *SFRP1* (70%), *MYOD1* (60.8%), *HIC1* (61.7%), and *SLIT2* (56.7%). In addition, *MGMT* methylation was detected at a high frequency (68.3%). *RASSF1A*, *APC*, and *CDKN2A* methylation frequencies were 42.5%, 25%, and 32.8%, respectively. K-means clustering analysis of the methylation events results in the clustering of the CRC samples into three groups depending on the level of methylation detected.

Conclusion: Group II (PCGT methylation and CIMP-negative) methylation signature carried a favorable prognosis for male patients, whereas older patients with group I rare methylation signature have a potentially poorer clinical outcome.

Impact: Methylation of the PCGT genes along with *RASSF1A*, *APC*, and *MGMT* can be potentially used as a new biomarker for the classification and prognosis of CRC tumors and independently of where the tumor has originated. *Cancer Epidemiol Biomarkers Prev; 21(11); 2069–75. ©2012 AACR.*

Introduction

CRC is the third most common type of cancer in the worldwide and the most common type in males in the Kingdom of Saudi Arabia (1). The age-standardized incidence rates (per 100,000) of CRC in Kingdom of Saudi Arabia vary between 9.8 in females to 14.3 in males (1) Although low compared with Western countries, CRC incidence rate in Kingdom of Saudi Arabia has almost tripled in less than 8 years (2). This increase in CRC incidence rates coincides with a shift towards Western world lifestyle including diet and daily activities (3, 4).

CRC is a heterogeneous disease with different molecular characteristics associated with the sites from which the tumors originate. Such heterogeneity is compounded by the multitude of genetic and epigenetic variations acting as passengers or drivers of the tumor (5). Majority of CRC develop via chromosomal instability (CIN) pathway. CIN is often exacerbated by inactivation of the Wnt signaling pathway "master regulator" *APC* gene (6), activating mutations of KRAS or BRAF oncogenes (7), or deletions of the 18q (8), and 17p (9) chromosomal regions with deleterious effects on the tumor suppressor genes *TP53* and *DCC*. Defective mismatch repair (MMR) pathway results in a subtler form of genomic instability, namely microsatellite instability (MSI). High levels of MSI (or MSI-H) in sporadic CRC are usually caused by hypermethylation of the *MLH1* promoter (10). In terms of methylation, the CpG island methylator phenotype (CIMP) pathway is the second most common pathway in sporadic CRC (11).

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CRC tumors with high levels of CIN have a poor prognosis, especially if they are in stage II or III (12). Conversely, MSI-H tumors have a better clinical outcome compared with microsatellite-stable (MSS) tumors (13). CIMP-positive (CIMP+) CRC tumors are usually associated with the proximal colon of older females. CIMP+ tumors also show positive association with *BRAF* mutations (14). CIMP+ CRC tumors have better prognosis if the tumors are also MSI-H. However, CIMP+ CRC tumors that are MSS have poor clinical outcome.

In this study, we investigate the methylation frequency of several genes including the CIMP markers (*IGF2*, *CACNA1G*, *NEUROG1*, *RUNX3*, *and SOCS1*), the MMR genes (*MSH2* and *MLH1*), tumor suppressor genes (*APC*, *RASSF1A*, and *CDKN2A*) in addition to *MGMT* DNA repair gene. The hypermethylation-mediated silencing of the polycomb group target (PCGT) genes (15) in cancer has been recently shown to be a hallmark of carcinogenesis (16). Although the methylation of such genes have been shown in ageing normal colon mucosa, their methylation is much more widespread and pronounced in cancerous samples (16–18). We have recently shown that methylation of *SLIT2*, *SFRP1*, *HIC1*, and *MYOD1* is frequent in sporadic breast cancer from Saudi Arabia with methylation of the latter a possible marker for poor prognosis (19). The contribution of the inactivation of MMR genes in CRC for this population is yet to be shown. Similarly, such information is lacking for the other genes analyzed in this cohort.

Patients and Methods

Patients

The material of the present study consist of a series of 120 CRC specimens, retrospectively collected from the archives of Anatomical Pathology Laboratory in King Abdulaziz University Hospital (Jeddah, Kingdom of Saudi Arabia), covering the period from January 2005 to December 2009. Serial sections were cut from paraffin blocks, stained with hematoxylin and eosin for routine histologic examination, classification, grading, and staging following the American Joint Committee on Cancer (AJCC) staging system (20). The pertinent clinicopathologic data (gender, age, grade, and lymph node status), and follow-up results were retrieved from the patients' records after obtaining the relevant ethical approvals. DNA was extracted from 10 μm thin formalin-fixed paraffin-embedded slices using the Qiagen QIAMP Formalin-fixed Paraffin-embedded Tissue DNA extraction kit, following the manufacturer's guidelines. *KRAS* and *BRAF* mutational status were determined according to the previously published reports (21). The microsatellite instability was determined according to Berg and colleagues (22).

Bisulfite DNA modification and MethyLight assay

Up to 0.5 microgram of DNA was used for bisulfite conversion using the Qiagen Epitect Bisulfite Conversion kit. DNA methylation analysis was conducted using MethyLight as described elsewhere (23). The methylation levels of *RASSF1A*, *APC*, *MGMT*, *CDKN2A*, *SLIT2*, *SFRP1*, *MYOD1*, *HIC1*, *MSH2*, *MLH1* and the CIMP markers *IGF2*, *SOCS1*, *RUNX3*, *CACNA1G*, *NEUROG1* were analyzed using the primer-probe combinations listed in Table 1 which were made according to previously published reports (24–27). A probe targeting bisulfite-modified Alu repeat sequences was used to normalize for input DNA. The specificity of the reaction was ascertained using *sssI*-treated and bisulfite-modified positive control DNA (Qiagen) and the negative control DNA (Qiagen). The percentage of fully methylated reference (PMR) was calculated by dividing the gene:Alu ratio of a sample by the gene:Alu ratio of the positive control DNA and multiplying by 100. Samples with PMR more than 10 were considered positive for methylation, whereas samples with PMR less than 10 were considered negative (i.e., unmethylated). The PMR more than 10 is considered positive as it indicates a very likely hypermethylation-mediated loss of expression for the genes analyzed.

Statistical analysis

All statistical tests were carried out using IBM SPSS Statistics version 19. Fisher exact test was used to identify statistical significance of correlation between methylation events and clinicopathologic factors. The primary endpoints of the study included overall disease-free survival (DFS) calculated from the date of diagnosis to the appearance of disease recurrence or the last recorded date of being alive or death caused by CRC. In calculating DFS, patients who died of other or unknown causes were excluded. All survival times were calculated by univariate Kaplan–Meier analysis, and equality of the survival functions between the strata was tested by log-rank (Mantel–Cox) test. Multivariate Cox regression analysis was conducted to disclose independent predictors of DFS. All tests were 2-sided, and P values < 0.05 were considered statistically significant. K-means clustering was conducted using the Gene CLUSTER 3.0 program and visualized using JavaTree software (28).

Results

We analyzed 120 patients with colorectal cancer selected on the basis of the availability of tissue material and clinical data. Mean age was 58 years (range, 24–96 years) with 34 patients (28.3%) being under 50 years. This cohort consisted of 72 male patients (60%) and 48 female patients (40%). Thirty eight (31.7%) tumors were in the right colon, 36 (30%) tumors were in the left colon, and 46 (38.3%) tumors were in the rectum. There is a significant association between the male gender and tumors from the right colon ($P = 0.016$). In addition, tumors from the left colon were more predominant in females ($P = 0.027$). Tumors from the right side are more likely to involve the lymph nodes ($P = 0.039$). There are no other significant differences between right, left, and rectal colon cancer in terms of age, grade, or recurrence.

Table 1. Primer and probe sequences used in this study

Gene	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
SFRP1	CAACTCCCGACGAAACGAA	CGCGAGGGAGGCGATT	6FAM-CACTCGTTACCACGTCCGTCACCG-BHQ1
MYOD1	GAGCGCGCCGTAGTTAGCG	TCCGACACGCCCTTTCC	6FAM-CTCCAACACCCGACTACTATATCCGCGAAA-BHQ1
HIC1	GTTAGGCGGTTAGGGCGTC	CCGAACGCCTCCATCGTAT	6FAM-CAACATCGTCTACCCAACACACTCTCCTACG-BHQ1
RASSF1A	ATTGAGTTGCGGGAGTT GGT	ACACGCTCCAACCGAATA CG	6FAM-CCCTTCCCAACGCGCCCA-BHQ1
CDKN2A	TGGAATTTTCGGTTGATT GGTT	AACAACGTCCGCACCTC CT	6FAM-ACCCGACCCCGAACCGCG-BHQ1
SLIT2	CAATTCTAAAAACGCACGACTCTAAA	CGGGAGATCGCGAGGAT	6FAM-CGACCTCTCCCTCGCCCTCGACT-BHQ1
IGF2	GAGCGGTTTCGGTGTCGTTA	CCAACTCGATTTAAACCGACG	6FAM-CCCTCTACCGTCGCGAACCCGA-BHQ1
NEUROG1	CGTGTAGCGTTCGGGTATTTGTA	CGATAATTACGAACACACTCCGAAT	6FAM-CGATAACGACCTCCCGCGAACATAAA-BHQ1
RUNX3	CGTTCGATGGTGGACGTGT	GACGAACAACGTCTTATTACAACGC	6FAM-CGCACGAACTCGCCTACGTAATCCG-BHQ1
SOCS1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA	6FAM-ACAATTCCGCTAACGACTATCGCGCA-BHQ1
CACNA1G	TTTTTCGTTTCGCGTTTAGGT	CTCGAAACGACTTCGCCG	6FAM-AAATAACGCCGAATCCGACAACCGA-BHQ1
MGMT	CTAACGTATAACGAAAATCGTAACAACC	AGTATGAAGGGTAGGAAGAATTCGG	6FAM-CCTTACCTCTAAATACCAACCCCAAACCCG-BHQ1
APC	TTATATGTCGGTTACGTGCGTTTATAT	GAACCAAAACGCTCCCCAT	6FAM-CCCGTCGAAAACCCGCCGATTA-BHQ1
MSH2	TTTTAGTGCGGAGGTACGGG	AAACGATCCTCCGAAACCAAA	6FAM-CCGCACAAACACCAACGTTCCG-BHQ1
MLH1	AGGAAGAGCGGATAGCGATTT	TCTTCGTCCCTCCCTAAAACG	6FAM-CCCGCTACCTAAAAAAATATACGCTTACGCG-BHQ1
ALU	GGTTAGGTATAGTGGTTTATAT TTGTAATTTTAGTA	ATTAACTAAACTAATCTTAAACT CCTAACCTCA	VIC-CCTACCTTAACCTCCC-MGBNFQ

The mutation status of *BRAF* codon 600 and *KRAS* at codon 12,13 was determined by sequencing. *BRAF* mutations were rare in our cohort ($n = 120$, 2.5%). *KRAS* mutations were more frequent ($n = 108$, 24.1%). There was no significant association between *KRAS* mutation and tumor location, age, sex, or grade. However, metastatic tumors are more likely to harbor a *KRAS* mutation ($P = 0.007$). The microsatellite instability (MSI) status was determined for 66 cases. A total of 22.7% patients had microsatellite stable (MSS) tumors, whereas 34.8% exhibited MSI-low status and 42.4% were MSI-high. In this cohort, it is more likely for the rectal cancer cases to be microsatellite stable ($P = 0.012$). We have determined the CpG island methylator phenotype (CIMP) by analyzing the methylation frequency of 5 genes; *IGF2*, *SOCS1*, *RUNX3*, *CACNA1G*, and *NEUROG1*. A case is considered CIMP+ if methylation of 2 or more genes can be detected. Overall, CIMP+ tumors were only 14.2% of the total cohort and are significantly associated with male patients ($P = 0.014$).

The methylation frequency of the 15 genes analyzed are shown in Fig. 1. The highest overall methylation frequency observed was for *SFRP1* (70%) and the lowest was for *MLH1* and *MSH2* (1.2% and 0%, respectively). The methylation levels for the PCGT genes are consistently higher in tumorous tissues compared with matching nonmalignant counterparts (Supplementary Fig. S1). There is no significant association between the methylation of any gene and tumor location (with the exception of the CIMP markers). When stratified according to tumor location, metastasis is associated with *SLIT2*, *SFRP1*, and *RASSF1A* methylation in tumors originating from the rectum ($P = 0.011$, $P = 0.003$, and $P = 0.039$, respectively). Also in the rectal tumors, *MYOD1* methylation is positively associated with *KRAS* mutations ($P = 0.003$). Rectal cancers from male patients exhibit a significant association with *APC* methylation ($P = 0.007$). Moreover, *APC* methylation positively associated with MSI-H rectal tumors ($P = 0.012$). Tumors originating from the left colon exhibit a positive association between *SFRP1* methylation ($P = 0.022$), *HIC1* methylation ($P = 0.022$), *RASSF1A* methylation ($P = 0.018$), and male patients. MSI-H right-sided tumors are positively associated with *RASSF1A* methylation ($P = 0.019$).

We have conducted K-means clustering analysis based on the methylation status of 13 genes and CIMP status (Fig. 2) to distinguish the subgroups of our cohort based on methylation events. As shown in Fig. 2, 3 distinct subgroups can be identified by K-means clustering. Group I (methylation-low, $n = 36$) is characterized by over representation of rectal cancer (50%) and being MSS or MSI-low (combined percentage is 70%). Group II ($n = 67$) is characterized by the prominent methylation of the PCGT genes (*SFRP1*, *SLIT2*, *HIC1*, *MYOD1*) in addition to the hypermethylation of *MGMT*, *RASSF1A*, and *APC*. Male patients were 59.7% of group II patients (Table 2). Group III (methylation-high, $n = 17$) is characterized by the predominance of male patients who in addition to

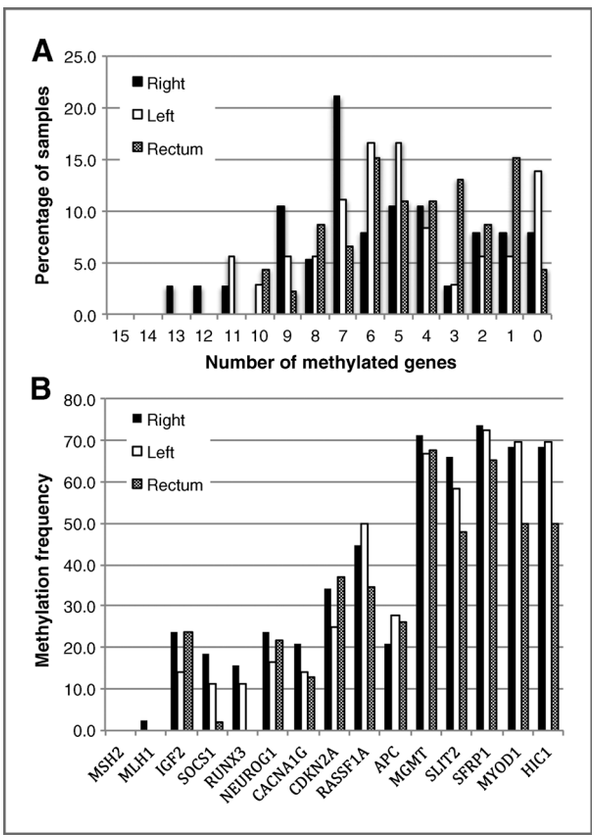

Figure 1. Methylation frequency in CRC. A, percentage of samples originating either from the right colon, left colon, or rectum that exhibit methylation events in the selected genes. B, methylation frequency of the genes analyzed in samples originating either from the right colon, left colon, or rectum.

methylation of the PCGT genes show positive CIMP status and frequent methylation of *RASSF1A*, *APC*, *MGMT*, and *CDKN2A*.

Group I exhibits week association with the female gender ($P = 0.07$) and tend to be well-differentiated tumors ($P = 0.052$). In addition, *KRAS* mutations show negative association with group I cases ($P = 0.053$). Group II is weekly but significantly associated with MSI-H status ($P = 0.048$). Group III is strongly associated with the male gender ($P = 0.014$) and poorly differentiated tumors ($P = 0.048$). None of the new groups show statistically significant association with age, tumor location, or *KRAS* mutation.

Next, we evaluated overall DFS in the 3 groups by univariate Kaplan–Meier analysis. When conducted on all patients, there was no significant effect of belonging into any of the groups on DFS. However, when stratified into young (<50 years old) versus old (>50 years old), worse DFS could be seen if the patient is older than 50 years old and displays group III methylation pattern ($P = 0.058$), Fig. 3A. When stratified according to the gender, group I patients display worse DFS in males only ($P = 0.073$). Interestingly, being a male patient displaying group II methylation pattern carries a favorable prognosis as significantly better DFS is observed ($P = 0.027$; Fig. 3B).

Multivariate Cox regression analysis was conducted with the variables like *KRAS* status, metastasis, age, sex, and tumor location in addition to grouping by K-means cluster analysis. As expected, metastasis is the strongest poor prognosis indicator with $P < 0.0001$ and HR of 8.837 [95% confidence interval (CI), 3.787–20.619]. However, group II methylation pattern is a good prognosis indicator with $P = 0.013$ and HR of 0.269 (95% CI, 0.095–0.761).

Discussion

In this study, we have analyzed 120 cases of sporadic colorectal cancer (CRC) originating from the right colon, left colon, and the rectum for the presence of *KRAS*, *BRAF* mutations, MSI, and CIMP. We have additionally analyzed the methylation frequency of the polycomb group target genes (PCGT) that are normally silenced in stem cells. Furthermore, we have determined the methylation frequency of *RASSF1A*, *APC*, *CDKN2A*, *MGMT*, *MLH1*, and *MSH2* in the same cohort.

The mutation rates of *KRAS* (24.1%) and *BRAF* (2.5%) were found to be lower than worldwide average, which

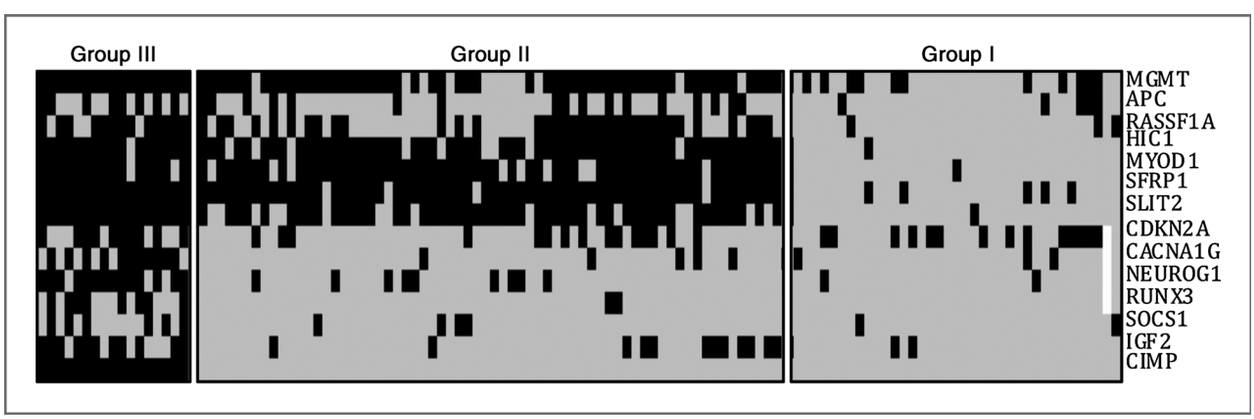

Figure 2. K-means clustering analysis based on methylation events shows 3 distinct subgroups. Black shades indicate positive methylation results, whereas gray shades indicate lack of methylation. White shades reflect missing data.

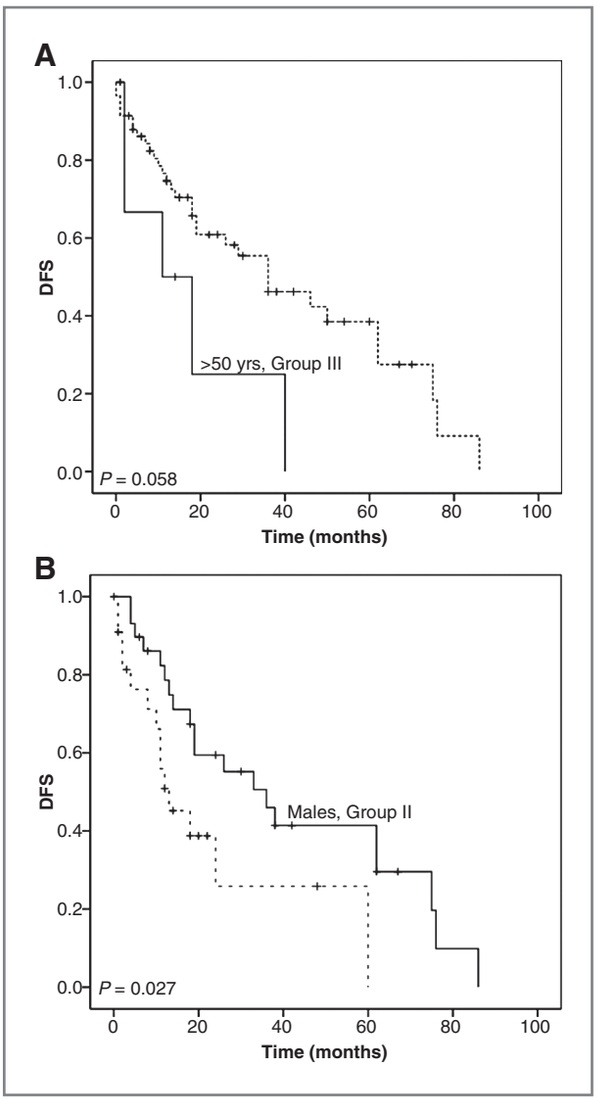

Figure 3. Univariate Kaplan–Meier blots for overall DFS. A, poor overall survival in older patients exhibiting group III methylation pattern (solid line). B, significantly better outcome for male patients with group II methylation pattern (solid line).

may reflect possible ethnic differences affecting the mutation rates of these 2 oncogenes (29). CIMP frequency (14.2%) is similar to previous reports (11). However, we could not detect any statistically significant association between CIMP+ status and mutations of *BRAF*, *KRAS*, or presence of MSI. Moreover, CIMP+ tumors were found to be associated with the male gender in our cohort ($P = 0.014$). Right-sided tumors were also more prominent in males ($P = 0.016$). Therefore, there is a correlation between CIMP and right-sided tumors although this association is not statistically significant ($P = 0.164$).

The most common events in our cohort were the methylation of the PCGT genes as well as the methylation of *MGMT* gene. Interestingly, it has been suggested the MGMT is also a target of the polycomb complex (15). The methylation of the MMR genes was rare in our cohort. This is could be because CRC follows a different route to tumorigenesis and we cannot exclude the presence of inactivating mutations in this pathway

We have conducted K-means clustering analysis to stratify our samples based on their methylation signature. Our cohort can be separated into 3 distinct groups, group I (low methylation), group II (intermediate methylation), and group III (high methylation). Low-methylation frequency in all the genes studied is the hallmark of group I which is mostly, but not exclusively, represented by tumors originating from the rectum. Group I cases are likely to be well-differentiated tumors harboring wild-type *KRAS* ($P = 0.052$ and $P = 0.053$, respectively). Group II cases are MSI-H ($P = 0.048$); however, they are exclusively CIMP negative. Group II cases do not show any statistically significant association with any other clinicopathologic parameter despite being the group with best representation in our cohort. CRC tumors exhibiting the most frequent methylation amongst the genes analyzed clustered together in Group III. These poorly differentiated and CIMP+ tumors ($P = 0.048$) originated mostly from male patients ($P = 0.014$) but had no other significant

Table 2. Clinicopathologic characteristics of group II patients in relations to all other patients

	Group II	Other groups	Total
Number of cases	67	53	120
Males	40	32	72
Females	27	21	48
Age			
Less than 50 years old	17	17	34
Tumor location			
Right colon	21	17	38
Left colon	23	13	36
Rectum	23	23	46
Lymph node status			
LN+	31	27	58
LN−	19	16	35
Grade			
Grade 1 (well differentiated)	8	11	19
Grade 2 (moderately differentiated)	47	30	77
Grade 3 (poorly differentiated)	2	4	6
MSI status			
MSS	6	9	15
MSI-L	10	13	23
MSI-H	19	9	28
Recurrence	35	23	58
KRAS mutation	16	10	26
BRAF mutation	1	2	3
CIMP+	0	17	17

associations. Belonging to any of the 3 groups can serve as a potential prognostic marker for overall survival depending on age or sex. Patients with CRC who are more than 50 years old and display group III methylation pattern have worse overall DFS compared with younger counterparts or patients displaying other methylation patterns. This is in line with previous reports of poor prognosis in CIMP+ cases that are not MSI-H (30). A potentially poorer clinical outcome is observed for male patients displaying group I methylation pattern. The reason for this observation is unknown as the presence and nature of genetic alterations in these tumors is not determined in this study. Unfortunately, the sample size of this group is too small to allow for a more definitive conclusion. Interestingly, however, is the observation that male patients with group II methylation pattern have a better clinical outcome compared with patients displaying other methylation pattern. This is perhaps not entirely surprising as MSI-H tumors generally have good prognosis (30). Multivariate Cox regression analysis confirmed this observation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Dallol, J. Al-Maghrabi, A. Buhmeida, M.S. Al-Ahwal, M.H. Al-Qahtani
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Dallol, J. Al-Maghrabi, A. Buhmeida, M.A. Gari, A.G. Chaudhary, M.S. Al-Ahwal, A. Sibiany
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Dallol, J. Al-Maghrabi, H.-J. Schulten
Writing, review, and/or revision of the manuscript: A. Dallol, J. Al-Maghrabi, A. Buhmeida, A.G. Chaudhary, M.S. Al-Ahwal, M.H. Al-Qahtani
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