Crosstalk Between DNA Methylation and Gene Mutations in Colorectal Cancer

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Colorectal cancer (CRC) is often characterized by mutations and aberrant DNA methylation within the promoters of tumor suppressor genes and proto-oncogenes. The most frequent somatic mutations occur within *KRAS* and *BRAF* genes. Mutations of the *KRAS* gene have been detected in approximately 40% of patients, while mutations in *BRAF* have been detected less frequently at a rate of 10%. In this study, the DNA methylation levels of 22 candidate genes were evaluated in three types of tissue: mucosal tumoral tissue from 18 CRC patients, normal adjacent tissues from 10 CRC patients who underwent surgical resection, and tissue from a control group of six individuals with normal colonoscopies. A differential methylation profile of nine genes (*RUNX3*, *SFRP1*, *WIF1*, *PCDH10*, *DKK2*, *DKK3*, *TMEFF2*, *OPCML*, and *SFRP2*) presenting high methylation levels in tumoral compared to normal tissues was identified. *KRAS* mutations (codons 12 or 13) were detected in eight CRC cases, and *BRAF* mutations (codon 600) in four cases. One of the CRC patients presented concomitant mutations in *KRAS* codon 12 and *BRAF*, whereas seven patients did not present these mutations (WT). When comparing the methylation profile according to mutation status, we found that six genes (*SFRP2*, *DKK2*, *PCDH10*, *TMEFF2*, *SFRP1*, *HS3ST2*) showed a methylation level higher in *BRAF* positive cases than *BRAF* negative cases. The molecular sub-classification of CRC according to mutations and epigenetic modifications may help to identify epigenetic biomarkers useful in designing personalized strategies to improve patient outcomes.

Keywords: colorectal cancer, DNA methylation, *KRAS*, *BRAF*, mutations

INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer and, even with advances in CRC screening and therapeutic strategies, it still remains the second deadliest malignancy for both sexes. CRC incidence has continued to increase in countries with medium to high human development indexes and in younger populations (1). This type of cancer is a highly heterogeneous disease that can be subtyped

according to anatomical location or pathological and molecular signatures. The development of CRC is influenced by both environmental risk factors (such as obesity, a sedentary lifestyle, an unhealthy diet, alcohol consumption, and smoking) (2) and genetic risk factors, with less than 10% of patients presenting inherited mutations that increase the risk of CRC onset (3) and 25% of patients with "familial" CRC (4). About 70% of CRC cases are sporadic; these cases are most common in patients over the age of 50 and seem to depend mainly on dietary and environmental factors.

In general, the development of sporadic CRCs involves the "normal-adenoma-dysplasia-carcinoma" sequence as described by Vogelstein and Fearon (5). This sequence includes genetic and epigenetic changes in the colonic epithelium that transform normal glandular epithelium into invasive adenocarcinomas. The progression from adenoma to carcinoma is a multistep process that involves three molecular pathways: the Chromosomal Instability (CIN) pathway, the Microsatellite Instability (MSI) pathway, and the CpG Island Methylator Phenotype (CIMP) pathway. CIN is characterized by loss or gain of chromosomal segments, chromosomal translocations, or gene amplifications, which result in gene copy number variations. In addition, mutations in specific oncogenes, including *KRAS* and *BRAF*, and in tumor suppressor genes, such as *APC* and *TP53*, can be detected (6). MSI occurs in 15–20% of sporadic CRC cases and comprises recurrent alterations in the microsatellite zone without structural and numerical changes in the genome. CIMP, reported in 20–30% of sporadic CRCs (7), is characterized by hyper-methylation of CpG islands located in promoters that regulate the activity of several tumor suppressor genes and other CRC related genes. It is well known that specific mutations can modify DNA methylation (8) and that DNA methylation changes can cause an increase in mutation rate (9). In CRC, 10–40% lower levels of absolute methylation than normal colon tissue within the whole genome have been determined (10). However, CpG islands in the promoters of CRC related genes show a hyper-methylated profile, resulting in repression of transcription of tumor suppressor genes (11). Therefore, the first aim of this study was to identify methylation differences in the promoters of 22 candidate genes in CRC tissues compared with control tissues (from non-CRC individuals) and with paired normal adjacent tissues. The second aim was to examine the relationship between promoter DNA methylation and the presence of mutations in *KRAS* and *BRAF* genes.

MATERIALS AND METHODS

Sample Collection
The DNA for the study was obtained from 18 CRC sporadic tumor tissues and 10 corresponding paired normal adjacent tissues (NAT) from CRC patients who underwent surgical resection. The NATs were collected approximatively 10 cm away from the tumors. For nine patients out of 10, the NAT was collected from the same anatomic segment. For one patient with tumor localization in the recto-sigmoid junction, the NAT was collected from the sigmoid. For five of the 18 CRC patients, blood samples were collected in EDTA tubes. The tissue specimens and the blood samples were collected at Fundeni Clinical Institute in Bucharest (Romania) and were stored at −80°C until DNA isolation. Six specimens from normal colonic mucosa as well as five blood samples from independent controls, from previous preclinical studies were available from the biobank of the Victor Babes Institute (**Table 1**). The present study was approved by the local ethics committee (registration number 291, 8 March 2016), carried out in accordance with the Declaration of Helsinki, and the individuals gave their written informed consent. Genomic DNA was isolated using the QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's instruction and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). All samples were examined by a pathologist, and clinical information of the patient's cohort are reported in **Table 2**.

Detection of *KRAS* and *BRAF* Mutations Detection and DNA Methylation Analysis
KRAS gene mutations (in codons 12, 13, 61) were detected by pyrosequencing as previously described (12). Mutations in codons 600 and 601 of the *BRAF* gene were assessed using the BRAF 600/601 StripAssay (ViennaLab Diagnostic GmbH, Vienna, Austria) by PCR followed by reverse hybridization according to the manufacturer's protocol. The DNA methylation levels of the promoters of the 22 genes involved in colorectal cancer (*APC, CDH1, CDKN2A, DKK2, DKK3, HIC1, HNF1B, HS3ST2, MGMT, MLH1, OPCML, PCDH10, RASSF1, RUNX3, SFRP1, SFRP2, SFRP5, SPARC, TMEFF2, UCHL1, WIF1,* and *WT1*) were analyzed using the EpiTect Methyl II PCR Array (Qiagen, Hilden, Germany) according to manufacturer's protocol. This array system is based on the detection of the input DNA that remains after digestion with a methylation-sensitive and/or methylation-dependent restriction enzyme using the EpiTect Methyl II DNA Restriction kit (Qiagen, Hilden, Germany). The relative amount of un-methylated (UM) and methylated (M) DNA was quantified by qPCR using the comparative cycle threshold method.

Statistical Analysis
The statistical analysis was conducted using the Statistical Package for Social Science (SPSS version 17.0). Categorical variables were tested using the chi-square test and continuous variables were tested using a t-test or Mann-Whitney U test. The

TABLE 1 | Socio-demographic data of the individuals involved in the study.

Group	Age ± SD	Sex (% Female)	p-value Age	p-value, Sex
T (N = 18)	65.72 ± 11.31	55.55	T *vs* C, p = 0.381	T *vs* C, p = 0.633, χ^2 = 0.229
NAT (N = 10)	62.40 ± 11.16	72.72	T *vs* PT, p = 0.461	T *vs* PT, p = 0.453, χ^2 = 0.562
C (N = 6)	60.66 ± 14.06	66.66	PT *vs* C, p = 0.789	PT *vs* C, p = 0.793, χ^2 = 0.069

TABLE 2 | Available clinical data and mutation status of CRC patients involved in the study.

Mutational status (KRAS/BRAF)	Number	Mutation type	Location	Hepatic metastasis
WT	7	//	Colon (n = 3) Rect/RSJ (n = 3) Sigmoid (n = 1)	1
KRAS12+	4	KRAS12_G12D (n = 2) KRAS12_G12V* (n = 2)	Colon (n = 3) Sigmoid (n = 1)	1*
KRAS13+	3	KRAS13_G13D (n = 3)	Colon (n = 1) Rect/RSJ (n = 1) Sigmoid (n = 1)	0
BRAF+	3	BRAF_V600E (n = 3)	Colon (n = 2) Rect/RSJ (n = 1)	1
KRAS12+BRAF+	1	KRAS12_G12D+ BRAF_V600E (n = 1)	Sigmoid (n = 1)	0

The patient with hepatic metastasis presented KRAS12_G12V mutation.

Mann-Whitney U test was used to identify differences in promoter methylation levels between CRC patients (T) and controls (C), normal adjacent tissue (NAT) and controls (C), as well as to compare samples from patients with different mutation status for *KRAS* and *BRAF*. Receiver operating characteristic (ROC) curves were created and the area under the curve (AUC) was calculated to determine the role of methylation in differentiating T from C. Logistic regression was used to build a diagnostic model that could explore whether varying combinations of differentially methylated genes could better differentiate T from C. A gene promoter was considered to be methylated if the methylation level was over 20% according to the instructions of the manufacturer. A further analysis of the 10 tumoral samples with their matched NAT samples was performed using the Wilcoxon Signed Rank test. All reported significant p values were two-sided, with $p < 0.05$.

RESULTS

In this study, colonic mucosa sample from 18 patients with CRC and six controls were investigated to evaluate the levels of DNA methylation of the promoters of 22 target genes. The tumors were located in the ascending and transverse colon (n = 9), in the sigmoid (n = 4), and in the rectum or the rectosigmoid junction (RSJ) (n = 5). At the moment of sample collection, three cases presented hepatic metastasis. Seven out of the 18 analyzed tumoral tissues presented mutations in the *KRAS* gene: four occurred in codon 12 and three in codon 13. Three patients had mutations in the *BRAF* gene, and one patient showed a double mutation, i.e., mutations in both *BRAF* and *KRAS* genes. *KRAS* and *BRAF* mutations were not found in the 10 normal adjacent tissues or in the seven tumoral tissues (WT) (**Table 2**).

Statistical analysis of the DNA methylation levels of the promoters was performed for 16 genes out of 22, because in six genes, the detected DNA methylation level in the tumoral tissue was below 20% (**Figure 1** and **Table 3**).

A significant differential methylation profile of nine genes (*RUNX3*, *SFRP1*, *WIF1*, *PCDH10*, *DKK2*, *DKK3*, *TMEFF2*, *OPCML*, and *SFRP2*) was detected in tumoral tissues when compared to control tissues (**Table 3**). No statistical difference was observed when comparing the methylation levels of NAT to

C ($p > 0.05$). To assess the potential diagnostic value in discriminating tumoral from normal tissues from controls, ROC analysis was performed. All the significant genes presented an AUC above 0.80 and a specificity and sensitivity between 77.8 and 100% (**Table 3**). When combining three selected potential biomarkers (*SFRP1*, *PCDH10*, and *DKK2*) the value of the AUC was 0.972 and $p = 0.001$ (**Figure 2**).

We also performed a paired analysis for the 10 tumoral samples with their matched NATs. The results showed that *RUNX3* and *TMEFF2* promoters were significantly hyper-methylated in tumoral tissue ($p = 0.017$ and $p = 0.037$, respectively) (**Figure 3**). *SFRP1*, *WIF1*, *PCDH10*, *DKK2*, *DKK3*, *OPCML*, and *SFRP2* maintained the same trend of hyper-methylation in T *vs.* C cases, without reaching statistical significance in the comparison with NAT.

When comparing methylation profiles according to *BRAF* mutation status, we found that *SFRP2*, *DKK2*, *PCDH10*, *TMEF2*, *SFRP1*, and *HS3ST2* showed methylation levels that were significantly higher in the *BRAF* positive cases (n = 4, including the case with *BRAF+* and *KRAS12+*) than in *BRAF* negative cases (WT and *KRAS+*; n = 14) (**Figure 4A**). No changes were identified comparing *BRAF+ vs* WT (n = 7).

Regarding *KRAS* mutation status, we found that the methylation level of *SFRP2* was lower in *KRAS*12+/13+ (n = 8) *versus KRAS*12 −/13− (n = 10) ($p = 0.027$). Even when the case with double mutation (*BRAF+* and *KRAS12+*) was excluded, the comparison remained significant ($p = 0.043$). Moreover, *OPCML* was less methylated in the comparison of *KRAS*12+ (n = 4) to *KRAS*12− (n = 14), ($p = 0.035$). No significant difference in the percentage levels of methylation was found in the following comparisons: *KRAS*12+ (n = 4) with WT (n = 7), *KRAS*13+ (n = 3) with WT (n = 7), *KRAS*12+/13+ (n = 8) with WT (n = 7). The methylation results of the tumoral samples grouped by mutational status are presented in the heat map of **Figure 4B**.

Furthermore, we quantified the methylation levels of the promoters in peripheral blood mononuclear cells (PBMC) from five controls and five CRC patients whose methylation profiles were performed in the tumoral tissue. None of the five patients presented BRAF/KRAS mutations in their blood. Except for *RUNX3*, which was highly methylated both in patients and controls, the other 21 gene promoters did not show a significant general level of methylation (data not shown).
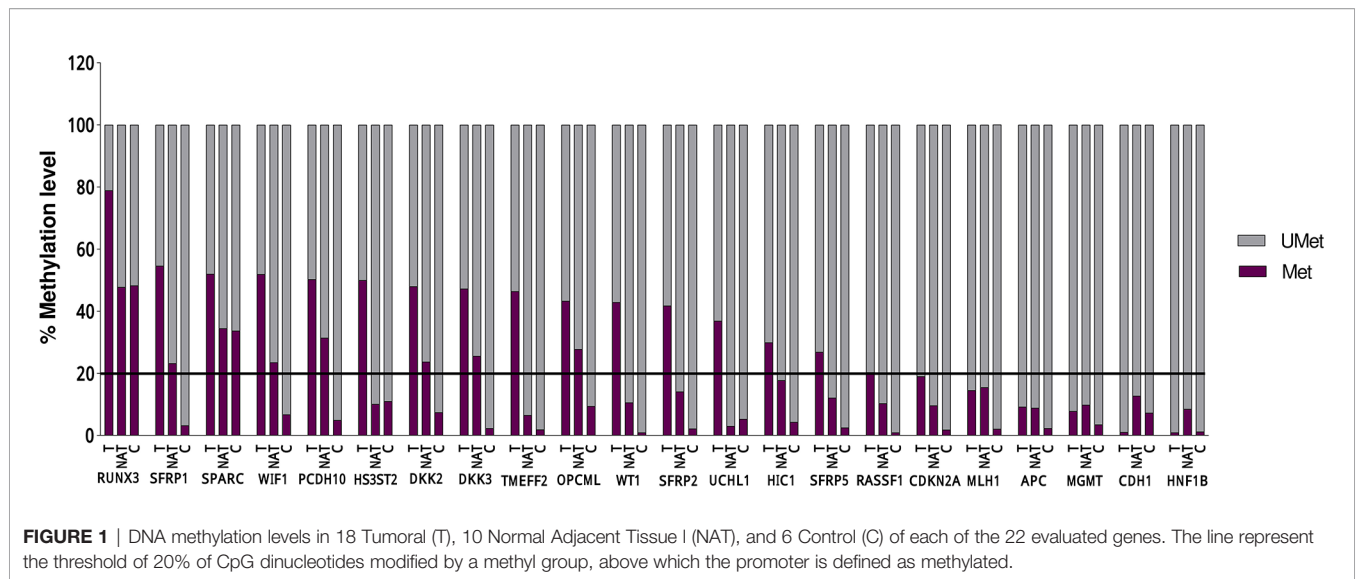
FIGURE 1 | DNA methylation levels in 18 Tumoral (T), 10 Normal Adjacent Tissue l (NAT), and 6 Control (C) of each of the 22 evaluated genes. The line represent the threshold of 20% of CpG dinucleotides modified by a methyl group, above which the promoter is defined as methylated.

TABLE 3 | Average of the methylation percentage (± SEM) in tumoral and control tissues along with the statistical results between the two groups and the ROC curve analysis.

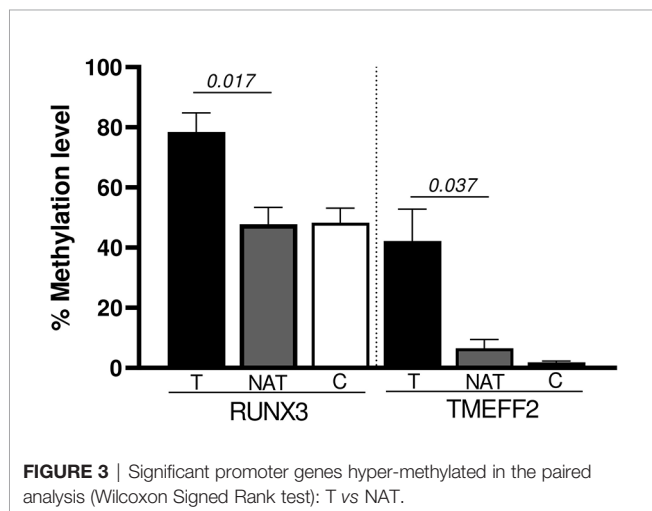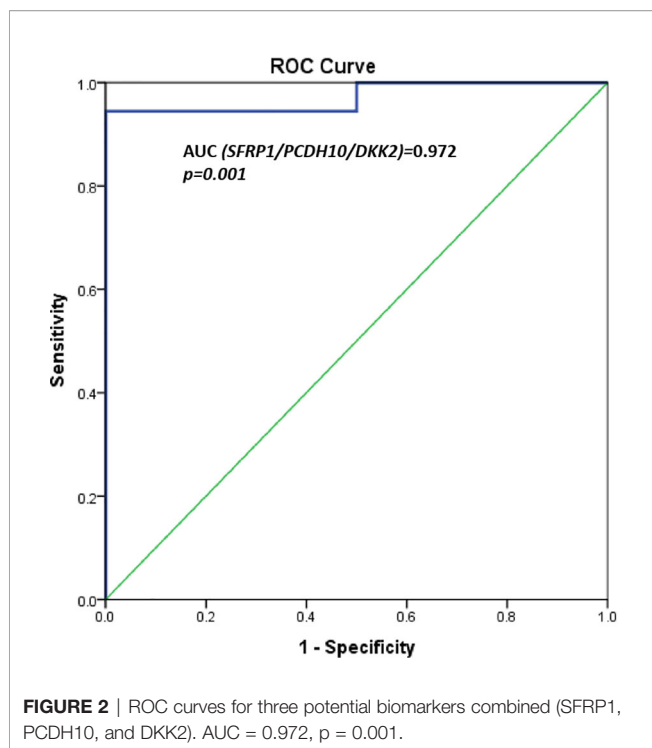| Gene	TUMORAL N = 18		CONTROL N = 6		% Methylation T vs C	ROC			
	Mean	SEM	Mean	SEM	p-value (Mann-Whitney U test)	AUC	Cut-off%	Sensitivity%/ Specificity%	p-value
RUNX3	78.87	4.05	48.31	4.83	<0.001	0.917	55.57	94.4–83.3	0.003
SFRP1	54.61	7.44	3.18	0.98	<0.001	0.944	10.51	94.4–100	0.001
SPARC	52.01	6.14	33.66	4.78	ns				
WIF1	51.95	7.13	6.66	1.69	0.003	0.889	15.69	88.9–100	0.005
PCDH10	50.31	7.07	4.95	1.01	0.001	0.935	12.03	88.9–100	0.002
HS3ST2	50.03	8.39	11.04	8.7	ns				
DKK2	47.96	6.68	7.38	1.58	<0.001	0.981	13.81	94.4–100	0.001
DKK3	47.22	8.65	2.35	0.55	0.027	0.806	3.92	77.8–83.3	0.028
TMEFF2	46.4	7.89	1.87	0.4	0.002	0.907	2.99	88.9–83.3	0.003
OPCML	43.28	5.37	9.38	2.69	0.003	0.889	15.20	83.3–83.3	0.005
WT1	42.9	9.09	0.9	0.23	ns				
SFRP2	41.84	7.51	2.18	0.66	0.015	0.833	10.84	83.3–100	0.016
UCHL1	36.95	7.6	5.28	2.65	ns				
HIC1	29.87	8.12	4.34	0.58	ns				
SFRP5	26.87	7.57	2.53	0.93	ns				
RASSF1	20.28	6.6	0.87	0.22	ns				
CDKN2A	19.03	7.09	1.84	0.38		Methylation <20%			
MLH1	14.48	6.88	2.05	0.95					
APC	9.2	3.36	2.28	0.58					
MGMT	7.81	3.38	3.5	1.54					
CDH1	1.08	0.1	7.35	2.38					
HNF1B	0.9	0.11	1.23	0.43					

The bold values refer to significant results.
NS, not significant.

DISCUSSION

In this study, we profiled DNA methylation of the promoters of 22 candidate genes in tumoral tissue, normal adjacent tissue from CRC patients, and normal mucosa from controls. In all tumoral samples, we also assessed the mutation status of *KRAS* and *BRAF* to investigate a possible association between hyper-methylation of these candidate genes and *KRAS* and *BRAF* mutations. Our study revealed the hyper-methylation of nine genes out of 22 in tumoral tissue from CRC patients compared to

normal mucosa and identified a specific methylation profile for patients with the *BRAF* V600E mutation.

In the case-control comparison, we found hyper-methylation of genes belonging to the Wnt signaling pathway (*SFRP1*, *SFRP2*, *DKK1*, *DKK2*, and *WIF1*), the TGF-Beta;/SMAD pathway (*RUNX3*), two tumor suppressor genes (*PCDH10* and *OPCML*), and a gene that acts both as an oncogene and a tumor suppressor, depending on the cellular context (*TMEFF2*). Many CRCs feature methylation of the extracellular inhibitors of Wnt signaling, such as *SFRP1*, *SFRP2* (13–15), and

FIGURE 2 | ROC curves for three potential biomarkers combined (SFRP1, PCDH10, and DKK2). AUC = 0.972, p = 0.001.

FIGURE 3 | Significant promoter genes hyper-methylated in the paired analysis (Wilcoxon Signed Rank test): T *vs* NAT.

genes belonging to the dickkopf (Dkk) family. Although genes from the Dkk family largely have an inhibitory effect on this pathway, there is evidence that *DKK2* can also activate Wnt signaling (16). In particular, *DKK2* and *DKK3* were hyper-methylated in tumoral tissue from CRC *versus* control tissues or paired adjacent healthy tissues (13, 17, 18). Furthermore, *DKK3* expression levels were negatively correlated with the rate of promoter methylation (19). Moreover, it has been suggested that *DKK2* and *DKK3* co hyper-methylation might be considered as an independent prognostic predictor (20). Aberrant methylation of *WIF1* was found in CRC (13, 14, 20, 21) with a statistically significant association with increasing tumor stage and tumor differentiation (22).

A high percentage of colorectal cancers contain mutations that disrupt signaling in the pathways of the TGF-β family that regulate the proliferation, differentiation, adhesion, and migration of cells (23). One of the transcriptional effectors involved in TGF-β/SMAD signaling is *RUNX3*. Its promoter has been found to be methylated in approximately 30% of CRCs (15) with high levels both in CRC tissues (17, 24) and in serum, where methylation levels increase with the advancement of pathological stage (25). *PCDH10* belongs to the protocadherin gene family and acts as tumor suppressor gene inhibiting cell proliferation and cell invasion in colorectal cancer development. The rate of *PCDH10* methylation in CRC tissue was significantly higher compared with normal mucosa in different studies (26, 27) as well as in other types of cancer such as lymphomas (28, 29), breast cancer (30) and medulloblastoma (31). Another tumor suppressor gene found hyper-methylated in our study was *OPCML* (Opioid Binding Protein/Cell Adhesion Molecule Like). It is involved in cell growth, invasion, and metastasis. This gene exhibits high promoter methylation and reduced expression levels in different cancers, including ovarian, bladder, nasopharyngeal, and cervical cancers, as well as hepatocellular carcinomas, and colorectal cancer (32, 33). Finally, our comparison between CRC tissue and controls revealed hyper-methylation of *TMEFF2*. In this case, our results are also in line with the literature as it shows aberrant methylation of this gene in CRC (34), suggesting its role as prognostic marker (35).

Mutations in *BRAF* and *KRAS* genes occur in about 10 and 40% of CRC cases respectively (36, 37) and affect different biological pathways. Functionally, these genes are linked to dysregulated DNA methylation (38) and miRNA expression (39). We found a specific methylation profile of tumoral tissues with the *BRAF* V600E mutation that showed increased methylation levels of *SFRP2*, *DKK2*, *PCDH10*, *TMEFF2*, *SFRP1*, and *HS3ST2* compared with tissues without this mutation.

The association of the *BRAF* V600E mutation and *SFRP2* methylation levels has been already shown by Bagci and colleagues (40). In line with the results of this research group, the association between *KRAS* mutations in codon 12 and codon 13 and hyper-methylation of *SFRP2* did not show significant results. Regarding the other identified genes, a specific association between *BRAF* V600E mutation and methylation levels has not, to our knowledge, been reported in the literature.

Epigenetic aberrations are reversible and therefore represent promising targets for novel approaches for cancer therapies (41, 42). The observation that *BRAF*-mutant CRCs displayed six hyper-methylated genes with tumor suppressive functions respect to *BRAF*-negative CRCs may shed light on their possible re-expression following treatment with demethylating agents. In CRC, epigenetic alterations may promote resistance to systemic drugs such as 5-fluorouracil (5-FU), oxaliplatin, and irinotecan (43). For this reason, it has been suggested that combined therapies with epigenetic agents may reverse drug resistance. It has been demonstrated that the use of a demethylating compound, such as 5-azacitidine (5-AC) improves sensitivity and reduces resistance in *BRAF*-mutant CRCs that are usually characterized by very aggressive
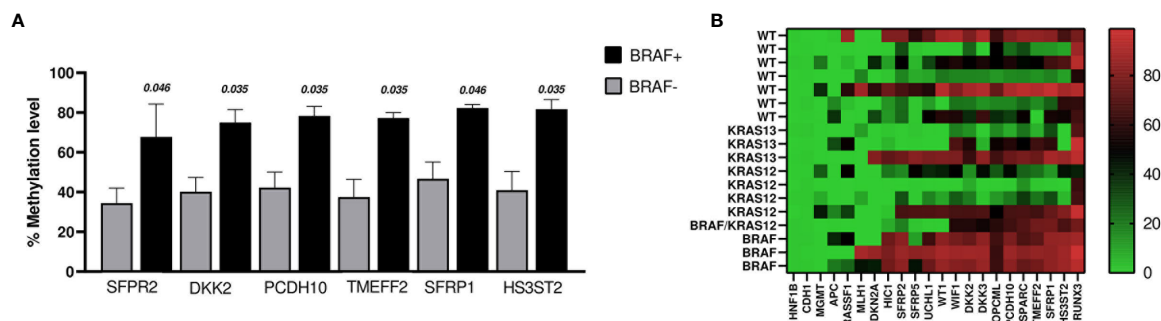
FIGURE 4 | (A) The graph shows the genes whose promoters methylation percentage were significantly higher in BRAF+ cases (n = 4) *vs* BRAF− cases (n = 14). The bars indicate the mean ± SEM. **(B)** Heat map of DNA promoter methylation according to mutation status. Methylation levels vary according to the color scale, ranging from light green (low) to red (high).

behavior, poor prognosis, and resistance to therapies with 5-FU or irinotecans (44). Mao et al. demonstrated that treatment with demethylating agents might prime CRC for *BRAF* inhibitor treatment. In a xenograft model of CRC, these authors showed that pretreatment with 5-AC significantly increased the efficacy of subsequent treatments with a *BRAF* inhibitor (45).

Our results indicate a panel of genes that could be considered for the identification of an epigenetic molecular signature of BRAF-mutant CRCs and thus potential molecular targets for selective epigenetic treatments. Several epigenetic drugs are currently under study in clinical trials for the treatment of CRC (46); epigenetic target-based therapy might be a promising approach in the future to improve the curative treatments of CRC.

Clearly, there were some limitations to this study. Firstly, the sample size was relatively small; secondly, mRNA data, which is useful to perform a correlation between methylation and gene expression, was not available.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Victor Babes National Institute of Pathology. The patients/participants provided their written informed consent to participate in this study.

REFERENCES

1. Wong MCS, Huang J, Lok V, Wang J, Fung F, Ding H, et al. Differences in Incidence and Mortality Trends of Colorectal Cancer Worldwide Based on Sex, Age, and Anatomic Location. *Clin Gastroenterol Hepatol* (2020) 19 (5):955–66.e61. doi: 10.1016/j.cgh.2020.02.026

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.697409/full#supplementary-material

2. Keum N, Giovannucci E. Global Burden of Colorectal Cancer: Emerging Trends, Risk Factors and Prevention Strategies. *Nat Rev Gastroenterol Hepatol* (2019) 16:713–32. doi: 10.1038/s41575-019-0189-8

3. Yamagishi H, Kuroda H, Imai Y, Hiraishi H. Molecular Pathogenesis of Sporadic Colorectal Cancers. *Chin J Cancer* (2016) 35:4. doi: 10.1186/s40880-015-0066-y

4. Montazeri Z, Li X, Nyiraneza C, Ma X, Timofeeva M, Svinti V, et al. Systematic Meta-Analyses, Field Synopsis and Global Assessment of the Evidence of Genetic Association Studies in Colorectal Cancer. *Gut* (2019) 69(8):1460–71. doi: 10.1136/gutjnl-2019-319313

5. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic Alterations During Colorectal-Tumor Development. *N Engl J Med* (1988) 319:525–32. doi: 10.1056/NEJM198809013190901

6. Nguyen HT, Duong H-Q. The Molecular Characteristics of Colorectal Cancer: Implications for Diagnosis and Therapy. *Oncol Lett* (2018) 16:9–18. doi: 10.3892/ol.2018.8679

7. Cionca FL, Dobre M, Dobrea CM, Iosif CI, Comănescu MV, Ardeleanu CM. Mutational Status of KRAS and MMR Genes in a Series of Colorectal Carcinoma Cases. *Rom J Morphol Embryol* (2018) 59:121–9.

8. Lee C-J, Ahn H, Jeong D, Pak M, Moon JH, Kim S. Impact of Mutations in DNA Methylation Modification Genes on Genome-Wide Methylation Landscapes and Downstream Gene Activations in Pan-Cancer. *BMC Med Genomics* (2020) 13:27. doi: 10.1186/s12920-020-0659-4

9. Robertson KD, Jones PA. DNA Methylation: Past, Present and Future Directions. *Carcinogenesis* (2000) 21:461–7. doi: 10.1093/carcin/21.3.461

10. Feinberg AP, Vogelstein B. Hypomethylation Distinguishes Genes of Some Human Cancers From Their Normal Counterparts. *Nature* (1983) 301:89–92. doi: 10.1038/301089a0

11. Kerachian MA, Kerachian M. Long Interspersed Nucleotide Element-1 (LINE-1) Methylation in Colorectal Cancer. *Clin Chim Acta* (2019) 488:209–14. doi: 10.1016/j.cca.2018.11.018

12. Dobre M, Comănescu M, Arsene D, Iosif C. Bussolati G. K-Ras Gene Mutation Status in Colorectal Cancer: Comparative Analysis of Pyrosequencing and PCR-RFLP. *Rom J Morphol Embryol* (2013) 54:567–74.

13. Voorham QJM, Janssen J, Tijssen M, Snellenberg S, Mongera S, van Grieken NCT, et al. Promoter Methylation of Wnt-Antagonists in Polypoid and Nonpolypoid Colorectal Adenomas. *BMC Cancer* (2013) 13:603. doi: 10.1186/1471-2407-13-603

14. Patai ÁV, Valcz G, Hollósi P, Kalmár A, Péterfia B, Patai Á, et al. Comprehensive DNA Methylation Analysis Reveals a Common Ten-Gene Methylation Signature in Colorectal Adenomas and Carcinomas. *PloS One* (2015) 10:e0133836. doi: 10.1371/journal.pone.0133836

15. Tse JWT, Jenkins LJ, Chionh F, Mariadason JM. Aberrant DNA Methylation in Colorectal Cancer: What Should We Target? *Trends Cancer* (2017) 3:698–712. doi: 10.1016/j.trecan.2017.08.003

16. Niehrs C. Function and Biological Roles of the Dickkopf Family of Wnt Modulators. *Oncogene* (2006) 25:7469–81. doi: 10.1038/sj.onc.1210054

17. Silva TD, Vidigal VM, Felipe AV, DE Lima JM, Neto RA, Saad SS, et al. DNA Methylation as an Epigenetic Biomarker in Colorectal Cancer. *Oncol Lett* (2013) 6:1687–92. doi: 10.3892/ol.2013.1606

18. Winther K. The Effect of Beta-Blockade on Platelet Function and Fibrinolytic Activity. *J Cardiovasc Pharmacol* (1987) 10 Suppl 2:S94–8. doi: 10.1097/00005344-198710011-00018

19. Zhao S, Hao C-L, Zhao E-H, Jiang H-M, Zheng H-C. The Suppressing Effects of Dkk3 Expression on Aggressiveness and Tumorigenesis of Colorectal Cancer. *Front Oncol* (2020) 10:600322. doi: 10.3389/fonc.2020.600322

20. Liu X, Fu J, Bi H, Ge A, Xia T, Liu Y, et al. DNA Methylation of SFRP1, SFRP2, and WIF1 and Prognosis of Postoperative Colorectal Cancer Patients. *BMC Cancer* (2019) 19:1212. doi: 10.1186/s12885-019-6436-0

21. Jalilvand A, Soltanpour MS. Promoter Hypermethylation of Wnt/Beta;-Catenin Signaling Pathway Inhibitor WIF-1 Gene and Its Association With MTHFR C677T Polymorphism in Patients With Colorectal Cancer. *Oman Med J* (2020) 35:e131. doi: 10.5001/omj.2020.49

22. Samaei NM, Yazdani Y, Alizadeh-Navaei R, Azadeh H, Farazmandfar T. Promoter Methylation Analysis of WNT/β-Catenin Pathway Regulators and its Association With Expression of DNMT1 Enzyme in Colorectal Cancer. *J BioMed Sci* (2014) 21:73. doi: 10.1186/s12929-014-0073-3

23. Jung B, Staudacher JJ, Beauchamp D. Transforming Growth Factor Beta; Superfamily Signaling in Development of Colorectal Cancer. *Gastroenterology* (2017) 152:36–52. doi: 10.1053/j.gastro.2016.10.015

24. Shin EJ, Kim HJ, Son MW, Ahn TS, Lee HY, Lim DR, et al. Epigenetic Inactivation of RUNX3 in Colorectal Cancer. *Ann Surg Treat Res* (2018) 94:19–25. doi: 10.4174/astr.2018.94.1.19

25. Nishio M, Sakakura C, Nagata T, Komiyama S, Miyashita A, Hamada T, et al. RUNX3 Promoter Methylation in Colorectal Cancer: Its Relationship With Microsatellite Instability and its Suitability as a Novel Serum Tumor Marker. *Anticancer Res* (2010) 30:2673–82.

26. Zhong X, Zhu Y, Mao J, Zhang J, Zheng S. Frequent Epigenetic Silencing of PCDH10 by Methylation in Human Colorectal Cancer. *J Cancer Res Clin Oncol* (2013) 139:485–90. doi: 10.1007/s00432-012-1353-5

27. Li M, Yan DG, Liu JL. Methylation Status of PCDH10 and RASSF1A Gene Promoters in Colorectal Cancer. *Zhonghua Yi Xue Za Zhi* (2016) 96:456–9. doi: 10.3760/cma.j.issn.0376-2491.2016.06.010

28. Narayan G, Xie D, Freddy AJ, Ishdorj G, Do C, Satwani P, et al. PCDH10 Promoter Hypermethylation Is Frequent in Most Histologic Subtypes of Mature Lymphoid Malignancies and Occurs Early in Lymphomagenesis. *Genes Chromosomes Cancer* (2013) 52:1030–41. doi: 10.1002/gcc.22098

29. Huang W, Xue X, Shan L, Qiu T, Guo L, Ying J, et al. Clinical Significance of PCDH10 Promoter Methylation in Diffuse Large B-Cell Lymphoma. *BMC Cancer* (2017) 17:815. doi: 10.1186/s12885-017-3810-7

30. de Ruijter TC, van der Heide F, Smits KM, Aarts MJ, van Engeland M, Heijnen VCG. Prognostic DNA Methylation Markers for Hormone Receptor Breast Cancer: A Systematic Review. *Breast Cancer Res* (2020) 22:13. doi: 10.1186/s13058-020-1250-9

31. Bertrand KC, Mack SC, Northcott PA, Garzia L, Dubuc A, Pfister SM, et al. PCDH10 is a Candidate Tumour Suppressor Gene in Medulloblastoma. *Childs Nerv Syst* (2011) 27:1243–9. doi: 10.1007/s00381-011-1486-x

32. Li C, Tang L, Zhao L, Li L, Xiao Q, Luo X, et al. OPCML is Frequently Methylated in Human Colorectal Cancer and its Restored Expression Reverses EMT *via* Downregulation of Smad Signaling. *Am J Cancer Res* (2015) 5:1635–48.

33. Abdel-Rahman WM, Lotsari-Salomaa JE, Kaur S, Niskakoski A, Knuutila S, Järvinen H, et al. The Role of Chromosomal Instability and Epigenetics in Colorectal Cancers Lacking β-Catenin/TCF Regulated Transcription. *Gastroenterol Res Pract* (2016) 2016:6089658. doi: 10.1155/2016/6089658

34. Gonzalo V, Lozano JJ, Muñoz J, Balaguer F, Pellisé M, Rodríguez de Miguel C, et al. Aberrant Gene Promoter Methylation Associated With Sporadic Multiple Colorectal Cancer. *PloS One* (2010) 5:e8777. doi: 10.1371/journal.pone.0008777

35. Lam K, Pan K, Linnekamp JF, Medema JP, Kandimalla R. DNA Methylation Based Biomarkers in Colorectal Cancer: A Systematic Review. *Biochim Biophys Acta* (2016) 1866:106–20. doi: 10.1016/j.bbcan.2016.07.001

36. Caputo F, Santini C, Bardasi C, Cerma K, Casadei-Gardini A, Spallanzani A, et al. BRAF-Mutated Colorectal Cancer: Clinical and Molecular Insights. *Int J Mol Sci* (2019) 20:5369. doi: 10.3390/ijms20215369

37. Dobre M, Dinu DE, Panaitescu E, Bîrlă RD, Iosif CI, Boeriu M, et al. KRAS Gene Mutations - Prognostic Factor in Colorectal Cancer? *Rom J Morphol Embryol* (2015) 56:671–8. doi: 10.26226/morressier.57a2f938d462b8028d88feff

38. Tanaka N, Huttenhower C, Nosho K, Baba Y, Shima K, Quackenbush J, et al. Novel Application of Structural Equation Modeling to Correlation Structure Analysis of Cpg Island Methylation in Colorectal Cancer. *Am J Pathol* (2010) 177:2731–40. doi: 10.2353/ajpath.2010.100361

39. Milanesi E, Dobre M, Bucuroiu AI, Herlea V, Manuc TE, Salvi A, et al. MiRNAs-Based Molecular Signature for KRAS Mutated and Wild Type Colorectal Cancer: An Explorative Study. *J Immunol Res* (2020) 2020:4927120. doi: 10.1155/2020/4927120

40. Bagci B, Sari M, Karadayi K, Turan M, Ozdemir O, Bagci G. KRAS, BRAF Oncogene Mutations and Tissue Specific Promoter Hypermethylation of Tumor Suppressor SFRP2, DAPK1, MGMT, HIC1 and P16 Genes in Colorectal Cancer Patients. *Cancer Biomark* (2016) 17:133–43. doi: 10.3233/CBM-160624

41. Suvà ML, Riggi N, Bernstein BE. Epigenetic Reprogramming in Cancer. *Science* (2013) 339:1567–70. doi: 10.1126/science.1230184

42. Baretti M, Azad NS. The Role of Epigenetic Therapies in Colorectal Cancer. *Curr Probl Cancer* (2018) 42:530–47. doi: 10.1016/j.currproblcancer.2018.03.001

43. Crea F, Nobili S, Paolicchi E, Perrone G, Napoli C, Landini I, et al. Epigenetics and Chemoresistance in Colorectal Cancer: An Opportunity for Treatment Tailoring and Novel Therapeutic Strategies. *Drug Resist Update* (2011) 14:280–96. doi: 10.1016/j.drup.2011.08.001

44. Sharma A, Vatapalli R, Abdelfatah E, Wyatt McMahon K, Kerner Z, A Guzzetta A, et al. Hypomethylating Agents Synergize With Irinotecan to Improve Response to Chemotherapy in Colorectal Cancer Cells. *PloS One* (2017) 12:e0176139. doi: 10.1371/journal.pone.0176139

45. Mao M, Tian F, Mariadason JM, Tsao CC, Lemos R, Dayyani F, et al. Resistance to BRAF Inhibition in BRAF-Mutant Colon Cancer can be Overcome With PI3K Inhibition or Demethylating Agents. *Clin Cancer Res* (2013) 19:657–67. doi: 10.1158/1078-0432.CCR-11-1446

46. Kishore C. Epigenetic Regulation and Promising Therapies in Colorectal Cancer. *Curr Mol Pharmacol* (2021). doi: 10.2174/1874467214666210126105345