RESEARCH PAPER

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Gene promoter methylation in colorectal cancer and healthy adjacent mucosa specimens

Correlation with physiological and pathological characteristics, and with biomarkers of one-carbon metabolism

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We evaluated the promoter methylation levels of the *APC, MGMT,* h*MLH1, RASSF1A* and *CDKN2A* genes in 107 colorectal cancer (CRC) samples and 80 healthy adjacent tissues. We searched for correlation with both physical and pathological features, polymorphisms of folate metabolism pathway genes (*MTHFR*, *MTRR*, *MTR*, *RFC1*, *TYMS*, and *DNMT3B*), and data on circulating folate, vitamin B12 and homocysteine, which were available in a subgroup of the CRC patients. An increased number of methylated samples were found in CRC respect to adjacent healthy tissues, with the exception of *APC*, which was also frequently methylated in healthy colonic mucosa. Statistically significant associations were found between *RASSF1A* promoter methylation and tumor stage, and between h*MLH1* promoter methylation and tumor location. Increasing age positively correlated with both h*MLH1* and *MGMT* methylation levels in CRC tissues, and with *APC* methylation levels in the adjacent healthy mucosa. Concerning gender, females showed higher h*MLH1* promoter methylation levels with respect to males. In CRC samples, the *MTR* 2756AG genotype correlated with higher methylation levels of *RASSF1A*, and the *TYMS* 1494 6bp ins/del polymorphism correlated with the methylation levels of both *APC* and h*MLH1*. In adjacent healthy tissues, *MTR* 2756AG and *TYMS* 1494 6bp del/del genotypes correlated with *APC* and *MGMT* promoter methylation, respectively. Low folate levels were associated with h*MLH1* hypermethylation. Present results support the hypothesis that DNA methylation in CRC depends from both physiological and environmental factors, with one-carbon metabolism largely involved in this process.

Introduction

Colorectal cancer (CRC) represents a serious health concern, with over one-million new cases diagnosed worldwide every year.[1] The disease occurs sporadically in most of the cases (75–80%) as a result of a multi-step process leading to the accumulation of genetic and epigenetic alterations in colon mucosa cells, primarily affecting oncogenes, tumor suppressor genes and DNA repair genes.[2] DNA methylation involves the covalent addition of a methyl group to the 5′ position on cytosine residues, usually in CpG dinucleotides, and is one of the most studied epigenetic marks in CRC.[3] Methylation of CpG islands (domains unusually enriched with CpG dinucleotides) in the promoter region of a gene is commonly associated with gene silencing as it inhibits the access of the transcriptional machinery to the promoter, while promoter demethylation is a condition enabling gene expression.[3]

The genes frequently methylated in CRC tissues, as well as the factors that can contribute to the methylation levels of those genes, including aging, gender, dietary habits, life-styles,

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Table 1. Demographic characteristics of the study population

Total subjects	Age (Mean ± SD)	Gender	Stage (TNM)	Tumor size	Location[a]	Methylated genes[b]
107	71.07 ± 12.98	M: 61	Adenoma: 11	T1: 3	C: 73	*APC*: 53 (49.5%)
		F: 46	I: 17	T2: 19	S: 12	*MGMT*: 45 (42.0%)
			II: 32	T3: 66	SR: 17	*CDKN2A*: 31 (29.0%)
			III: 33	T4: 8	R: 3	h*MLH1*: 18 (16.8%)
			IV: 14		M: 1	*RASSF1A*: 18 (16.8%)

[a]Location: C, colon; S, sigma; SR, sigma-rectum; R, rectum; M, mixed. [b]Methylated genes: number (percent) of methylated samples in CRC tissues

Table 2. Methylation data observed in CRC and adjacent healthy tissues in terms of numbers of methylated samples and percentage for each of the studied genes

Total patients = 80 (Females = 34; Males = 46)	Cancer tissue N° (%)	Adjacent healthy tissue N° (%)
APC	39 (48.7)	37 (46.2)
MGMT	36 (45.0)	14 (17.5)[a]
CDKN2A	24 (30.0)	5 (6.25)[a]
h*MLH1*	13 (16.2)	1 (1.25)[a]
RASSF1A	10 (12.5)	1 (1.25)[a]

[a]Significant difference CRC vs. Adjacent healthy tissue ($P < 0.01$, Chi square or Fisher Exact Test).

environmental agents, and medications, have been recently reviewed.[2-5] The ever-growing number of genes that show impaired methylation in CRC emphasizes the crucial role of epigenetic alterations for future diagnosis, prognosis and choice of therapeutic strategies, and active research is currently ongoing to develop rapid, cost effective and reproducible tools for the detection of epigenetic marks.[3,4]

DNA methylation is largely dependent on folate bioavailability, and impairments within the folate (one-carbon) metabolic pathway can be of relevance for cancer development.[5] There is consensus in the literature indicating that individuals who habitually consume the greatest quantities of folate, or who have the highest concentrations of blood folate, are at decreased risk for the development of CRC.[6,7] However, folate derivatives are cofactors in nucleotide synthesis and high levels of the vitamin could therefore promote the proliferation of rapidly dividing cells.[6,7] Indeed, the current opinion is that higher folate intake is protective against CRC development in nearly all circumstances except for those individuals who consume an excessive amount of the vitamin and have existing neoplastic lesions.[6,7]

Given its pivotal role in DNA methylation processes, one-carbon metabolism has been largely investigated as a potential modulator of DNA methylation in CRC. Indeed, researchers started addressing the relationship between promoter methylation of CRC-related genes and folate intake at the end of the last century.[8] Those studies were followed by several papers aimed at addressing the possible contribution of polymorphisms of genes involved in folate metabolism as modulators of DNA methylation changes in CRC.[9-12]

In the present study we assessed the methylation levels in the promoters of five key CRC genes (*APC, MGMT,* h*MLH1, RASSF1A,* and *CDKN2A/p16*) in both surgically resected cancer tissues and healthy adjacent mucosa of a group of diagnostically confirmed sporadic CRC individuals. We then searched for correlation between the methylation levels of each gene and age, gender, tumor size, cancer stage, and nine functional polymorphisms of six genes (*MTHFR, MTR, MTRR, RFC1 [SLC19A1], TYMS,* and *DNMT3B*) involved in one-carbon metabolism. Moreover, in a subgroup of the patients, data on circulating levels of folate, homocysteine, and vitamin B12 were available, and we searched for correlation between those biomarkers and the methylation profiles of the genes under investigation.

Results

Comparison between tumor and healthy tissues in CRC patients

Table 1 shows the demographic and clinical characteristics of the 107 CRC patients recruited for the present study, and the number of methylated samples for each of the studied genes in the CRC tissue. For 80 out of 107 CRC patients (34 females, 46 males) both tumor and healthy adjacent tissue specimens, located near the cancerous lesion (about 20 cm distance), were collected to analyze the methylation levels in the promoters of the chosen genes: *APC, MGMT,* h*MLH1, RASSF1A,* and *CDKN2A/p16* (**Table 2**).

Table 2 shows the methylation data observed in CRC and adjacent healthy tissues in terms of numbers of methylated samples for each of the studied genes. For all of them, with the exception of *APC*, we observed a statistically significant increased number of methylated samples in CRC vs. adjacent healthy tissues (Chi square or Fisher exact test $P < 0.01$). By contrast, *APC* showed a similar frequency of methylated samples in both CRC and healthy tissues.

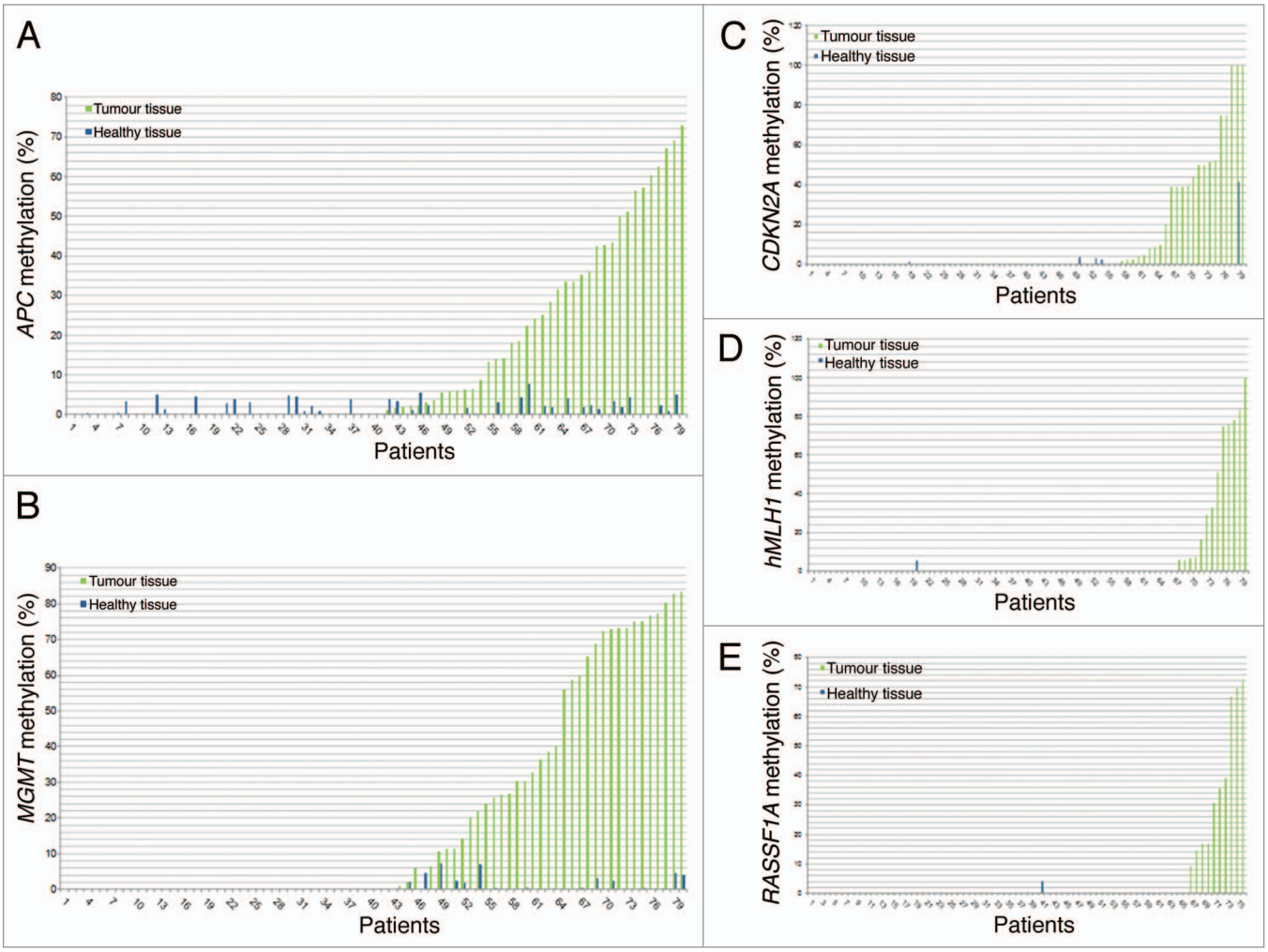

Figure 1. *APC* (**A**), *MGMT* (**B**), *CDKN2A* (**C**), h*MLH1* (**D**) and *RASSF1A* (**E**) methylation in tumor and adjacent healthy tissue for each patient.

For each of the studied subjects and for each of the analyzed genes the percentage of promoter methylation observed in both CRC and healthy specimens is shown (**Fig. 1**). *APC* and *MGMT* resulted frequently methylated in healthy tissues; however, promoter methylation levels were relatively low and not higher than 10% (**Fig. 1A and B**). Similar results were obtained also for the other three genes, which, however, resulted methylated only in very few healthy tissue samples (**Fig. 1C–E**). Interestingly, only for *CDKN2A*, one subject showed almost 40% promoter methylation also in the normal mucosa. That patient showed 100% promoter methylation in the cancerous tissue and experienced CRC recurrence (**Fig. 1C**). No correlation between the methylation levels in CRC and healthy tissues was observed (linear regression $P > 0.05$) for each of the studied genes.

Correlation between methylation levels and both clinicopathological and physical features

For each of the studied genes we analyzed the correlation between promoter methylation and both physical and clinicopathological features of the CRC patients such as age, gender, TNM stage, tumor size and tumor location in all the 107 CRC specimens and also assessed the contribution of age and gender with respect to gene promoter methylation in the 80 healthy tissues analyzed. This last analysis was restricted to *APC* and *MGMT* genes, since they were those most frequently methylated in healthy tissues (**Table 2**).

A statistically significant association between *RASSF1A* promoter methylation and stage was found ($P = 0.01$), with stages I and III showing higher methylation than adenomas and/or other stages (**Fig. 2A**). A statistically significant association between h*MLH1* promoter methylation and tumor location was observed ($P = 0.04$). Particularly, after stratification of the samples into three groups (right colon, left colon, and sigma/rectum) higher h*MLH1*methylation was observed in the right colon with respect to cancers of the sigma/rectum (**Fig. 2B**).

By correlating promoter methylation status with gender, a statistically significant association between h*MLH1* promoter methylation in CRC tissues and gender was found. Particularly, females showed higher methylation levels with respect to males ($P = 0.03$) (**Fig. 3**). No statistically significant association between the methylation levels of other genes and gender was found in either CRC or adjacent healthy tissues (data not shown). A statistically significant association between *MGMT* and h*MLH1*

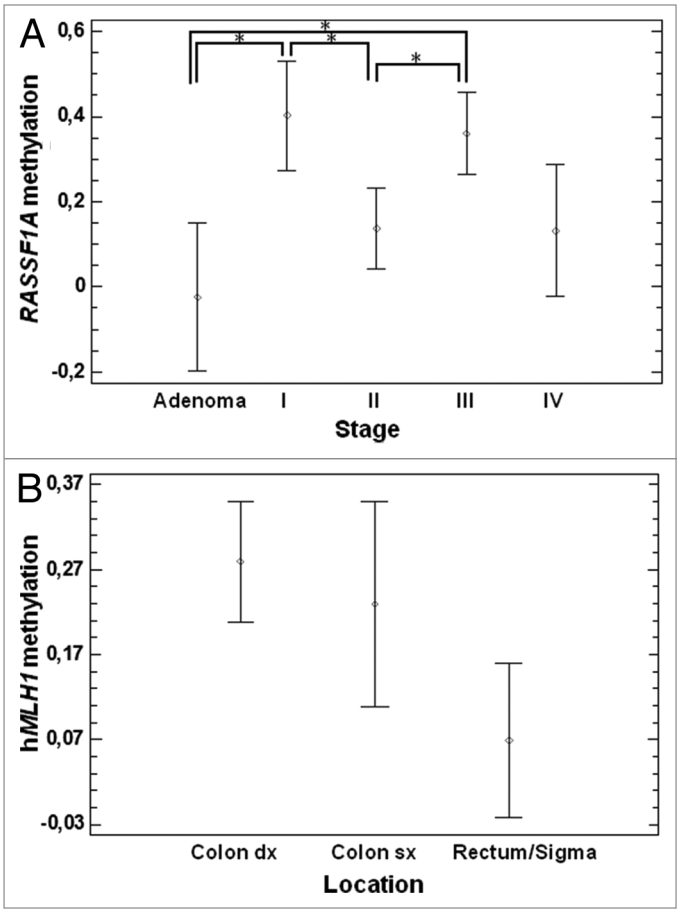

Figure 2. Correlation between gene promoter methylation, tumor stage and location. (**A**) A correlation between stages and *RASSF1A* promoter methylation was found, and particularly stage I and III showed hypermethylation with respect to adenomas and/or other stages ($P = 0.01$). (**B**) Correlation between location and h*MLH1* promoter methylation. We observed a significant difference between right colon and sigma/rectum ($P = 0.04$).

promoter methylation and age ($P = 0.002$ and $P = 0.0006$, respectively) has been observed in CRC tissues. Particularly, an increase of methylation levels of these two genes with aging was noticed (**Fig. 4A and B**). Moreover, a significant correlation between age and *APC* promoter methylation in adjacent healthy tissues was observed ($P = 0.01$, **Fig. 4C**).

Polymorphisms in one-carbon metabolism genes and gene promoter methylation

Allele and genotype frequencies of each of the studied polymorphisms (*MTHFR* 677C > T, *MTHFR* 1298A > C, *MTRR* 66A > G, *MTR* 2756A > G, *RFC1* 80G > A, *TYMS* 28 bp repeats, *TYMS* 1494 6 bp ins/del, *DNMT3B* -149C > T, and *DNMT3B* -579G > T) are shown in **Table 3**, and their correlation with CpG island methylation of *APC*, *MGMT*, h*MLH1*, *RASSF1A* and *CDKN2A* gene promoters was tested in CRC samples, as well as in the adjacent healthy tissues (only for *APC* and *MGMT* promoters). A statistically significant correlation was found between the *MTR* 2756A > G polymorphism and: (1) *RASSF1A* in CRC samples ($P = 0.02$, **Fig. 5A**), (2) *APC* in

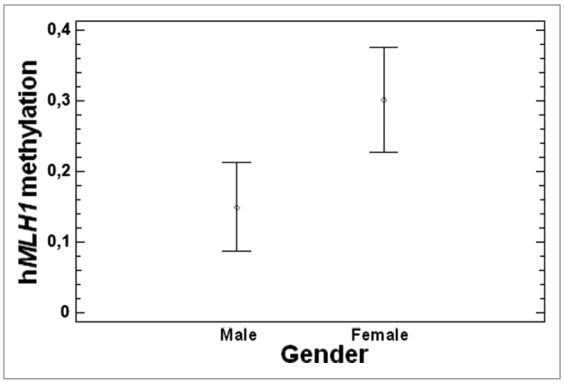

Figure 3. Correlation between gender and h*MLH1* promoter methylation ($P = 0.03$).

the healthy mucosa samples ($P = 0.03$, **Fig. 5B**). Particularly, the AG genotype correlates with increased *RASSF1A* and *APC* promoter methylation with respect to the AA one (**Fig. 5**). Moreover, we observed a statistically significant correlation between the *TYMS* 1494 6bp ins/del polymorphism and both *APC* and h*MLH1* promoter methylation levels in CRC tissues ($P = 0.02$ and 0.03, respectively, **Fig. 6A and B**), and *MGMT* promoter methylation in the healthy tissue ($P = 0.02$, **Fig. 6C**). In CRC specimens the presence of the 6bp del/del genotype correlates with decreased promoter methylation levels, while in the normal mucosa with increased methylation.

Folate and homocysteine values in CRC patients and their correlation with gene promoter methylation

Folates, homocysteine, and vitamin B12 values have been measured in blood at colonoscopy. For some patients these biomarkers were not available, due to technical or other reasons as explained in details in the materials and methods section. Overall, we collected those values for about 40 CRC patients (**Table 4**). We observed a correlation ($P = 0.05$) between plasma folate levels and h*MLH1* promoter methylation, showing individuals with folate levels below the normal range higher methylation (**Fig. 7**).

Immunohistochemical analysis for MLH1 protein and correlation with the *BRAF* V600E mutation

Immunohistochemical analysis was performed to evaluate the correlation between h*MLH1* promoter methylation and MLH1 protein expression (**Fig. 8**). A total of 30 samples was assessed, 15 of them showing h*MLH1* promoter methylation and the other 15 with 0% promoter methylation. 80% of the subjects showing h*MLH1* promoter methylation were negative for MLH1 immunostaining. Moreover, those few subjects (20%) showing h*MLH1* promoter methylation and positive MLH1 immunostaining had very low percentages of gene promoter methylation (**Table 5**). By contrast, 93% of the subjects showing 0% promoter methylation were positive for MLH1 immunostaining (**Table 5**). Present results indicate a very good correlation between h*MLH1* promoter methylation and protein expression ($P < 0.001$). In addition, 10 out of the 15 samples with h*MLH1* promoter methylation were carriers of the *BRAF* V600E mutation, most of them showing also lack of MLH1

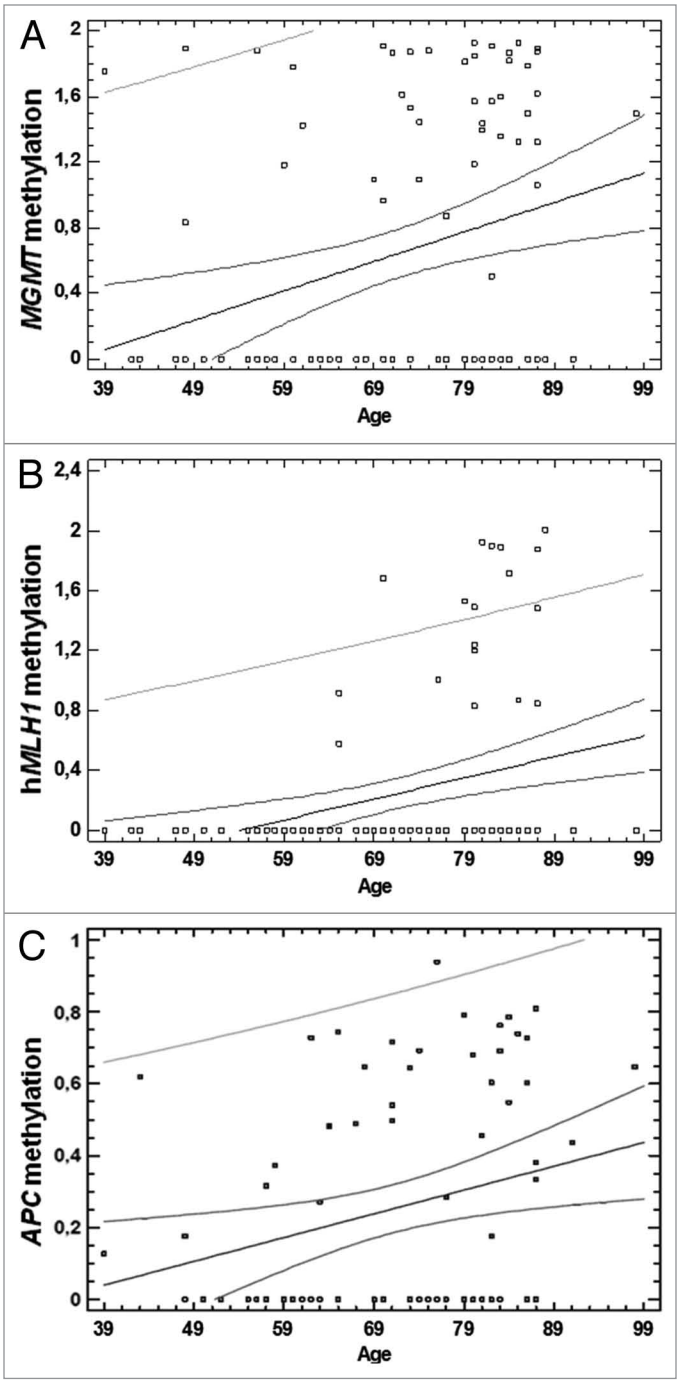

Figure 4. Correlation between age and gene promoter methylation. (**A**) Correlation between age and *MGMT* promoter methylation in CRC tissues ($P = 0.002$). (**B**) Correlation between age and h*MLH1* promoter methylation in CRC tissues ($P = 0.0006$). (**C**) Correlation between age and *APC* promoter methylation in adjacent healthy tissues ($P = 0.01$).

protein and an elevated number of methylated genes among the five under investigation in the present study (**Table 5**). The *BRAF* V600E mutation was not found among the 15 individuals showing 0% h*MLH1* promoter methylation (**Table 5**).

Table 3. Distribution of genotypes

Polymorphisms	Total	Genotype frequencies		
RFC1 80G > A	93	GG (32.3%)	GA (50.5%)	AA (17.2%)
MTHFR 677C > T	93	CC (34.4%)	CT (47.3%)	TT (18.3%)
MTHFR 1298 A > C	94	AA (55.3%)	AC (36.2%)	CC (8.5%)
MTRR 66A > G	94	AA (41.5%)	AG (39.4%)	GG (19.1%)
MTR 2756A > G	94	AA (72.3%)	AG (27.7%)	GG (0.0%)
TYMS 1494 6bp ins/del	92	$6^{+}6^{+}$ (34.8%)	$6^{+}6^{-}$ (51.1%)	$6^{-}6^{-}$ (14.1%)
TYMS 28 bp repeats	92	3R3R (33%)	3R2R (46%)	2R2R (21%)*
DNMT3B -149C > T	92	CC (41.3%)	CT (44.6%)	TT (12.0%)
DNMT3B -579G > T	92	GG (45.7%)	GT (44.6%)	TT (9.7%)

*For four patients the genotypes were 2R4R and for two other patients 3R4R and 3R5R, respectively.

Discussion

It is now largely accepted that cancer is a multi-step process resulting from the accumulation of both genetic and epigenetic alterations of the genome.[13] Gene mutations and epigenetic modifications have been initially viewed as two separate mechanisms participating in carcinogenesis. However, recent evidence points to a crosstalk between these two mechanisms in cancer formation, suggesting that gene mutations have the potential of disrupting several epigenetic patterns and that epigenetic modifications can drive genome instability and mutagenesis.[14,15] For example, the epigenetic inactivation of DNA repair genes, such as h*MLH1*, *MGMT*, and others is often associated with genome instability and increased frequency of point mutations in cancer-related genes.[14] Physiological aging and gender differences have been often associated with changes of epigenetic patterns, that can also be induced by exposure to environmental or lifestyle factors.[2-5] Among dietary habits linked to changes of DNA methylation, particular attention has been dedicated to the folate metabolic pathway, given its pivotal role in providing one-carbon moieties for DNA methylation reactions.[5]

To further address this issue, here we evaluated the methylation profiles of five CRC-related genes, namely *APC*, *MGMT*, h*MLH1*, *RASSF1A*, and *CDKN2A* by means of MS-HRM technique in cancer tissues of 107 CRC patients as well as in the healthy adjacent mucosa of 80 of them. We then searched for correlation of the promoter methylation profiles of the selected genes with both physiological and pathological characteristics of the patients, and with a panel of biomarkers of one-carbon metabolism.

Our analysis revealed that *APC* was the most frequently methylated gene in both CRC (49.5%) and healthy mucosa tissues (46.2%), although the level of promoter methylation in healthy tissues was relatively low and not higher than 10%. No correlation between the methylation levels in CRC and healthy tissues was observed, as indicated by several subjects showing low levels of methylation in the healthy tissue and no methylation in the CRC one, or high levels of methylation in the CRC tissue but no or very low promoter methylation in the adjacent healthy mucosa.

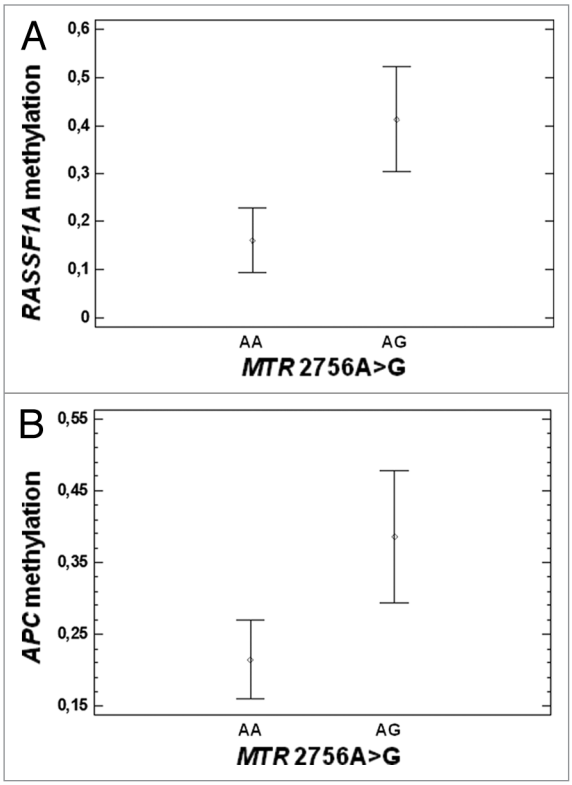

Figure 5. Correlation between the *MTR* 2756A > G polymorphism and gene promoter methylation. (**A**) Correlation with *RASSF1A* promoter methylation in CRC tissues ($P = 0.02$). (**B**) Correlation with *APC* promoter methylation in healthy tissues ($P = 0.03$).

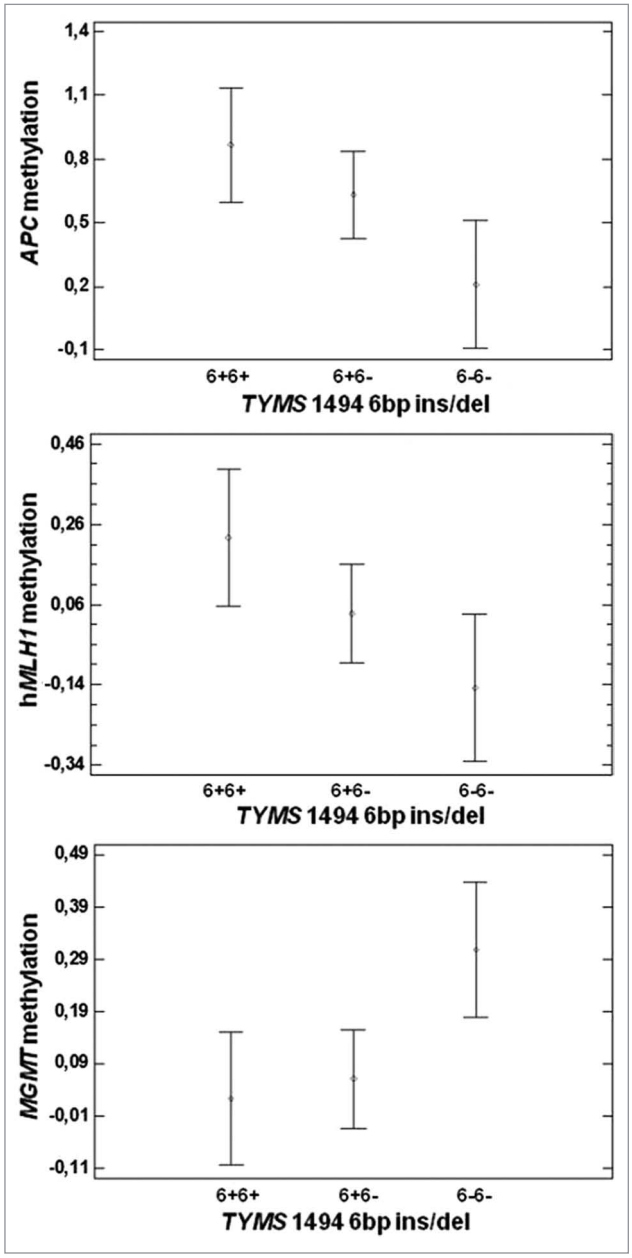

Figure 6. (**A**) Correlation between *TYMS* 1494 6bp ins/del polymorphism and *APC* promoter methylation in CRC tissues ($P = 0.02$). (**B**) Correlation between *TYMS* 1494 6bp ins/del polymorphism and h*MLH1* promoter methylation in CRC tissues ($P = 0.03$). (**C**) Correlation between *TYMS* 1494 6bp ins/del polymorphism and *MGMT* promoter methylation in healthy tissues ($P = 0.02$).

The tumor suppressor gene *APC* is one of the key components of the Wnt pathway, germline mutations in *APC* are associated with hereditary familial adenomatous polyposis (FAP) or attenuated FAP, and somatic mutations are common in sporadic CRC.[16] *APC* hypermethylation is also frequent in sporadic CRC and may cause transcriptional silencing occurring early during colon neoplasia progression, but the reported methylation status of the *APC* promoter varies greatly among studies performed in different populations.[16-19] Moreover, hypermethylation of the *APC* promoter has been shown to be relatively common in the normal colonic mucosa.[19] Present results are in agreement with recent reports in Swedish and Vietnamese CRC patients, where methylation of the *APC* gene was detected with similar frequencies between the cancerous and normal tissues.[19] We also observed that *APC* methylation in normal colonic mucosa increased significantly with age. This is in agreement with some previous reports of an age-related methylation of tumor suppressor genes in normal colorectal mucosa, that might represent physiological changes and/or constitute pre-neoplastic lesions.[20,21]

The *MGMT* gene is involved in DNA repair processes (mismatch repair) and in our cohort resulted hypermethylated in more than 40% of CRC tissues and in 17.5% of normal colonic mucosa. Again, we observed higher methylation levels in the affected tissue than in the adjacent healthy mucosa. Several previous reports of the literature suggest that *MGMT* hypermethylation results in gene silencing and increased rate of mutations in normal colonic mucosa that might represent an initiating step in the development of mismatch repair-deficient CRC.[22-25] We also observed an age-related increase in *MGMT* methylation in CRC tissues in agreement with previous reports by Tserga et al.[26] that showed a correlation between age and *MGMT* promoter methylation in breast cancer specimens, and Menigatti et al.[21] that showed an age related increase of *MGMT* methylation in healthy colonic mucosa samples.

Table 4. Folate, homocysteine and vitamin B12 values in CRC patients (mean and standard deviation)

	Folate (ng/ml)			Homocysteine (μmol/l)			Vitamin B12		
	Total	4.6–18.7 ng/ml[a]	< 4.6 ng/ml[b]	Total	4.3–11.1 μmol/l[a]	>11.1 μmol/l[c]	Total	191–663 pg/ml[a]	> 663 pg/ml[c]
N° of patients	36	23	13	37	22	15	39	35	4
Mean ± SD	5.5 ± 1.7	6.5 ± 1.1	3.6 ± 0.6	10.8 ± 4.3	8.0 ± 1.9	14.3 ± 3.2	447 ± 152	414 ± 119	738 ± 226

[a]Normal range, [b]below the normal range, [c]above the normal range.

CDKN2A was methylated in 29% of CRC samples and both *RASSF1A* and h*MLH1* in 16.8% of them; by contrast, they resulted methylated only in a few healthy mucosa tissues. Also the reported methylation frequencies of those genes varied within studies. For example, concerning the DNA repair h*MLH1* gene, Huang et al.[27] observed h*MLH1* methylation in 20% of the CRC tissues analyzed, and others reported that it was methylated only in 1.8% of 112 adenomas.[24] Arai et al.[28] observed that the proportion of gastric and colorectal carcinomas with h*MLH1* hypermethylation increases with age, reaching 25–30% of all carcinomas in elderly subjects, and those cancers are usually characterized by microsatellite instability and favorable prognosis. We also observed a significant increased h*MLH1* promoter methylation with aging, as well as gender differences with females showing higher h*MLH1* methylation than males. Those data are in agreement with results by Menigatti et al.[21] that showed increased h*MLH1* methylation with both increasing age and female gender in the healthy colonic mucosa. Ramírez et al.[29] observed that methylation of the DNA repair genes h*MLH1* and *MGMT* in normal mucosa correlated significantly with microsatellite instability and k-ras activation in the neighboring cancerous mucosa tissue, suggesting that epigenetic alterations in the mucosa surrounding cancerous neoplastic lesions might be due to a "field effect" occurring in early stages of carcinogenesis and working as a substrate for the subsequent accumulation of genetic alterations.

We also observed a strong correlation between h*MLH1* promoter methylation and MLH1 protein levels evaluated by means of immunohistochemical analysis. Moreover, almost 70% of the samples showing h*MLH1* promoter methylation were also carriers of the *BRAF* V600E mutation. Promoter hypermethylation of the mismatch repair gene h*MLH1* is associated with microsatellite instability (MSI) and *BRAF* mutations in CRC.[30] Indeed, h*MLH1* methylation, MSI, and the *BRAF* V600E mutation, are often observed in CIMP high tumors, a specific subgroup of CRC denoted as the "CpG island methylator phenotype" as it displays extensive levels of methylated genes.[30] Therefore, it is reasonable to speculate that factors linked to h*MLH1* methylation are also linked to the CIMP high status. In this regard, we observed increased h*MLH1* methylation in females than males, as well as increased h*MLH1* methylation with age and in right colon tumors than in those of the sigma/rectum. Present data are in agreement with several previous reports suggesting that CIMP high tumors are associated with older age, female gender, proximal tumor location, microsatellite instability, *BRAF* mutation, and h*MLH1* methylation.[28,30,31] However, the panel of biomarkers used to evaluate the CIMP high status is not yet standardized,[30] we had no opportunity to include all the most commonly studied CIMP biomarkers in the present investigation, and we only had a limited number of subjects with both h*MLH1* methylation and *BRAF* mutation. Therefore, present factors linked to h*MLH1* methylation are only indicative of a possible link to the CIMP high condition.

Also, *CDKN2A* methylation has been largely studied in CRC tissues and adjacent healthy mucosa.[29] A recent large-scale study revealed that *CDKN2A* methylation in the normal mucosa can range from 0 to > 90% in some cases.[32] Indeed, we observed a patient with more than 40% *CDKN2A* methylation in the normal mucosa. Similarly to the present study, *CDKN2A* methylation was observed in 24.8% of CRC specimens of Spanish CRC patients.[33] Despite some reports suggesting that *CDKN2A* methylation might be associated with good prognosis,[33] recent large-scale studies and a literature meta-analysis revealed that it is a marker of poor prognosis in CRC and other surgically treated cancers.[32,34]

RASSF1A methylation levels have been also largely investigated in CRC tissues. Nillson et al.[35] reported it to be methylated in 14% of Swedish CRC tissues and 0% of the adjacent healthy mucosa, values that are really similar to the present 16.8% in CRC tissues and 1.25% in adjacent mucosa. Gene promoter methylation was also associated with poor prognosis in a 20 y follow up study of those patients.[35] Also in this case, *RASSF1A* methylation levels varied with studies ranging from 10–20% of CRC subjects up to 40–50%, with some authors observing *RASSF1A* methylation in early stages of CRC, and others reporting it more frequently methylated in later stages.[35-37] Some correlations with tumor stage were also observed in the present study.

The correlation between polymorphisms of genes involved in folate metabolism and DNA methylation has been investigated by several authors, and we recently reviewed the literature in the field.[5]

A very interesting finding of the present study was the association between the *MTR* 2756A > G polymorphism and promoter methylation levels of *RASSF1A* in CRC tissues and *APC* in healthy mucosa. The possible contribution of the *MTR* 2756A > G polymorphism to CRC risk has been largely investigated, often with conflicting results among studies, and a recent meta-analysis suggests that it could be associated with increased CRC risk in alcohol consumers.[38] Methionine synthase is the enzyme that catalyzes the transmethylation of hcy to methionine, which is then used to form *S*-adenosylmethionine (SAM), the major intracellular methylating agent, and alcohol consumption reduces MTR activity and the production of SAM.[39] Noteworthy, 89% of our CRC patients reported to be daily alcohol consumers, usually

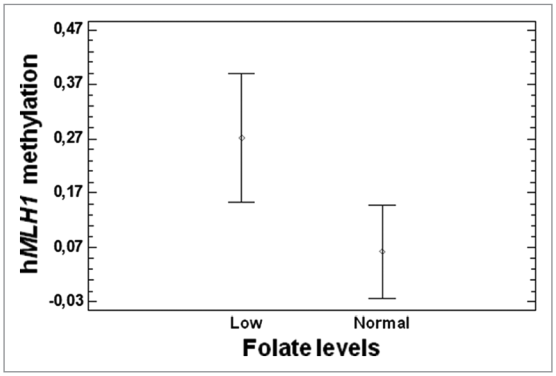

Figure 7. Correlation between plasma folate levels and h*MLH1* promoter methylation ($P = 0.05$).

wine twice a day. Association of the *MTR* 2756A > G polymorphism with CRC risk has been also reported in smokers,[38] but in this case only a few of our patients (7.5%) were smokers or ex-smokers from less than 10 y.

Another interesting finding was a correlation between the *TYMS* 1494 6bp ins/del polymorphism and both *APC* and h*MLH1* promoter methylation in CRC tissues, and with *MGMT* methylation in the healthy mucosa. Thymidylate synthase is required for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymine monophosphate (dTMP) in the de novo synthesis of pyrimidines, and the studied polymorphism is believed to impair TYMS mRNA expression or stability, ultimately leading to reduced protein levels in del/del homozygous individuals.[40] Reduced TYMS levels in del/del individuals might impair the one-carbon metabolic pathway, particularly in rapidly dividing cells such as cancerous ones,[41] thereby resulting in impaired methylation of certain genes, as we observed. Differences between cancer tissue and normal mucosa cells might reflect the different rate of cellular division in the two tissues and the consequent different need of TYMS activity and DNA precursors. In addition, genetic association studies and their meta-analysis suggest a contribution of *TYMS* polymorphisms to CRC risk.[42,43]

Data on folate, vitamin B12 and homocysteine, were available only for a small subgroup of the patients, however we observed a significant correlation between folate levels and h*MLH1* promoter methylation in CRC tissues. Complex interactions among folate, hcy, vitamin B12, other B group vitamins and polymorphisms of one-carbon metabolic genes are known to affect DNA methylation in CRC,[5] that we could not evaluate due to the scarce availability of biochemical data.

The method used in the present study does not allow to get information on the methylation status of each single CpG within the region under investigation, but provides an average methylation value of all the CpG sites in the fragment analyzed.[44] Our recent investigation of the methylation levels of *APC, MGMT,* h*MLH1*, and *CDKN2A* promoter regions by means of pyrosequencing revealed that all the CpG sites analyzed tended to be methylated and no difference in mean methylation levels among different sites was observed for *APC*, h*MLH1*, and *CDKN2A* promoters,[45] while a few CpG sites in the promoter of the *MGMT* gene tended to be less methylated (average 35–40% methylation) than the others (average 60–70% methylation).[45]

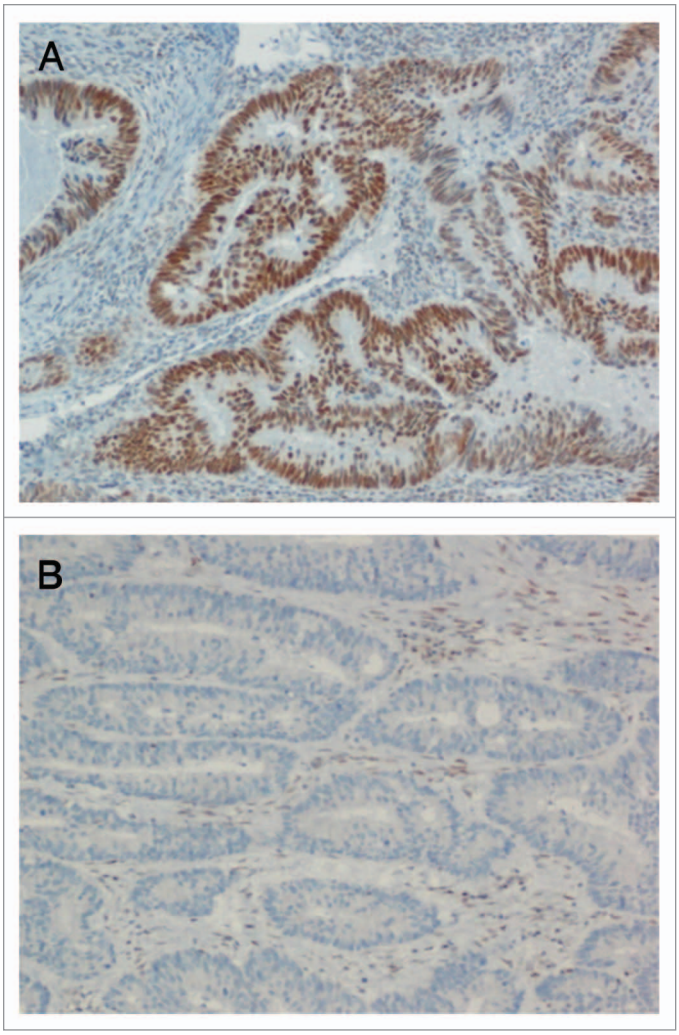

Figure 8. Immunohistochemical analysis showing the correlation between h*MLH1* promoter methylation and MLH1 protein expression. (**A**) Unmethylated sample showing positive MLH1 nuclear staining (nuclei of brown color). (**B**) Methylated sample showing lack of MLH1 nuclear staining (absence of brown color in the nuclei).

Conclusion

In summary, we screened a large cohort of CRC and healthy adjacent tissues by means of MS-HRM, a rapid and cost-effective technique for DNA methylation analyses. Our screening confirmed several previously reported observations in CRC, such as the high frequency of *APC* promoter methylation in both CRC tissues and healthy mucosa, and the correlation between h*MLH1* promoter methylation with both age, gender, lack of MLH1 protein in the nuclei, and *BRAF* mutations among others. We also noticed several interesting correlations between markers of one-carbon metabolism and gene promoter methylation in CRC, further supporting both the contribution of this pathway to CRC

Table 5. Immunohistochemical analysis and *BRAF* V600E mutation in individuals with different percentage of h*MLH1* methylation

Gender	Age	h*MLH1* methylation %	MLH1 IHC[a]	N° of methylated genes[b]	*BRAF* mutation[c]
F	80	30.0	-	4	V600E
F	71	0	+	0	
F	79	0	+	0	
M	87	0	+	1	
M	81	83.5	-	4	V600E
F	64	0	+	1	
F	82	0	+	2	
F	82	78.4	-	5	V600E
F	62	0	+	0	
M	81	0	+	2	
F	84	51.1	-	2	V600E
F	87	6.0	-	3	V600E
F	85	6.4	+	2	
F	70	47.3	-	4	V600E
F	84	0	+	1	
M	91	0	-	1	
M	87	29.3	-	4	
F	79	32.9	-	2	
M	80	0	-	1	
F	83	76.5	-	4	V600E
F	65	7.3	-	1	
M	88	100	-	4	V600E
F	87	75	-	5	V600E
F	77	0	+	2	
F	80	14.8	+	3	
M	81	0	+	0	
F	79	0	+	1	
M	80	16.4	+	3	V600E
F	83	0	+	0	
M	76	0	+	0	

[a]IHC, immunohistochemical analysis (+, positive sample; -, negative sample). [b]Number of methylated genes among the five investigated in the present study. [c]Carriers of the V600E mutation are indicated, wild type subjects are left blank.

pathogenesis and the evidence that DNA methylation in colonic mucosa cells is a multifactorial trait depending from both physiological and environmental factors.

Materials and Methods

Study population

DNA was obtained from both surgically resected tumor tissues of 107 patients (**Table 1**) and the adjacent normal tissue (at 20 cm of distance), available from 80 of them (**Table 2**). CRC diagnosis was performed by Medical Doctors at Department of Surgery, Medical, Molecular and Critical Area Pathology, University of Pisa, that provided tissue specimens. Staging was assessed after pathological examination of specimens based on TNM classification (**Table 1**). Family history of CRC was ascertained and all the subjects included in the present study had no family history of the disease. The study was approved by the ethical committee of the Pisa University Hospital. The individuals gave their written informed consent.

Extraction of genomic DNA

Genomic DNA was extracted using QIAmp DNA blood Mini Kit (Qiagen) according to the manufacturer's instruction. The extracted DNA was quantified using a Nano Drop ND 2000c spectrophotometer (NanoDrop Thermo scientific).

Bisulfite modification

An amount 200 ng of DNA from each sample were treated with sodium bisulfite using the EpiTectH Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Sodium bisulfite

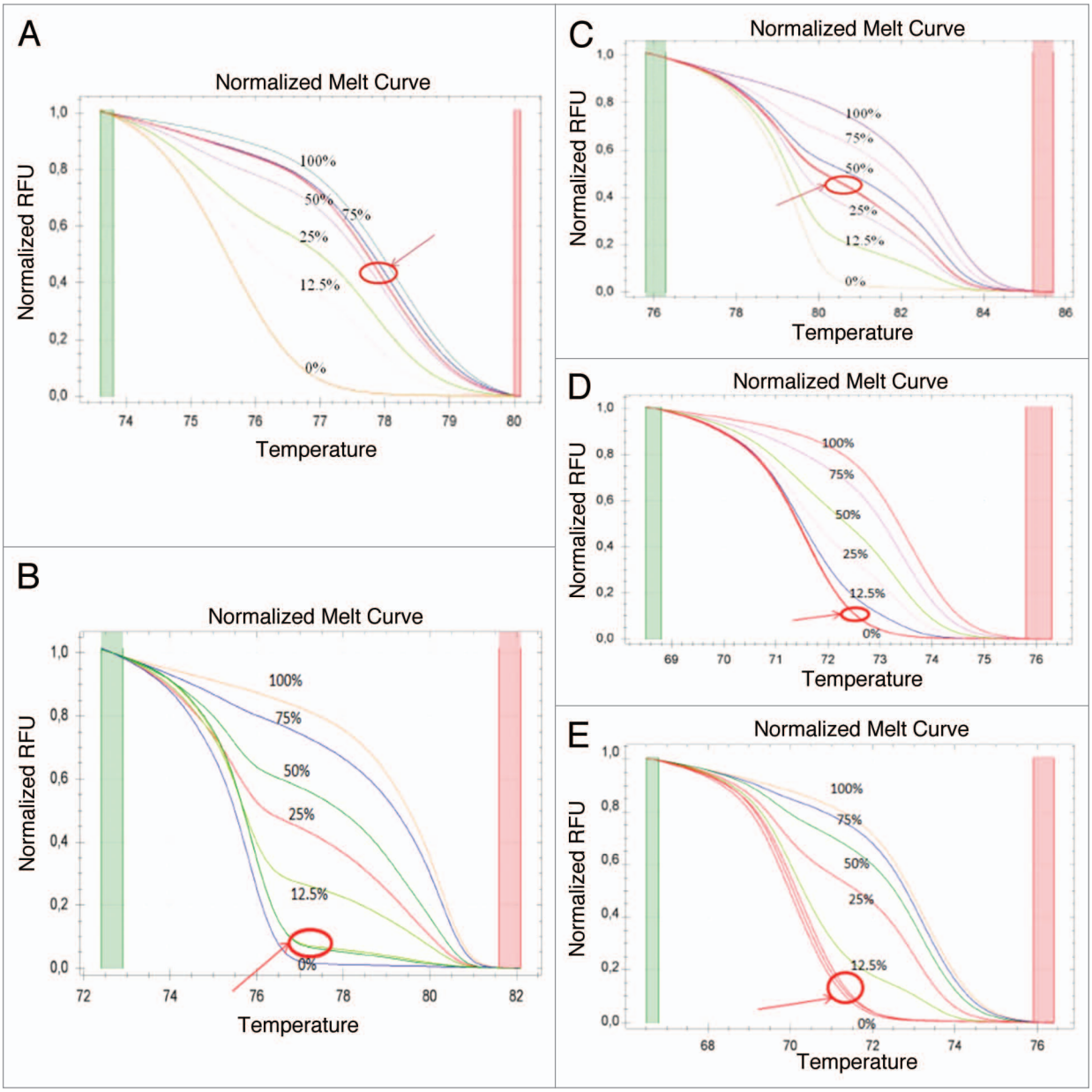

Figure 9. Melting curves for each of the studied genes. (**A**) *APC*. (**B**) *MGMT*. (**C**) *CDKN2A*. (**D**) *RASSF1A*. (**E**) h*MLH1*. Each curve shows the standards (0%, 12.5%, 25%, 50%, 75% and 100% methylation) and a sample in duplicate (indicated with an arrow).

treatment converts all unmethylated cytosines into uracil, while methylated cytosines are left unchanged.

Methylation sensitive-high resolution melting (MS-HRM) analysis

Promoter methylation was assessed by means of methylation sensitive-high resolution melting (MS-HRM) analysis in a CFX96 Real-Time PCR detection system (Bio-Rad). For the MS-HRM analysis we developed in-house protocols according to literature recommendations, using methylation independent primers (MIP).[46,47] All analyses were run according to the following conditions: 1 cycle of 95 °C for 12 min, 60 cycles of 95 °C for 30s, Ta for 30s and 72 °C for 15s; followed by an HRM step of 95 °C for 10s and 50 °C for 1 min, 65 °C for 15 s, and continuous acquisition to 95 °C at one acquisition per 0.2 °C. PCR was performed in a final volume of 25 ml, containing 12.5 ml of master mix (Qiagen), 10 pmol of each primer and 1 ml (almost 10 ng) of bisulfite modified DNA template. Each reaction was performed in duplicate. We analyzed 10% of the samples independently on separate occasions to verify the inter-assay variability and we observed a good reproducibility. **Table 6** shows the conditions (primers, annealing temperature, CpG sites, and amplicon length) used for each gene. Fully methylated and unmethylated DNA (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen) were mixed to obtain the following ratios of methylation: 0%, 12,5%, 25%, 50%, 75%, 100%. Standard curves with known methylation ratios were

Table 6. Primers and annealing temperature (Ta) used during MS-HRM analysis, as well as amplicon length and number of CpG sites for each of the studied genes

Gene	Primer sequences: 5′–3′	Ta	CpG sites	Amplicon lenght
APC	F: CGGGGTTTTG TGTTTTATTG R: TCCAACGAAT TACACAACTA C	56 °C	4	71 bp
MGMT	F: GCGTTTCGGA TATGTTGGGA TAAGT R: AACGACCCAA ACACTCACCA AA	58 °C	12	110 bp
hMLH1	F: GGTTATAAGA GTAGGGTTAA R: ATACCAATCA AATTTCTC	56 °C	5	81 bp
RASSF1A	F: TCGGGTTTTA TAGTTTTTGT ATTTAGGTTT T R: CCTCCCCCAA AATCCAAACT AA	60 °C	7	87 bp
CDKN2A	F: CGGAGGAAGA AAGAGGAGGG GT R: CGCTACCTAC TCTCCCCCTC T	62 °C	7	93 bp

included in each assay and were used to deduce the methylation ratio of each tumor and normal sample (**Fig. 9**). Validation of the MS-HRM assays was performed by means of pyrosequencing, as detailed elsewhere.[44] In order to obtain single methylation percentage values from MS-HRM assays, rather than a range, we applied an interpolation method recently developed and described by us, that allowed to obtain precise HRM methylation values comparable to those obtained by pyrosequencing.[44]

Genotyping

Genotyping for *SLC19A1* (*RFC1*) 80A > G (rs1051266), *MTHFR* 677C > T (rs1801133), *MTHFR* 1298A > C (rs1801131), *MTRR* 66A > G (rs1801394), *MTR* 2756A > G (rs1805087), *TYMS* 28bp repeats (rs34743033), *TYMS* 1494 6bp ins/del (rs34489327), *DNMT3B* -149C > T (rs2424913), and *DNMT3B* -579G > T (rs1569686) were performed according to PCR-RFLP methods previously described by us.[48,49] They are all functional polymorphisms, commonly studied in CRC genetic association studies, and with reported minor allele frequencies ranging from 15% to 49% in healthy Caucasians.[48-50] Internal quality control samples with confirmed genotypes were added to each PCR-RFLP reaction. Digestion fragments were visualized after electrophoresis on a 3% agarose gel stained with ethidium bromide. Genotyping was possible only on 92–94 of the total CRC samples (**Table 3**) due to DNA run-out during MS-HRM analyses.

Biochemical analyses

Peripheral blood samples from CRC patients had been collected before surgery. Plasma was immediately separated and stored in freezer at -80 °C. All the analyses were performed with standard protocols at the diagnostic laboratory of the Pisa University Hospital, as detailed elsewhere.[49] Those data are available for a subgroup of the patients (n = 39 for vitamin B12, n = 37 for hcy, and n = 36 for folates) because of blood drawings for biochemical markers was not possible for all of them or for technical problems during analyses, and also because we excluded individuals taking vitamin supplements, metformin, or other drugs known to interfere with those biomarkers.

Immunohistochemical analysis

5-μm sections from formalin-fixed, paraffin-embedded tissue were stained using the avidin-biotin complex method of Ventana Medical System (Ultraview DAB detection kit, Ventana Medical System) and using the BenchMark XT (Ventana Medical System) automated immunohistochemical stain. Staining was performed using antibodies to MLH1 (clone M1, Ventana Medical System). Nuclear immunostaining of normal colonic mucosal epithelial cells, lymphocytes and stromal cells served as internal positive controls. Positive nuclear staining of more than 10% of tumor cells was considered positive for MLH1 protein expression (**Fig. 8**). Loss of expression was recorded when all malignant cells showed absent nuclear staining or when less than 10% of tumor cells showed positive nuclear staining (**Fig. 8**). A total of 30 samples was assessed, 15 showing h*MLH1* methylation and 15 showing 0% h*MLH1* promoter methylation (**Table 5**).

Microdissection and DNA extraction for the evaluation of *BRAF* mutation

Serial 5-μm sections were taken from the above described formalin fixed paraffin embedded tissues. The last section was stained with hematoxilin-eosin; the tumor area was marked and the percentage of tumor cells was estimated by a pathologist. The tumor tissue was manually microdissected from one to three unstained sections previously submitted to xylene deparaffination and was lysed overnight at 56 °C in 180 μl of ATL buffer and 20 μl of proteinase K. DNA was purified using the spin column procedure (QIAamp DNA Mini Kit, QIAGEN) and finally reconstituted in 40 μl of AE buffer. DNA content was measured with a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and was kept at 4 °C before use.

Detection of *BRAF* V600E mutation by Real Time PCR

Five microliters of genomic DNA concentrated 10 ng/μl was tested for *BRAF* V600E mutation using the CE-marked Easy® BRAF kit (Diatech Pharmacogenetics), a real-time PCR based assay that uses allele-specific primers and probes in association with a mutant enrichment technique, following the manufacturer's instructions. The kit enables the detection of a low percentage of mutant in a background of wild-type genomic DNA and the oligo mix allows the co-amplification of *BRAF* mutated target sequence and an endogenous control gene. The latter is used for the assessment of the quality and quantity of DNA in the sample. All reactions was performed on Rotor-Gene™ 6000 instrument (Corbett Research) as follows: hold at 95 °C for 2 min; denaturation at 95 °C (10 s) and annealing at 58 °C (60 s) for 40 cycles. The mutation status of sample was determined by considering the ΔCt values in according to the manufacturer's recommendations.

If the ΔCt value was less than 10, the sample was considered as V600E positive, otherwise as negative (**Table 5**).

Statistical analyses

Differences in the number of methylated samples in CRC vs. healthy adjacent tissues have been evaluated by means of chi square analyses or Fisher exact test. Linear regression analysis was performed to search for correlation between age and methylation data, as well as to search for correlation between methylation levels in CRC samples and methylation levels in the adjacent healthy mucosa. The effect of gender on mean methylation levels was assessed by means of analysis of variance (ANOVA), including age at sampling, the number of methylated genes and the levels of methylation of each of the other genes as covariates. Multifactorial analysis of variance (MANOVA), including age at sampling, gender, the number of methylated genes for each sample (ranging from 0 to 5), and the levels of methylation of each of the other four genes as covariates, was used to correlate methylation data with tumor size, staging and with each of the studied polymorphisms (SNPs). By means of MANOVA we evaluated simultaneously the effect of the nine SNPs on the methylation levels of a certain gene. In such a way the number of tests performed has been significantly reduced, and the analysis allowed correcting the effect of each given SNP for the presence of all the other ones that were tested simultaneously. Since methylation data were not normally distributed, natural logarithm transformation of all values was done before analysis. Folate, vitamin B12, and hcy levels were available only for about 40 out of 107 individuals. For each of the studied markers the patients were stratified into two groups (i.e., low folates [< 4.6 ng/ml] and normal folates [4.6–18.7 ng/ml], normal vitamin B12 [191–663 pg/ml] and high vitamin B12 [> 663 pg/ml], and normal hcy [4.3–11.1 μmol/l] and high hcy [> 11.1 μmol/l] [**Table 4**]) and mean methylation differences among the two groups have been evaluated for each of the studied genes by means of MANOVA, as explained above. Analyses were performed with the STATGRAPHICS 5.1 Plus software package for Windows.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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