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Epigenetic events in normal colonic mucosa surrounding colorectal cancer lesions

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ABSTRACT

Gene inactivation by promoter hypermethylation has been demonstrated in the colonic mucosa of colorectal cancer (CRC) patients. However, current data do not prove direct involvement of this epigenetic modification in the early stages of CRC. Promoter methylation profiles of *E-cadherin*, *hMLH1*, *MGMT*, $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$; mutations of *K-ras*, *B-raf* and *TP53* and microsatellite instability (MSI) were examined in normal and cancerous colonic mucosal tissue in 82 CRC patients using methylation-specific PCR assays. Methylation of *hMLH1* and *MGMT* in normal mucosa correlated significantly with MSI and *K-ras* activation in neighbouring cancerous mucosal tissues. Similarly, poorly differentiated tumours were associated with methylated $p16^{INK4a}$ and *E-cadherin* in neighbouring normal colonic tissues (NCTs). Our results indicate that epigenetic changes in mucosa surrounding colorectal neoplastic lesions may describe a 'field cancerisation' phenomenon that may occur previous to genetic alterations in early stages of carcinogenesis.

1. Introduction

Colorectal cancer (CRC) is initiated by alterations in the *Wnt* and *TGFβ* signalling pathways, activation of proto-oncogenes such as *K-ras* and inactivation of tumour suppressor genes such as *APC* and *TP53*[1–3]; over 50% of CRCs can be explained by these mechanisms.[4] Moreover, the multistep carcinogenesis model in colon cancer serves as the classical model of genetic alterations in cancer.[1] Other CRCs (approximately 13% of sporadic colorectal carcinomas) have defective mismatch repair processes, resulting in increased intragenic mutation rates, characterised by generalised instability of short tandem DNA repeat sequences (microsatellites).[5,6] Therefore, suppressor and mutator pathways have been proposed in the development of CRC.

Another potential mechanism underlying CRC progression is epigenetic silencing associated with promoter hypermethylation.[7] A 'CpG island methylator phenotype (CIMP)' has been proposed to describe a subset of CRCs with mismatch repair deficiency that appears to display a high incidence of human MutL homologue (*hMLH1*), cyclin-dependent kinase inhibitor 2A ($p16^{INK4a}$) and *THBS1* methylation. This epigenetic phenomenon may be a potential third pathway in the carcinogenesis of colon cancer.[8–10] In fact, more data exist regarding the hypermethylation of tumour suppressor genes in cancer cells than in normal cells in CRC patients.[11,12] However, studies in normal colonic tissue (NCT) are relatively few, and have produced contradictory results regarding the percentage of *hMLH1*, $p16^{INK4a}$ or $p14^{ARF}$ methylation, proposing in some cases that methylation of these genes is tumour

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specific[13,14]; other studies have found high methylation levels in NCT.[4,15,16]

To explain the role of hypermethylation in the normal mucosa of CRC patients, many authors have introduced the concept of 'field cancerisation'.[17–19] According to this model, precancerous cells in proximity to the cancer cells harbour some, but not all, of the genetic alterations that are present in fully developed cancer.[20] In CRC, an epigenetic inactivation of O^6-methylguanine-DNA methyltransferase (*MGMT*) has been described in NCT adjacent to cancerous colonic tissue, suggesting a field defect. Thus, the discovery of a marker of this field defect may be useful in early detection and in risk assessment in colon cancer.[19]

To clarify the role of epigenetic events in normal and cancerous tissue, and to study how epigenetic events could be involved in field cancerisation, we investigated the methylation status of six genes involved in colon cancer development [E-cadherin (*CDH1*), *hMLH1*, *MGMT*, $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2B ($p15^{INK4b}$) and cyclin-dependent kinase inhibitor 2A, alternated reading frame ($p14^{ARF}$)] in normal and cancerous tissues and in their relationship to patients' genetic and clinico-pathological features.

2. Materials and methods

2.1. Study population

Study participants were recruited from the Oncology Department of Municipal Hospital (Badalona, Spain). Eligible patients had histologically confirmed diagnosis of colonic adenocarcinoma. Tumour samples and corresponding non-tumour colonic mucosa were obtained from 82 surgical specimens (53 males and 29 females) aged between 42 and 92 years at the time of diagnosis. All patients provided signed informed consent. Tissue samples were stored at –80 °C until nucleic acid extraction. Total genomic DNA and RNA were extracted from tumour samples and from corresponding normal samples contiguous with the cancerous tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clinical and pathological data were collected; a summary of this series is shown in Table 1. Tumours were staged according to the American Joint Cancer Committee (AJCC) criteria. The adjacent NCT were obtained from 20 cm to the tumour, and diagnosis of normal mucosa was confirmed histologically. Histologic grade and presence or absence of mucinous pattern were determined by reviewing pathology reports.

Table 1 – Clinico-pathological profiles of the CRC patients (N = 82)

Clinico-pathological features	Number of cases
Sex	
Female	29
Male	53
Age at diagnosis (years)	
⩽65	33
>65	49
Location	
Proximal	20
Distal	62
Cell type	
Well differentiated	28
Poorly differentiated	54
Mucinous histology	
No	60
Yes	22
Stage	
I–II	46
III–IV	36
Lymph node involvement	
No	48
Yes	34
Metastasis	
No	61
Yes	21
Pre-existent polyps	
No	58
Yes	24

2.2. Methylation analysis

The methylation states of the *CDH1*, *hMLH1*, *MGMT*, $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ gene promoter regions were examined in DNA extracted from normal and cancerous tissues. The DNA methylation state of CpG islands was determined by methylation-specific polymerase chain reaction (MSP) after genomic DNA (1 μg) was subjected to sodium bisulfite treatment using the CpGenome DNA modification kit, as recommended by the manufacturer (Epigentek, Brooklyn, NY). This process converted all unmethylated cytosine residues from genomic DNA to uracil residues. For MSP, the modified DNA was amplified with primers capable of annealing both melthylated and unmethylated cytosine residues. Primer sequences, polymerase chain reaction (PCR) conditions and product sizes for each methylation marker analysed, and the specificity of the MSP assays, have been described previously.[13,21,22] PCR products were visualised after gel electrophoresis in 2% agarose w/v containing ethidium bromide. Commercially modified DNA (Chemicon International, Temecula, CA) and normal blood DNA were used as positive controls for methylated and unmethylated alleles, respectively. In order to summarise the results of methylation, data were grouped in non-methylated-loci, one or two methylated-loci and three or more methylated-loci.

2.3. Analysis of K-ras, B-raf and TP53 mutations

Allele-specific PCR was performed on 100 ng DNA samples to identify *K-ras*, *B-raf* and *TP53* mutations. Mutation analysis included *K-ras* exons 2 and 3, *B-raf* exons 11 and 15 and *TP53* exons 5–8. PCR primers conditions for mutation analysis have been previously described.[23,24] The PCR products were analysed by agarose gel electrophoresis (2% w/v), purified and used in two sequencing reactions per exon (forward and reverse), using the Big Dye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems Inc., Foster City, CA). Reactions were loaded into an ABI-3100 DNA sequencer (Applied Biosystems). All sequences were analysed with the SeqScape

software (Applied Biosystems). A second method, TheraScreen® *K-ras* Mutation Test Kit (DxS Ltd., Manchester, UK), which can detect approximately 1% of mutant DNA in a background of wild-type genomic DNA, was used to screen the *K-ras* mutation in a more sensitive way. Cycling conditions were 95 °C for 4 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Data were interpreted according to the manufacturer's instructions. All tumour samples were compared with their corresponding normal samples.

2.4. MSI analysis

Microsatellite analysis of all matched normal and tumour tissues was performed by PCR amplification using the panel of five National Cancer Institute (NCI) workshop-recommended markers, including two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D2S123, D5S346 and D17S250) microsatellite markers.[25] Cycling conditions were 95 °C, 10 min; followed by 35 cycles of 95 °C for 45 s, 55 °C for 45 s, 70 °C for 1 min; and finally 72 °C for 7 min. Fluorescent dye-labelled samples were separated on an ABI-3100 DNA sequencer and analysed with GeneScan analysis software (Applied Biosystems). Changes in the electrophoretic mobility of PCR-amplified DNA were used to assess microsatellite instability (MSI). Tumours were classified as high-frequency MSI (MSI-H) if they possessed two or more of the Bethesda consensus panel markers. Tumours with one marker were classified as low-frequency MSI (MSI-L), and microsatellite-stable (MSS) cancers did not show MSI in any markers. MSI-L/MSS tumours were grouped together for statistical analysis, because they have similar clinico-pathological features, and do not differ in clinical outcome.

2.5. Statistical analysis

Correlations between methylation state, genetic events and clinico-pathological features were assessed using χ^2 or Fisher's Exact probability tests as appropriate. All p-values presented are two-sided. A p-value < 0.05 was regarded as statistically significant. All statistical tests were performed using the SPSS software package (version 12.0, SPSS, Chicago, IL).

3. Results

3.1. Comparison of CpG methylation frequency in tumour and non-tumour colon samples

In normal colonic mucosa, non-methylated genes were observed in the 47% of normal samples and 41% of these had one or two methylated-loci. Three or more methylated-loci were observed in 12% of normal colon tissues. In contrast, three or more methylated-loci were observed in 33% of tumour samples. *CDH1* was the most prevalent methylated gene in non-tumour tissues followed by $p16^{INK4a}$ and *hMLH1* loci. In our cohort, *hMLH1* was the least frequent methylated gene in CRC tissues. *MGMT* and $p14^{ARF}$ were the most frequently methylated genes in CRC lesions and the least commonly methylated in the corresponding non-tumour tissue (Supplementary Table 1). Methylation state in the assessed loci did not show statistical differences in the normal and tumour tissues. Furthermore, 71% of the patients had similar methylation patterns among normal and tumour tissues with changes in a maximum of two loci only. Statistical analysis revealed a significant association between methylation states of $p16^{INK4a}$ and *CDH1* promoters in the normal samples and presence of methylation in the tumour tissue ($p = 0.032$ and $p = 0.018$, respectively) (Table 2).

3.2. Statistically significant correlation between epigenetic events in normal tissue and genetic events in contiguous tumour tissue

The correlation between promoter gene methylation and the presence of mutations in *K-ras* and *TP53* were analysed. *K-ras* mutations were detected in 22 of 82 patients (27%; 16 patients had a G-to-A transition), and *TP53* gene mutations were detected in 27 of 82 patients (33%). Both genes were mutated in only six patients. We also analysed the presence of mutations in *B-raf* gene, but only one patient with a mutation at position 599 was found (V599E). In contrast to the methylation state, neither *K-ras*, *B-raf*, nor *TP53* mutations were detected in the corresponding normal mucosa samples.

Notably, in spite of the low numbers of patients with *MGMT* methylation in NCT, a significant correlation between *K-ras* mutations and *MGMT* methylation was detected ($p = 0.024$) (Fig. 1). All subjects with *MGMT* methylation in the normal mucosa had *K-ras* mutations in the corresponding tumour tissue. When we studied the presence of microsatellite instability, 60% of the samples with *hMLH1* methylation in NCTs demonstrated a high-frequency of MSI ($p = 0.029$). The rest of the genes studied had no correlation with this genetic alteration (Fig. 1). We did not find microsatellite instability in NCTs. Associations detected between the methylation states of *MGMT* and *hMLH1* in non-tumour tissue and genetic events in tumour tissue were not present when both types of events were analysed in the cancerous tissue only.

3.3. In normal tissue, methylation of $p16^{INK4a}$ and CDH1 genes demonstrated statistically significant correlation with the clinico-pathological features in patients with CRC

The correlation between the methylation of these six genes and the clinico-pathological features of primary CRC (including age, gender, tumour location, tumour-differentiation grade, mucinous histology, T-staging, node involvement and

Table 2 – Association between methylated $p16^{INK4a}$ and CDH1 loci in normal samples and presence of this methylation state in tumour tissue

	Tumour-tissue		
	Non-methylated cases (%)	Methylated cases (%)	p
$p16^{INK4a}$ ($N = 10$)	3 (30)	7 (70)	0.032*
CDH1 ($N = 21$)	6 (28.6)	15 (71.4)	0.018*

* p-Value < 0.05. All statistical analyses were calculated using the χ^2 or Fisher's test.

presence of metastasis) was evaluated. Eleven of 18 female patients (61%) showed higher *CDH1* methylation than male patients (27%; N = 10/37) in NCT ($p = 0.015$). All patients with *hMLH1* methylation in NCT were older than 65 years, but this association with age was not significant ($p = 0.093$). The rest of the genes analysed demonstrated no statistically significant gender- or age-related correlations with the methylation state in the normal and tumour tissues. Regarding other clinico-pathological features, 76% (N = 16/21) of normal colon samples with *CDH1* methylation and 63% (N = 5/8) of normal colon samples with $p16^{INK4a}$ methylation showed a poorly or moderately differentiated cell type in the paired tumour ($p = 0.026$ and $p = 0.035$, respectively).

With respect to colorectal tumours, proximal tumours were associated with *hMLH1* ($p = 0.018$) and $p14^{ARF}$ ($p = 0.030$) gene methylation. Tumours with mucinous histology were associated with methylation of $p16^{INK4a}$ ($p = 0.034$) and $p14^{ARF}$ ($p = 0.030$). $p16^{INK4a}$ Methylation was also associated with the presence of lymph node involvement ($p = 0.025$) (Table 3). *hMLH1* Methylation also demonstrated a trend with the development of second neoplasias ($p = 0.054$). No associations were found between the methylation states of the other genes and clinico-pathological features.

3.4. *Pre-existent polyp, mucinous histology and poor differentiation*

In the samples tested, we detected statistically significant correlations between *K-ras* and *TP53* mutations, microsatellite instability and clinico-pathological CRC parameters. Patients with pre-existent polyps had more frequent mutations in the *K-ras* gene [50% (N = 12/24) versus 20% (N = 11/58), $p = 0.028$], and demonstrated a higher-frequency of MSI [42% (N = 10/24) versus 9% (N = 5/58), $p = 0.018$]. However, any association between preexistent polyps and methylated-loci does not exist in these patients. Moreover, tumours with mucinous histology demonstrated a higher-frequency of *K-ras* mutations [47% (N = 10/22) versus 19% with non-mucinous histology (N = 11/60), $p = 0.018$]. On the other hand, mutations of the *TP53* gene were associated with the poorly differentiated tumours ($p = 0.025$). None of the other clinico-pathological features demonstrated a statistically significant relationship with genetic alterations of the tumour.

4. Discussion

Multiple studies regarding the role of the methylation process in colon cancer have been performed, and many genes that could be altered by aberrant promoter methylation rather than DNA structural changes have been studied. Toyota and colleagues[8] classified the methylation of some genes, such as the *MyoD* and oestrogen receptor (*ERα*) genes, as Type A (age-related methylation) and others, such as *TIMP3*, as Type C (cancer-specific methylation). The possibility that methylation of some genes is cancer-specific could be discussed regarding our results. $p16^{INK4a}$ and *hMLH1* have been suggested by other authors as examples of genes with Type C methylation because they are methylated exclusively in the tumour tissue; however, in our data both these genes and *CDH1* have the highest-frequencies of methylation in normal colonic mucosa (15%, 12% and 39%, respectively). This increase in the methylation of $p16^{INK4a}$ and *hMLH1* in the normal colonic mucosa of patients with CRCs has been previously reported.[15]

Like Toyota, Herman and colleagues suggest that DNA methylation constitutes a positive and stable marker that cannot be masked by the presence of NCTs.[26] However, in our group of patients 53% have some methylated genes in apparently normal colonic mucosa. This percentage demonstrates that the early appearance of this epigenetic alteration in normal cells without a tumour phenotype may be a molecular marker of cancer progression. This hypothesis is supported by statistically significant correlations found between epigenetic events in NCTs and some genetic alterations and clinico-pathological features in our patients. Regarding genetic alterations, we have found a statistically significant correlation between *MGMT* methylation in normal cells and *K-ras* mutations in cancer cells ($p = 0.024$). This epigenetic alteration may cause susceptibility to *K-ras* mutation during the carcinogenesis process. Similarly, it has been described that

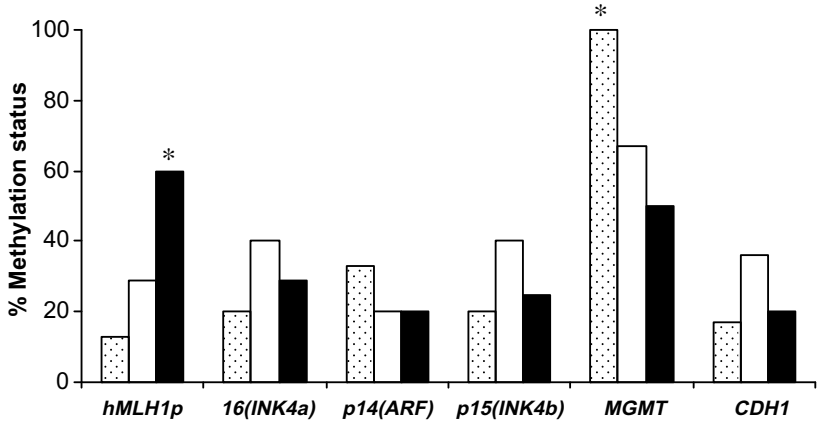

Fig. 1 – *hMLH1* and *MGMT* methylations in normal colon tissue correlated significantly with MSI-H tumours ($p = 0.029$) (dark bars) and with the presence of *K-ras* mutation ($p = 0.024$) (light bars), respectively. No associations were found between the methylation states of normal tissues and *p53* mutations (open bars). *p-Value < 0.05.

Table 3 – Correlation between DNA methylation status of hMLH1, p16^{INK4a} and p14^{ARF} and clinical pathological parameters of tumour samples

Clinico-pathological features	Methylation status					
	hMLH1		*p16*^{INK4a}		*p14*^{ARF}	
	%	*p*	%	*p*	%	*p*
Sex						
Female	21	NS	35	NS	40	NS
Male	29		33		39	
Age at diagnosis (years)						
⩽65	20	NS	28	NS	37	NS
>65	26		38		41	
Location						
Proximal	47	$p = 0.018$*	41	NS	61	$p = 0.030$*
Distal	16		32		32	
Cell type						
Well differentiated	31	NS	23	NS	42	NS
Poorly differentiated	20		40		37	
Mucinous histology						
No	26	NS	27	$p = 0.034$*	32	$p = 0.030$*
Yes	18		56		61	
Stage						
I–II	23	NS	23	$p = 0.022$*	34	NS
III–IV	26		50		48	
Lymph node involvement						
No	22	NS	23	$p = 0.025$*	32	NS
Yes	28		50		48	
Metastasis						
No	24	NS	31	NS	40	NS
Yes	21		48		40	
Pre-existent polyps						
No	25	NS	33	NS	34	NS
Yes	22		33		50	

* *p*-Value < 0.05. All statistical analyses were calculated using the χ^2 or Fisher's test.

O^6-alkylguanine-DNA alkyltransferase activity in NCT from individuals with CRC who had a GC → AT transition in the *K-ras* oncogene was significantly lower than in individuals without this mutation.[27] Many authors have also shown that *MGMT* methylation in colonic tumours is strongly linked to the accumulation of *K-ras* mutations or G-to-A transition mutations, which can affect genes required for genomic stability.[19,28] Furthermore, we found that the methylation level of *hMLH1* in NCTs from CRC patients is correlated in a statistically significant fashion with a high-frequency of MSI. Kawakami and colleagues[15] described this same association. However, they also found a significant correlation between CIMP+ tumours and *hMLH1* methylation, while in our study the methylation level of *hMLH1* in tumours had no apparent association with CIMP+ phenotype. This difference could be explained by the fact that the CIMP phenotype included different tumour suppressor genes in our series compared to the Kawakami's series. The fact that other authors have found that in the MSI(–) cases hypomethylation of the *hMLH1* gene was demonstrated in the tumour, and hypermethylation in normal mucosa can be justified by the method used to study methylation.[29] Actually, the most widely used technique is the methylation-specific PCR analysis of DNA after bisulfite treatment. However, this method has notable shortcomings as qualitative nature, and false-positive signals at high PCR cycles. Real-time methodology remedies these deficiencies and would lead to perform a quantitative analysis. This characteristic leads to assert that methylation pattern is translated in a decreased production of specific mRNA.

An interesting result of our study is the statistical correlation found between some epigenetic events in normal mucosa and clinico-pathological features. Normal mucosa surrounding moderately or poorly differentiated tumours showed a certain degree of methylation in *CDH1* and *p16*^{INK4a} genes ($p = 0.026$ and $p = 0.035$). In addition, we found that *p16*^{INK4a} and *CDH1* genes had the highest-frequencies of methylation in normal colonic mucosa (15% and 39%, respectively) and this methylation state was maintained by a high proportion of subjects in the tumour samples ($p = 0.032$ and $p = 0.018$, respectively). Previous studies have shown that *CDH1* and *p16*^{INK4a} methylation frequencies are lower in non-neoplastic tissue of patients with sporadic serrated adenomas or with aberrant crypt foci (AFC) (13% versus 39% and 2% versus 15%, respectively).[16,21] Therefore, all these characteristics may reveal information about the direct involvement of *CDH1* and *p16*^{INK4a} genes in the specific early carcinogene-

sis process in CRC, as well as potential imprinting in normal cells of the surrounding mucosa. The down-regulation of *CDH1* levels *in vivo* and the $p16^{INK4a}$ methylation of tumour cells have been associated with cell indifferentiation in CRC.[30,31] However, Ye and colleagues[16] did not find association between the methylation states of *hMLH1*, *MGMT* or $p16^{INK4a}$ genes in non-neoplastic mucosa from patients with CRC and adenoma characteristics. These differences could be a result of the different primer sequences used to evaluate these genes in both studies. It would be recommended for future studies to consider a list with standard sequence primers and PCR temperature conditions.

Associations between epigenetic/genetic alterations and epigenetic events/clinico-pathological features were not found when we study the tumour tissue of patients, except in the case of the *hMLH1*, $p14^{ARF}$ and $p16^{INK4a}$ genes, whose methylation is significantly correlated with some clinico-pathological features. $p16^{INK4a}$ Methylation has a clear correlation with mucinous histology, more advanced stage and lymph node involvement. Other authors have also found similar results in their studies and revealed an association between $p16^{INK4a}$, poor prognosis and more advanced T-stage.[32,33] These last data may give information regarding the significance of this gene to the whole process of CRC carcinogenesis, and the importance of pathways involved in its regulation.

All these statistically significant correlations may be brought into question by the possibility that NCTs were contaminated with tumour cells. However, this is unlikely given that in the normal-appearing tissue, we found no samples with *K-ras*, *B-raf* or *TP53* mutations, or with microsatellite instability. Furthermore, normal mucosa was histologically confirmed.

In summary, the different degrees of methylation found among the genes studied in normal colonic mucosa could cause a selective gene inactivation by means of not genetic mechanisms, suggesting its relevance during the early steps of carcinogenesis process. For the first time, in this report we detect epigenetic alterations in the absence of genetic events, which reveal a statistically significant correlation with different tumour characteristics that include molecular features and clinico-pathological data. Therefore, these associations could suggest that CRC arises from a region of cells with a 'field defect'; cells that appear normal but have an underlying molecular defect.[34] Methylation field effects of DNA repair genes (*hMLH-1* and *MGMT*), tumour suppressor genes ($p16^{INK4a}$) and one gene related to metastasis and invasion (*CDH1*) would confer increased risk for CRC development, as it has been proposed by Giovannucci and colleagues.[35] Thus, in any tumour it is possible to find simultaneous inactivation of several pathways by aberrant methylation compromising the described function. By this reason, it is necessary to know the risk of suffering epigenetic effect of each gene during cancerous process.

The fact that significant associations found in NCT have not been detected in tumour mucosa could be explained by the great number of molecular alterations in cancer cells. Tumour regulation can be attributed to both independent and dependent factors including changes in receptor expression, availability of downstream signalling components, pro-inflammatory cytokines and others. These factors could alter the genetic and epigenetic patterns present in the NCT of CRC patients. Meanwhile, it has been described that high levels of hypomethylation are associated with cancer genotype (genomic damage) and with cancer phenotype (prognosis) in cancer cells.[36] The absence of a statistically significant correlation in the tumours of our study may also be due to tumour microenvironment and hypomethylation mechanism parallel to the carcinogenesis process. It is necessary to find a better strategy to look for standard epigenetic sensitive risk markers in normal mucosa. Conventional primary therapy for CRC is complete surgical resection; analysis of methylation pattern in NCT would offer information about relapse disease possibility in these patients. More studies are needed to clarify differences with respect to methylation state between NCT of healthy subjects and cancer patients, and to describe the mechanisms that can promote a normal cell to suffer a field cancerisation.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.09.004.

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