EPIGENETIC SILENCING OF MLH1 AND p16[INK] AND THEIR RELATION TO CERTAIN CLINICO-PATHOLOGICAL FEATURES IN PATIENTS WITH COLORECTAL CANCER

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ABSTRACT

Introduction: Promoter methylation as a cause of gene inactivation has been studied mainly by qualitative methods. MLH1 and p16[INK] are important tumor-suppressor genes that are often silenced in cancer. **Aim:** To study the level of mlh1 and p16 and to correlate the data with certain clinico-pathological features. **Materials and methods:** 181 colorectal cancer patients were included in the study. DNA was extracted from all of them, subjected to bisulfite treatment, PCR and pyrosequencing allowing quantitative methylation analysis. Results on MSI status, TNM classification and histology were previously available. **Results:** Only high level and dense mlh1 methylation caused loss of protein expression. Those tumors tended to be proximal, low differentiated, had elevated mucin production, and were seen in elderly patients. No correlation between the level of p16[INK] methylation and tumor location, age, sex, stage and degree of differentiation was found. However, a group of tumors with T4, presence of lymph node and liver metastasis exhibited higher level of p16[INK] methylation. About 6% of the cases showed dense and elevated methylation in both genes. These were proximal, low differentiated, had high mucin production, and were present in older and female subjects. None of them had liver metastasis which did not reach statistical significance. **Conclusions:** Different levels of promoter methylation in the studied genes are observed. These molecular features are associated with certain clinico-pathological parameters and allow additional stratification of tumors.

Key words: colorectal cancer; promoter methylation; quantitative analysis; pyrosequencing

Two basic concepts – the genetic and epigenetic, are believed to be the major players in colorectal cancer (CRC) formation. Genetic changes are sequence-based and qualitative, that are perpetuated through cell division (13).

They include chromosomal instability (CIN) in which frequent cytogenetic abnormalities and chromosomal gains or losses are seen (5), and microsatellite instability (MSI) whose hallmark is the accumulation of numerous mutations in repetitive non-encoding microsatellite regions (9). In epigenetics no structural changes in DNA sequence are encountered but alteration in gene expression level. The latter is achieved by enzymatic methylation of cytosine residues in CpG islands that are found in the promoter regions of ~50% of human tumor-suppressor genes. This methylation is transmitted through cell division (24). DNA methylation is now recognized as one of the most common malignancies in human neoplasms (2). In the context of CRC, in which age is a serious risk factor, an age-related promoter methylation in certain genes is detected, such as ER gene (10) and some bystander genes: IGF2, MYOD1, N33, PAX6, CSPG2 (1,11).

MLH1 and p16[INK] are important tumor-suppressor genes, the dysfunction of which leads to two separate ways of tumor formation. Human MutL homologue or MLH1 is a member of the mismatch repair system whose function is to replicate the genome faithfully (1). Germline mutations in MLH1 lead to MSI in HNPCC cases (16). MSI is also present in 10-15% of sporadic cases (22), albeit that mutations in MLH1 are rarely detected. Kane et al. described originally that methylation of CGI 5' to hMLH1 causes its inactivation (14). Later on it was demonstrated that methylation only in the region -248 to -175 from transcriptional start correlates with loss of protein expression (8).

p16[INK] is located in the locus 9p21 and encodes a cell-cycle regulatory element that blocks progression through the G_1/S restriction point, e.g. before the DNA synthesis phase (15). Deletions or mutations in the p16 result in abnormal cell cycling and growth (17). Furthermore two independent groups reported a significant correlation between p16[INK] CGI methylation and its transcriptional silencing (6,7).

An interesting notion is that methylation could be a

progressive process, in contrast to sudden appearance of mutations. This means that several 'waves' of dysregulated methylation are necessary to produce dense hypermethylation (4). Song et al. proposed also that a 'seeding' of methylation exists and this subsequently attracts more methylation (20). In keeping with the above mentioned we studied the level of promoter methylation of MLH1 and p16[INK] and tried to correlate our data with certain clinical and pathological features of patients with colorectal caner. We used pyrosequencing as a method of choice since it offers semiquantitative, high throughput and reliable method for detection of CpG island methylation (3).

MATERIALS AND METHODS:

Patients: A total of 181 patients treated surgically for colorectal cancer were included in our study. Ninety-eight from them presented to MHAT "St. Marina", Medical University, Varna, Bulgaria for surgical treatment, and the rest of them were recruited in Medical University of Bonn, Germany. Demographic, clinical and pathological characteristics of each patient were available, and detailed histology provided histopathological staging. The study was approved by ethical committees in each hospital unit. All the cases were analysed previously for microsatellite instability status and mlh1 immunohistochemistry.

Tissue collection: Eighty-two samples were intraoperatively obtained. Tumor samples about 5 cm^3 were excised within the tumor and were immediately stored at -50°C. Normal tissue was excised prior to tumor resection about 10 cm away from the primary lesion to prevent contamination. The remaining tissues were obtained from formalin-fixed paraffin embedded blocks.

DNA extraction and bisulfite treatment:

Fresh-frozen normal and tumor tissues were defrosted and 2 cm^3 were put into Eppendorf tubes containing 80мl PBS buffer and were homogenized. DNA was extracted using Proteinase K according to QIAGEN protocol (QIAGEN Ltd.). Formalin-fixed paraffin embedded blocks were initially sliced in 10 мм, hematoxilin-eosin stained and microdissected allowing separation of normal from tumor tissue. Subsequently sections were deparaffinised in a heater by 37°C overnight, and rehydrated in ethanol in a descending order. Tissues were scratched and placed in Eppendorf tubes following the same steps as for fresh-frozen samples. The quantity of DNA was measured by Spectrophotometer-NanoDrop® ND-1000 (PEQLAB Biotechnologie GmbH). Bisulfite treatment of 1мg of each sample was undertaken using the QIAGEN EpiTect® kit.

PCR and pyrosequencing methylation analysis: Forward-, reverse- and sequencing primers were manufactured by Biotage. For amplification 2 мl bisulfite treated DNA and Platinum® Taq DNA polymerase by Invitrogen were used. For MLH1 we amplified a region between -209 and -188 from transcriptional start using the following PC conditions: initial denaturation by 95°C for 10 min, 40 cycles of: denaturation by 94°C for 45 sec, annealing by 54°C for 45 sec, elongation by 72°C for 45 sec, and final product elongation by 72°C for 10 min. For p16 the amplified region of interest was in the first exon (between +148 and +161) following the same PCR conditions. Conformation of PCR product quality and freedom from contamination was established on 2% agarose gel with ethidium bromide staining. Pyrosequencing was carried out using the PSQ96MA System (Biotage) according to manufacturer's protocol, including single strand binding protein (PyroGold reagents).

Statistical analysis: For each sample a methylation index or an average value of $^mC/(^mC+C)$ for all examined CpGs in the gene was calculated. The mean values of the methylation index and the clinical and pathological variables were correlated using the Statistica 6.0 software for Windows. A p<0.005 was assumed as statistically significant.

RESULTS:

Pyrosequencing is a quantitative method allowing detection of different levels of methylation in each of the four CpG islands within the gene promoters. Hence we were able to observe variations in the methylation of the two studied genes: mlh1 and p16.

The incidence of mlh1 methylation according to our results was 56 of 181 (31%). We observed two clusters of methylation according to the level: the first from 1.4% to 10%, and second from 26% to about 60% (Fig.1). Only the second group was found to be associated with lack of protein expression. We had one case (0.6%) which showed high level of only one CpG island (27%) but still expressed mlh1 protein. When we used a cut-off value of 10% our incidence was 8% (14/181). There was a good correlation between the level of mlh1 methylation and microsatellite instability (MSI) status (Table 1). Even the low levels of mlh1 methylation were more often seen in MSI than in MSS tumors (p<0.00001).

We observed some positive correlations between the level of mlh1 methylation and some clinical and pathological features such as age, tumor location and histology (Table 1). The mean age of the patients with MSI tumors occurring through mlh1 methylation was 73.5 years (49ч81) as opposed to patients with mlh1 mutations whose mean age was 35.9 (16ч53) (p<0.0001). We found out that tumors with higher levels of mlh1 methylation tended to be proximal in location (p=0.009), low differentiated (p=0.05), and with elevated mucin production (p=0.05). There was no significant correlation between presence of lymphocyte infiltration in the tumor (p=0.89), and gender (p=0.38). No association was seen also for tumor stage (p=0.92). Interestingly we noticed increase of the level of mlh1 methylation with depth of wall invasion (p=0.00003), and a trend towards a decrease in the

level with increase of the number of lymph node and presence of liver metastases, although this did not reach statistical significance for the latter two – p=0.29 and p=0.19 respectively.

For p16 the cases showing any CpG island methylation were 44% (79/181). There was a similar to mlh1 methylation clustering and about 28% (50/181) had methylation level below 10% (Fig.2). We did not see any association between the level of p16 methylation and age, gender, location, differentiation, and tumor stage. We observed higher level of methylation in T1 in contrast to T2, 3 and 4 (p=0.05), and also a trend towards a stepwise lowering of the level of methyaltion with increase of the number of lymph node metastases. A higher level of p16 methylation was seen in the cases with liver metastases. Again the latter two did not reach statistical significance. Statistical significance was reached only when we divided the tumors in two groups: T1/2/3 vs. T4/N/M (p<0.001).

Ten cases (6%) showed dense methyaltion of all CpGs in both mlh1 and p16. These tumors were proximal, low differentiated, had elevated mucin production, and were more common in women (Table 2). No liver metastases were found in them although this was not statistically significant (p=0.25).

DISCUSSION:

In the 'multistep' carcinogenesis model proposed by Fearon and Vogelstein the transformation of the normal large bowel mucosa through aberrant crypt foci, adenoma with different size and carcinoma is accompanied by accumulation of mutations in the APC, k-ras, DCC and p53 genes (21). No such a pathway with gradual accumulation of methylated genes has been discovered so far. Furthermore, Esteller (4) and Song (20) proved that methylation is a quantitative event, e.g. it starts with low levels with probably no physiological function, and attracts more methylation until critical level for gene silencing is achieved (4,20). Therefore it is plausible to search for certain differences in the level of methylation in certain genes taking into account their function, and to correlate them with some tumor characteristics.

The first of the two observed clusters of methylation for the mlh1 gene – the one with low level, is probably related to the sensitivity of the method. Pyrosequencing is able to detect very low amounts of DNA that is methylated even in a single CpG island. The biological function of the low level of methylation remains unknown. Several hypotheses could be driven from this: it could be due to misidentification of the unmethylated CpGs by the DNMTs; could represent the heterogeneity of the tumor, e.g. different ways of tumor formation act simultaneously; or is a part of the ageing large bowel mucosa. There is some interplay between mlh1 methylation and microsatellite instability since even these low levels are seen more often in MSI tumor

according to our results. The higher levels of mlh1 promoter methylation, and more importantly the dense methylation is able to suppress gene transcription by sequestration of methyl-CpG-binding proteins, local histone deacetylation and chromatin condensation (19). Also mlh1 dysfunction through methylation results in deficiency of the mismatch repair system leading to microsatellite instability (14). This was also seen in our samples. This is the mechanism of gene inactivation of sporadic colorectal cancers with MSI, and also in elderly patients. Interestingly these tumors share some clinico-pathological features that allow them to be differentiated. They are: proximal tumor location (above the splenic flexure), low differentiation, elevated mucin production. These have been proven also by other studies (12, 18), as well as female preponderance and presence of lymphocyte infiltration which we did not observe.

p16[INK] is an important tumor-suppressor gene that is incorporated in cell-cycle regulation (15). We did not find out any correlation between the level of p16 methylation and age, gender, tumor location or differentiation. In contrast one large study has found such an association (23). We believe that p16[INK] promoter methylation is an early epigenetic phenomenon since it is present in throughout all stages of cancer: from T1 to T4, from stage I to IV. The fact that there was a slight prevalence in p16 methylation in T1 is probably due to the inefficient number of patients in T1 – only 2. However, we did observe a difference in the methylation level when we divided our sampled in 2 gropus: T1/2/3 and T4/N/M. This is not a coincidence since patients stratified in the latter group are subjected to adjuvant chemotherapy. We support the idea that this difference could be also a result of the more aggressive tumor behavior and the presence of more tumor cells containing methylated DNA. As a whole tumors exhibiting p16 methylation form a distinct subgroup. It will be of great interest to follow them and to find out whether p16 could serve as a marker for higher or lower 5-year survival.

The presence of 10 cases (6%) with dense promoter methylation of both mlh1 and p16 is intriguing. These tumors share some features of the MSI tumors: proximal location, low level of differentiation, mucin production, female sex, and older age. Interestingly no liver metastasis was found suggesting that probably methylation needs time to cause sufficient gene suppression or that there is some mechanism of host protection against tumor invasion and spreading.

In conclusion, we think that methods for detection of DNA methylation should not be restricted only to the presence or absence of methylation. It seems that low levels that are detected by qualitative methods as positive are not strong enough to cause gene inactivation. More detailed studies analyzing the level of methylation with the degree of gene expression by RT-PCR (e.g. mRNA) or even immunohistochemistry could help in revealing the paradigm of gene regulation.

Table 1. Clinical and pathological correlations

	MLH1 (MtI)	p	p16 (MtI)	p
MSI	0.16	<0.0001†	0.13	0.97 †
MSS	0.02		0.16	
Location		0.05 †		0.53 †
proximal	0.14		0.13	
distal	0.11		0.17	
Differentiation		0.05 †		0.11 †
low	0.07		0.09	
high	0.02		0.06	
Mucin production		0.05 †		
yes	0.06			
no	0.02		—	-
Lymphocyte infiltration		0.89 †		
yes	0.03			
no	0.03		—	-
Gender		0.38 †		0.65 †
male	0.11		0.16	
female	0.15		0.13	
T		0.00003†		0.05 †
1	0.00		0.56	
2	0.13		0.13	
3	0.07		0.13	
4	0.24		0.18	
N		0.29 †		0.54 †
0	0.13		0.15	
1	0.20		0.15	
2	0.08		0.15	
3	0.03		0.00	
M		0.19 †		0.28 †
0	0.03		0.06	
1	0.03		0.09	
Stage		0.92 †		0.67 †
I	0.13		0.17	
II	0.13		0.13	
III	0.12		0.13	
IV	0.15		0.20	
T1/2/3 vs. T4/N/M	-	-	0.13 vs. 0.17	<0.001‡

‡ Mann-Whitney test; † ANOVA

Table 2. Comparison between tumors showing methyaltion in both genes vs. unmethylated ones

	Both genes methyl.	Unmethylated	p †
Number of cases	10 (6%)	74 (41%)	
Mean age	71.2	60	0.02
Proximal location	9 (90%)	25 (34%)	0.001
Differentiation-low	5 (50%)	14 (19%)	0.03
Mucin production	7 (70%)	16 (22%)	0.02

Gender
 	
    male
    female
	
	3 (30%)
	7 (70%)
		
		49 (66%)
		25 (34%)
			
			0.03

Liver metastasis	0 (0%)	9 (12%)	0.25

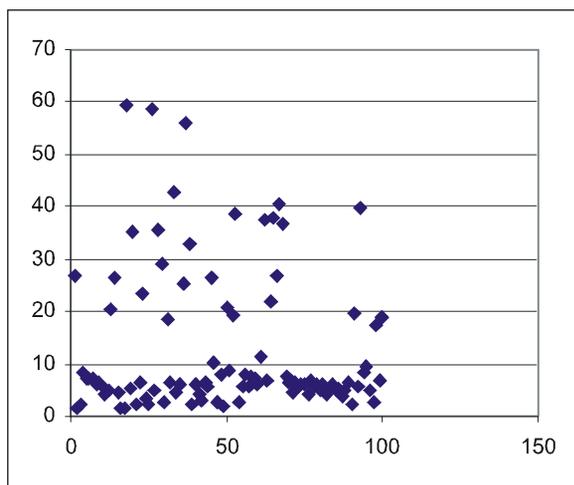† Comparison of proportions
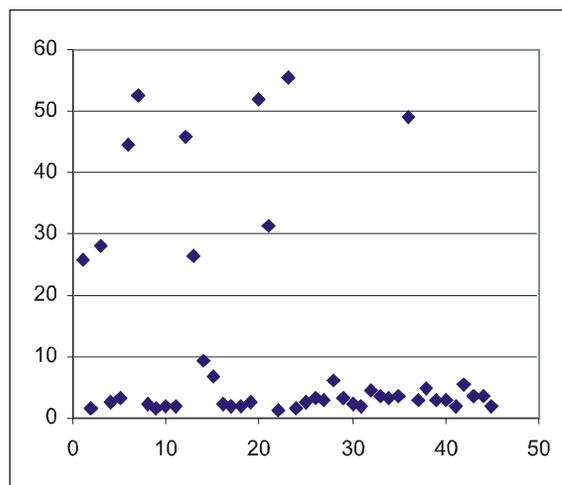

Fig. 1. MLH1 Methylation

Fig. 2. p16 Methylation

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