Expression and promoter methylation status of *hMLH1*, *MGMT*, *APC*, and *CDH1* genes in patients with colon adenocarcinoma

Christina Michailidi¹, Stamatios Theocharis², Gerasimos Tsourouflis³, Vasiliki Pletsa⁴, Gregorios Kouraklis³, Efstratios Patsouris², Athanasios G Papavassiliou¹ and Constantinos Troungos¹

¹Department of Biological Chemistry, University of Athens Medical School, Athens 11527, Greece; ²First Department of Pathology, University of Athens Medical School, Athens 11527, Greece; ³Second Department of Propedeutic Surgery, University of Athens Medical School, Athens 11527, Greece; ⁴Institute of Biology, Medicinal Chemistry and Biotechnology, Division of Biological Research & Biotechnology, National Hellenic Research Foundation, Athens 11635, Greece

Corresponding authors: Constantinos Troungos. Email: ktrougkos@med.uoa.gr; Athanasios G Papavassiliou. Email: papavas@med.uoa.gr

Abstract

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide. CRC development is the result of genetic and epigenetic alterations accumulation in the epithelial cells of colon mucosa. In the present study, DNA methylation, an epigenetic event, was evaluated in tumoral and matching normal epithelium in a cohort of 61 CRC patients. The results confirmed and expanded knowledge for the tumor suppressor genes *hMLH1*, *MGMT*, *APC*, and *CDH1*. Promoter methylation was observed for all the examined genes in different percentage. A total of 71% and 10% of the examined cases were found to be methylated in two or more and in all genes, respectively. mRNA and protein levels were also evaluated. Promoter methylation of *hMLH1*, *MGMT*, *APC*, and *CDH1* genes was present at the early stages of tumor's formation and it could also be detected in the normal mucosa. Correlations of the methylated genes with patient's age and tumor's clinicopathological characteristics were also observed. Our findings suggest that DNA methylation is a useful marker for tumor progression monitoring and that promoter methylation in certain genes is associated with more advanced tumor stage, poor differentiation, and metastasis.

Keywords: Colon cancer, epigenetics, methylation, CpG islands, methylation-specific PCR

Experimental Biology and Medicine 2015; 240: 1599–1605. DOI: 10.1177/1535370215583800

Introduction

Colorectal cancer (CRC) is worldwide the third most common cancer in men and the second in women forming the third leading cause of cancer-related deaths in the United States,¹ with the incidence and mortality rate similar to those observed in Europe.² Despite the high mortality rates, colon cancer is often treatable when is detected early, with a five-year survival rate around 90%. Colonoscopy is the gold standard for the disease diagnosis but due to the invasiveness of the procedure most of the patients will not present in clinic before clinical symptoms being prominent. In developed countries, only 40% of colon cancer patients are diagnosed at early stage when treatment is most likely to be successful.²

The biology of colon cancer is well established. Two different genetic pathways have been characterized and linked to hereditary colon cancer. The first pathway includes among others Adenomatous Polyposis Coli (APC) germline

mutations and is correlated with the familial adenomatous polyposis (FAP) syndrome.³ The second pathway covers the hereditary non-polyposis colon cancer (HNPCC) which incorporates alterations in mismatch repair (MMR) genes like *hMLH1*.⁴ Both of the above-mentioned syndromes (FAP and HNPCC) are reflections of germline mutations. Mutations observed in somatic cells consist a different category of tumors, the microsatellite instability (MSI) ones, a type that is more frequent, responsible for sporadic tumors of the colon.⁵ The common linker in all the above altered pathways is the impairment of DNA repair systems that predisposes cells to genetic instability, a hallmark of cancer.⁶

During the last decades, epigenetic alterations have been reported to play an important role in cancer initiation, progression, and metastasis.^{7,8} DNA methylation, among various sites within a gene sequence, can be found in dense CpG areas like the CpG islands in the promoter region of tumor suppressor genes resulting in their silencing.

In the present study on colon cancer patients, the promoter methylation of the tumor suppressor genes hMLH1, MGMT, APC, and CDH1 involved in DNA repair and cellular adhesion mechanisms was examined. Moreover, we attempted to identify if this epigenetic effect is present at the early stage of tumor formation and if it could also be detected in the normal mucosa, in an effort to evaluate whether methylation in the selected genes could be used as a screening biomarker to differentiate benign from cancer cases. Additionally, mRNA and protein levels were evaluated for all the genes included in the study and correlated with clinicopathological parameters important for patients' management.

Materials and methods

Patients and nucleic acid preparation

Sixty-one patients with colon cancer operated from 2007 to 2012 in Second Department of Propedeutic Surgery at Medical School of National and Kapodistrian University of Athens constituted the group of our study. All patients with available tissue during this period were included in the study. In this sample cohort, there was also a rectal case which is not further discussed. The study was approved by the ethical committee of Laikon General Hospital and written informed consent was obtained from all patients included in the study.

Thirty-six (59%) of the patients were men and 25 (41%) were women with a mean age of 69.13 ± 1.62 years. Tumors were classified and staged according to the revised guidelines set by American Joint Committee on Cancer and International Union Against Cancer. Three levels of differentiation were used to clarify histopathological grading as: well in 12 (19.7%); moderately in 35 (57.3%), and poorly in 14 (23%) differentiated cases, respectively. The resected tumors were histologically staged according to Dukes' classification as: A in 11 (18%), B in 23 (37.8%), C in 23 (37.8%), and D in 4 (6.4%) cases, respectively.

All tissue samples were kept in RNA later reagent, sheltered from RNases activity (Ambion, AM7021). Samples of normal-appearing mucosa were also derived from sites adjacent to, but at least 1 cm away from the tumor. Tissues were then homogenized and genomic DNA (QIAamp DNA Mini Kit, Qiagen) and total RNA (Nucleospin RNA II, Macherey-Nagel) were extracted according to manufacturer's protocol. Paraffin-embedded tumor tissue sections from the same patients were also used for immunohistochemical studies.

Bisulfite treatment of the DNA and methylation-specific PCR

DNA from both tumoral and normal tissue specimens was subjected to bisulfite chemical modification (EZ DNA Methylation-Gold Kit, Zymo Research), according to manufacturer's instructions. Two hundred ng of modified DNA followed methylation-specific PCR amplified by primers pair specific for methylated and unmethylated sequences, as previously described.⁹ Primers were designed with Meth-Primer software, based on the human

genes' promoter sequence found in GenBank. DNA from human placenta served as positive control for the unmethylated sequences (Sigma Aldrich). As positive control for the methylated sequences either fully methylated DNA obtained after treatment to placental DNA with an excess of M. SssI methyltransferase (New England Biolabs, U.S.) or standard methylated DNA {S}¹⁰ commercially available (CpGenome Universal Methylated DNA, Chemicon International) was used. Samples were also amplified with the wild-type primer set to verify completed chemical modification, as unmodified DNA produces a specific DNA (Figure 1(a) and (b)). Water was used as negative control. Primer sequences and the specific annealing temperatures for each set of primers are shown in Supplementary Table S1.

The generated fragments were analyzed on 2% agarose gel (Agarose low EEO, Applichem), stained with ethidium bromide and visualized under UV. DNA marker to evaluate fragment size was also used (GelPilot DNA Molecular Weight Marker 100 bp, Qiagen).

Results were also evaluated with sequencing technique that revealed the methylated cytosines' position in methylated samples.

Semi-quantitative RT-PCR

One μ g of total RNA was subjected to first-strand cDNA synthesis by using Omniscript Reverse Transcriptase (Omniscript, Qiagen) and random hexamers (Qiagen), according to manufacturer's protocol. PCR was performed with 100 ng of the cDNA template. The specific primers and PCR conditions for each of the examined genes are shown in Supplementary Table S2. The RT-PCR products were analyzed on 3% agarose gel, stained with ethidium bromide and visualized under UV. Water served as negative control (Figure 2). Although RT-PCR is not a quantitative assay, a degree of quantitation was achieved by normalization with *abl* gene. mRNA was evaluated in all malignant and non-malignant adjacent lesions.

Immunohistochemistry (IHC)

A three step (avidin-biotin-peroxidase) immunohistochemical method was applied for the detection of hMLH1, MGMT, APC, and CDH1 proteins on formalin-fixed, paraffin-embedded colon cancer tissue sections as previously described.¹¹ Mouse monoclonal anti-MLH1 (CM 220 CK, Biocare Medical), anti-MGMT (sc-56432, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-APC (sc-289, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-CDH1 (sc-56527, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilutions 1:150, respectively, were used as primary antibodies. Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant antiserum. As a positive control, colon cancer tissue sections with known increased hMLH1, MGMT, APC, and CDH1 immunoreactivity were used (Figure 3(a) to (d)). Immunohistochemistry was performed only in the cancer cases.

Figure 1 (a) Electrophoresis of MSP-PCR products for *hMLH1* gene. Starting from the left we can see the DNA marker and then follow the samples (sample 1, 2, 3). Each sample was amplified with both unmethylated and methylated primers pair as well as with the wild-type primer for certification of fully chemical modification. DNA that has not undergone modification gives a band with wild-type primers as shown in the last well. The amplified samples were not methylated. To the right (positions 11–15) are the controls. Placental DNA with unmethylated primers (position 11) serves as positive control for unmethylated sequences. Fully methylated DNA (F,S; positions 13–14) with methylated primers set serves as the positive control for methylated DNA. Water also served as the negative control (positions 12, 15). The expected product sizes are also shown in the figure; (b) Electrophoresis of MSP-PCR products for *MGMT*, *APC*, and *CDH1* genes. Starting from the left we can see the DNA marker and then follow the samples. Each sample was amplified with both unmethylated and methylated primers pair. Some samples were methylated and other unmethylated. Patients could be homozygous or heterozygous for methylation as indicated in red rectangles. Positive controls for unmethylated and methylated primers are following. The expected product sizes are shown in the figure. Water served as the negative control

Figure 2 Electrophoresis of RT-PCR products for genes *abl*, *hMLH1*, *MGMT*, *APC*, and *CDH1*. *Abl* gene was used for quantification of cDNA. On the left, DNA size marker is distinguished, followed by amplified samples. Water served as the negative control in all cases. The intensity of the band related to mRNA expression differs between samples. As shown in figure (red rectangles), samples that were not methylated gave a strong band (*CDH1*), and patient samples that were methylated for a gene gave a low intense band (*APC*) or no band (*hMLH1*) which certifies that methylation is associated with genes' silencing. The expected product sizes are also shown in the figure

Figure 3 Representative immunostainings for hMLH1 (a), MGMT (b), APC (c), and CDH1 (d) in colon cancer tissues (original magnification $\times 200$). Protein pattern of distribution: nuclear for hMLH1 and MGMT, cytoplasmic for APC and membranous for CDH1

Evaluation of immunohistochemistry

The percentages of positively stained colon cancer cells were obtained by counting at least 1000 cells in each case by two independent observers (S.T. and E.P.) blinded to the clinical data, with complete observers' agreement. Specimens were considered "positive" for MLH1, MGMT, APC, and CDH1 proteins when more than 5% of the colon cancer cells were stained. We consider that over-expression exists when the percentage of positive stained cancer cells overpass the percentage of the mean average stain for each probe. The intensity of immunostaining was estimated semi-quantitative as low, medium, or intense, based on previously reported studies for other immunohistochemical markers on colon cancer samples.¹¹

Statistical analysis

Chi-square tests were used to assess the associations between the methylation status of MLH1, MGMT, APC, and CDH1 and clinicopathological characteristics of the tumor lesions, as well as to assess the associations between MLH1, MGMT, APC, and CDH1 immunoreactivity and clinicopathological characteristics in patients with malignant colon lesions. One-way ANOVA was used for all age correlations since age followed normal distribution. A two-tailed $p < 0.05$ was considered (statistically) significant. Statistical analyses were performed using the software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA).

Results

Promoter DNA methylation in colon cancer tissue and adjacent normal tissue

For hMLH1 gene, our analysis detected promoter methylation in 15 out of 61 (25%), for MGMT gene 40 out of 61 (66%), for APC gene 18 out of 61 (30%), and for CDH1 gene 53 out of 61 (87%) tumor cases, respectively. Forty-three out of 61 cases (71%) were found to be methylated in two or more genes and six out of 61 (10%) ones were found to be methylated in all of the examined genes.

Methylation in the promoter region of the examined genes was also detected in the normal matching tissue. Six out of the 14 (43%) cases presented also methylation in the normal mucosa for hMLH1 gene, 22 samples out of 27 (81.5%) for MGMT gene, 12 out of the 14 (86%) for APC, and 23 out of 31 (74.2%) for CDH1 gene, respectively). Normal pairing tissue, adjacent to the tumor, was missing for one of the methylated for hMLH1 gene samples.

Promoter methylation and correlations with patients' demographics and mRNA expression

The correlations between patients clinicopathological characteristics and the genes methylation status are depicted in Supplementary Table 3. Methylation in hMLH1 and MGMT genes was found to be statistically associated with patients' age ($p = 0.04$ and $p = 0.003$, respectively). No statistical significant associations were observed for hMLH1, MGMT, and APC genes when compared with tumor stage and histological grade. Statistically significant correlations

were found for *CDH1* gene. Promoter methylation of *CDH1* gene was associated with Dukes' stage when classifying the tumors as lower (A and B) versus higher stage (C and D) or (A, B, C versus D) ($p=0.02$ and $p=0.0001$, respectively). Promoter methylation of *CDH1* gene was also correlated with the histological tumor grade ($p=0.031$). Again, when tumor cases were subdivided as well and moderately differentiated versus poorly differentiated ones promoter methylation was more frequently observed in the poorly differentiated cases ($p=0.005$). The low number of stage D ($n=4$) and grade III cases ($n=14$) should also be considered when looking at the statistical analysis results as this may have contributed to the inexistence of strong correlations between the methylation status and the clinico-pathological variables of the tumors in the rest of the genes or a more balance distribution of the samples could have possibly provided a stronger correlation for *CDH1* gene with tumors' stage and grade.

Methylation in two or more of the examined genes was found to be statistically associated with tumor grade ($p=0.04$), as also was able to discriminate the samples when well and moderately differentiated cases were examined versus the poorly differentiated ones ($p=0.005$). Methylation in two or more genes was not correlated with Dukes' stage ($p=0.176$) but when examining two different groups: stages A and B versus C and D, methylation in two or more genes was more frequently presented in the latter ones, $p=0.047$. Moreover, methylation in two or more of the genes included in our study was associated with the age of the patients ($p=0.018$).

Lower expression levels or no expression at all was observed in all the methylated samples when mRNA was amplified by RT-PCR for each of the examined genes (Figure 2).

Protein expression—correlations with tumor characteristics

Positivity for *hMLH1* was noted in 32 out of 46 cases (69.6%), for *MGMT* in 31 out of the 47 cases (66%), for *APC* in all ($n=44$) examined samples (100%), and for *CDH1* in nearly all ($n=44$) examined cases (98.0%). The protein pattern of distribution was nuclear for *hMLH1* and *MGMT*, cytoplasmic for *APC*, and membranous for *CDH1*, respectively (Figure 3(a) to (d)). *hMLH1* protein positivity, extent of expression, and intensity of staining were correlated with patients' age ($p=0.047$, $p=0.003$, and $p=0.003$, respectively). *hMLH1* protein positivity was correlated with Dukes' stage ($p=0.030$). *hMLH1* protein intensity of staining was correlated with patients' age ($p=0.005$) and histological grade ($p=0.023$). *MGMT* protein positivity was correlated with tumors histological grade ($p=0.029$). *MGMT* protein extent of expression and intensity of staining was correlated with patients' age ($p=0.03$ and 0.006 , respectively). *MGMT* protein intensity of immunostaining was correlated with patients' Dukes stage ($p=0.003$). *CDH1* protein intensity of staining was correlated with patients' gender, age, and tumor histological grade ($p=0.013$, $p=0.062$, and $p=0.005$,

respectively). IHC results and correlations with patients' demographics per gene are shown in Supplementary Tables S4 to S7.

Integration of promoter methylation with protein expression

When cross examining methylation with immunohistochemical variables (positivity, staining percentage, and intensity) no significant associations were noted. However, the vast majority of the positive for *hMLH1* and *APC* proteins samples were not methylated. Furthermore, the percentage and the intensity of the stain for both proteins were lower in the methylated cases compared to the unmethylated ones. *MGMT* positivity and stain intensity were somewhat evenly distributed between methylated and unmethylated lesions. On the contrary, *CDH1* protein exhibited high levels of expression and staining intensity in all the methylated cases.

Discussion

CRC development is the phenotype of the accumulation of genetic and epigenetic alterations in the epithelial cells of the colon mucosa.¹² The vast majority of CRCs have several chromosomal abnormalities, the so-called chromosomal instability phenotype. This phenotype is caused by defects in DNA repair machinery and more specific the MMR system.¹³ The genetic defects in the adenocarcinoma sequence are very well characterized.¹⁴ Epigenetic alterations involve histone modification, DNA methylation and chromatin remodeling, non-self-disqualifying events. During the last decades, epigenetic alterations have been the area of interest for many cancer studies.

Our study was focused on epigenetic alterations in CRCs. Genes that have been reported to play an important role in colorectal carcinogenesis were selected for further exploration. Genes with characterized mutations linked to hereditary colon cancer syndromes, as also genes involved in DNA repair machinery, adhesion, and metastasis. On that angle *hMLH1*, *MGMT*, *APC*, and *CDH1* genes were studied. Our aim was first to study the promoter methylation of the selected genes in combination with the mRNA and protein expression levels in an effort to identify correlations between methylation and expression levels in cancer cases and additionally identify possible positive associations with tumor characteristics. Second, we wanted to explore the time period of occurrence for these epigenetic events by examining tissue from the normal adjacent mucosa for an available number of cancer cases.

hMLH1 is a gene involved in the MMR machinery and is linked to the HNPCC formation and the MSI tumors. Promoter methylation was observed in 25% of the cancer cases and mRNA and protein levels were in accordance with the gene's silencing frequency due to methylation. Our results are consistent with what has been reported so far in sporadic CRCs.¹⁰ Although we didn't perform an MSI testing, most of the studies support the notion that an antibody against *hMLH1* protein can allow a diagnosis of MSI

with a 95% sensitivity and that MSI-H tumors have high hMLH1 methylation frequency which is also an early event associated with low protein levels detected by IHC.^{4,15,16} Based on the above-mentioned reasons we can speculate that cancer cases found hypermethylated in hMLH1 promoter can be related to MSI. Forty-three percent of the methylated cases exhibit promoter methylation in the adjacent normal mucosa. This is not unexpected since hMLH1 hypermethylation was also found in 16% of polyps in young patients.¹⁷

MGMT gene codes the direct repair protein O6Methylguanine Methyltransferase which protects the human genome against mutagenesis by removing methyl adducts from O6 guanine.¹⁸ Methylation of MGMT is an initiating step in the development of MMR deficient colorectal carcinomas.^{19,20} Furthermore, aberrant methylation of hMLH1 and MGMT has been shown to silence genes and result in cancer progression with an MSI phenotype.²¹ Our study identified 66% of the cancer cases being methylated in MGMT promoter region, a frequency consistent with that of others^{10,20,22} and 81.81% of these cases were also methylated in the normal adjacent mucosa, findings that are in accordance with these of Shen *et al.*²³ where methylation was observed in 81% in the normal adjacent epithelium. mRNA and protein levels were also in accordance with the methylation findings and MGMT staining intensity correlated with Dukes' stage ($p = 0.003$) providing an inefficiency in DNA repair capacity in the advanced tumor stage. This assumption is also supported by the high positivity that was noted in higher grade lesions ($p = 0.029$).

APC is a tumor suppressor gene related with the FAP syndrome. Inactivation of APC is a frequent event in CRC mainly due to truncating mutations. In our discovery set, we observed a consistency between the 30% methylation levels and mRNA expression pretty close to the 28% that have been reported previously¹² but not with the protein levels, as IHC revealed a 100% positivity. This is not unexplained as there are studies suggesting that APC protein levels were independent of the presence of APC methylation²⁴ and that APC methylation does not result in complete gene inactivation and does not act as a second hit in carcinogenesis, based on Knudson's two hit hypothesis.²⁵ APC has a central role in WNT signaling, activation of which results in increased cell proliferation.

Cadherin type 1 (CDH1) is a calcium-dependent cell-cell adhesion glycoprotein further stimulating WNT. In our cohort a high methylation frequency was observed with 87% of the cancer cases being methylated in the promoter region. Among them 74.2% were also methylated in the adjacent normal mucosa and IHC revealed a 100% positivity. Our results showed higher methylation levels than others²⁶ but there are several lines of explanation. One can be attributed to the high sensitivity of PCR-based techniques that can detect methylation even when it occurs in a small cell population. Furthermore, methylation was detected only in one allele in 95% of the cases, so the protein is still expressed. Another explanation is that the gene promoter region we targeted by MSP is not the most strongly correlated with gene silencing. Thus, maybe a promoter

region that was not associated with the protein expression levels was targeted. CRC is an heterogeneous disease. Gene amplification can also be the reason resulting in an increase in the number of copies of a gene sequence.

Our study also revealed associations between methylation and patients' age for hMLH1 and MGMT genes ($p = 0.04$ and 0.003 , respectively). Methylation observed in two or more of the examined genes was also correlated with patients' age ($p = 0.018$). Positive correlation between promoter hypermethylation and age has been reported by many groups.^{27,28} Suzuki *et al.* have reported that global DNA demethylation in gastrointestinal cancer is age dependent and proceeds genomic damage.²⁷ DNA methylation undergoes global hypomethylation (especially within the repetitive sequences in the gene body) and a simultaneous hypermethylation in the promoter region of certain genes.

Another interesting finding of our analysis was the high methylation frequency observed in the normal mucosa for the samples found to be methylated in the cancer cases suggesting that DNA methylation is an early event in carcinogenesis. DNA methylation in the normal colorectal mucosa is associated with pathway-specific predisposition to cancer.²⁹ Epigenetic alterations opposite to the genetic alterations occur in normal cells and after neoplastic transformation they are exposed in the tumors by clonal expansion. Therefore, epigenetic alterations occur prior to genetic alterations. Based on our findings, there is strong evidence to propose that methylation in normal lesions can be used as a prognostic marker, an idea that has also been reported by other groups.³⁰

CDH1 methylation was associated with tumor grade ($p = 0.031$). Poorly differentiated tumors were more frequently methylated. This observation, along with the trend with Dukes' stage (cases of stage C and D were more frequently methylated) and the statistically significant correlations between methylation in two or more genes and tumor stage and grade, propose CDH1 as a very important gene in CRC, related with more aggressive tumors.

In summary, our study validated and expanded previously published data in tumor suppressor genes in CRC. Our findings suggest that DNA methylation is a useful marker and that promoter methylation in certain genes is associated with more advanced tumor stages, poor differentiation, and metastasis. Thus, promoter methylation can have an application as a risk assessment tool or as a marker of recurrence to help oncologists decide on the aggressiveness of the treatment. Our results support the notion that future comparison between colon tumor, adjacent colon mucosa, and non-colon tissue may be useful to assess differential epigenetic alterations. Additionally, larger studies in more centers on these and other genes are needed to delineate our findings in a more extended patient population. Moreover, since promoter methylation was identified with high frequency in normal adjacent mucosa it will be worthy to evaluate if these genes can serve as a biomarker for early detection in bodily fluids such as plasma, serum, or urine and provide a non-invasive method to clinicians to stratify patients of higher risk for CRC that should be monitored more closely.

Authors' contributions: All authors have contributed significantly in this article. All authors have approved the article's publication.

ACKNOWLEDGEMENTS

This work and Dr C. Michailidi were supported by the Stavros S. Niarchos Foundation.

CONFLICTS OF INTEREST

The authors of this research have no personal financial relationships with people or organizations that could inappropriately influence the impartiality of this work.

REFERENCES

- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* n.d.;**62**:10-29
- Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 2013;**49**:1374-403
- Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;**61**:759-67
- Miyakura Y, Sugano K, Konishi F, Ichikawa A, Maekawa M, Shitoh K, Igarashi S, Kotake K, Koyama Y, Nagai H. Extensive methylation of hMLH1 promoter region predominates in proximal colon cancer with microsatellite instability. *Gastroenterology* 2001;**121**:1300-9
- Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;**260**:816-9
- Michailidi C, Papavassiliou AG, Troungos C. DNA repair mechanisms in colorectal carcinogenesis. *Curr Mol Med* 2012;**12**:237-46
- Carmona FJ, Esteller M. Epigenomics of human colon cancer. *Mutat Res* 2010;**693**:53-60
- Venkatachalam R, Ligtenberg MJL, Hoogerbrugge N, de Bruijn DRH, Kuiper RP, Geurts van Kessel A. The epigenetics of (hereditary) colorectal cancer. *Cancer Genet Cytogenet* 2010;**203**:1-6
- Tsellou E, Michailidi C, Pafiti A, Troungos C. DNA methylation-independent regulation of p16 in epithelial cells during mouse mammary gland development. *Epigenetics* n.d.;**3**:143-8
- Psafaki V, Kalogera C, Tzambouras N, Stephanou D, Tsianos E, Seferiadis K, Kolios G. Promoter methylation status of hMLH1, MGMT, and CDKN2A/p16 in colorectal adenomas. *World J Gastroenterol* 2010;**16**:3553-60
- Giaginis C, Georgiadou M, Dimakopoulou K, Tsourouflis G, Gatzidou E, Kouraklis G, Theocharis S. Clinical significance of MCM-2 and MCM-5 expression in colon cancer: association with clinicopathological parameters and tumor proliferative capacity. *Digest Dis Sci* 2009;**54**:282-91
- Kim MS, Lee J, Sidransky D. DNA methylation markers in colorectal cancer. *Cancer Metastasis Rev* 2010;**29**:181-206
- Lind GE, Thorstensen L, Løvig T, Meling GI, Hamelin R, Rognum TO, Esteller M, Lothe RA. A CpG island hypermethylation profile of primary colorectal carcinomas and colon cancer cell lines. *Mol Cancer* 2004;**3**:28
- Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;**319**:525-32
- Menigatti M, Truninger K, Gebbers J-O, Marbet U, Marra G, Schär P. Normal colorectal mucosa exhibits sex- and segment-specific susceptibility to DNA methylation at the hMLH1 and MGMT promoters. *Oncogene* 2009;**28**:899-909
- van Roon EHJ, van Puijbroek M, Middeldorp A, van Eijk R, de Meijer EJ, Erasmus D, Wouters KAD, van Engeland M, Oosting J, Hes FJ, Tops CMJ, van Wezel T, Boer JM, Morreau H. Early onset MSI-H colon cancer with MLH1 promoter methylation, is there a genetic predisposition? *BMC Cancer* 2010;**10**:180
- Koh DC, Luchtefeld MA, Kim DG, Attal H, Monroe T, Ingersoll K. Microsatellite instability and MLH1 hypermethylation - incidence and significance in colorectal polyps in young patients. *Colorectal Dis* 2007;**9**:521-6
- Pegg AE. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* 1990;**50**:6119-29
- Svrcek M, Buhard O, Colas C, Coulet F, Dumont S, Massaoudi I, Lamri A, Hamelin R, Cosnes J, Oliveira C, Seruca R, Gaub M-P, Legrain M, Collura A, Lascols O, Turet E, Fléjou J-F, Duval A. Methylation tolerance due to an O6-methylguanine DNA methyltransferase (MGMT) field defect in the colonic mucosa: an initiating step in the development of mismatch repair-deficient colorectal cancers. *Gut* 2010;**59**:1516-26
- Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;**61**:3225-9
- Poynter JN, Siegmund KD, Weisenberger DJ, Long TI, Thibodeau SN, Lindor N, Young J, Jenkins MA, Hopper JL, Baron JA, Buchanan D, Casey G, Levine AJ, Le Marchand L, Gallinger S, Bapat B, Potter JD, Newcomb PA, Haile RW, Laird PW. Molecular characterization of MSI-H colorectal cancer by MLH1 promoter methylation, immunohistochemistry, and mismatch repair germline mutation screening. *Cancer Epidemiol Biomarkers Prev* 2008;**17**:3208-15
- Lee K-H, Lee J-S, Nam J-H, Choi C, Lee M-C, Park C-S, Juhng S-W, Lee J-H. Promoter methylation status of hMLH1, hMSH2, and MGMT genes in colorectal cancer associated with adenoma-carcinoma sequence. *Langenbecks Arch Surg* 2011;**396**:1017-26
- Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa J-PJ. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst* 2005;**97**:1330-8
- Chen J, Röcken C, Lofton-Day C, Schulz H-U, Müller O, Kutzner N, Malfetheriner P, Ebert MPA. Molecular analysis of APC promoter methylation and protein expression in colorectal cancer metastasis. *Carcinogenesis* 2005;**26**:37-43
- Segditsas S, Sieber OM, Rowan A, Setien F, Neale K, Phillips RKS, Ward R, Esteller M, Tomlinson IPM. Promoter hypermethylation leads to decreased APC mRNA expression in familial polyposis and sporadic colorectal tumours, but does not substitute for truncating mutations. *Exp Mol Pathol* 2008;**85**:201-6
- Miotto E, Sabbioni S, Veronese A, Calin GA, Gullini S, Liboni A, Gramantieri L, Bolondi L, Ferrazzi E, Gafà R, Lanza G, Negrini M. Frequent aberrant methylation of the CDH4 gene promoter in human colorectal and gastric cancer. *Cancer Res* 2004;**64**:8156-9
- Suzuki K, Suzuki I, Leodolter A, Alonso S, Horiuchi S, Yamashita K, Peruchio M. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell* 2006;**9**:199-207
- Boks MP, Derks EM, Weisenberger DJ, Strengman E, Janson E, Sommer IE, Kahn RS, Ophoff RA. The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS One* 2009;**4**:e6767
- Worthley DL, Whitehall VLJ, Buttenshaw RL, Irahara N, Greco SA, Ramsnes I, Mallitt K-A, Le Leu RK, Winter J, Hu Y, Ogino S, Young GP, Leggett BA. DNA methylation within the normal colorectal mucosa is associated with pathway-specific predisposition to cancer. *Oncogene* 2010;**29**:1653-62
- Auclair J, Vaissière T, Desseigne F, Lasset C, Bonadona V, Giraud S, Saurin J-C, Joly M-O, Leroux D, Faivre L, Audouyoud C, Montmain G, Ruano E, Herceg Z, Puisieux A, Wang Q. Intensity-dependent constitutional MLH1 promoter methylation leads to early onset of colorectal cancer by affecting both alleles. *Genes Chromosomes Cancer* 2011;**50**:178-85

(Received December 10, 2014, Accepted March 12, 2015)