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Original article

Analysis of O6-methylguanine-DNA methyltransferase methylation status in sporadic colon polyps

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ABSTRACT

Background/aim: The aim of our study was to check how MGMT methylation status together with known factors influenced the risk of colon cancer development.

Materials and methods: We examined patients with colon polyps. Information concerning gender, age, lifestyle, diet, anthropometry and medical information, including cancer and family history of cancer, was analyzed. Polymorphism variety of MGMT gene was investigated in another study. Genetic analysis for MGMT methylation assessment was performed for polyp tissue samples from 143 patients.

Results: Positive methylation MGMT status was found in 55 patients. There was no correlation between gender and MGMT methylation status ($p = 0.43$). We did not find correlation between patients younger and older than 60 ($p = 0.87$). There was no correlation between smoking and MGMT methylation status ($p = 0.36$). We did not find correlation between BMI and MGMT methylation status ($p = 0.86$). We did not find correlation between MGMT methylation status and colon cancer in familial history ($p = 0.45$).

Conclusion: Our study showed no correlations between methylation status of MGMT polymorphisms and clinical features like age, gender, polyp localization, smoking status, or obesity. It has been shown previously that MGMT methylation status may show nonspecific methylation in colon polyps. Gene methylation status in adenoma tissues has also been associated by other authors with the adenoma's size, histology, and degree of atypia. In our study, we evaluated the gene methylation status in colon polyps and found no association with adenoma characteristics. The present study showed no correlation for MGMT methylation in polyps in different regions of colon.

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1. Background

MGMT promoter methylation is presented in approximately 20–30% of colorectal cancers and in many of them it coexists with or in some cases is independent of CIMP status. Transcriptional inactivation by promoter CpG island methylation tumor suppressor genes is an important mechanism in human carcinogenesis.[1–3] Epigenetic suppressor gene silencing has commonly been involved in all types of human tumors.[4–6] The inactivating of tumor suppressor genes may affect many important cellular processes, such as the cell cycle, the TP53, the WNT signaling pathways, DNA repair, apoptosis pathways and the metastasizing process.[1–3] The human MGMT gene is located on chromosome 10q26 and consists of 5 exons. MGMT is a DNA repair protein that removes adduct from 0^6 –G in DNA. The same effect is observed in many types of human tumors where the lack of MGMT expression results in higher frequency of mutations in genes critical for carcinogenesis, such as K-ras2 and p53.[7–9] Mutations in MGMT have rarely been found, and it has been suggested that MGMT inactivation is primarily manifested through hypermethylation-induced silencing of its promoter in human cancers, including those of the colon.[10–12]

We explored these potential biomarkers using methylation specific PCR (MSP) assay approach to measure the methylation status of MGMT gene in colon polyps. Then we examined the MGMT methylation status and correlations between clinical features like age, gender, polyp localization, smoking status, obesity and sporadic polyps characteristics.

2. Aim

The aim of the study was to establish the role of methylation status in colon polyps.

3. Materials and methods

The cases were recruited from colonoscopy department of the Greater Poland Cancer Centre between the years 2004–2008. Patients were chosen randomly; there was only one criterion for inclusion into the study: having a colon polyp. Volunteers were outpatients with no known gastrointestinal pathology who had undergone colonoscopy as a diagnostic procedure, typically to investigate nonspecific symptoms, such as abnormal bowel habit or unexplained rectal bleeding. Ethical approval for the project was received from the Institutional Review Board at Poznan University of Medical Sciences (local Research Ethics Committee, project reference 965/08) and the consent was obtained in advance of the expected date of endoscopy. Information concerning gender, age, lifestyle, diet, anthropometry and medical information, including cancer and family history of cancer, was obtained from a questionnaire. Experimental biopsies were collected from the endoscopy patients in the endoscopy department, immediately after procedure. All colon polyps were divided into two parts, one underwent histopathological estimation and the other one served as material for DNA extraction. All polyps were examined twice by two independent pathologists. Polymorphism variety of MGMT gene was investigated in another study.

3.1. Genetic analysis

Methylation-specific PCR. Bisulfite modification: DNA (1 μg) in volume of 50 μl was denatured by NaOH to final concentration of 0.2 M for 10 min at 37 °C. Thirty microliters of 10 nM hydroquinone and 520 μl of 3 M sodium bisulfite at pH 5 were mixed and added to the sample and incubated at 50 °C for 16 h. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer protocol (Promega) and eluted into 50 μl of water. Modification was completed by NaOH (final concentration of 0.3 M) incubation for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and before PCR reaction, concentration of DNA was measured.

MGMT amplification: The following sets of primers were used for amplification: UM F: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT; UM R: 5′-AACTCCACACTCTTCCAAAAACAAAACA; M F: 5′-TTTCGACGTTCGTAGGTTTTCGC and M R: 5′-GCACTCTTCCGAAAACGAAACG.

PCR-SSCP analysis was performed for polyp tissue samples from 143 patients according to the MSP method. We used TaqPol (Applied Biosystem) polymerase, and primer sequences of *MGMT* for the unmethylated reaction F were: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (upper primer) and R: 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (lower primer) and for the methylated reaction F: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (upper primer) and R: 5′-GCACTCTTCCGAAAACGAAACG-3′ (lower primer). Each PCR mixture contained a buffer (10×), $MGCl_2$ (2 nM), dNTP (300 μM), F (50 pM), R (50 pM), TaqPol (5 U), and DNA matrix (200 ng). Volume of reaction was 10 μl. The annealing temperature was 59 °C.

3.2. Statistical analysis

The Pearson's chi-square (χ^2) and Fisher's exact tests were used to test the differences in genotype and allele (respectively) distribution between patients and control subjects. Statistical analyses were performed using Statistica v.7.1 software (Statsoft, USA). For polymorphisms containing less than 5 observations per cell, the Fisher–Freeman–Halton exact test was performed using StatsDirect statistical software v.2.6.2. Logistic regression was employed to calculate odds ratios (OR) and 95% confidence intervals (95%CI) and used to calculate interactions. Odds ratios were calculated using a demonstration version of GraphPad InStat 3.

4. Results

Our study group consisted of 74 males (51.75%) and 69 females (48.25%). The median age in our group was 60.32. The main aim of our study was to check how MGMT methylation status together with known factors influenced the risk of colon cancer development. Positive methylation MGMT status was found in 55 patients. There was no correlation between gender and MGMT methylation status ($p = 0.43$). Patients were divided

Table 1 – Clinical and histipatological factors and MGMT methylation status.

Factor		All	Methylated	p	OR, 95%CI
Gender	Female	69	23	0.4314	0.7708
	Male	74	32		0.4112–1.448
Age	≤60	68	25	0.874	0.9191
	>60	75	30		0.4924–1.716
BMI	≤25	98	39	0.8638	1.119
	>25	45	16		0.5666–2.211
Tobacco users	Smokers	39	11	0.3623	0.6667
	Non-smokers	104	44		0.3129–1.42
Familial history	Colon cancer	17	4	0.4449	0.5813
	Others	126	51		0.1865–1.812
Familial history	Non colon cancer	84	31	0.8724	0.9072
	Others	59	24		0.4839–1.701
Polyps	Hyperplastic	42	14	0.599	0.8
	Adenomatous	96	41		0.3939–1.625
Polyps size	≤5 mm	122	48	0.8227	1.18
	>5 mm	21	7		0.4711–2.957
Polyps localization	Distal colon	108	41	1	0.9491
	Proximal colon	35	14		0.4635–1.943

into two groups: aged 60 and younger and older than 60. We did not find correlation between patients younger and older than 60 ($p = 0.87$). In the study group, there were 39 smokers and 104 non-smokers. There was no correlation between smoking and MGMT methylation status ($p = 0.36$). Obesity was also regarded as a risk factor of polyp formation. 98 patients were found to have a BMI higher than 25 and we did not find correlation between BMI and MGMT methylation status ($p = 0.86$). Moreover, all patients followed the same diet, typical for Central Europe.

In our group, the polyps were localized in the rectum (39; 27.4%), sigmoid colon (60; 41.9%), descending colon (9; 6.3%), transverse colon (17; 11.9%), ascending colon (12; 8.4%) and cecum (6; 4.2%). We expected the methylation status of MGMT gene to show association with proximal localization but no statistical evidence was noticed to prove it ($p = 1.00$). Among histological types of polyps, the biggest group was adenomas recognized in 96 (67.1%) cases, followed by hiperplastic polyps with 42 (29.4%) cases. Other types, such as mixed polyps and serrated polyps, were recognized in 2 (1.4%) and 3 (2.1%) cases, respectively (2 sessile serrated adenoma and 1 traditional serrated adenoma).

Adenomatous and hiperplastic polyps share the same risk factors, such as age, sex, smoking and alcohol consumption. Age is the risk factor for adenomatous polyps while smoking for hyperplastic. We analyzed how MGMT methylation together with age and smoking modulated the risk of developing each of polyp type, but found no correlation between age, smoking, MGMT methylation status and type of colon polyps.

For further statistical analysis, the two groups of high risk polyps were appreciated based on size. The first group included 21 polyps larger than 5 mm; no correlation was found between this group and smoking ($p = 1.00$), age (for patients older than 60, $p = 0.38$), gender ($p = 0.72$) and MGMT methylation status was. Among 84 patients who declared cancer cases in close relatives, 17 colon cancers were found in familial history. Moreover, 3 patients fulfilled the criterion of HNPCC syndrome. Familial colorectal cancer was defined broadly to include colorectal cancer patients who have at least one first-degree relative with colorectal cancer. We did not find correlation between MGMT methylation status and colon cancer in familial history ($p = 0.45$). Nor did we find any association between other types of cancers reported in family history ($p = 0.87$). Results of the statistical analysis are presented in Tables 1 and 2.

5. Discussion

Colorectal cancers develop as a result of the transformation of normal mucosal epithelium to cancer through precursor lesions with genetic and epigenetic changes. Promoter methylation of several genes has been found to be limited to colorectal cancers. It has been observed in tumor suppressor genes and DNA repair genes such as MGMT.[13,14] Ogino et al.[15] investigated the connection between MGMT germline SNPs and promoter methylation in colorectal cancer. A strong association was found between c.−56C > T and promoter methylation and MGMT silencing. Unfortunately, non-promoter polymorphisms, as L84F, did not show such significant correlation.

The aging process and varying exposures to environment have been hypothesized to cause significant changes in methylation profiles.[22] Recent research has shown an overall trend of increased methylation in several genes associated with older age in normal colon tissues.[20] Age-dependent methylation in healthy human colon may contribute to an increased risk of colorectal cancer.[20,23] In some studies, authors observed the contrary, that is they found no correlation with age.[19,24] Yamashita at al. proposed that all

Table 2 – Colon polyps localization and MGMT methylation status.

Polyps		Methylated	*p*	OR, 95%CI
Recti	39	12	0.4733	0.7442
	104	43		0.3556–1.557
Sigmae	60	25	0.7488	1.153
	83	30		0.6163–2.156
Descending colon	9	4	0.7573	1.168
	134	51		0.3443–3.961
Transverse colon	17	6	1.000	0.9076
	126	49		0.3380–2.437
Ascending colon	12	7	0.4194	1.592
	131	48		0.5920–4.282
Ceci	6	1	0.6758	0.4228
	137	54		0.04971–3.597

methylation statuses in colorectal cancers were related only to the aging process, non neoplastics.[25] Our data did not suggest that MGMT was methylated in an age-dependent manner. There was no correlation between MGMT methylation status and age in our study group. Our data were in accordance with the observation of Eckhardt et al.[26] There was no changes in DNA methylation over time. In that study, values were averaged across individuals for a given age group.

These findings may indicate that there are differences between populations, perhaps because of genotyping variation or differing exposure to environmental factors, such as diet. Bjornsson et al. suggest more complex associations.[24] These authors found both increased and decreased intra-individual global methylation levels (promoter regions) in peripheral blood cell DNA over time. In this context, it is crucial to more extensively characterize the contribution of aging and the environment to tissue-specific epigenetic profiles.

Ahlquist et al.[27] identified methylation profiles of normal colorectal tissues, adenomas and carcinomas. The results demonstrated a stepwise increase in CpG island promoter methylation towards malignancy. Taken together, these observations suggest that methylation levels in the normal colonic mucosa could serve as markers of risk for the development of colon adenomas and colorectal cancer. Animal studies have shown that a loss of DNA methylation increases intestinal adenoma initiation and a gain of DNA methylation increases adenoma progression.[28] Similarly, both hypomethylation and hypermethylation could lead to autoimmune disease by activating autoreactivity genes or silencing histocompatibility genes.[29] Other previous studies have evaluated the relationship between methylation and other clinicopathological characteristics of adenomas.[30]

In our study, colon polyps were divided according to distal or proximal colon localization, localization according to the colon topography, size and histological types of polyps (hiperplastic versus adenoma). We did not observe correlation between MGMT methylation status and these markers.

Another salient finding of our analysis was the higher frequency of MGMT methylation among subjects who were overweight or obese (BMI $\geq$ 25). To our knowledge, this study is the first to report an association between overweight/obesity and an increased frequency of *MGMT* methylation. We did not observe correlation between MGMT methylation and overweight or obesity, but these results are interesting and warrant investigation in future studies with a larger group of patients.

Several reports have described an association between the methylation status of genes and a familial tendency to colorectal cancer.[31] However, Ward et al. found no evidence that patients with heavily methylated colorectal cancers were more likely to develop a second malignancy or have a positive family history of cancer.[32] Aberrant methylation may result from an inherited defect in the methylation. In this study, there were no differences between the groups with or without familial history of colorectal cancer who presented the positive MGMT methylation status. It still remains to be elucidated whether promoter methylation in some genes is one of the main mechanisms to evoke cancer or is purely coincidental.

To acquire significance to DNA methylation in cancer and various other diseases, assays to measure DNA methylation proved very useful to both research and clinical practice. DNA methylation of some tumor suppressor genes in breast cancer has been shown to be predictive of responsiveness to tamoxifen therapy.[33] It has been proved that methylation and silencing of the *MGMT* gene in glioblastoma are associated with an increased benefit from temozolomide treatment.[6] Regarding all evaluated genes, patients with multiple lesions exhibited a higher degree of methylation in tumor samples than those with solitary tumors.[34] This epigenetic change can also be detected in precancerous lesions and seemingly normal peritumor tissues,[35] thus suggesting its potential involvement in the initial carcinogenetic process. A series of studies investigating the fecal DNA of patients with colorectal cancer and adenomas in comparison with samples from normal individuals revealed a higher rate of methylation of the target genes in the patients with cancer.[36] This fact clearly demonstrates the need to carefully analyze any newly detected gene in the context of methylation-silencing in terms of its role in carcinogenesis. Early cancer detection and removal of premalignant adenomatous polyps has been shown to be of fundamental importance and consequence in preventing death due to colorectal cancer and reducing colorectal cancer incidence.[37] For patients with colon polyps,

sometimes with unclear clinical manifestation, with family history, however detailed histopatological examination of all presenting polyps, we search instruments to discern whether any of the polyps are at risk from cancerogenesis.

6. Conclusion

Unexpectedly, our study showed no correlations between methylation status of MGMT polymorphisms and clinical features like age, gender, polyp localization, smoking status, or obesity. It has been shown previously that MGMT methylation status may show nonspecific methylation in colon polyps. Gene methylation status in adenoma tissues has also been associated by other authors with the adenoma's size, histology, and degree of atypia. In our study, we evaluated gene methylation status in colon polyps and found no association with adenoma characteristics. The present study showed no correlation for MGMT methylation in polyps in different regions of the colon. The probable involvement of MGMT SNPs is possible via cumulative action of different environmental factors or by synchronous effect of different polymorphisms in other genes. These possibilities ought to be considered to recognize the true role of MGMT and MGMT methylation status in polyps and colon cancer formation.

Conflict of Interest

None declared.

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