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PROMOTER METHYLATION OF CANCER-RELATED GENES IN GASTRIC CARCINOMA

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Genetic changes associated with gastric cancer are not completely known, but epigenetic mechanisms involved in this disease seem to play an important role in its pathophysiology. One of these mechanisms, an aberrant methylation in the promoter regions of genes involved in cancer induction and promotion, may be of particular importance in gastric cancer. ***Aim:*** **To analyze the methylation status of eight genes: *Apaf-1, Casp8, CDH1, MDR1, GSTP1, BRCA1, hMLH1, Fas* in gastric cancer patients.** ***Methods:*** **The methylation pattern of the genes was assessed by methylation specific restriction enzyme PCR (MSRE-PCR) in gastric tumors taken during surgery of 27 patients and compared with the methylation pattern in material obtained from biopsy in 25 individuals without cancer and pre-cancerous lesions.** ***Results:*** **We observed a promoter hypermethylation in the *Casp8*, *hMLH1*, *CDH1* and *MDR1* in gastric cancer patients as compared with the controls. Additionally, we investigated the relationship between promoter hypermethylation and age, gender, smoking and gastric cancer family history. The hypermethylation of the *hMLH1* gene occurred more frequently in female than in men, and the hypermethylation of the *CDH1* gene was observed preferentially in smoking than in non-smoking individuals.** ***Conclusion:*** **The data obtained indicate that changes in DNA methylation may contribute to gastric carcinogenesis.**

Key Words: **DNA hypermethylation, gastric cancer, cancer-related genes, MSRE-PCR.**

DNA methylation is recognized as the most important epigenetic change in the malignant transformation. It includes global hypermethylation and the hypermethylation of CpG islands localized in the regulatory regions of most human genes [1, 2]. Methylated cytosine may undergo spontaneous deamination, producing a C → T transition, which, if in the promoter, may significantly affect the level of expression of a gene and eventually lead to its silencing. Methylated sequences in DNA are also targeted by specific proteins, which can recruit chromatin remodeling proteins changing the accessibility of a gene for transcription machinery and, again, affecting the level of its expression.

An aberrant methylation in cancer-related genes is frequently detected in materials from gastric tumors, suggesting its involvement in the induction/promotion of gastric cancer [3–5]. The identification of methylated genes may provide an insight in the molecular mechanisms of tumor development and might reveal new tools to define markers of prognostic significance. In addition, identification of hypermethylated genes may be useful in cancer therapy, by more specific targeting cancer cells on the basis of their methylation status. To investigate the methylation pattern in gastric cancer we examined the methylation of the promoter of 8 tumor-suppressor genes: *Apaf-1, Casp8, CDH1, MDR1, GSTP1, BRCA1, hMLH1* and *Fas*. The products of these genes may protect the genome from mutagenesis (*hMLH1, BRCA1, GSTP1, MDR1*), impede deregulated progression through the cell cycle (*BRCA1)*, induce apoptosis in cells that escape normal cell cycle controls *(Apaf-1, Casp8*, *Fas*), and inhibit cellular migration and metastasis (*CDH1*) [6].

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The human *Apaf-1* gene encodes a cytoplasmic protein involved in the mitochondrial apoptosis pathway [7]. The gene may be closely related to some proto-oncogenes and tumor suppressor genes, including *p53* and *Bcl-2* [8, 9]. The lowered expression of *Apaf-1* gene or its inactivation was associated with methylation silencing in acute leukemia and laryngeal squamous carcinoma [10, 11]. The caspase 8 protein encoded by *Casp8* gene plays also a major role in the process of apoptosis [7]. Because many anticancer drugs induce apoptosis by the activation of the caspase cascade, the deactivation of this pathway by silencing of *Casp8* may lead to drug-resistance of cancer cells. The protein encoded by the *Fas* gene is a member of the family of death receptors that induce apoptosis in sensitive cells upon binding to their specific death ligands [12]. Silencing of the Fas receptor by DNA methylation was reported in several cancers [13]. The *CDH1* gene encodes E-cadherin, a protein involved in cellular adhesion, tumor growth, invasion and metastasis [4, 14]. The *hMLH1* gene encodes the DNA mismatch protein MLH1, playing a role in familial colorectal cancer and in sporadic gastric carcinomas that display microsatellite instability phenotype [3, 5, 15, 16]. BRCA1 is a breast cancer susceptibility protein, playing an essential role in the repair of DNA double strand breaks [17, 18]. The *MDR1* gene codes for glycoprotein P, involved in cellular transport and its aberrant expression can be important for etiology and progression of various cancers [19]. GSTP1 is an enzyme, important for the detoxification of mutagens/carcinogens [20, 21].

In the present work we have also checked the association between the hypermethylation of and age, gender, smoking and family history of gastric cancer.

MATERIALS AND METHODS

Tissue samples. Tissue samples were taken from 27 patients with gastric cancer during gastrectomy

performed in 2006 and 2007 in the Nicolas Copernicus Hospital, Lodz, Poland. The patients did not undergo chemotherapy. Blood samples from age-matched healthy individuals, who had no current or previous diagnosis of cancer, were obtained from 25 patients of Polish Mother's Memorial Institute, Lodz, Poland and Community Health Centre, Rzgow, Poland. A local ethic committee approved the study and each patient enrolled gave a written consent.

Chemicals. The genomic DNA from gastric tumors and lymphocytes of peripheral blood was isolated with GeneMATRIX Blood DNA Purification Kit and GeneMATRIX Tissue DNA Purification Kit (EurX, Gdansk, Poland) respectively. Methylation-specific restriction enzyme *Hin*6I was obtained from Fermentas (Abo, Gdansk, Poland). All reagents for PCR reaction were from Qiagen (GmbH, Hilden, Germany). Electrophoresis was conducted in TAE buffer.

Methylation specific restriction enzyme digestion PCR. The assessment of methylation status was conducted by methylation specific restriction enzyme digestion PCR (MSRE-PCR) [22]. Digestion of genomic DNA was preformed with methylation-specific enzyme *Hin*6I. The enzyme recognizes and digests the unmethylated 5'-GCGC-3' sequence, whereas its methylated variant is left intact. 150 ng of genomic DNA was mixed with 100 pg of pUC19 plasmid and digested with 40 U of the enzyme in 37 °C for 72 h in a total volume of 50 ml and PCR reaction was run. The fragments of the promoters of the gene under study contained at least one, but no more than nine, *Hin*6I recognition sites located in CpG islands were selected for amplification. The sequences of primers are given in Table 1. After digestion with the enzyme, a PCR reaction with primers specific to a 457 bp fragment of pUC19 was performed. The primers flanked a region containing four *Hin*6I recognition sites. Results of this reaction served as a quality control of the digestion. Next, a separate PCR reaction was conducted for each gene. In three of examined genes (*Apaf-1, CDH1, GSTP1)* the standard PCR reaction mix was enriched by Q-Solution buffer (Qiagen, GmbH, Hilden, Germany). The thermocycler was programmed for 35-cycles with annealing temperature 60 °C (except for *CDH1,* where it was 65 °C). Control samples with non-digested DNA were included for each PCR reaction to ensure that a lack of the product in digested samples was a result of enzymatic degradation of unmethylated template, and not of the PCR reaction failure. Only those samples which yielded products in undigested control where taken for further analysis.

Statistical analysis. Differences between groups were examined using Fisher's exact test and those at $p < 0.05$ were considered significant. The statistical analysis was preformed using STATISTICA 6.0 package from StatSoft (Tulsa, OK, USA).

RESULTS

We determined the status of the promoter methylation of the *Apaf-1, Casp8, CDH1, MDR1, GSTP1, BRCA1, hMLH1* and *Fas* genes in 27 gastric cancer patients and 25 healthy individuals. Two of the genes, *Apaf-1* and *Fas,* were unmethylated in both gastric cancer patients and the controls. Four of eight genes were more frequently methylated in gastric cancer patients than in the control (*Casp8* 81% *vs* 0%, *MDR1* 100% *vs* 28%, *hMLH1* 22% *vs* 0% and *CDH1* 74.1% *vs* 28%). These results are presented in Table 2. It has been recently reported that concurrent hypermethylation of multiple tumor-related genes, including *hMLH1,* is detected frequently in various carcinomas. It was suggested that silencing of *hMLH*1 led to changes in CpG-island methylation pattern and resulting silencing of other genes may contribute to neoplastic transformation [23, 24]. We did not find statistically significant differences between groups in concurrent methylation of *hMLH1* and two or more other methylated genes (Table 3). We also analyzed the methylation changes in the gastric cancer tissue and the questionnaire data obtained from the patients (Table 4). The promoter hypermethylation of *GSTP1* and *hMLH1* were more frequent in female than in male (100% *vs* 5.26% for *GSTP1* and 71.43% *vs* 5.26% for *hMLH1*). When we compared the methylation in smoking and nonsmoking groups, we found a statistically relevant difference for *CDH1* (100% *vs* 66.67%). We did not find any correlation between promoter hypermethylation and age and gastric cancer family history.

Table 2. The frequency of DNA hypermethylation in gastric cancer and control group

	Frequency of hypermethylation (%)							
	Casp 8	*Apaf-1*	*MDR1*	*GSTP1*	*BRCA1*	*hMLH1*	*Fas*	*CDH1*
Cases	81*	0	100*	3.7	3.7	22**	0	74.1**
Control	0	0	28	0	0	0	0	28

*$p < 0.001$; **$p < 0.05$.

Table 3. Concurrent hypermethylation of the *hMLH1* gene with other genes

hMLH1	Concurrent hypermethylation (> 1 genes)
Methylated 6	6 (100%)
Unmethylated 21	18 (85%)

DISCUSSION

We demonstrated that DNA hypermethylation at the promoter regions of the *Casp8, hMLH1, CDH1* and *MDR1* genes occur more frequently in gastric cancer than in normal tissue. The loss of *Casp8* expression in

Table 1. Sequences of primers used in MSRE-PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product length
Apaf-1	GCGCCTTCCACTGCGATATTGC	TTCCCACCAATGCCGGACTC	154 bp
Casp8	CCATCTGGTAGATACCAGGCATGA	TGAGCTCCAAGTCCACTCTGTT	443 bp
CDH1	CAAGGCAGGAGGATCGCTTCAG	CTGACTTCCGCAAGCTCACAGG	658 bp
MDR1	AGAGGTGCAACGGAAGCCAGAAC	GCTTGGAAGAGCCGCTACTCGAATG	218 bp
GSTP1	TCCGGGATCGCAGCGGTCTTAGG	TCTTCTGGAGGGTCCCGCGGACT	262 bp
BRCA1	TGAGAGGCTGCTGCTTAGCGGTAG	AAATCCACTCTCCCACGCCAGTACCC	266 bp
hMLH1	CGCCACATACCGCTCGTAGTATTC	GCTGTCCGCTCTTCCTATTGGTTC	419 bp
Fas	CGTCTGTGAGCCTCTCATGT	CTCCAGCCAAGTCACTCGTA	523 bp

cancer cells may result in resistance to drug-induced apoptosis, and may be important for therapeutic strategy [25–27]. Recent studies demonstrated that demethylation of CpG islands of *Casp8* gene in resistant cancer cells sensitized them to chemotherapeutic agents [28, 29]. Moreover, inactivation of *Casp8* expression by promoter hypermethylation may result in cancer progression. *Casp8* methylation was detected in 90% of medulloblastoma samples by nonquantitative methylation-specific PCR. Cancer progression and invasion can be also correlated with the loss of *CDH1* expression. Recent data indicate that E-cadherin level is decreased in wide spectrum of tumors, including head and neck, breast and gastric cancer, and that this event correlates with DNA methylation in *CDH1* promoter region [4, 14, 30]. Methylation of *CDH1* promoter in gastric cancer occurs in early steps of carcinogenesis and may be initiated by *H. pylori* infection, which colonizes gastric mucosa cells and introduces many cytotoxic agents which may act as hypermethylating agents [31, 32].

Table 4. Association between the characteristics of the subject and promoter hypermethylation

Variables	Frequency of hypermethylation (%)					
	Casp 8	*MDR1*	*GSTP1*	*BRCA1*	*hMLH1*	*CDH1*
Sex						
Female	100	100	100*	14.29	71.43*	85.71
Male	73.68	100	5.26	0.00	5.26	73.68
Age						
< 60	91.67	100	8.33	0	18.18	90.91
> 60	71.43	100	0	7.14	28.57	71.43
Smoking						
Yes	87.5	100	0	0	25.00	100*
No	77.78	100	5.56	5.56	22.22	66.67
Family history**						
Yes	66.67	100	16.67	0	0	66.67
No	85.00	100	0	5.00	30.00	80.00

*p values < 0.05; **one or more first-degree relatives with gastric cancer.

The role of the *MDR1* gene in carcinogenesis is rather controversial. The lack of its expression was reported in prostate cancer [33] and seems to contribute to the progression of neuroblastoma [34]. Other reports correlate *MDR1* expression with cancer initiation and progression rather than the gene silencing [35]. The correlation reported in our study between gastric cancer and DNA methylation of the *MDR1* promoter needs further studies performed on a larger group.

Previous reports suggest that there is a concurrent hypermethylation of multiple genes in neoplastic diseases, including acute myeloid leukemia, colorectal, pancreatic, lung and gastric carcinoma [14, 24, 36–39]. Tumors with concurrent methylation in multiple *loci* have been called the CpG-island methylator phenotype. The presence of concurrent hypermethylation may imply a mechanism leading to aberrant methylation of CpG-island that contributes to cancer development. We tried to compare the hypermethylation pattern between *hMLH1* and other genes used in this study. We showed that concurrent hypermethylation of *hMLH1* and other genes was a common event in gastric cancer. Unfortunately we do not have data on the microsatellite instability status of studied cases of gastric carcinoma. Thus, more detailed studies performed on a larger group with more detailed characteristics are needed to establish an association between concurrent hypermethylation of *hMLH1* and other genes.

We also investigated an association between promoter hypermethylation and age, gender, smoking and family history. We found that hypermethylation of *GSTP1* and *hMLH1* promoter region was more common in women than in men. Our data suggest that some genes show gender specific pattern, what is in agreement with other reports [40]. The explanation for this fact remains unknown. Previous studies showed that the number of methylated gene promoters increased with age [41, 42]. We did not confirm these data but a limitation of our study is the number of analyzed cases. The study performed on a larger group should establish the correlation between DNA hypermethylation, aging and cancer. We also examined correlation between promoter hypermethylation and smoking. The proportion of promoter methylation of *CDH1* gene was significantly increased in smoking group. Our data are consistent with previous results suggesting that DNA hypermethylation pattern may be influenced by smoking [42].

In conclusion, our study regarding promoter hypermethylation of tumor-related genes in gastric cancer showed the high frequency of methylation of the *Casp8*, *MDR1, CDH1* and *hMLH1* genes. This study also demonstrated that aberrant DNA methylation can be associated with female gender and smoking.

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МЕТИЛИРОВАНИЕ ПРОМОТОРОВ ОПУХОЛЬАССОЦИИРОВАННЫХ ГЕНОВ ПРИ РАКЕ ЖЕЛУДКА

Генетические изменения, ассоциированные с опухолью желудка, изучены не в полной мере. В то же время эпигенетические механизмы скорее всего играют ключевую роль и лежат в основе возникновения этого заболевания. Один из таких механизмов – нарушения метилирования промоторов генов, которые регулируют злокачественную трансформацию и прогрессирование опухолевого процесса, может быть особенно важным в развитии рака желудка. ***Цель:*** **проанализировать статус метилирования промоторов восьми генов:** ***Apaf-1, Casp8, CDH1, MDR1, GSTP1, BRCA1, hMLH1, Fas*** **у больных раком желудка.** ***Методы:*** **метилирование промоторов генов изучали с помощью специфической к сайтам метилирования рестрикцией с ПЦР (MSRE-PCR) на хирургическом материале (опухоли желудка) 27 пациентов. В качестве контроля использовали биопсийный материал, полученный от 25 больных, у которых не было выявлено рака или предраковых состояний.** ***Результаты:*** **отмечали гиперметилирование промоторов генов** ***Casp8***, ***hMLH1***, ***CDH1*** **и** ***MDR1*** **в опухолевой ткани желудка по сравнению с контрольными образцами. Кроме того, нами была прослежена взаимосвязь между гиперметилированием промоторов генов и возрастом, полом пациентов, курением и семейной историей заболевания раком желудка. Гиперметилирование гена** ***hMLH1*** **выявляли чаще у женщин, чем у мужчин, а гиперметилирование гена** ***CDH1*** **— в основном у курильщиков.** ***Выводы:*** **полученные данные свидетельствуют о том, что метилирование ДНК может играть важную роль при развитии рака желудка.**

Ключевые слова: **гиперметилирование ДНК, рак желудка, опухольассоциированные гены, MRSE-PCR.**