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Silencing of MGMT expression by promoter hypermethylation in the metaplasia–dysplasia–carcinoma sequence of Barrett’s esophagus

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Abstract

To determine the relevance of MGMT in Barrett’s carcinogenesis, we analyzed promotor hypermethylation and expression of MGMT in Barrett’s adenocarcinomas and its paired precursor lesions from 133 patients using a methylation-specific PCR, real-time RT-PCR and immunohistochemistry. Hypermethylation was detected in 78.9% of esophageal adenocarcinomas, in 100% of Barrett’s intraepithelial neoplasia, in 88.9% of Barrett’s metaplasia, but only in 21.4% of normal esophageal mucosa samples ($P < 0.001$) and correlated significantly with downregulation of MGMT transcripts ($P = 0.048$) and protein expression ($P = 0.02$). Decrease of protein expression was significantly correlated with progressed stage of disease, lymph node invasion and tumor size. We conclude, that aberrant promoter methylation of MGMT is a frequent and early event during tumorigenesis of Barrett’s esophagus. High prevalence of MGMT

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hypermethylation may represent a candidate marker for improved diagnosis and targeted therapy in Barrett's adenocarcinoma.

Keywords

O⁶-methylguanine-DNA methyltransferase (MGMT); Hypermethylation; Carcinogenesis; Barrett's metaplasia; Barrett's adenocarcinoma

1. Introduction

Esophageal adenocarcinoma arises in the setting of Barrett's esophagus, which is a premalignant condition in which the native squamous epithelium of the lower esophagus is replaced by an abnormal metaplastic columnar epithelium (Barrett's mucosa, BE). BE represents the most serious histological consequence of chronic gastroesophageal reflux disease. The incidence of BE has been increasing rapidly in the United States and many regions of Western Europe [1]. It is well known that Barrett's carcinogenesis is a multistage and progressive process which includes reflux esophagitis, intestinal metaplasia, low and high grade intraepithelial neoplasia (IN) and advanced Barrett's adenocarcinomas (BA) [2]. Chronic inflammatory status is believed to predispose development of intraepithelial neoplasia and BA. To date, the exact cellular and molecular mechanisms leading to the malignant transformation/neoplastic progression in Barrett's epithelium have not been fully understood. Thus, hypermethylation of CpG islands located in the promoter of putative tumor suppressor genes is now firmly established as an important mechanism for gene inactivation and is gradually becoming a focus in carcinogenesis of BA [3–6].

O⁶-methylguanine-DNA methyltransferase (MGMT), a cellular DNA repair protein, provides protection from the mutagenic and carcinogenic effects of alkylating agents. MGMT transfers alkyl groups at the O⁶-position of guanine to a cysteine residue within its own sequence in an auto-inactivating reaction [7]. If not removed, O⁶-methylguanine can preferentially mispair with thymine rather than cytosine during replication, thus causing G:C to A:T mutations, which may initiate carcinogenesis if the mutations occur in tumor-related genes [8]. Indeed, it has been shown that MGMT inactivation is associated with G:C to A:T mutations in *K-ras* and *p53* in a variety of human primary carcinomas such as colorectal and gastric carcinoma, non-small cell lung cancer and astrocytomas [9–13].

MGMT protein is expressed in all human tissues at various levels, and is decreased in some tumors in comparison to normal tissue [14–16]. Several reports have demonstrated that MGMT function is frequently lost in association with hypermethylation of the CpG islands within the MGMT promoter in several human malignancies [9,17–20]. Promoter hypermethylation of MGMT and its association with loss or reduction of MGMT protein expression have also been reported to occur in gastrointestinal malignancies, including esophageal squamous-cell carcinoma, sporadic and ulcerative colitis-associated colorectal carcinomas, and gastric cancers [11,16,21–24]. Only few studies have examined MGMT gene promoter hypermethylation in Barrett's adenocarcinoma [25–29]. However, little is known about the status of MGMT promoter hypermethylation and inactivation in early

stages of Barrett's carcinogenesis, and there are no data on MGMT inactivation in Barrett's metaplasia–dysplasia–carcinoma sequence.

Based on these previous studies, we sought to determine the timing of this epigenetic event and the potential role MGMT may play in tumor initiation and stepwise progression in Barrett's carcinogenesis. Therefore, we analyzed the MGMT promoter hypermethylation in a series of BA and paired Barrett's mucosa and intraepithelial neoplasia, as well as in normal esophageal squamous mucosa, and examined whether promoter inactivation has an impact on MGMT protein expression. In addition, we investigated the relation of loss of MGMT protein to clinicopathological factors.

2. Material and methods

2.1. Patients and tissue samples

Specimens of esophageal tissue analyzed in this retrospective study were obtained from two principal sources and comprised 133 patients:

Firstly, formalin-fixed, paraffin-embedded specimens consisting of 47 BA, 27 BE, 13 low or high grade intraepithelial neoplasia (IN) and 28 samples from non-dysplastic esophageal squamous mucosa (NT) were analyzed by both methylation-specific PCR and immunohistochemistry. The specimens were obtained from mucosal and surgical resections from 71 patients recorded in the archives of the Institutes of Pathology Bayreuth, Regensburg and Magdeburg, Germany. This group had a median age of 64.2 years (range 43–79) and consisted of 63 males and 8 females. Thirty-four of the 47 investigated BA (72%) represented mucosal carcinoma or carcinoma of early tumor stages (Table 1).

Secondly, formalin-fixed, paraffin-embedded specimens obtained from Barrett's adenocarcinoma and paired non-cancerous esophageal mucosa of 62 patients, who had undergone surgery at the University of Virginia, were submitted to immunohistochemical tissue microarray analysis. This study group comprised 57 male and 5 female patients, ranging in age from 51 to 79 years (median, 62.7 years), and included carcinoma of advanced tumor stages (clinicopathological features are summarized in Table 2).

Histopathological diagnosis was verified on the basis of hematoxylin-eosin-stained sections blinded to all clinical data independently by at least three pathologists specialized in gastrointestinal pathology (D.K., M.S., M.V., A.H., A.R.). Carcinomas were classified according to the recent guidelines of the UICC TNM classification system. Tumors pretreated with chemotherapy or radiation were excluded. The local ethics committee approved this study.

2.2. Tissue microarray design

For immunohistochemical analysis, representative regions of carcinoma and paired normal esophageal mucosa were selected on hematoxylin-eosin-stained slices for inclusion in a tissue microarray. The arrays were assembled by taking core needle biopsies with a diameter of 0.6 mm from the areas of interest in pre-existing paraffin-embedded tissue blocks and re-

embedding them in an arrayed recipient block (Beecher Instruments, Silver Spring, MD, USA). Samples were punched in triplicate.

2.3. DNA extraction

The areas of interest were separated by macro- or laser microdissection (P.A.L.M) of approximately three to seven 10 µm thick tissue slices according to the labeling on a mirror-imaged H & E-stained section. Genomic DNA of formalin- fixed, paraffin-embedded tissue was isolated using the Nucleospin DNA extraction kit according to the instructions of the manufacturer (Machery and Nagel, Dueren, Germany).

2.4. Bisulfite treatment of DNA and methylation-specific PCR (MSP)

Extracted DNA was bisulfite-modified using the CpGenome DNA modification kit (Intergen, Purchase, NY). The modified DNA was subjected to MSP with the following specific primers: for methylated sequence (sense 5'-TTC GAC GTT CGT AGG TTT TCG C-3' and antisense 5'-GCA CTC TTC CGA AAA CGA AAC G-3') and for unmethylated sequence (sense 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' and antisense 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3'); both generate PCR products of 80 bp and 94 bp, respectively.

For PCR, 2 µl of bisulfite-modified DNA was amplified in a total volume of 25 µl containing 1 × PCR buffer, 3 mM MgCl₂, 12.5 pmol of each primer, 160 µM dNTPs, and 0.5 U of Hot-Goldstar Taq-polymerase (Eurogentec, Seraing, Belgium). PCR conditions were as follows: 95 °C for 10 min, 35 cycles of 95 °C for 1 min, annealing with 61 °C (methylated) or 58 °C (unmethylated) for 1 min, followed by a final extension step at 72 °C for 10 min. Methylated standard DNA (Intergen) was used as a positive control for methylation, and placenta DNA was used as a negative control. PCR products were electrophoresed on polyacrylamide gels and visualized by silver staining.

2.5. Quantitative real-time RT-PCR (9 samples of Group A)

One microgram of total RNA isolated by the RNA isolation kit (RNeasy kitIM, Qiagen, Hilden, Germany) was reversely transcribed (Promega, Mannheim, Germany). Quantitative real-time PCR (qPCR) was performed using Sybr green technology with an iCycler (Bio-Rad, Hercules, CA, USA) with a threshold cycle number determined by use of iCycler software, version 3.0. For qRT-PCR, single-stranded cDNA was synthesized using the Advantage RT-PCR Kit (Clontech, Palo Alto, CA, USA). Gene-specific primers for mRNA specific sequence of MGMT were designed. The forward primer sequence was 5'-CCGTTTGCGACTTGCTACTT -3' and the reverse primer sequence was 5'-CCCAGGAGCTTTATTTCGTG -3'. These primers were obtained from Integrated DNA Technologies (IDT Inc., Coralville, IA, USA). A single-melt curve peak was observed for each product, thus confirming the purity of all amplified cDNA products. The qRT-PCR results were normalized to HPRT1, which had minimal variation in all normal and neoplastic samples tested. Fold over-expression was calculated according to the formula, $2^{(R_t - E_t)/2(R_n - E_n)}$, as described earlier [30] where R_t is the threshold cycle number for the reference gene observed in the tumor, E_t is the threshold cycle number for the experimental gene observed in the tumor, R_n is the threshold cycle number for the reference

gene observed in the normal sample, and E_n is the threshold cycle number for the experimental gene observed in the normal sample. R_n and E_n values were averages of the corresponding normal analyzed samples.

2.6. Western blotting (6 samples of Group A)

Lysates were prepared from frozen tissue samples of six Barrett's adenocarcinomas. Forty micrograms of proteins were loaded onto 12% SDS-PAGE. After transfer to nitrocellulose membranes immunodetection was processed with anti-MGMT (Ab-1, clone MT3.1; Biocarta, San Diego, CA, USA), anti- β -actin (Sigma, St. Louis, MI, USA) for control equal loading and protein quality and with secondary antibodies (anti-mouse IgG peroxidase conjugated; Pierce, Rockford, IL). Bound antibodies were detected by incubating the blots in West Pico chemiluminescent substrate (Pierce, Rockford, IL). The level of immunoreactivity was measured as peak intensity using an image capture and analysis system (GeneGnome, Syngene, UK).

2.7. Immunohistochemical analysis of MGMT

Immunohistochemical studies were performed in all cases of NT, BE, IN and in 35 BA investigated in Group A and on the tissue microarray of group B using the avidin-biotin complex immunostaining method with the automated immunohistochemistry slide staining system by Ventana NexES (Ventana Medical System, Strasbourg, France). Three micrometer thick formalin-fixed, paraffin-embedded tissue sections were deparaffinised and dehydrated. For antigen retrieval, the sections were treated by microwave heating in 1 mM sodium citrate buffer (30 min, 600 W, pH 6.0). Incubation with monoclonal mouse anti-MGMT antibody (mouse monoclonal antibody, clone MT 23.2, dilution 1:30, Zymed Laboratories Inc., San Francisco, CA, USA) was conducted at 37 °C for 32 min and followed by PBS-washing. Positive immunohistochemical reactions were revealed using the iVIEW™ DAB Detection Kit (Ventana, Germany) as chromogen substrate. Specimens were counterstained with hematoxylin and mounted with DEPEX™.

Samples were examined by two different reviewers (D.K., I.K.) blinded to other data. Correlation between the two observers was calculated using Cohen's kappa statistic. Immunostaining was evaluated for nuclei of the epithelium in NT, IN, BE and carcinoma. Staining intensity ([SI], 1 = weak, 2 = moderate, 3 = strong) and the percentage of positive cells ([PC], 1 = <10%, 2 = 10–50%, 3 = 51–80%, 4 = >80%) were assessed semiquantitatively, resulting in an immunoreactive score [$IRS = SI \times PC$] with a possible maximum of 12 points. An average immunoreactive score was finally estimated for each group. MGMT positivity of inflammatory cells, mainly lymphocytes, served as an internal positive control.

2.8. Statistical analysis

All statistical calculations were made using SPSS 13.0 for Windows. The frequency of MGMT promoter methylation in BA, BE, IN and NT was compared using Fisher's exact test. An analysis between various subgroups concerning the association between the MGMT methylation status or protein expression and clinicopathological data was made using two

sided One-way ANOVA test as well as paired *t*-test for averages. All reported *P*s are two sided, and all significant associations were considered if $P \leq 0.05$.

3. Results

3.1. MGMT promoter hypermethylation

To assess the methylation status of the MGMT promoter in BA and its precursor lesions and in non-neoplastic esophageal squamous mucosa, 115 samples from 71 cases were analyzed. Representative examples of the MSP products analyzed for the MGMT gene are displayed in Fig. 1B. In all methylated cases, unmethylated bands were also visible in the samples investigated, which may result from methylation of only one allele, heterogeneity in tumor cell populations, or admixed normal cells within or adjacent to the lesions of interest.

MGMT showed promotor hypermethylation among all groups (Table 1 and Fig. 1A). In BA, MGMT methylation was detected in 37/47 cases (78.9%), whereas the epigenetic modification of the gene promoter occurred only in 6/29 cases (21.4%) of the non-dysplastic esophageal squamous mucosa ($P < 0.001$). In BE without or with intraepithelial neoplasia, MGMT methylation occurred in 24/27 cases (88.9%) and in 13/13 cases (100%), respectively.

Among 27 paired BA and their adjacent metaplastic BE and/or normal esophageal squamous mucosa, 22 BA exhibited MGMT promoter hypermethylation. Of these, only 33% also had this change in their adjacent normal tissue compared to 100% of the paired non-neoplastic BE. As expected, among the remaining five BA without methylation, all cases were unmethylated in their matched metaplastic or normal tissue samples. Although the difference among BE, IN and BA was not statistically significant, methylation occurred with an increasing frequency along neoplastic progression, with the largest increase found between NT and non-dysplastic BE, and a further increase in the development of intraepithelial neoplasia and BA.

3.2. Association between MGMT promoter methylation and its mRNA and protein expression

To determine the significance of promoter CpG island methylation for gene expression, MGMT expression was analyzed both at the mRNA and the protein level. In general, hypermethylation was tightly correlated with the loss of MGMT mRNA and protein expression, underlining the validity of our methylation analysis.

Methylation of the MGMT promoter in BA was associated with a concomitant decrease in the level of MGMT mRNA expression compared to unmethylated samples (Fig. 1C and Table 1). Statistical analysis was not performed due to the low number of unmethylated cases investigated.

Fig. 2 shows representative immunohistochemical staining patterns of MGMT in NT, BE, IN and BA. Within a given lesion, a nearly homogeneous expression was demonstrated. MGMT was detectable by immunohistochemistry in all unmethylated specimens examined. Regarding interobserver reliability, there was a kappa coefficient with linear weighting of

0.79 (95% CI 0.44 – 1.15) for the two observers. Inverse correlation was found between methylation and immunohistochemical protein expression with a significant reduction of the MGMT protein level in methylated samples compared to unmethylated samples ($P = 0.02$, Fig. 1D). In the group of methylated lesions, 5/27 cases of BE, 3/13 cases of IN and 7/47 cases of BA appeared to be completely immunonegative. Western blot-analysis of each three samples of methylated and unmethylated BA clearly reduced MGMT protein expression, which indicates that the results of the immunohistochemistry studies are reliable (Fig. 1E). But a selected change with functional consequences.

3.3. Correlation with clinicopathological data

Because the majority of the carcinomas of group A ($n = 35$) available both for methylation and immunohistochemical protein expression were in a very early tumor stage, we performed an immunohistochemical study of 62 additional cases with advanced tumor stages (group B, $n = 62$). Available clinicopathological data on all merged patients investigated by immunohistochemistry ($n = 97$) and their tumor characteristics at the time of resection were reviewed and correlated with MGMT protein expression. Clinical data and immunoreactive scores are summarized in Table 2. The major results are depicted in Fig. 3. Reduced MGMT protein expression was detected more frequently in poorly differentiated adenocarcinomas than in well and moderately differentiated adenocarcinomas ($P = 0.04$). For clinicopathological correlation we merged the TNM and pT category into early stage (stage II; pT2) and late stage (>stage II; >pT3). In cancers demonstrating advanced local invasion and progressed tumor spread, the incidence of reduced MGMT expression was higher than in less invasive carcinomas and carcinomas at early tumor stage (pT1/2 vs pT3/4, $P = 0.04$; stage I/II vs stage III/IV, $P = 0.021$). Furthermore, loss of protein expression was present more frequently in carcinomas with than in those without lymph vessel invasion ($P = 0.025$). In contrast, differences in MGMT expression regarding gender, age, evidence of lymph node metastasis or distant metastasis did not exist ($P = 0.397$, $P = 0.196$, $P = 0.839$ and $P = 0.23$, respectively).

4. Discussion

There is increasing evidence that inactivation and loss of MGMT are involved in the pathogenesis of different gastrointestinal malignancies [11,16,21–24]. For Barrett's adenocarcinoma, it has been accepted that genetic and epigenetic events, including promoter hypermethylation of tumor suppressor genes, are frequent in the multistep process of Barrett's carcinogenesis [3–6], and play a critical role not only in tumorigenesis but also in clinical progression, as well as in their responsiveness to therapy.

We report a methylation frequency of MGMT of 21.4% for NT, 88.9% for BE, 100% for IN and 78.9% for BA. Our results, which demonstrate that promoter hypermethylation is a very frequent event in Barrett's carcinogenesis, are in good agreement with reported prevalences of MGMT hypermethylation in BA found in other series [25–29]. The number of methylated NT samples in our cohort is in line with previous reports about Barrett's esophagus in the literature [26,28]. Also, in other tumors, such as esophageal squamous mucosa, promoter hypermethylation of MGMT occurred in histologically normal-appearing tissue adjacent to the tumors [21]. It still needs to be clarified in future studies whether methylation of normal

mucosa reflects an epigenetic field effect in the surrounding areas of cancer or points towards an elevated risk of developing into metaplastic and neoplastic lesions. The slightly higher frequency of MGMT hypermethylation for intraepithelial neoplasia compared to BA in our study may be attributed to the fact that the samples were microdissected from paraffin sections, and there were a lower number of unmethylated stromal cells in the samples compared to BA.

Only one previous study has evaluated the methylation of MGMT across the histological progression of normal, Barrett's metaplasia, IN and BA tumor tissues [5]. Remarkably, these authors found comparable methylation frequencies for all stages of Barrett's carcinogenesis, but they did not investigate progression within one patient. To the best of our knowledge, this is the first report investigating MGMT promoter hypermethylation in a series of BA and paired Barrett's mucosas and dysplastic lesions, as well as in normal esophageal mucosa. Interestingly, for samples obtained within one individual, MGMT hypermethylation tended to increase in prevalence with increasing histological severity of disease. Furthermore, most of the analyzed BA exhibited a significantly lower MGMT protein expression than the corresponding normal, metaplastic or dysplastic lesion obtained from the same individual. Hypermethylation in normal tissue was always accompanied by MGMT methylation in matching tumor tissue. However, due to the relatively small sample size, differences could not be tested statistically.

We report an accumulation of hypermethylation during the metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. Methylation of MGMT tended to increase with histological progression, the clearest and most consistent increase occurs between NT and BE. Our findings suggest that MGMT methylation occurs at the metaplastic step, rather than being cancer-specific. Because MGMT methylation occurs commonly and early in Barrett's metaplasia, it may be specific to transformed premalignant or malignant epithelium. Furthermore, the findings of our current study emphasize that MGMT inactivation is an early event in Barrett's carcinogenesis and support the functional role MGMT hypermethylation seems to play in esophageal neoplastic transformation in the pathogenesis of BA.

In view of the poor prognosis of BA and the risk of malignant transformation of BE to dysplasia and BA, recent research has focused on markers of progression in Barrett's esophagus. The identification of genes inactivated by promotor hypermethylation in BA and its precursor lesions might enable focused screening of risk patients and could contribute to the improvement of diagnosis and prognosis. In our study, we could show a gradual increase in the frequency of MGMT hypermethylation with histopathological and clinical progression of the disease. These data suggest that loss of MGMT may promote tumorigenesis and implies a possible prognostic value of promoter hypermethylation of a certain gene profile which might include MGMT. Because MGMT is frequently methylated in BE, DYS and BA, it may find application in screening or surveillance as part of a panel of markers. However, to identify patients with Barrett's esophagus at increased risk of progression to dysplasia or BA, and to explore the value of methylated MGMT as an independent risk factor for Barrett's esophagus and BA, further methylation studies containing a detailed endoscopic surveillance program are needed.

It is now firmly believed that promoter CpG island hypermethylation represents an alternative mechanism, as opposed to genetic factors, in the inactivation of important genes. Loss of MGMT protein expression is not commonly caused by deletion, mutation or rearrangement of the MGMT gene or mRNA instability [31–33]. In parallel, MGMT has been frequently observed to be down-regulated by promoter hypermethylation [11,16,17,21,22,24] and is an early epigenetic alteration in a wide variety of human tumor malignancies [4,34,35]. Our data provide compelling evidence for the strong relationship between MGMT promoter methylation and gene silencing in Barrett's adenocarcinoma.

Interestingly, in our study, loss of MGMT protein expression was significantly associated with lymph vessel invasion, progressed tumor stage and depth of invasion. Since methylation of MGMT in general is a frequent event of already early tumor stages, further loss of protein expression in progressed tumor stages might be caused by additional epigenetic or genetic alterations of the MGMT expression, by an increase in the number of tumor cells methylated, and/or by certain methylation pattern within the MGMT promoter. To clarify this issue, further studies using quantitative methylation methods such as COBRA or Methyl-Light might be useful. Based on our observation of a correlation between promoter methylation and decreased protein expression of MGMT, increase in the methylation frequency and quantity of MGMT might be correlated with a more aggressive biological behavior of BA. Also for gastric cancer, a significant association of MGMT methylation with progressed disease could be shown, but methylation was not an independent predictor of survival in multivariate analysis [11,36]. In contrast, for non-small cell lung cancer and head and neck cancer, MGMT hypermethylation proved to be a useful prognostic marker for tumor outcome and patient's survival, and was associated with poorer prognosis [17,34,37]. Furthermore, in a complex methylation study of BA, there was a strong trend towards poorer survival and earlier tumor recurrence for patients, whose tumors were methylated for a group of genes, including MGMT [29]. In our study, regarding survival and the prognostic value of MGMT hypermethylation, no definitive conclusions could be drawn since follow-up of the cohort was still ongoing, and survival data are not available yet.

Alkylating agents are highly reactive mutagens and carcinogens, and their analogous compounds are used for the treatment of human malignancies. The lethal and mutagenic effects of these compounds are inhibited by the cellular DNA repair enzyme MGMT. Conversely, MGMT silencing through methylation of its promoter induces low expression of MGMT protein and decreases its DNA repair activity. Several experimental studies have shown that tumor cell lines with MGMT promoter methylation and/or lacking MGMT activity are highly sensitive to the toxic effect of chemotherapeutic alkylating agents [38,39]. MGMT is most probably the major player in NF- κ B-mediated chemoresistance to alkylating agents [40]. MGMT gene expression is known as a major contributing factor for the development of resistance to alkylating chemotherapeutic agents, and MGMT inactivation is accepted as a relevant prognostic marker and predictor of chemosensitivity in glioblastomas, head and neck cancer, and lymphomas [34,35,38,39,41,42]. Thus, targeted inactivation of MGMT could improve the effectiveness of currently used alkylating agents against cancer [43]. For BA, the high prevalence of MGMT promoter hypermethylation in

progressed tumor stages may suggest a more favorable response of these tumors, which should be treated by alkylating chemotherapeutic agents.

We conclude that, MGMT promoter hypermethylation is an early event in Barrett's multistep carcinogenesis and therefore plays a critical role in the tumorigenesis. Detection of MGMT inactivation by promoter methylation might potentially provide new targets for both chemotherapeutic intervention and improved diagnosis of Barrett's adenocarcinoma.

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Fig. 1.

Results of methylation-specific PCR analysis of the MGMT promoter and correlation with MGMT protein and mRNA expression. (A) Frequency of hypermethylation of the MGMT promoter in non-tumorous, premalignant and malignant esophageal tissue. Each bar illustrates the portion of samples of a certain lesion classified as “methylated” or “unmethylated”. (B) Representative MSP results of the MGMT gene promoter of three patients (B06, B44, B27), including samples of non-tumorous squamous epithelium (NT), Barrett’s metaplasia (BE), Barrett’s intraepithelial neoplasia (IN) and Barrett’s adenocarcinoma. (BA). (C) Methylated cases showed a lower mRNA transcript level than the unmethylated cases investigated. (D) Correlation analyses of MGMT promoter methylation and immunohistochemical protein expression demonstrated as immunoreactive score. Methylation resulted in a remarkable reduction of MGMT immunostaining. The number of cases investigated (n) is given below the graph. un, unmethylated; meth, methylated. (E) Six Barrett’s adenocarcinomas were analyzed for MGMT protein expression by immunoblotting. MGMT protein expression was significantly reduced in methylated cases (B43, B50, B53) compared to unmethylated cases (B14, B48, B71).

Fig. 2.

Immunohistochemical analysis for protein expression of MGMT in esophageal tissues representative for non-, meta- and neoplastic alterations. Methylation of MGMT promoter resulted in decreased and partial loss of MGMT protein expression. Non-tumorous esophageal squamous-cell epithelium with high (A)/low expression (B), Barrett's metaplasia with high (C)/low expression (D), and Barrett's adenocarcinoma with high (E)/low expression (F) of MGMT. Magnification 100×/400×.

Fig. 3. Correlation of immunohistochemical MGMT protein expression and clinicopathologic data. Immunohistochemical protein expression of MGMT, indicated by an immunoreactive score (= IRS), was significantly altered depending on: (A) Tumor differentiation (grading), (B) depth of invasion (pT), (C) tumor stage, and (D) lymphatic vessel invasion. A marked decrease in MGMT protein expression correlates with progressively advanced stages of tumor disease.

Table 1

PCR-specific promoter methylation pattern of MGMT.

Samples	Sex/age	pTNM	Grading	Methylation pattern			mRNA fold expression	
				NT	BE	IN	BA	
B01	M/70	T1N0Mx	3	Meth		Meth	Meth	
B02	M/43			Meth	Meth			
B03	F/71			Un				
B04	M/50				Un			
B05	M/72	T1N0Mx	2	Meth	Meth	Meth		
B06	M/72	T1N0Mx	2		Meth	Meth		
B07	F/70	T1N0Mx	1				Un	
B08	F/70	T1N0Mx	1				Un	
B09	M/61	T1N0Mx	1				Meth	
B010	M/49	T1N0Mx	1				Meth	
B011	M/64					Meth		
B012	M/46				Meth			
B013	M/49	T1N0Mx	1		Meth			Meth
B014	M/73	T1N0Mx	1				Un	
B015	M/58	T1N0Mx	1				Un	
B016	M/65	T1N0Mx	2				Meth	
B017	F/69	T1N0Mx	2				Meth	
B018	M/57	T1N0Mx	2				Meth	
B019	M/69	T1N0Mx	1				Meth	
B020	M/69	T1N0Mx	1				Meth	
B021	M/52	T1N0Mx	1	Un			Un	
B022	F/69	T1N0Mx	1		Un		Un	
B023	F/54				Meth			
B024	M/60					Meth		
B025	M/60			Meth				
B026	M/50	T1N0Mx	1		Meth			Meth
B027	M/71	T1N0Mx	1	Un		Meth		

Samples	Sex/age	pTNM	Grading	Methylation pattern				mRNA fold expression	
				NT	BE	IN	BA		
B028	M/48	T1N0Mx	1	Meth	Meth		Meth		
B029	M/73	T1N0Mx	2		Meth				
B030	M/59	T1N0Mx	1	Un			Meth		
B031	M/79	T1N0Mx	1	Meth			Meth		
B032	M/62			Un	Un	Meth			
B033	F/53	T1N0Mx	1		Meth		Meth		
B034	M/74	T1N0Mx	1		Meth				
B035	M/64	T1N0Mx	1	Un					
B036	M/72	T1N0Mx	2		Meth		Meth		
B037	M/67	T1N0Mx	1		Meth	Meth			
B038	M/63			Un					
B039	M/77				Meth	Meth			
B040	M/70	T1N0Mx	3	Un	Meth				
B041	M/67	T1N0Mx	2		Meth				
B042	M/78	T1N0Mx	1		Meth				
B043	M/64	T2N1M0	3	Un			Meth		-39.4
B044	M/62	T3N1M1	3	Un			Meth		-16.0
B045	M/68	T1N0Mx	2	Un			Un		-16.22
B046	M/69	T1N0Mx	1	Un			Meth		-20.29
B047	M/66	T2N1M0	3	Un			Un		
B048	M/64	T1N1M0	2	Un			Un		-9.85
B049	M/69	T2N0M0	2						-13.32
B050	M/60	T3N3M0	3				Meth		-58.28
B051	M/59	T1N0Mx	1	Un			Meth		-11.31
B052	F/65	T1N0Mx	2	Un			Meth		-8.28
B053	M/65	T3N1M0	3	Un			Meth		-24.93
B054	M/75	T1N0Mx	1			Meth			
B055	M/71	T4N1M0	3	Un	Meth				
B056	M/71	T4N1M0	2	Un					
B057	M/66	T4N0M0	2	Un					

Samples	Sex/age	pTNM	Grading	Methylation pattern				mRNA fold expression
				NT	BE	IN	BA	
B058	M/75	T1N0Mx	1		Meth	Meth	Meth	
B059	M/51				Meth	Meth		
B060	M/51	T1N0Mx	1					
B061	M/60	T3N1M1	2		Meth	Meth	Meth	
B062	M/66	T3N1M1	3		Meth	Meth	Meth	-6.06
B063	M/62	T3N1M1	3	Un				
B064	M/74	T3N1M0	3	Un				
B065	M/78	T1N1M0	1				Meth	
B066	M/52	T1N0Mx	2				Meth	
B067	M/72	T2N0M0	3				Meth	
B068	M/70	T1N0Mx	1				Meth	
B069	M/73	T1N0Mx	2				Meth	
B070	M/54	T1N0Mx	2				Meth	
B071	M/71	T1N0Mx	2				Meth	

PCR-specific promoter methylation pattern of MGMT according to histopathological diagnosis and clinical characteristics (study group A, n = 71). M, male; F, female; pTNM, postsurgical histopathological tumor classification; Un, unmethylated; Meth, methylated.

Table 2

Clinicopathological features and immunoreactive scores of Barrett's adenocarcinomas investigated by immunohistochemistry for MGMT protein expression.

Clinicopathological factor	<i>n</i>	IRS mean ± SD	<i>P</i> -value
Age, years (median ± SD)	67 (64.4 ± 8.9)		
<i>Sex</i>			
Male	86	5.23 ± 3.79	0.062
Female	8	2.63 ± 3.02	
<i>Tumor differentiation (Grading)</i>			
1	22	6.45 ± 3.90	0.04
2	35	5.37 ± 3.77	
3	39	3.94 ± 3.68	
<i>Depth of invasion (pT)</i>			
1	41	5.73 ± 3.94	0.04
2	23	5.43 ± 3.90	
3	17	4.11 ± 4.08	
4	16	3.75 ± 2.89	
<i>Lymph node metastasis (pN)</i>			
Negative	64	5.05 ± 4.03	0.834
Positive	17	4.82 ± 3.34	
<i>Lymphatic vessel invasion</i>			
Negative	36	6.11 ± 3.98	0.025
Positive	34	4.03 ± 3.58	
<i>Metastasis (pM)</i>			
Negative	63	5.24 ± 4.08	0.239
Positive	8	3.50 ± 1.60	
<i>Stage</i>			
I	41	5.44 ± 3.91	0.021
II	30	5.80 ± 4.30	
III	17	3.82 ± 3.08	
IV	9	3.11 ± 1.90	

Group of Barrett's adenocarcinomas (*n* = 97) used for correlation of clinicopathological data and immunohistochemical MGMT protein expression. This group includes all 62 carcinomas of study group B and 35 carcinomas of study group A.