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Prognostic and Predictive Roles of MGMT Protein Expression and Promoter Methylation in Sporadic Pancreatic Neuroendocrine Neoplasms

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Key Words

Pancreatic neuroendocrine neoplasm · Pancreatic neuroendocrine tumour · Neuroendocrine tumour · O6-methylguanine-methyltransferase · Prognosis · Response prediction · Temozolomide · Immunohistochemistry · Promoter methylation

Abstract

Background/Aims: O6-methylguanine-methyltransferase (MGMT) is an important enzyme of DNA repair. *MGMT* promoter methylation is detectable in a subset of pancreatic neuroendocrine neoplasms (pNEN). A subset of pNEN responds to the alkylating agent temozolomide (TMZ). We wanted to correlate *MGMT* promoter methylation with MGMT protein loss in pNEN, correlate the findings with clinico-pathological data and determine the role of MGMT to predict response to TMZ chemotherapy. ***Methods:*** We analysed a well-characterized collective of 141 resected pNEN with median follow-up of 83 months for MGMT protein expression and promoter methylation using methylation-specific PCR (MSP). A second collective of 10 metastasized, pretreated and progressive patients receiving TMZ was used to examine the predictive role of MGMT by determining protein expression and promoter methylation using primer extension-based quantitative PCR. ***Results:*** In both collectives there was no correlation between MGMT protein expression and promoter methylation. Loss of MGMT protein was associated with an adverse outcome, this prognostic value, however, was not independent from grade and stage in multivariate analysis. Promoter hypermethylation was significantly associated with response to TMZ. ***Conclusion:*** Loss of MGMT protein expression is associated with adverse outcome in a surgical series of pNET. *MGMT* promoter methylation could be a predictive marker for TMZ chemotherapy in pNEN, but further, favourably prospective studies will be needed to confirm this result and before this observation can influence clinical routine.

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Introduction

Recent studies have shown that several tumour suppressor genes are inactivated by methylation in pancreatic neuroendocrine neoplasms (pNEN) [1–4]. *MGMT* has been found to be one of the most often methylated genes in pNEN. However, data regarding the frequency of *MGMT* promoter methylation in pNEN are conflicting [1–4]. The *MGMT* gene is located on 10q26 and codes for

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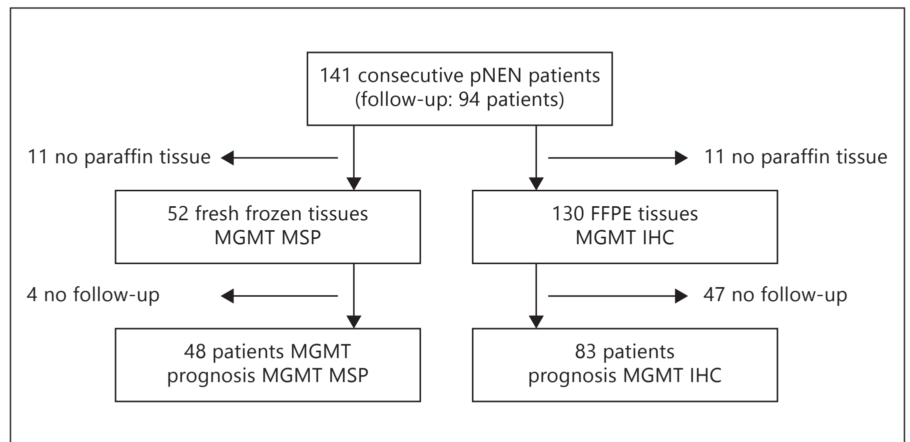

Fig. 1. Specification of tissue specimens included in the prognostic part of the study.

the O[6]-methylguanine-methyltransferase. MGMT is an important enzyme of DNA repair [5] removing the mutagenic alkyl group from O[6]-guanine in DNA and transferring it to an active cysteine [6], thus counteracting a crucial step in carcinogenesis. In gliomas, *MGMT* promoter methylation is known to be both a strong predictor of survival [7, 8] and of response to alkylating agents [9–11]. Glioma patients demonstrating *MGMT* promoter methylation in tumour tissue therefore show a significant advantage in survival. Only recently similarly promising results could be demonstrated in metastatic malignant melanoma with significantly better response rates to the alkylating agent temozolomide (TMZ) in melanomas showing *MGMT* promoter methylation [12]. The concept is that silencing of MGMT by promoter methylation is reflected by a loss of MGMT protein expression leading to increased sensitivity of tumours to alkylation-induced DNA damage. However, most studies comparing *MGMT* promoter methylation analysis with loss of MGMT protein expression as a potential surrogate marker for an inactivation of the *MGMT* gene described only a poor or lacking correlation [12–16], except for a study by Sonoda et al. [17] that reported a good correlation between the two methods.

In a subset of low- and intermediate-grade metastasized pNEN, TMZ showed promising effects with response rates of up to 70% [18–20]. *MGMT* promoter methylation was described between 0 and 40% of pNEN [1–4], however these studies did not indicate any follow-up data related to chemotherapy, response or prognosis of the patients. Two studies by Ekeblad et al. [21] and Kulke et al. [20] correlated response to TMZ with MGMT protein expression and a correlation between loss of MGMT protein expression by immunohistochemistry and response to TMZ was found only by the latter. In the absence of any prognostic data on MGMT, it is theoretically possible that the findings by Kulke et al. [20] could reflect a prognostic rather than a predictive effect. In order to claim an effect predictive for therapy response, the prognostic value of a marker independent from the therapy needs to be known.

Therefore, we performed a study using two different patient cohorts and comprising a prognostic and a predictive part which aims at comparing *MGMT* promoter methylation to MGMT protein loss, testing if *MGMT* promoter methylation and/or loss of MGMT protein expression are of prognostic value in pNEN, and if *MGMT* promoter methylation and/or loss of MGMT protein expression can predict response to a chemotherapy with TMZ in pNEN.

Materials and Methods

Tumour Specimens Prognostic Part

Altogether, tissue specimens of 141 patients with pNEN from the archives of the Institute of Pathology at the University of Zurich were included in the study. These comprised formalin-fixed and paraffin-embedded (FFPE) tissues of 130 patients and fresh frozen tissues from 52 patients. From 41 patients both paraffin blocks and fresh frozen tissues were available. Figure 1 gives an overview of the tissue specimens included in the prognostic part of the study.

The available paraffin specimens comprised 47 non-functioning tumours (36%), 39 insulinomas (30%), 11 glucagonomas (9%), 9 VIPomas (7%) and 8 gastrinomas (6%). In 16 cases (12%), the hormonal status was unknown. According to the WHO 2010 classification, 86 cases (66%) were pancreatic neuroendocrine tumours G1 (pNET G1), 37 cases (28%) were pancreatic neuroendocrine tumours G2 (pNET G2) and 2 cases (2%) were pancreatic neuroendocrine carcinomas (pNEC). 5 cases (4%) could not be

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Table 1. Overview of patients' characteristics and results

Patient No.	Sex	Age	Site of primary tumor	Grade	Prior therapy	MGMT IHC	MGMT MSP	MGMT qPCR, %	TTP, months	Remission (RECIST)
1	m	39	pancreas	3	Cis/Eto/ACE/CarboEtoIrino/Ev	+	neg nd nd	0 nd 0	4	PD
2	m	61	pancreas	2	Gem/5-FU-FS/Ev	–	neg neg neg	26 73 57	27	PR
3	m	52	pancreas	2	SSA/STZ-FU/Ev/Sut	n.a.	nd nd	0 nd	4	PD
4	f	39	pancreas	2	Thalid/STZ-5-FU/SSA	+	neg	0	3	PD
5	m	36	pancreas	2	STZ/5-FU, CAPOX	+	neg	0	3	PD
6	m	49	pancreas	n.a.	STZ-5-FU/CisEto	–	neg	0	7	PR
7	m	44	pancreas	2	STZ/5-FU	+	nd	62	7	SD
8	m	44	pancreas	2	STZ/5-FU (2x), FOLFOX	–	neg neg	0 10	26	PR
9	m	65	pancreas	2	SSA/IFN/STZ-5-FU	+	neg	0	3	PD
10	m	58	pancreas	3		–	neg neg neg	0 0 0		† after 1 month

MSP = Methylation-sensitive PCR; qPCR = primer extension based quantitative PCR; TTP = time to progression; PD = progressive disease; SD = stable disease; PR = partial remission; n.a. = not assessable; Cis = cisplatin; Eto = etoposide; ACE = adriamycin, cyclophosphamide, etoposide; CarboEtoIrino = carboplatin, etoposide, irinotecan; Ev = everolimus; Gem = gemcitabine; (5-)FU = (5-)fluorouracil; SSA = somatostatin analogue; STZ = streptozotocin; Sut = sutent; Thalid = thalidomide; CAPOX = capecitabine, oxaliplatin; FOLFOX = folinic acid, 5-FU, oxaliplatin; IFN = interferon.

reclassified retrospectively. Survival data were available for 83/130 (63.8%) patients.

Fresh frozen tissues of 52 patients were available for methylation-specific PCR (MSP). These specimens comprised 12 non-functioning tumours (23.1%), 28 insulinomas (53.8%), 3 glucagonomas (5.8%), 4 VIPomas (7.7%) and 5 gastrinomas (9.6%). According to the WHO 2010 classification, 31 cases (60%) were pancreatic neuroendocrine tumours G1 (pNET G1), 15 cases (29%) were pancreatic neuroendocrine tumours G2 (pNET G2), and 6 cases (11%) could not be classified. Survival data were available for 48/52 patients (92%).

Tumour Specimens Predictive Part

FFPE tissue specimens of a cohort of 10 consecutive patients with progressive metastasized pNEN followed up at the Charité University Hospital Berlin and receiving TMZ chemotherapy after one to four prior treatment lines were included in the study. From 5 patients, multiple tissue blocks obtained either at different time points during tumour progression (1 case) or obtained from different body sites (4 cases) were available. According to WHO and ENETS criteria, 7 tumours (70%) were G2 and 2 tumours (20%) were G3. Grading was not possible in 1 case. Radiological tumour response was classified according to the Revised Response Evaluation Criteria In Solid Tumours (RECIST 1.1) [22]. An overview of the patients' characteristics is given in table 1.

Immunohistochemistry Prognostic Part

A 4-μm section from a previously described tissue microarray (TMA) [23] was stained with an antibody against MGMT. The immunohistochemical staining was performed on an automated staining system (Bond Refine, Vision BioSystems Ltd., Newcastle upon Tyne, UK). The following antibody was used: MGMT clone MT 3.1 (Gene Tex Inc., Irvine, Calif., USA), dilution 1:160. Antigen retrieval was performed by heating (H2(60), EDTA-based pH 9.0 Bond Epitope Retrieval Solution, 30 min). Visualization was performed using the avidin-biotin complex (ABC) method leading to a brown staining signal. The stainings were evaluated by two pathologists (A.M.S. and A.B.). Interobserver agreement was almost perfect (κ = 0.92). Only cases with a positive internal control in non-neoplastic cells were evaluated for MGMT protein expression. As control, glioblastoma tissues with known MGMT protein expression status were used.

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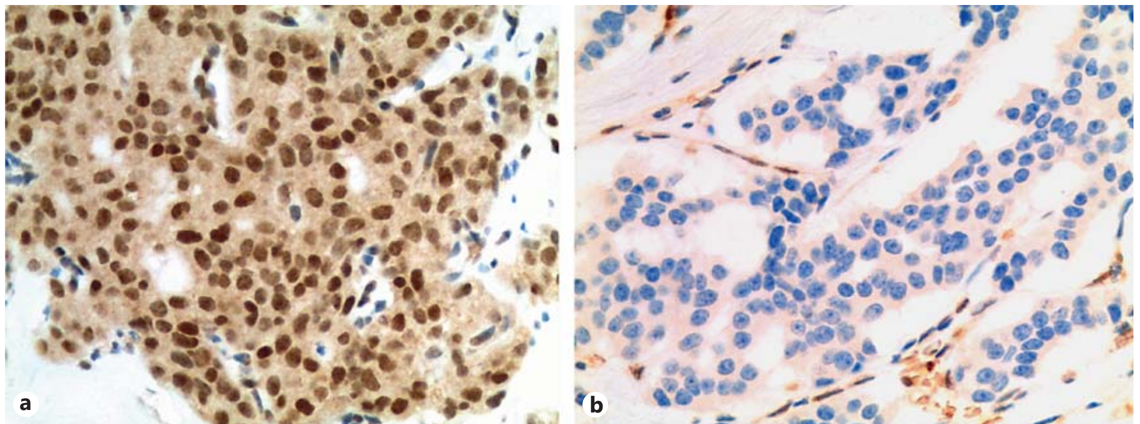

Fig. 2. a pNEN with positivity for MGMT in most of the tumour nuclei. MGMT immunohistochemistry was scored positive when >5% of tumour cell nuclei stained positive. **b** pNEN negative for MGMT. Note positive staining in endothelial cells as positive intern control.

Immunohistochemistry Predictive Part

A TMA containing tissue specimens from all 10 patients described above was constructed. MGMT immunohistochemistry was performed as described in the section above. In addition, the TMA was stained with an antibody against Mib1. The immunohistochemical staining was performed manually. The following antibody was used: clone MIB-1 (Dako, Glostrup, Denmark), at a concentration of 0.8 µg/ml, diluted in TBS with 0.5% casein and 5% normal goat serum. Antigen retrieval was performed by boiling in 10 mM citrate buffer at pH 6.0 in a pressure cooker. Visualization was performed using a polymer-based visualization system with horseradish peroxidase (Envision+; Dako) leading to a nuclear brown staining signal. The proliferation index was assessed by generating the percentage of positively staining nuclei per total tumour nuclei contained in the corresponding TMA punch. Grading was performed according to the WHO 2010 and ENETS guidelines [24].

MSP Prognostic Part

In all 52 tumours a one-step MSP of the *MGMT* promoter region was informative. DNA from fresh frozen tissue was modified by bisulfite treatment (Qiagen, Valencia, Calif., USA), converting all unmethylated cytosine residues to uracil [25]. This bisulfite treatment was followed by amplification with primers specific for methylated and unmethylated DNA as described [10]. The annealing temperature was 62 and 58 °C, respectively. After 40 cycles, PCR amplification of template DNA was performed.

MSP Predictive Part

DNA was extracted from FFPE tissues. MSP of the *MGMT* promoter region was carried out as described in the section above except for using DNA extracted from paraffin tissue. The analysis was informative in 13 tissue samples from 8 patients.

Primer Extension-Based Quantitative PCR Predictive Part

Primer extension-based quantitative PCR, adapted to FFPE tissues, was performed as described [26]. In brief, the bisulfite-converted DNA was amplified using primers designed to amplify both methylated and unmethylated DNA. In a second step, fluorescently labelled forward primers only specific for methylated (green) and unmethylated (blue) were added to the product for primer extension. After capillary electrophoresis the relative amount of methylated DNA was normalized to the ratio of signal intensities obtained for a control containing equal amounts of methylated and unmethylated DNA performed in the same experiment. The assay was informative in 16 of 18 tissue samples from all 10 patients included in the study. A tumour sample was considered to be hypermethylated if ≥10% of the tumour DNA demonstrated a hypermethylation of the *MGMT* promoter.

Statistical Analysis

Kaplan-Meier curves were used for survival analysis. Methylation status and immunohistochemistry were correlated using cross-tabulation. Multivariate analysis was calculated by the Cox proportional hazard regression model using SPSS software, version 21 (SPSS Software, Chicago, Ill., USA). The level of significance was defined as $p < 0.05$.

Ethics

The investigations of the prognostic part were approved by the local ethical committee. All patients of the predictive part gave broad consent according to the guidelines of the Charité University Hospital. The patients alive at the time point of the study additionally gave informed consent.

Results

Prognostic Part

MGMT Immunohistochemistry

MGMT protein expression was evaluated semiquantitatively. According to the literature a pNEN was scored positive when ≥5% of tumour nuclei stained positive (fig. 2). Non-neoplastic cells always showed a nuclear

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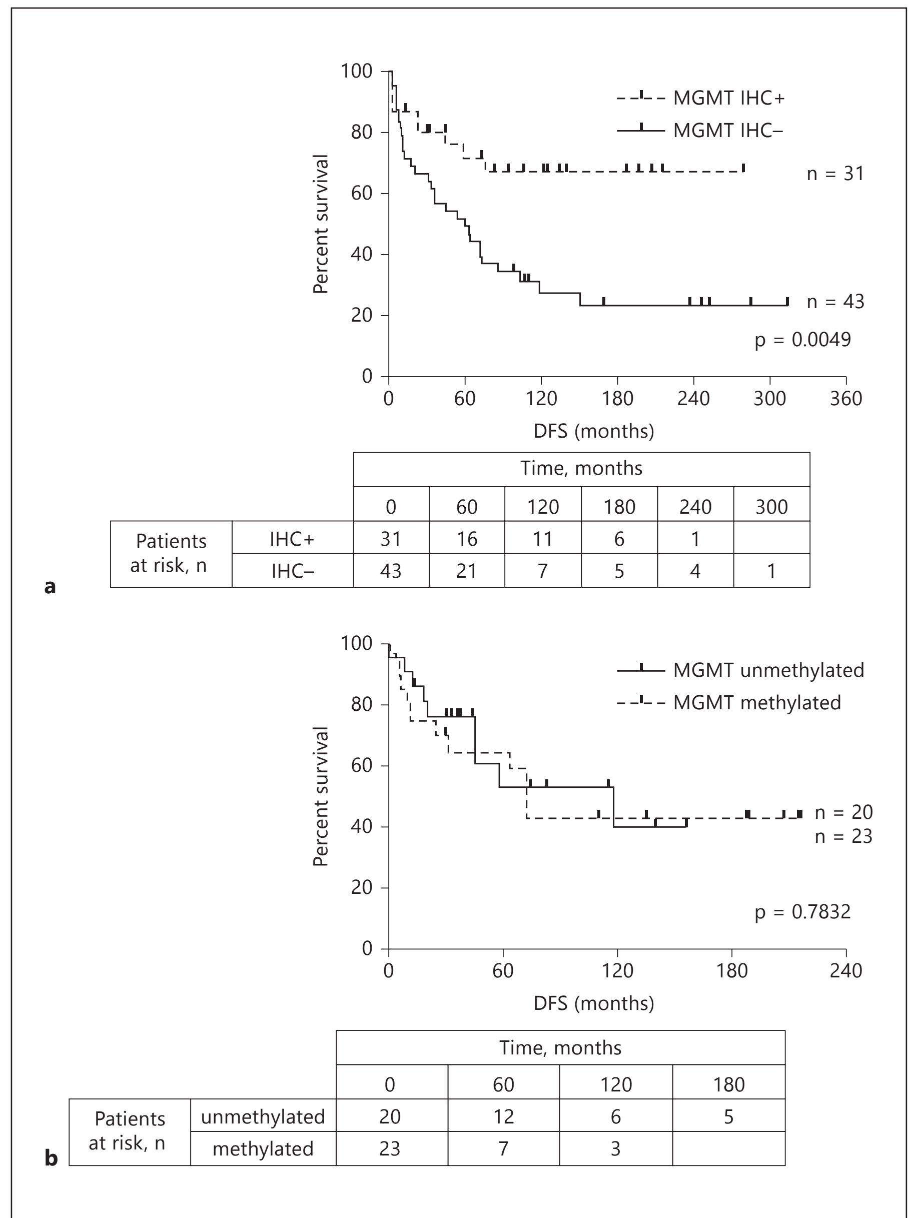

		Time, months					
		0	60	120	180	240	300
Patients at risk, n	IHC+	31	16	11	6	1	
	IHC–	43	21	7	5	4	1

		Time, months			
		0	60	120	180
Patients at risk, n	unmethylated	20	12	6	5
	methylated	23	7	3	

Fig. 3. a Kaplan-Meier curve depicting a significant longer disease-free survival (DFS) for MGMT-immunopositive PET in comparison with MGMT-immunonegative PET. **b** In contrast, no significant difference in DFS between tumours with methylated and with unmethylated *MGMT* promoter.

staining. 86/130 (66%) of the examined pNEN showed a complete loss of MGMT protein expression, while 44/130 (34%) of the examined pNEN showed a nuclear staining in >5% of tumour cells.

DNA Analysis

Promoter methylation of the *MGMT* gene was detected by MSP in 29/52 (56%) of the examined pNEN. A tumour was scored as methylated when a PCR product specific for methylated DNA could be detected on an agarose gel.

Statistical Analysis

Concordance of MGMT protein expression and promoter methylation status by MSP was found in 20/41 tumours (48.8%), and discordance was found in 21/41 tumours (51.2%). In contrast to MGMT methylation status (p = 0.8), loss of MGMT protein expression correlated significantly with a shortened disease-free survival (p = 0.005) (fig. 3) and with a decreased disease-specific survival (p = 0.03) in a univariate analysis. In a multivariate analysis including the widely recognized and best supported prognostic markers stage and grade [27] as well as

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Table 2. Prognostic part: results of uni- and multivariate analysis

	Univariate analysis		Multivariate analysis			
	DFS p	DSS p	DFS p	Exp(B) p	DSS p	Exp(B)
CK19+	0.007	0.000	n.s.		0.000	11.641
MGMT–	0.005	0.03	n.s.		n.s.	
Stage	0.000	0.000	0.000	2.112	n.s.	
Grade	0.000	0.000	0.000	4.981	0.000	6.818

DFS = Disease-free survival; DSS = disease-specific survival; Exp(B) = hazard ratio; CK19+ = expression of CK19 by immunohistochemistry; MGMT– = loss of MGMT protein expression by immunohistochemistry; n.s. = not significant.

CK19 [23], loss of MGMT protein expression did not retain its significance with regard to disease-free or tumour-specific survival, only proliferation and CK19 remained significant (table 2).

Predictive Part

MGMT and Mib1 Immunohistochemistry

MGMT protein expression was evaluable in 9/10 cases (90%). Immunohistochemistry could not be evaluated in 1 case (10%) (no nuclear staining in non-neoplastic cells). Four of the 9 cases that could be evaluated showed a complete loss of MGMT protein expression, while the remaining 5 cases showed a nuclear staining in >5% of tumour cells and were thus scored MGMT-positive.

Two cases (20%) (1 with an initial proliferation index of 10% but with a proliferation index of 40% in the metastasis at the beginning of the TMZ therapy; the other case with a proliferation index of 35%) were graded as G3 according to the WHO 2010 classification, all other tumours were graded as G2. Importantly, these two tumours showed a well-differentiated morphology.

DNA Analysis

Promoter methylation of the *MGMT* gene was absent by MSP in all pNEN with informative results (8/10), but this assay is not optimized for FFPE tissue.

Promoter methylation of the *MGMT* gene was detected in tissue samples of 3 of 10 patients (30%) by primer extension-based quantitative PCR. The percentage of methylated DNA ranged between 10 and 73%. In none of the 5 patients with multiple tumour samples obtained at different time points or from different body sites were there differences between these tumour samples, reflecting a certain stability of *MGMT* methylation during tumour progression. Examples of each one methylated and unmethylated sample as detected by primer extension-based quantitative PCR are depicted in figure 4.

Statistical Analysis

Concordance of MGMT protein expression and promoter methylation status by primer extension-based quantitative PCR was obtained in 6/9 tumours (66%), and discordance was obtained in 3/9 tumours (33%). However, the correlation did not reach statistical significance (p = 0.5). *MGMT* promoter methylation, as assessed by primer extension-based quantitative PCR, correlated significantly with response according to RECIST criteria. p values for progressive disease vs. stable disease vs. partial response and for progressive disease vs. stable disease or partial response were 0.033. No significant correlation could be observed between MGMT protein expression and response to TMZ (p value for progressive disease vs. stable disease or partial response 0.206), however the p value for progressive disease vs. stable disease vs. partial response missed the level of significance only marginally (p = 0.056).

Discussion

Correlation of MGMT Protein Expression and Promoter Methylation

We did not find a significant correlation between MGMT protein expression by immunohistochemistry and *MGMT* promoter methylation by MSP in both cohorts. This finding is in line with the results of previous studies on gliomas and malignant melanoma by other authors describing only a partial correlation or no correlation at all between the two methods [12–16, 28–31]. This points to other mechanisms in addition to promoter methylation that control *MGMT* expression.

Notably, *MGMT* was shown to be regulated transcriptionally by p53 via the transcription factor Sp1 in human cell lines [32]. Consistent with these findings, binding sites for Sp1 as well as for other transcription factors such as glucocorticoid-responsive elements and AP-1 [33] as well as NF-κB [14] were identified in the *MGMT* promoter region, and binding of these factors to the promoter region was confirmed experimentally in mouse and human cell lines. In addition, *MGMT* expression can be regulated by histone methylation in cell lines [34]. These ad-

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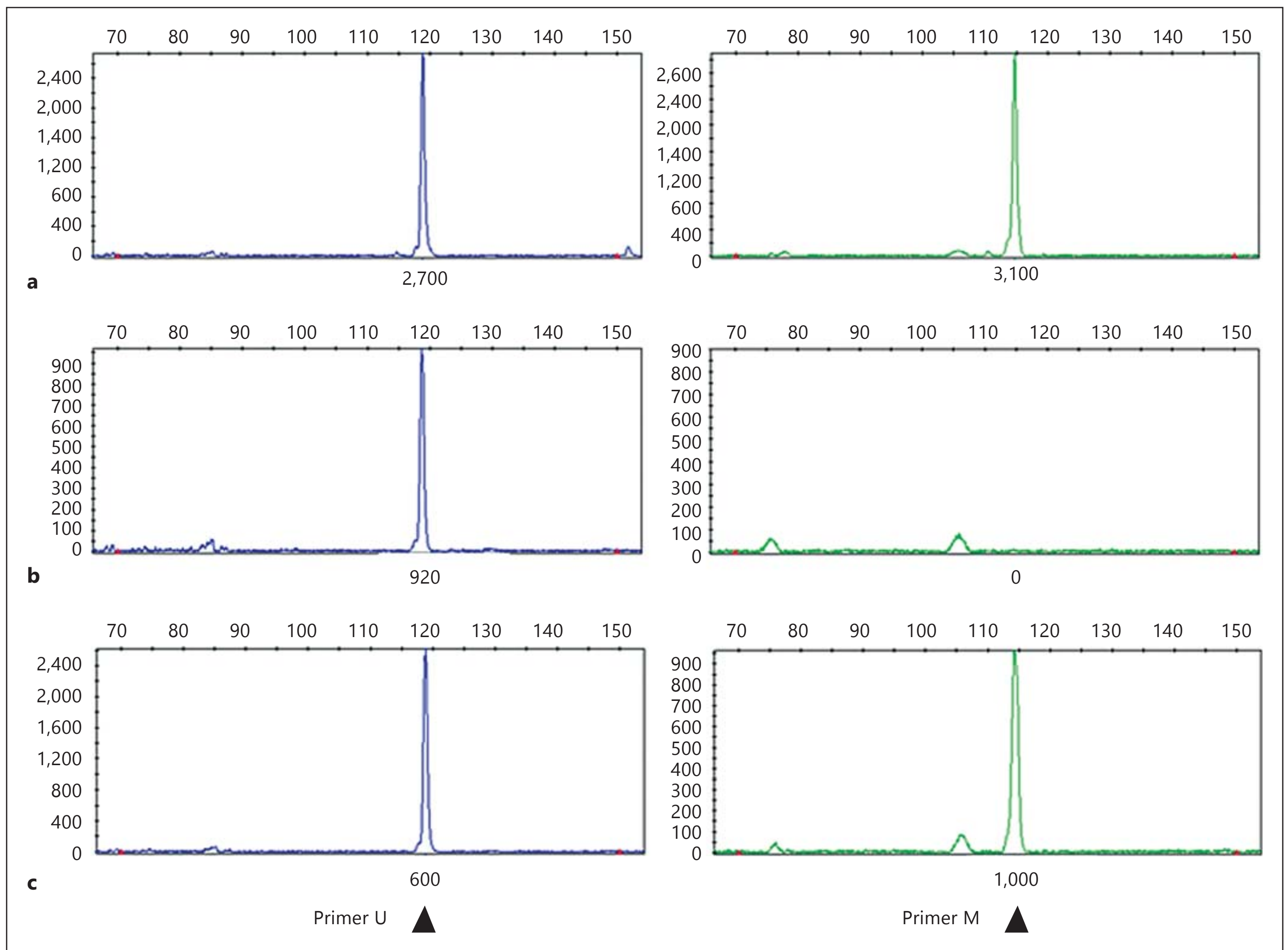

Fig. 4. Examples of capillary electrophoresis analysis of primer extension-based PCR: (**a**) control (50% methylated; 3,100/5,800 = 53%); (**b**) unmethylated sample (patient No. 4; 0/920 = 0%), and (**c**) methylated sample (patient No. 7; 1,000/1,600 = 63%). Primer U = Primer specific for unmethylated DNA; Primer M = primer specific for methylated DNA. Boxes underneath peaks indicate signal intensity. Intensity of both methylated and unmethylated signals is used for quantification.

ditional regulatory mechanisms may explain the inconsistent results in MGMT immunohistochemistry and MSP. Yet another possible explanation could be, as hypothesized by Clark and Melki [35], that hypermethylation itself is not the reason, but rather the consequence of downregulation or silencing of particular genes, making them more susceptible to de novo methylation at specific CpG sites.

From a methodological point of view, several authors have claimed that in gliomas MGMT immunohistochemistry and MSP are not interchangeable [15, 29, 30]. On the one hand, immunohistochemistry holds the immanent risks provided by the use of different antibodies between laboratories as well as interobserver variability [15]. On the other hand, MSP can lead to false-negative results due to tumour heterogeneity with the presence of cells with methylated and non-methylated promoters [36–38]. Another source of error regarding MSP may be the site of selected primers. Everhard et al. [39] compared MGMT mRNA expression and *MGMT* methylation by pyrosequencing and concluded that the region investigated by the most commonly used MSP primers [5] was not among the regions that best correlated with expression, thus supporting the notion that some CpG sites may be more important than others with regard to expression [40]. We think, based on the following arguments, that methodological problems alone cannot explain the observed differences between protein expression and methylation.

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We found the same difference in two independent cohorts. All experiments were repeated twice. MSP in the first cohort was only done on fresh frozen tissues, where the methodological issues are minimal. Therefore, other mechanisms of *MGMT* regulation such as the ones described in other tumours could play a role in pNEN and need to be explored.

A possible explanation for this apparent discrepancy could be the following: a general CpG island methylator phenotype, CIMP, is detected by *MGMT* promoter methylation analysis. Such a phenotype, including methylation of MGMT, has been postulated [2, 4]. This discrepancy would also explain the differential role of MGMT protein expression and promoter methylation with regard to prognosis and prediction of response to TMZ.

Prognostic Part

In the prognostic part of the present study, we show that loss of MGMT protein expression by immunohistochemistry, but not *MGMT* promoter methylation, is an adverse prognostic marker in sporadic pNEN after surgical resection. To our knowledge, the prognostic impact of the loss of MGMT protein in pNEN has not been investigated to date. MGMT is an important enzyme of the DNA repair machinery and as such plays a central anticancer role, according to the present data also in pNEN. Although our study demonstrates that loss of MGMT protein expression correlates significantly with an adverse outcome in univariate analysis, this significance is not retained in multivariate analysis including the widely accepted prognostic markers grade and stage as well as CK19. Increased proliferation is a hallmark of more aggressive pNET, and MGMT inactivation could be one molecular way of achieving this phenotype. This indicates that loss of MGMT protein expression is not to be proposed as an additional prognostic marker in daily routine practice. However, it will be important to keep in mind this adverse prognostic role of MGMT protein loss for interpreting a potential predictive role as discussed in the following section.

Predictive Part

In the present study, based on a limited cohort of 10 patients, we provide evidence that MGMT promoter methylation might serve as a predictor for response to chemotherapy with the alkylating agent TMZ in pNEN. While the examined series is small, we need to keep in mind that from our prognostic collective we have the information of adverse outcome of patients with MGMT protein loss.

Kulke et al. [20] have described a correlation between MGMT protein expression and response to TMZ; unfortunately, no methylation assay was performed. In contrast, in a study by Ekeblad et al. [21], no correlation between MGMT protein expression and response to TMZ was reported. In the present series, we were not able to confirm the findings of Kulke et al. on protein level, however the correlation between MGMT protein expression and response to TMZ missed the level of significance only marginally. Although it has to be stated that the lack of association of loss of MGMT protein expression with response to TMZ in our series might well be due to the limited size of the cohort investigated, our findings are similar to the situation in gliomas where *MGMT* promoter methylation and MGMT protein expression are not interchangeable and *MGMT* promoter methylation but not MGMT protein expression predicts response.

In conclusion, in the present study we demonstrate that MGMT immunohistochemistry but not *MGMT* promoter methylation assessed by MSP is an adverse prognostic marker in sporadic untreated pNEN after surgical resection. In contrast, we provide evidence that *MGMT* promoter methylation, possibly as a surrogate marker for CIMP, as well as possibly MGMT protein expression might serve as a predictor of response to TMZ in sporadic pNEN. Against the background of an unfavourable prognosis of MGMT-deficient untreated pNEN, it seems likely that the survival benefit of MGMT-deficient pNEN after TMZ treatment reflects the ability of the MGMT status to predict response rather than being the consequence of its prognostic effect. Further, favourably prospective studies will be needed to validate these findings and to confirm the correlation between *MGMT* promoter methylation, CIMP and response to TMZ before this observation can influence clinical routine.

Acknowledgement

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Disclosure Statement

A.S., T.R., H.D., A.B., P.K. and E.V. have nothing to declare. A.P. and M.P. have received honoraria for presentations and consultancy/advisory boards from Novartis, IPSEN and Pfizer. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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