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Evaluation of *MGMT* Gene Methylation in Neuroendocrine Neoplasms

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Unresectable neuroendocrine neoplasms (NENs) often poorly respond to standard therapeutic approaches. Alkylating agents, in particular temozolomide, commonly used to treat high-grade brain tumors including glioblastomas, have recently been tested in advanced or metastatic NENs, where they showed promising response rates. In glioblastomas, prediction of response to temozolomide is based on the assessment of the methylation status of the *MGMT* gene, as its product, O^6-methylguanine-DNA methyltransferase, may counteract the damaging effects of the alkylating agent. However, in NENs, such a biomarker has not been validated yet. Thus, we have investigated *MGMT* methylation in 42 NENs of different grades and from various sites of origin by two different approaches: in contrast to methylation-specific PCR (MSP), which is commonly used in glioblastoma management, amplicon bisulfite sequencing (ABS) is based on high-resolution, next-generation sequencing and interrogates several additional CpG sites compared to those covered by MSP. Overall, we found *MGMT* methylation in 74% (31/42) of the NENs investigated. A higher methylation degree was observed in well-differentiated tumors and in tumors originating in the gastrointestinal tract. Comparing MSP and ABS results, we demonstrate that the region analyzed by the MSP test is sufficiently informative of the *MGMT* methylation status in NENs, suggesting that this predictive parameter could routinely be interrogated also in NENs.

Key words: Neuroendocrine neoplasms; *MGMT* gene methylation; Methylation-specific PCR; Amplicon bisulfite sequencing; Temozolomide

INTRODUCTION

With an estimated prevalence of around 1/10,000, the incidence of neuroendocrine neoplasms (NENs) is constantly rising. NENs originate from embryonic neuroendocrine cells. Combining characteristics of endocrine, hormone-producing, and nerve cells, neuroendocrine cells are present in several body regions and are more abundant in the lung, intestine, pancreas, where they play fundamental physiological roles.

Diagnosis of NENs can be challenging due to their high biological as well as cellular heterogeneity. Consequently, NENs often have little relation with each other despite their putative common cells of origin[1]. Moreover, diagnosis is often delayed because NENs present with non-specific, extremely variable (and sometimes absent) symptoms; thus, in 20% of cases, the tumors are already in an advanced or metastatic stage at the time of diagnosis[2]. Histologically, NENs can be divided into at least two subtypes: well differentiated and poorly differentiated,

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with undifferentiated tumors being characterized by poor hormonal production[3]. Surgery is the therapy of choice; when not feasible, several therapeutic approaches such as chemotherapy, target therapies, biological therapies, and peptide receptor radionuclide therapy are exploited. The most frequently used chemotherapeutic scheme is platinum-based agents plus etoposide, but prognosis of unresectable, undifferentiated NENs remains dismal[4].

Alkylating agents, in particular temozolomide, have been tested for the treatment of NENs unresponsive or resistant to commonly used therapeutic approaches[5–8]. Temozolomide is currently used in the clinical management of different types of tumors, in particular lymphomas and melanomas, and, in addition to surgical resection and radiotherapy, as first-line treatment of glioblastomas[9–13]. The cytotoxic mechanism of temozolomide action is based on the addition of a methyl group to purine bases of DNA, in O^6-guanine, N7-guanine, and N3-adenine. The principal cytotoxic lesion is *O*^{6}-methylguanine (O6-MeG), which is consequently recognized as adenine, inducing a mismatch during DNA replication[14]. The mismatch can be repaired by *O*^{6}-methylguanine-DNA methyltransferase (MGMT), a demethylating enzyme mainly involved in this type of DNA repair. The *MGMT* gene product directly removes the methyl group from O^6 of guanine, transferring it on itself, thus acting as a "suicide enzyme"[15]. Therefore, temozolomide effects are severely counteracted by MGMT functions. Accordingly, assessment of *MGMT* epigenetic silencing by regulatory region hypermethylation is commonly used in clinical practice as a predictive and prognostic marker for response to temozolomide in the management of glioblastomas[14–16].

Given the encouraging results of the recent clinical trials evaluating the potential use of temozolomide in NENs[4–8], we here investigated the methylation profiles of the *MGMT* gene in a heterogeneous group of NENs, including both well-differentiated (G1 and G2) and poorly differentiated (G3 and metastatic) tumors. To this aim, we used two different technical approaches: methylation-specific polymerase chain reaction (PCR) (MSP), routinely used in clinical practice for glioblastomas, and amplicon bisulfite sequencing (ABS), which allows a wider and ultradeep analysis of the methylation status of several CpG sites at the *MGMT* gene. In addition, by comparing the results obtained by the two tests, we investigated whether the *MGMT* methylation status of NENs can be straightforwardly assessed by the same MSP test routinely utilized in glioblastoma management.

MATERIALS AND METHODS

Tumor Samples

Neuroendocrine tumor samples ($n = 42$) were obtained from and characterized by the Department of Pathology, National Cancer Institute, IRCCS-Foundation "G. Pascale" in Naples.

Glioblastoma samples ($n = 12$) were obtained from CEINGE-Biotecnologie Avanzate, in Naples and characterized by the Pathology Unit of the University of Naples "Federico II." After biopsy or surgical resection, the tissues were fixed in 10% formalin and embedded in paraffin. Sample characteristics are shown in Table 1. All patients participating in this study provided informed consent. Ethical statement: Ethical Committee of the National Cancer Institute, IRCCS-Foundation "G. Pascale" in Naples (reference EC:263/2019).

DNA Extraction and Bisulfite Conversion

Human DNA was extracted from tumor tissues using the FFPE DNA Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality was checked using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Genomic DNA (500 ng) was converted by sodium bisulfite with the EZ DNA Methylation Gold Kit (Zymo Research, Irvine, CA, USA) and eluted in 20 µl of RNase- and DNase-free water according to the manufacturer's instructions.

MSP Analysis

Methylation analysis of samples was performed using a nested PCR. The first PCR step was performed using bisulfite-specific primers. Reactions were performed in 30 µl of total volume: 3 µl of 10X reaction buffer, 0.6 µl of 10 mM dNTP mix, 1.5 µl of 4 mM forward primers (PAN forward: 5′-GGATATGTTGGGATATAGTT-3′), 1.5 µl of 4 mM reverse primers (PAN reverse: 5′-CCATCCACAATCACTACAAC-3′), 5 µl of bisulfite template DNA, 0.3 µl of FastStart Taq, and H_2O up to the final volume. A PCR sample mix, without DNA, was used as reaction control. PCRs were performed under these temperature conditions: 95°C for 15 min; 45 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and elongation at 72°C for 50 s; 72°C for the final 10 min. After the first PCR, the amplification was performed both with specific primers for methylated DNA, producing an amplified of 81 base pairs (bp) (from +66 to +147 bp), and with specific primers for nonmethylated DNA, producing an amplified of 91 bp (+61 to +154 bp)[9]. The *MGMT* methylated primers were as follows: forward, 5′-GCACTCTTCCGAAAACGAAACG-3′; reverse, 5′-GCACTCTTCCGAAAACGAAACG-3′. The *MGMT* unmethylated primers were as follows: forward, 5′-TTT GTGTTTTGATGTTTGTAGGTTTTTGT-3′; reverse, 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′. Two reaction mixes were performed, one for the methylated primer set and the other for the unmethylated primer set, in 30-µl total volume: 3 µl of 10X reaction buffer, 0.6 µl of 10 mM dNTP mix, 1.5 µl of 4 mM forward primers,

Table 1. Clinical and Histological Information About NEN Samples Analyzed

Patients	Gender	WHO Classification	Grading	Tissue Site	Cromogranine	Ki-67	Tissue Origin
NEN-1	Female	NEC	G3	GI	-	60%	PT
NEN-2	Female	NET	G1	GI	+	1%	PT
NEN-3	Female	NET	G2	P	+	15%	PT
NEN-4	Male	NET	G3	L	+	40%	MT
NEN-5	Male	NEC	G3	GI	+	70%	PT
NEN-6	Male	NET	G2	GI	+	8%	PT
NEN-7	Female	NET	G3	GI	+	2%	MT
NEN-8	Male	NET	G1	GI	+	1%	PT
NEN-9	Female	NET	G2	GI	+	6%	PT
NEN-10	Female	NET	G2	GI	+	7%	PT
NEN-11	Male	NEC	G3	GI	-	70%	PT
NEN-12	Male	NET	G2	LMP	+	3%	PT
NEN-13	Male	NEC	G3	LV	+	70%	MT
NEN-14	Female	NET	G2	L	+	7%	PT
NEN-15	Male	NET	G1	GI	+	2%	PT
NEN-16	Male	NET	G2	GI	+	15%	PT
NEN-17	Male	NET	G2	LMP	+	15%	PT
NEN-18	Male	NEC	G3	GI	+	90%	PT
NEN-19	Male	NET	G1	GI	+	1%	PT
NEN-20	Male	NET	G2	LMP	+	15%	PT
NEN-21	Male	NEC	G3	GI	+	80%	MT
NEN-22	Male	NET	G2	P	+	5%	PT
NEN-23	Male	NET	G1	GI	+	1%	PT
NEN-24	Male	NEC	G3	GI	+	90%	PT
NEN-25	Male	NET	G2	LMP	+	3%	MT
NEN-26	Female	NEC	G3	GI	+	70%	PT
NEN-27	Male	NEC	LNEC	LV	+	80%	MT
NEN-28	Female	NET	G1	P	+	1%	PT
NEN-29	Male	NET	G3	P	+	20%	PT
NEN-30	Female	NEC	G3	GI	+	80%	PT
NEN-31	Male	NEC	G3	LMP	+	90%	MT
NEN-32	Male	NET	G2	P	+	6%	PT
NEN-33	Female	NEC	G3	GI	+	90%	PT
NEN-34	Male	NEC	G3	LMP	+	70%	MT
NEN-35	Female	NET	G2	P	+	5%	PT
NEN-36	Male	NEC	G3	GI	+	60%	PT
NEN-37	Female	NEC	G3	GI	+	70%	PT
NEN-38	Female	NET	G3	GI	+	50%	PT
NEN-39	Male	NET	G2	LV	+	5%	MT
NEN-40	Female	NEC	G3	GI	-	60%	PT
NEN-41	Male	NET	G1	GI	+	1%	PT
NEN-42	Male	NEC	G3	GI	+	80%	MT

GI: gastrointestinal tract; P: Pancreas; L: lung; LV: liver; LMP: lymph nodes; PT: primitive tumor; MT: metastatic tumor.

5 µl of bisulfite template DNA, 0.3 µl of FastStart Taq (Qiagen), and H_2O up to the final volume. The second PCRs were performed under these temperature conditions: 95°C for 15 min; 32 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 50 s; 72°C for the final 10 min. We used, as a positive control, a methylated commercial DNA for *MGMT* methylated alleles and, as a negative control, a nonmethylated commercial control (EpiTEC controls from Qiagen). Controls without DNA were used for each set of MSP assays. Ten microliters of each 30-µl MSP product was loaded directly onto 3% agarose gels, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA), and examined under ultraviolet illumination (Bio-Rad, Hercules, CA, USA).

Amplicon Library Preparation and Sequencing

We generated an amplicon library for sequencing as described[17]. Briefly, bisulfite-treated DNA underwent a double amplification strategy. The first PCR step was

performed using bisulfite-specific *MGMT* primers. The reaction protocol was as follows, in 30-µl total volume: 3 µl of 10X reaction buffer, 0.6 µl of 10 mM dNTP mix, 1.2 µl of 5 mM forward primers (5′-TCGTCGGCAGCG TCAGATGTGTATAAGAGACAGgatatgttgggatagtt-3′), 1.2 µl of 5 mM reverse primers (5′-GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAGcaatccacaatcactacaa c-3′), 5 µl of bisulfite template DNA, 0.3 µl of Hot Start Taq (Qiagen), and H_2O up to the final volume. The PCRs were performed with the following temperature conditions: 95°C for 15 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 50 s; 72°C for 6 min. The first PCR was purified using magnetic Beads (Beckman-Coulter, Brea, CA, USA) according to the manufacturer's instructions. A second step of PCR was performed to add multiplexing indices to first amplicons. The second PCR protocol was performed in 50-µl final volume: 25 µl of Master Mix KAPA Uracil plus (Roche, Basel, Switzerland), 3 µl of forward and reverse "Nextera XT" primers (Illumina, San Diego, CA, USA), 5 µl of first PCR product, and H_2O up to the 50-µl final volume. The PCRs were performed with the following temperature conditions: 95°C for 3 min; 12 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 30 s, and elongation at 72°C for 50 s; 72°C for 5 min. After the second PCR step, we purified the PCR product using AMPure purification magnetic beads (Beckman-Coulter) following the manufacturer's protocol. All amplicons were quantified using Qubit® 2.0 Fluorometer. We generated an equimolar amplicon library and then diluted to a final concentration of 8 pM. Phix control library (Illumina) [10% (v/v)] was added to increase diversity of base calling during sequencing. Amplicon's library was subjected to sequencing using V2-nano reagent kits on the Illumina MiSeq system (Illumina).

Sequence Handling and Bioinformatics Analyses

Paired-end reads were obtained from the Illumina Miseq sequencer platform. The obtained reads were assembled together using PEAR tool[18], with a minimum of 40 overlapping residues as threshold. FASTA reads were obtained converting FASTQ using PRINSEQ tool[19]. Sequences derived from bisulfite-DNAs were analyzed with ampliMethProfiler pipeline software (https://sourceforge.net/projects/amplimethprofiler), specifically designed for deep-targeted bisulfite amplicon sequencing. AmpliMethProfiler produces quality filtered FASTA files for all samples and directly extracts methylation average and methylation profiles. As output, the pipeline generates also a summary file with information about the number of reads passing filters, the methylation percentage of each C in CpG sites, and the bisulfite efficiency for each C in non-CpG sites. Since we analyzed DNA samples from brain tumors, we did not apply any bisulfite filter in order to also save sequences with non-CpG methylation.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7.0. Graphs were generated with GraphPad. Differences in single CpG site methylation were calculated by multiple *t*-test in GraphPad, followed by Bonferroni correction. In this study, results were considered statistically significant with a value of $p < 0.05$. Correlation between MSP and ABS methylation at single CpG sites was performed using Pearson correlation test.

RESULTS

To investigate the methylation status of the *MGMT* gene, we collected 42 NEN samples obtained through surgical operations or biopsies. Sample characteristics are shown in Table 1. Samples were analyzed by MSP, routinely used in clinical practice for characterizing *MGMT* gene methylation in glioblastomas, as well as by ABS, based on high-resolution, next-generation sequencing. DNA samples from glioblastomas ($n = 12$), whose degree of methylation was already known, were used as controls for both strategies.

ABS Reveals That the MGMT Gene Is Methylated in a High Percentage of NENs

We analyzed *MGMT* DNA methylation by ABS including a higher number of CpG sites as well as the same CpG sites analyzed by MSP. Since we investigated DNA methylation of brain tumors, we could not exclude the presence of non-CpG methylation in the *MGMT* analyzed region. For this reason, we decided to remove the pipeline bisulfite filter, preventing the loss of sequences that passed the bioinformatic analysis. As shown in Figure 1A, the ABS technical approach allows covering 27 CpG sites (from +45 to +249 nucleotide positions from TSS) in the *MGMT* gene and provides a quantitative assessment of the methylation degree of each CpG analyzed. As shown in Figure 1B, when investigated by ABS, in all of the 42 processed samples, each of the 27 analyzed CpG had a different degree of methylation. We arbitrarily set the average methylation cutoff value at 25% to separate positive (>25%) from negative (<25%) samples. The choice of the cutoff value was based on the methylation state evaluated in our laboratory for diagnostic purpose by qualitative MSP (see also Materials and Methods). The value arbitrarily divides the heatmap into two parts: positive samples (over the cutoff line) and negative samples (below the cutoff line). ABS analysis showed, among positive samples, an average of methylation of 45% considering all the 27 CpG sites. As shown in Figure 1C, *MGMT* gene was found to be methylated in 76% of the NENs analyzed. Among the 27 CpGs analyzed by ABS, we extrapolated the 9 CpG sites interrogated by the MSP assay routinely used in glioblastoma clinical management. As shown in Figure 1D and E,

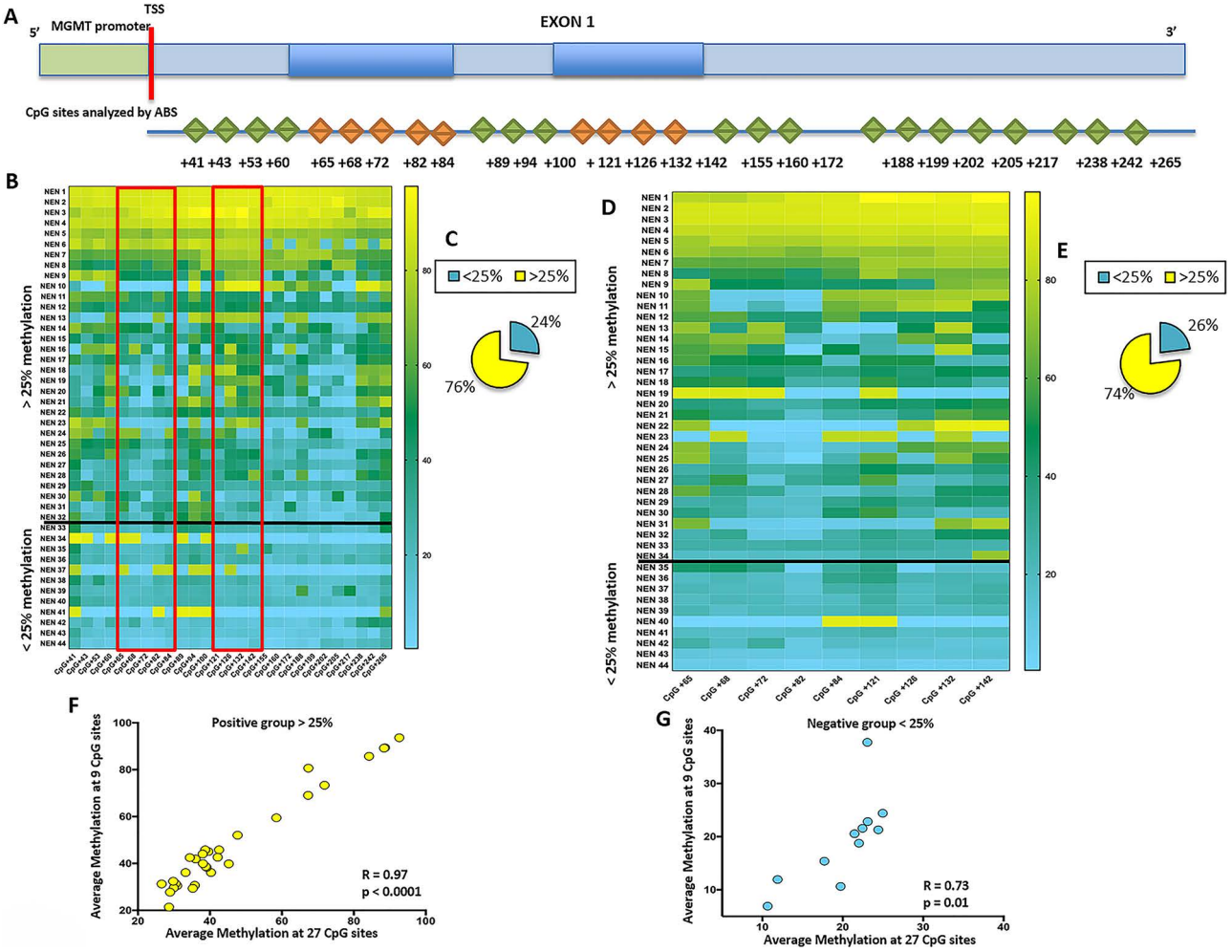

Figure 1. (A) Graphic representation of the *MGMT* gene regulatory region analyzed by methylation-specific polymerase chain reaction (PCR) (MSP) and amplicon bisulfite sequencing (ABS). Upper figure: *MGMT* gene promoter (green) and exon 1 (light blue) are represented. The regions spanned by MSP primers are highlighted within exon 1 (dark blue). Lower figure: The CpG sites analyzed by ABS are shown as rhombuses; in orange, the CpG sites interrogated also by the MSP test. CpG numbering is referred to nucleotide positions from the transcriptional start site (TSS). (B) DNA methylation heatmap of the 42 analyzed neuroendocrine neoplasms (NENs); in rows, the ID of each tumor sample, and in columns, each CpG site analyzed by ABS, with nucleotide position from TSS. Among CpGs, those in the red box are the CpG sites analyzed by both MSP and ABS. Colors related to grade of methylation (from light blue, nonmethylation, to yellow, high methylation) are shown on the right. The horizontal black line represents the cutoff value (25% of average methylation). (C) Graphic representation of the percentage of positive and negative samples based on all analyzed CpG. In yellow, the percentage of samples with average methylation at all 27 CpG sites >25%; in light blue, the percentage of samples with average methylation at all 27 CpG sites <25%. (D) DNA methylation heatmap of the CpG sites covered by both ABS and MSP; in rows, the ID of each sample, and in columns, each CpG site. The adjacent line indicates colors related to grade of methylation (from light blue, nonmethylation, to yellow, high methylation). The horizontal black line represents the cutoff value (25% of average methylation) and divides the map in two zones: methylated samples (over the cutoff line) and nonmethylated samples (under the cutoff line). (E) Graphic representation of the percentage of positive and negative samples based on nine CpG sites covered by both ABS and MSP: in yellow, the percentage of samples with average methylation at nine CpG sites >25%; in light blue, the percentage of samples with average methylation at nine CpG sites <25%. (F) Correlation between the average methylation at all 27 analyzed CpG sites and the average methylation at 9 analyzed CpG in positive samples (>25%). Correlation plot indicates on the *x* axis the average methylation at 27 CpG sites for each sample and on the *y* axis the average methylation at 9 CpG sites for each sample with a total average methylation >25%. (G) Correlation between the average methylation at all 27 analyzed CpG sites and the average methylation at 9 analyzed CpG in negative samples (<25%). Correlation plot indicates on the *x* axis the average methylation at 27 CpG sites for each sample and on the *y* axis the average methylation at 9 CpG sites for each sample with a total average methylation <25%.

the percentage of NENs positive for *MGMT* methylation ranged from 76%, resulting from the analysis of all the 27 CpGs together, to 74%, when taking into account only the 9 common CpG sites. We then correlated the average methylation at the 9 CpG sites with the average methylation at all 27 CpG sites for all samples and for both groups (<25% and >25%). As shown in Figure 1F and G, we found that, in positive samples (>25%), the average methylation at the 9 CpG sites strongly correlated (Pearson correlation, $R = 0.97$, $p < 0.0001$) with the average observed in the analysis of all 27 CpG sites. Moreover, also in the negative group (<25%), the correlation between the average methylation at 9 CpG sites and all 27 CpG sites was significantly robust (Pearson correlation, $R = 0.73$, $p = 0.01$). Altogether, our results demonstrate that the *MGMT* gene is methylated in up to 76% of the analyzed NENs. Moreover, as the average methylation of the 27 CpGs interrogated by ABS and the 9 CpGs usually investigated by MSP in glioblastomas is significantly correlated, our results suggest that the much more feasible MSP approach could be sufficiently informative for evaluating the *MGMT* methylation status also in NENs. From our bioinformatics analyses, we noticed a non-CG methylation ranging from 1% to 21%. However, in our experimental condition, we were not able to discriminate between the presence of non-CpG methylation events, described in several types of tumors[20], and partial bisulfite conversion.

MGMT Gene Methylation Status Can Be Easily and Efficaciously Assessed by MSP in NENs

In order to better investigate the reliability of the MSP in evaluating *MGMT* methylation in NENs, 18 samples, previously analyzed by ABS, were also analyzed by MSP. MSP is a qualitative technique, the results of which are based on the presence/absence of methylation in the regions where primers anneal. To perform MSP, we used specific pairs of primers previously described and routinely used in glioblastoma diagnostics[9]. An extrapolation of results is shown in Figure 2A.

As shown in Figure 2B, by comparing the results obtained with the two technical strategies, we found high concordance in 17/18 (94%) of the analyzed NEN samples. Therefore, we conclude that MSP could be a valid analytical technique in evaluating the *MGMT* methylation status also in NENs.

The MGMT Gene Is More Often Methylated in Differentiated NENs

To investigate whether the *MGMT* methylation status correlates with NEN grading, we divided samples into two groups: group 1 ($n = 21$), including G1 and G2 (well-differentiated) tumors, and group 2 ($n = 21$), including G3 (poorly differentiated) and metastatic tumors. As shown in Figure 3A, although the data are not statistically significant (chi-square test, $p = 0.35$), we found that *MGMT* was substantially more often methylated in group 1 (76% of cases) compared to group 2 (62% of cases). As shown in Figure 3B, the analysis of each CpG site covered by MSP, although again not statistically significant (multiple *t*-test), demonstrated a higher degree of methylation for the differentiated tumor group.

A High Percentage of Gastrointestinal NENs Harbors MGMT Gene Methylation

NENs have a very heterogeneous distribution, being found in many organs and body regions. Taking advantage of the fact that our samples came from different organs (gastrointestinal tract, pancreas, lung, and lymphoid organs), we investigated whether tumors of different origin showed different *MGMT* methylation states. As shown in Figure 3C, we did not find a relationship between methylation status and body region, except for tumors originating in the gastrointestinal tract. Indeed, as shown in Figure 3D, 19/25 (76%) of the gastrointestinal NENs showed a methylated *MGMT* gene.

DISCUSSION

In several clinical trials, encouraging results have been obtained with temozolomide in the treatment of advanced or metastatic, more aggressive, and less differentiated NENs[4–8]. Because of the high biological heterogeneity of NENs, establishing biomarkers for predicting response to temozolomide is crucial to avoid unnecessary, if not detrimental, therapies. While *MGMT* gene methylation is critical for predicting response to temozolomide-based therapy in glioblastoma, when such a correlation was searched for NENs, the reported results were conflicting[7,21–26]. Our study shows that, in a small cohort of NENs, the most informative CpGs for investigating *MGMT* gene methylation are evaluable by MSP, a simple and low-cost technique routinely used in glioblastoma diagnostics. Our results encourage future studies, recruiting larger numbers of patients, to finally establish the validity of MSP for the molecular characterization of NENs. If definitively proven effective also in NENs, *MGMT* gene methylation assessed by MSP could be utilized to better guide treatment choices, especially when temozolomide is a therapeutic option.

Recently, in NENs, *MGMT* gene methylation has been investigated also by pyrosequencing (PYR)[24,26]. PYR, by giving a quantitative methylation percentage for each analyzed CpG, is not subject to individual interpretation once the cutoff value has been set with still, however, little consensus on the optimal one[27]. We decide to use MSP, compared with ABS, because we envision MSP as a technique that, in the future, could be more easily, than PYR, included in the routine clinical management

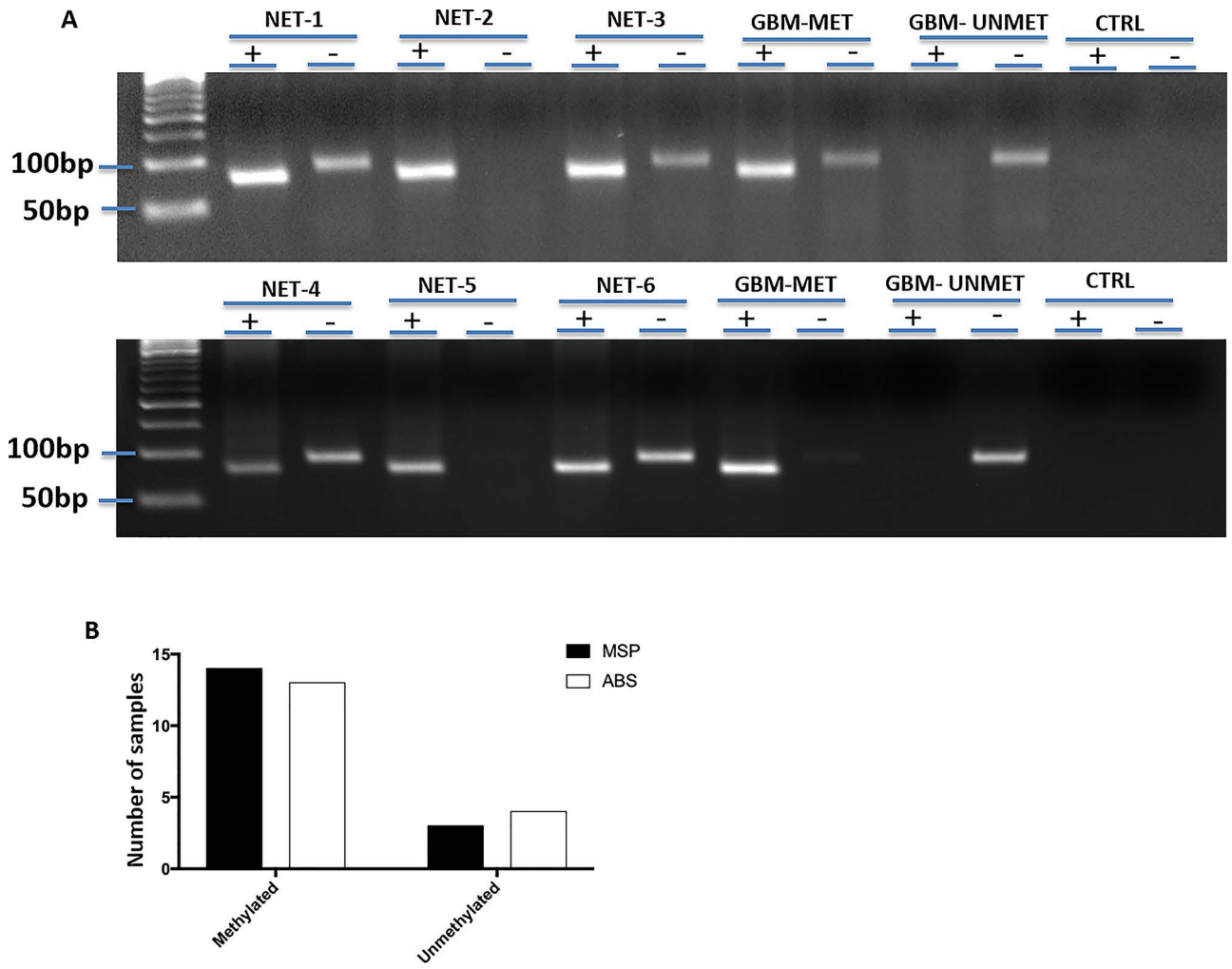

Figure 2. Evaluation of *MGMT* methylation by MSP. (A) Electrophoresis on agarose gel of the MSP: six NEN samples, four glioblastoma (GBM) samples (used as internal methylated and nonmethylated controls), and CTRL (controls without DNA) were loaded, each including a lane signed with + (methylated DNA–specific primers) and a lane signed with – (unmethylated DNA-specific primers). In the first lane: DNA ladder 50 base pairs (bp). (B) The histogram shows the number of samples (18) analyzed by both MSP and ABS (on *y* axis) presenting methylation or nonmethylation (on *x* axis) of *MGMT* CpG sites analyzed with MSP (in black, 14 samples show methylation and 4 samples are unmethylated) and with ABS (in white, 13 samples show an average of methylation >25% and 5 samples do not pass the cutoff). Please note that samples analyzed by ABS were included among the methylated or not methylated according to the cutoff value, as defined in the text.

of NENs. We recognize that MSP can be a method difficult to standardize, but, in clinical practice, it is already widely used to analyze *MGMT* gene methylation in glioblastomas[27]. Nevertheless, in the future, it will be interesting to broaden our analysis to compare, in addition to MSP and ABS, also PYR even though the concordance between MSP and PYR has already been reported high, at least in glioblastomas[28].

Overall, in our study, we found *MGMT* gene methylation in 74% (31/42) of the NENs investigated. In all our experiments, the *MGMT* methylation status in NENs was compared to glioblastoma samples and not to paired normal tissues as *MGMT* gene methylation is expected to occur only in a low percentage of nonneoplastic tissues, usually not even affecting protein expression or activity[29]. A higher methylation degree was observed in well-differentiated tumors and in tumors originating in the gastrointestinal tract. However, because of the small number of patients and the tumor heterogeneity in our cohort, the reported correlative findings should be taken as purely informative, pending future investigation.

In conclusion, our data suggest that MSP could be a promising technique for assessing *MGMT* gene methylation and, in turn, for predicting response to temozolomide also in NENs, as already well established for glioblastomas.

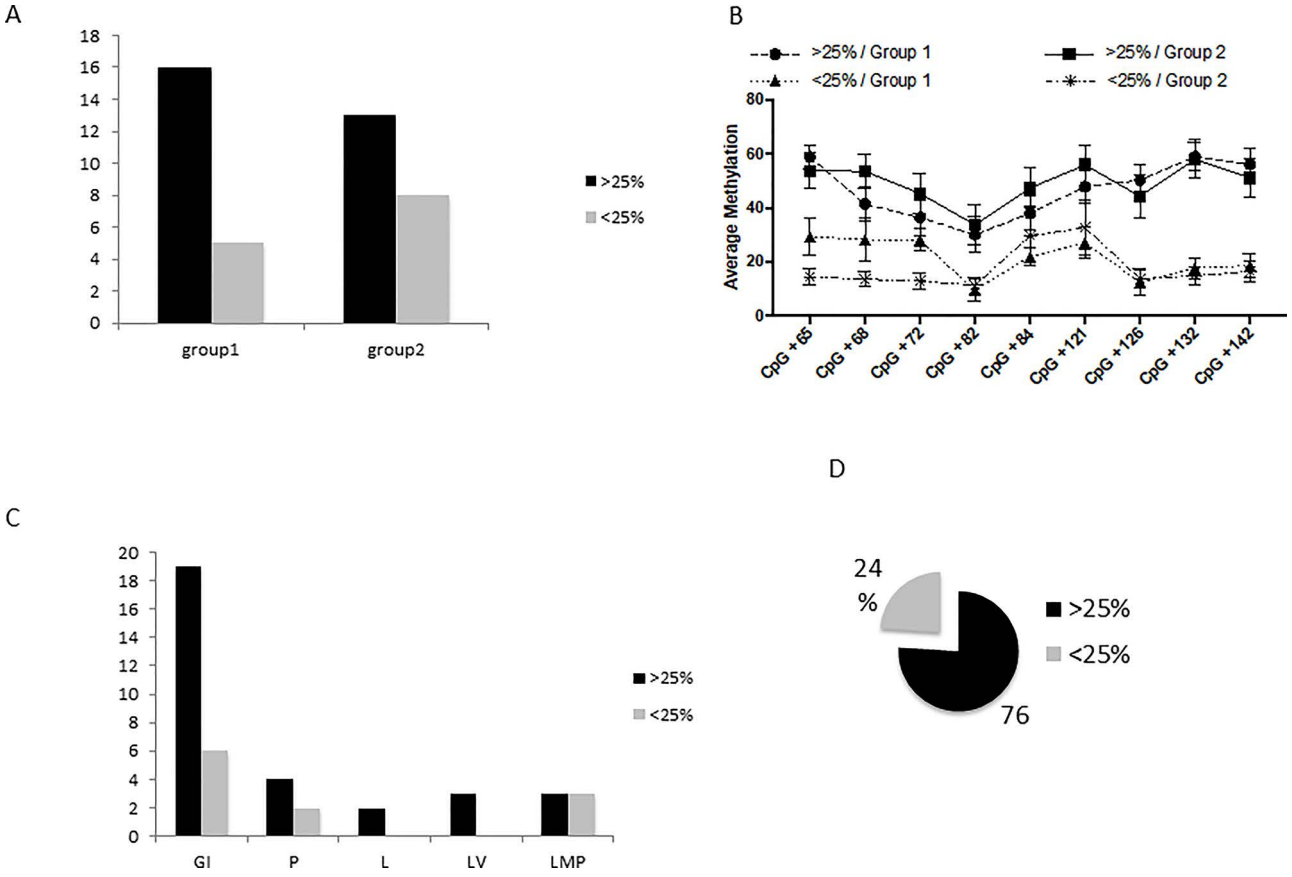

Figure 3. Evaluation of *MGMT* methylation in well differentiated versus undifferentiated NENs. (A) Average methylation in well differentiated (group 1 = 21 samples) and undifferentiated (group 2 = 21 samples) NENs, analyzed by ABS: average methylation of samples (on *y* axis) for each group (on *x* axis). Methylated samples in group 1 (16 samples) (black) and group 2 (13 samples) (black) and nonmethylated samples in group 1 (5 samples) (light gray) and group 2 (8 samples) (light gray) are shown. (B) Average methylation of each CpG covered by MSP. Methylated CpGs in group 1 and group 2 and nonmethylated CpGs in group 1 and group 2 are shown. (C) Methylation of samples relatively to primary location of NEN. The histogram shows primary location of NEN on the *x* axis and the number of samples on the *y* axis: 25 samples from the gastrointestinal tract (GI), 6 samples from the pancreas (P), 2 samples from the lung (L), 3 samples from the liver (LV), and 6 samples from the lymph node (LMP) presenting methylated (in black) or nonmethylated (light gray) *MGMT* gene. (D) In black, the percentage of *MGMT* methylated gastrointestinal NENs; in light gray, the percentage of *MGMT* nonmethylated gastrointestinal NENs.

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