

## Pyrosequencing Analysis of *MGMT* Promoter Methylation in Meningioma

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**Abstract.** *Background:* Methylation of the *O*<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) gene promoter is a well-established predictor of response to the DNA-alkylating agent temozolomide in patients with glioblastoma. *Materials and Methods:* Pyrosequencing analysis was used to determine the *MGMT* promoter methylation status in 61 meningiomas, to clarify whether it might have a predictive role. *Results:* Only two tumors (3%) had a mean methylation frequency higher than the cut-off value of 10% for the four CpG sites examined. *Conclusion:* The methylation of the *MGMT* promoter is uncommon, or occurs at a low frequency in meningiomas. There is no convincing rationale to test such tumors for their *MGMT* methylation status in a clinical setting.

*O*<sup>6</sup>-Methylguanine-DNA methyltransferase (*MGMT*; DNA-*O*<sup>6</sup>-methylguanine:protein-L-cysteine S-methyltransferase, EC 2.1.1.63) is an enzyme which repairs the *O*<sup>6</sup>-methylguanine residues of DNA by removing a methyl group (1). The protein is encoded by a single gene (*MGMT*) located on chromosome band 10q26 (2). The promoter of *MGMT* lacks TATA and CAAT boxes but contains a CpG island with multiple CpG dinucleotides (3). Many studies have shown

that methylation of cytosine to 5-methylcytosine in CpG dinucleotides in the promoter region of *MGMT* reduces expression of the gene (4-9).

Methylation of the *MGMT* gene promoter regulates transcription and is a well-established predictor of response to the DNA-alkylating agent temozolomide in patients with glioblastoma (1). Meta-analyses of *MGMT* promoter methylation in glioblastomas have shown that patients with methylated promoter in their tumor cells have better overall survival than those with unmethylated promoter when they were treated with temozolomide in addition to radiotherapy (10-12). The pattern of CpG site methylation varies among tumors. It is believed that methylation of CpG sites located in the first non-coding exon and enhancer is critical for loss of *MGMT* expression (1, 7, 13, 14). Thus, in a clinical setting, most assays for detection of *MGMT* methylation are designed to investigate these regions (1, 7, 13, 14).

The three most commonly used methods for detection of *MGMT* methylation are methylation-specific polymerase chain reaction (MSP), quantitative real-time polymerase chain reaction (PCR) or the similar MethyLight methylation-specific quantitative real-time PCR (MethyLight qMSP), and pyrosequencing (14-20). All the above-mentioned assays are based on the treatment of single-stranded DNA with sodium bisulfite, which results in conversion of unmethylated cytosine residues into uracil, whereas methylated cytosines are left unchanged (21, 22) (Figure 1). This treatment gives rise to different DNA sequences for methylated and unmethylated DNA (Figure 1), sequences which can be used as templates for the detection of unmethylated/methylated cytosine residues. In subsequent PCR amplification and sequencing, the uracil residues of the unmethylated DNA are recognized as thymine, whereas methylated cytosines are amplified as cytosine (21, 22). MSP is a qualitative method yielding a

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Table I. The methylation status of the *O*<sub>6</sub>-methylguanine-DNA methyltransferase (*MGMT*) promoter in meningiomas reported in the literature and in the current study.

Reference	Meningiomas, methylated/studied				Methodology
	Grade I	Grade II	Grade III	Total	
Bello <i>et al.</i> (30)	9/68	7/27	0/3	16/98 (16%)	MSP-1
Liu <i>et al.</i> (31)	1/16	1/19	1/13	3/48 (6%)	MSP-1
de Robles <i>et al.</i> (32)	NA	NA	NA	0/32 (0%)	MSP-1
Brokinkel <i>et al.</i> (33)	NAz	0/11	1/44	1/55 (2%)	MSP-2
Aydemir <i>et al.</i> (34)	1/16	2/17	1/3	4/36 (11%)	MSP-1
Jabini <i>et al.</i> (35)	0/156	0/68	0/6	0/230 (0%)	Quantitative MSP-1
Larijani <i>et al.</i> (36)	2/9	9/25	3/7	14/41 (34%)	MSP-1
Bujko and Kober (37)	0/28	0/9	0/5	0/42 (0%)	Targeted bisulfite sequencing
Current study	1/53	1/7	0/1	2/61 (3%)	Pyrosequencing (Therascreen <i>MGMT</i> Pyro Kit)

NA: Not available; NAz: not analyzed. MSP-1/MSP-2: methylation-specific polymerase chain reaction based on the primers published in Esteller *et al.* (7)/Beier *et al.* (38) (see Figure 1).

yes/no answer, whereas quantitative real-time PCR, MethyLight qMSP, and pyrosequencing provide the frequency of methylation for the examined CpG sites (14-20, 23).

Meningiomas are most often benign, intracranial neoplasms that can be cured by surgery alone (24). However, some of these tumors are more aggressive, such as high-grade meningioma, or may be inoperable because of their location, or may recur even in the absence of histological signs of atypia (25). Histopathological grading of these neoplasms, along with the presence or absence of postoperative residual tumor, is used to estimate the risk of recurrence and, hence, the need for further tumor management (24, 26-28). The decision whether or not to irradiate the neoplastic lesion is of particular interest as radiotherapy carries the risk of side-effects. Therefore, refinement of stratification criteria is warranted (24, 26-28).

In 2004, Chamberlain *et al.* (29) reported a prospective phase II study of temozolomide which was conducted on 16 patients with refractory meningioma. None of the patients showed complete or partial neuroradiographic response. In the same year, using MSP methodology, Bello *et al.* studied the promoter-methylation status of 10 tumor-related genes, among them *MGMT*, in a series of 98 meningiomas. The promoter of *MGMT* was found to be methylated in 16 out of the 98 examined meningiomas (30).

To date, the methylation status of the promoter region of *MGMT* in meningiomas has been examined in eight published studies (30-37). Table I summarizes their results and the methodology used in these studies. In six of them, only few meningiomas (up to 6%) had methylated *MGMT* promoter. However, in two studies, methylated *MGMT* promoter was found in 16% (30) and 34% of meningiomas (36). In six of the published works, MSP methodology was used (30-34, 36). In the seventh study, a methylation-specific

and SYBR-green-based quantitative PCR technique was used (36), whereas in the eighth, targeted bisulfite sequencing was performed to detect *MGMT* promoter methylation (37). For the MSP methodology, the primers described by Esteller *et al.* (7) were used in five of the published works (30-32, 34, 36), whereas the primers described by Beier *et al.* (38) were used in the sixth study (33) (Figure 1).

In the present study, pyrosequencing was used to determine the *MGMT* gene promoter methylation frequencies in 61 meningiomas. Pyrosequencing is regarded as a very robust technique for analysis of *MGMT* promoter methylation and its clinical utility has been validated in several independent studies (15-18, 20, 39, 40). Pyrosequencing provides the frequency of methylated alleles of each CpG site analyzed whereupon the mean of the different sites is used to classify tumors as 'methylated' or 'unmethylated' (40, 41). The Therascreen *MGMT* Pyro Kit (Qiagen, Hilden, Germany) which was used in the present study, has been tested and validated; it has shown a strong analytical performance (40, 41). The kit is used for quantitative measurement of methylation at four CpG sites in exon 1 of the human *MGMT* gene CGACGCCCGCAGGTCCTCG [genomic sequence on chromosome 10 from 131265519-131265537 on Human Feb. 2009 (GRCh37/hg19) assembly, and sequence from 72 to 90 on the *MGMT* mRNA sequence with accession number NM\_002412.4] (Figure 1).

## Materials and Methods

*Patients and samples.* Tumor samples from 61 patients who underwent surgery at the Department of Neurosurgery, Oslo University Hospital between January 2014 and December 2016 were included in this study. Information about the patients' gender and age, diagnosis, and tumor subtype is given in Table II. The study was approved by the Regional Committee for Medical and Health

## Genomic sequence

**A** GTGCCCTCG GCCCGCCCC CGCGCCCGG ATATGCTGGG ACAGCCCGC CCCCTAGAAC  
 GCTTTGCGTC **CGACGCCC** CAGGTCCTCG CGGTGCGCAC CGTTTGCAC TTGgtgagtg  
 tctgggtcgc ctcgctccg gaagagtgcg gagctctccc tctgggacggt ggcagcctcg

## Bisulfite treatment-Unmethylated cytosines

**B** GTGUUUUTUG GUUUUGUUUU UGUGUUUGG ATATGUTGGG AUAGUUUGUG UUUUTAGAAT  
 GTTTTGTGTT TTGAT → TGTG TTTT TAGAAT  
 TTTGTGTT TTGATGTTG TAGGTTTTT T →  
 GUTTTGUGTU UUGAUGUUUG UAGGTUUTUG UGGTUGUAU UGTTTUGAU TTGgtgagtg  
 tutgggtugu utugutuug gaagagtgcg gagututuuu tugggacggt gguaguutug  
 ← aca aaacaaaaac cttctcacac ctcaa  
 ← ac ctcaaaaaaa aaccctacca ccatc

## Bisulfite treatment-Methylated cytosines

**C** GTGUUUUTCG GUUUCGUUUU CGCGUUUCGG ATATGUTGGG AUAGUUCGCG UUUUTAGAAC  
 GTTTTGCGTT TCGAC → G TTTT TAGAAC  
 TT TCGACGTTTCG TAGGTTTTTCG C →  
 GUTTTGCGTU UCGACGUUCG UAGGTUUTCG CGGTGCGUAU CGTTTGCAGU TTGgtgagtg  
 tutgggtcgu utcgutuucg gaagagtgcg gagututuuu tctgggacggt gguaguutcg  
 ← gca aagcaaaagc cttctcacg  
 ← gc ctcaaaaaaa agccctgccac

Figure 1. The region of the *O*<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) promoter examined for methylation in meningiomas. **A**: The genomic sequence for the chromosome band 10q26 at position 131,265,448-131,265,627 [Human Feb. 2009 (GRCh37/hg19) assembly]. The capital letters indicate sequence from exon 1 of *MGMT* gene, whereas lower-case letters indicate intronic sequence. The starting codon ATG is highlighted in blue. Methylated cytosines (C) are given in blue type. The sequence in grey was studied with targeted bisulfite sequencing by Bujko and Kober (37). The target sequence of the Therascreen *MGMT* Pyro Kit is shown in the black box. **B**: The results of sodium bisulfite treatment on single-stranded DNA when all Cs are unmethylated. The treatment results in conversion of unmethylated C into uracil (U in red). **C**: The results of sodium bisulfite treatment on single-stranded DNA with methylated Cs. The treatment results in conversion of unmethylated C into U, whereas methylated Cs are left unchanged. In amplification and sequencing during polymerase chain reaction, U will be recognized as T, whereas the remaining methylated C will be amplified as C. The primers published by Esteller *et al.* (7) are shown in green. The primers described by Beier *et al.* (38) are shown in orange. Arrows indicate primer orientation.

Research Ethics South-East Norway (S-06046) and written informed consent to publication of the case details was obtained from all patients. The Ethics Committee's approval included a review of the consent procedure. All patient information has been de-identified.

**DNA isolation and bisulfite conversion.** Genomic DNA was extracted from tumor samples using the Maxwell 16 Instrument System and the Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA). The concentration and purity of DNA were

measured using a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences, Oslo, Norway).

Unmethylated cytosine residues were converted to uracil by bisulfite treatment of 500 ng DNA using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) and the QiaCube automated purification system (Qiagen) according to the manufacturer's recommendations.

**Pyrosequencing analysis.** The Therascreen *MGMT* Pyro Kit and the PyroMark Q24 system (both from Qiagen) were used to assess the

Table II. Frequency of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) promoter methylation (% units) at four CpG sites in exon 1 in 61 meningiomas. Designation of MGMT promoter methylation positivity was set using a cut-off for all four CpG sites of 10%. MGMT promoter methylation-positive cases are shown in bold.

Sample	Grade	Subtype	Gender	Age, years	CpG1	CpG2	CpG3	CpG4	Mean
1	I	Meningothelial	F	53	0.72	1.04	2.42	2.44	1.66
2	I	Meningothelial	M	65	1.01	1.18	2.08	2.24	1.63
3	I	Meningothelial	F	55	1.07	1.02	2.33	2.85	1.82
4	I	Fibrous	F	78	0.6	0.82	1.8	2.62	1.46
5	I	Fibrous	F	77	0.89	1.08	1.97	2.54	1.62
6	I	Meningothelial	F	50	1.19	1.31	2.6	3	2.02
7	I	Meningothelial	M	61	0.81	1.34	1.81	2.22	1.54
8	I	Meningothelial	F	53	1.72	2.25	4.4	5.2	3.39
9	III (Anaplastic)		F	62	1.03	1.34	2.65	2.08	1.78
10	I	Meningothelial	F	72	1.1	1.08	2.67	2.39	1.81
11	I	Meningothelial	F	61	1.49	1.85	3.38	3.26	2.50
12	I	Fibrous	F	67	3.99	4.81	6.16	6.63	5.40
13	II (Atypical)		F	56	1.29	1.74	2.66	3.21	2.22
14	I	Microcystic	M	60	1.58	2.15	2.47	2.87	2.27
15	I	Transitional	F	40	1.91	2.08	3.38	3.89	2.82
16	I	Transitional	F	69	5.65	5.01	7.04	7.38	6.27
17	I	Meningothelial	M	73	2.81	2.85	3.54	4.68	3.47
<b>18</b>	<b>II (Atypical)</b>		<b>M</b>	<b>63</b>	<b>34.08</b>	<b>30.85</b>	<b>31.87</b>	<b>35.35</b>	<b>33.04</b>
19	I	Fibrous	F	48	1.43	1.89	3.9	4.47	2.92
20	I	Meningothelial	F	69	3.95	4.18	9.71	10.12	6.99
21	I	Meningothelial	F	87	5.07	5.4	6.31	4.69	5.37
22	II (Atypical)		M	72	2.38	1.89	2.39	4.74	2.85
23	I	Meningothelial	F	80	1.39	1.21	2.5	3.74	2.21
24	I	Meningothelial	F	76	1.73	1.83	3.68	4.83	3.02
25	II (Atypical)		M	68	1.28	2.29	3.26	4.79	2.90
26	I	Meningothelial	F	64	1.33	1.73	3.97	4.72	2.94
27	I	Transitional	F	52	1.73	2.05	4.73	5.44	3.49
28	I	Meningothelial	F	71	1.34	1.81	4.38	10.84	4.59
29	I	Psammomatous	F	76	1.4	1.69	3.74	3.03	2.46
30	I	Meningothelial	M	52	1.49	1.72	3.54	4.09	2.71
31	I	Fibrous	F	58	0.77	1.51	4.09	3.43	2.45
32	I	Meningothelial	F	71	1.24	1.73	3.67	4.13	2.69
33	I	Meningothelial	M	70	1.42	1.55	3.95	4.39	2.83
34	I	Meningothelial	F	55	1.2	0.89	3.69	3.51	2.32
35	I	Angiomatous	M	70	1.1	1.05	2.33	2.69	1.79
36	I	Meningothelial	F	75	1.66	1.4	3.33	3.08	2.37
<b>37</b>	<b>I</b>	<b>Meningothelial</b>	<b>F</b>	<b>48</b>	<b>43.08</b>	<b>13.29</b>	<b>5.99</b>	<b>8.54</b>	<b>17.72</b>
38	I	Transitional	F	74	2.3	1.23	2.84	2.42	2.20
39	I	Meningothelial	M	47	1.72	1.72	3.24	4.61	2.82
40	I	Transitional	F	72	0.77	1.36	2.54	1.6	1.57
41	I	Meningothelial	F	40	1.46	1.66	4.43	4.52	3.02
42	I	Meningothelial	F	63	1.37	1.72	3.17	4.17	2.61
43	I	Meningothelial	M	63	1.63	1.08	4.59	4.4	2.92
44	II (Atypical)		M	69	1.29	1.1	2.71	3.27	2.09
45	I	Angiomatous	F	67	2.94	3.58	5.03	6.91	4.62
46	I	Transitional	F	59	1.36	1.99	3.77	4.04	2.79
47	I	Meningothelial	F	65	1.41	1.25	3.66	3.94	2.56
48	I	Meningothelial	F	76	1.9	2.2	4.8	5.2	3.52
49	I	Secretory	F	54	2.02	1.85	3.48	4.77	3.03
50	I	Fibrous	F	46	1.76	3.17	6.01	4.45	3.85
51	I	Meningothelial	F	73	1.33	1.4	3.83	3.84	2.60
52	I	Metaplastic	F	52	2.66	2.44	2.97	5.38	3.36
53	II (Atypical)		F	72	1.05	2.3	5.12	4.45	3.23
54	I	Meningothelial	M	63	1.48	1.46	3.89	3.87	2.68
55	I	Psammomatous	F	62	1.43	2.17	2.57	3.63	2.45
56	I	Fibrous	F	54	0.97	2.06	3.15	3.56	2.44
57	I	Meningothelial	M	71	1.79	1.87	1.57	3.75	2.24
58	I	Secretory	F	67	0.32	0.61	0.6	2.66	1.05
59	I	Meningothelial	F	60	1.59	2.02	4.3	4.12	3.01
60	I	Meningothelial	F	79	1.25	1.78	2.81	3.92	2.44
61	II (Atypical)		F	40	1.17	1.12	3.35	3.17	2.20

methylation status of the *MGMT* gene promoter. In brief, bisulfite-converted genomic DNA was amplified by PCR, the amplicons were immobilized on streptavidin beads, and single-stranded DNA was prepared, sequenced, and finally analyzed on the PyroMark Q24 system. Detailed information about the procedure can be found in the following links: <https://www.qiagen.com/no/resources/resourcedetail?id=29031fd2-6d22-4152-b544-288665bc5abc&lang=en>, <https://www.qiagen.com/no/resources/resourcedetail?id=59f0275d-e60f-4517-b786-b0e0ca13952e&lang=en>, <https://www.qiagen.com/no/resources/resourcedetail?id=a06f1196-2bd0-40af-87d5-45c80c285b48&lang=en>. According to the company's information, the limit of blank values represent methylation frequencies obtained from healthy blood donor samples with a probability of 95%: 1.5, 1.8, 3.2, and 3.4 for CpG sites 1, 2, 3, and 4, respectively (mean for CpG sites 1 to 4=2.5). In our assays, the cut-off frequency for accepting methylation as positive for all four CpG sites was set to 10%.

## Results

The methylation frequencies of the four analyzed CpG sites in exon 1 of *MGMT* in the 61 meningiomas are presented in Table II. In only two tumors was the mean methylation frequency of the four CpG sites higher than 10%. In case 18, which was an atypical meningioma, the mean methylation frequency was 33% and it was higher than 30% for all four CpG sites. In case 37, which was a grade I meningioma, the mean methylation frequency was 17%, but with marked differences among the four CpG sites: CpG site 1 had the highest methylation frequency (43%), followed by site 2 (13%), site 4 (8.5%), and site 3 (6.0%). The other meningiomas with histomorphological signs of aggressiveness, including six atypical and one anaplastic meningioma, had a methylation frequency of below 10.0%, similarly to the other 52 grade I meningiomas.

## Discussion

Our results suggest that the methylation frequency of the *MGMT* gene promoter in general is low in meningiomas, with 59 tumors (97%) having a mean methylation frequency at the four examined CpG sites of below 7%. These data are in line with previous studies that described no *MGMT* promoter methylation or a low frequency using MSP or targeted bisulfite sequencing methodologies (31-33, 35, 37).

The two cases with methylated *MGMT* promoter, *i.e.*, for which the mean methylation frequency of the four CpG sites was higher than 10%, had different methylation patterns for the four CpG sites. In the atypical meningioma (case 18, grade II tumor), the methylation frequency was higher than 30% for all four CpG sites. In the grade I meningioma (case 37), the methylation frequencies among the four CpG sites showed marked differences: CpG site 1 had the highest methylation frequency (43%), followed by site 2 (13%), site 4 (8.5%), and site 3 (6.0%). Bujko and Kober (37), using targeted bisulfite sequencing, reported a high methylation

frequency (of over 75%) for single CpGs within the *MGMT* promoter region in five tumor samples. However, the average methylation level for the entire region was very low in those samples.

Bello *et al.* (30), Aydemir *et al.* (34), and Larijani *et al.* (36) found that the *MGMT* promoter was methylated in 16%, 11%, and 34% of the examined meningiomas, respectively. In these three studies, the same principal methodology was used, namely MSP with primers published by Esteller *et al.* (7). In contrast, and using the same method as above, Liu *et al.* (31) and de Robles *et al.* (32) showed that the promoter of *MGMT* was methylated in 6% and 0% of their examined meningiomas. Jabini *et al.* (35) used quantitative MSP, but again with the primers used by Esteller *et al.* (7), finding that none of 230 examined meningiomas had methylated *MGMT* promoter. The reason behind the reported differences in the frequency of methylated meningiomas, measured using the same MSP methodology, is unknown. MSP does produce false-positive as well as false-negative results under some circumstances, especially when performed on DNA of low quality or quantity, including DNA extracted from formalin-fixed and paraffin-embedded tissue (15, 42-44). In addition, mosaic methylation patterns and incomplete bisulfite conversion may lead to mispriming and lower sensitivity and specificity (15, 42-47).

Based on the results of our study and taking into consideration the already published data, amounting to 643 meningiomas altogether, we conclude that the methylation frequency of the *MGMT* promoter in meningioma is low (6%). Consequently, there is no convincing rationale for testing such tumors for their *MGMT* methylation status in a clinical setting.

## Conflicts of Interest

The Authors declare that they have no conflict of interests in regard to this study.

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## References

- 1 Dullea A and Marignol L: *MGMT* testing allows for personalised therapy in the temozolomide era. *Tumour Biol* 37: 87-96, 2016.
- 2 Natarajan AT, Vermeulen S, Darroudi F, Valentine MB, Brent TP, Mitra S and Tano K: Chromosomal localization of human *O*<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) gene by *in situ* hybridization. *Mutagenesis* 7: 83-85, 1992.
- 3 Harris LC, Potter PM, Tano K, Shiota S, Mitra S and Brent TP: Characterization of the promoter region of the human *O*<sup>6</sup>-methylguanine-DNA methyltransferase gene. *Nucleic Acids Res* 19: 6163-6167, 1991.

- 4 von Wronski MA, Harris LC, Tano K, Mitra S, Bigner DD and Brent TP: Cytosine methylation and suppression of *O*<sup>6</sup>-methylguanine-DNA methyltransferase expression in human rhabdomyosarcoma cell lines and xenografts. *Oncol Res* 4: 167-174, 1992.
- 5 Qian XC and Brent TP: Methylation hot spots in the 5' flanking region denote silencing of the *O*<sup>6</sup>-methylguanine-DNA methyltransferase gene. *Cancer Res* 57: 3672-3677, 1997.
- 6 Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS and Futscher BW: Methylation of discrete regions of the *O*<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) CpG island is associated with heterochromatinization of the *MGMT* transcription start site and silencing of the gene. *Mol Cell Biol* 17: 5612-5619, 1997.
- 7 Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG: Inactivation of the DNA repair gene *O*<sup>6</sup>-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 59: 793-797, 1999.
- 8 Bhakat KK and Mitra S: CpG methylation-dependent repression of the human *O*<sup>6</sup>-methylguanine-DNA methyltransferase gene linked to chromatin structure alteration. *Carcinogenesis* 24: 1337-1345, 2003.
- 9 Nakagawachi T, Soejima H, Urano T, Zhao W, Higashimoto K, Satoh Y, Matsukura S, Kudo S, Kitajima Y, Harada H, Furukawa K, Matsuzaki H, Emi M, Nakabeppu Y, Miyazaki K, Sekiguchi M and Mukai T: Silencing effect of CpG island hypermethylation and histone modifications on *O*<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) gene expression in human cancer. *Oncogene* 22: 8835-8844, 2003.
- 10 Zhang K, Wang XQ, Zhou B and Zhang L: The prognostic value of *MGMT* promoter methylation in glioblastoma multiforme: A meta-analysis. *Fam Cancer* 12: 449-458, 2013.
- 11 Zhao H, Wang S, Song C, Zha Y and Li L: The prognostic value of *MGMT* promoter status by pyrosequencing assay for glioblastoma patients' survival: a meta-analysis. *World J Surg Oncol* 14: 261, 2016.
- 12 Binabaj MM, Bahrami A, ShahidSales S, Joodi M, Mashhad MJ, Hassanian SM, Anvari K and Avan A: The prognostic value of *MGMT* promoter methylation in glioblastoma: A meta-analysis of clinical trials. *J Cell Physiol* 233: 378-386, 2018.
- 13 Everhard S, Tost J, El Abdalaoui H, Criniere E, Busato F, Marie Y, Gut IG, Sanson M, Mokhtari K, Laigle-Donadey F, Hoang-Xuan K, Delattre JY and Thillet J: Identification of regions correlating *MGMT* promoter methylation and gene expression in glioblastomas. *Neuro Oncol* 11: 348-356, 2009.
- 14 Cankovic M, Nikiforova MN, Snuderl M, Adesina AM, Lindeman N, Wen PY and Lee EQ: The role of *MGMT* testing in clinical practice: a report of the association for molecular pathology. *J Mol Diagn* 15: 539-555, 2013.
- 15 Christians A, Hartmann C, Benner A, Meyer J, von Deimling A, Weller M, Wick W and Weiler M: Prognostic value of three different methods of *MGMT* promoter methylation analysis in a prospective trial on newly diagnosed glioblastoma. *PLoS One* 7: e33449, 2012.
- 16 Håvik AB, Brandal P, Honne H, Dahlback HS, Scheie D, Hektoen M, Meling TR, Helseth E, Heim S, Lothe RA and Lind GE: *MGMT* promoter methylation in gliomas-assessment by pyrosequencing and quantitative methylation-specific PCR. *J Transl Med* 10: 36, 2012.
- 17 Quillien V, Lavenu A, Karayan-Tapon L, Carpentier C, Labussiere M, Lesimple T, Chinot O, Wager M, Honnorat J, Saikali S, Fina F, Sanson M and Figarella-Branger D: Comparative assessment of 5 methods (methylation-specific polymerase chain reaction, MethyLight, pyrosequencing, methylation-sensitive high-resolution melting, and immunohistochemistry) to analyze *O*<sup>6</sup>-methylguanine-DNA-methyltransferase in a series of 100 glioblastoma patients. *Cancer* 118: 4201-4211, 2012.
- 18 Quillien V, Lavenu A, Sanson M, Legrain M, Dubus P, Karayan-Tapon L, Mosser J, Ichimura K and Figarella-Branger D: Outcome-based determination of optimal pyrosequencing assay for *MGMT* methylation detection in glioblastoma patients. *J Neurooncol* 116: 487-496, 2014.
- 19 Lattanzio L, Borgognone M, Mocellini C, Giordano F, Favata E, Fasano G, Vivenza D, Monteverde M, Tonissi F, Ghiglia A, Fillini C, Bernucci C, Merlano M and Lo Nigro C: *MGMT* promoter methylation and glioblastoma: A comparison of analytical methods and of tumor specimens. *Int J Biol Markers* 30: e208-216, 2015.
- 20 Quillien V, Lavenu A, Ducray F, Joly MO, Chinot O, Fina F, Sanson M, Carpentier C, Karayan-Tapon L, Rivet P, Entz-Werle N, Legrain M, Zalcman EL, Levallet G, Escande F, Ramirez C, Chiforeanu D, Vauleon E and Figarella-Branger D: Validation of the high-performance of pyrosequencing for clinical *MGMT* testing on a cohort of glioblastoma patients from a prospective dedicated multicentric trial. *Oncotarget* 7: 61916-61929, 2016.
- 21 Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL and Paul CL: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 89: 1827-1831, 1992.
- 22 Clark SJ, Harrison J, Paul CL and Frommer M: High-sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 22: 2990-2997, 1994.
- 23 Karayan-Tapon L, Quillien V, Guilhot J, Wager M, Fromont G, Saikali S, Etcheverry A, Hamlat A, Loussouarn D, Campion L, Campone M, Vallette FM and Gratas-Rabbia-Re C: Prognostic value of *O*<sup>6</sup>-methylguanine-DNA methyltransferase status in glioblastoma patients, assessed by five different methods. *J Neurooncol* 97: 311-322, 2010.
- 24 Rogers L, Barani I, Chamberlain M, Kaley TJ, McDermott M, Raizer J, Schiff D, Weber DC, Wen PY and Vogelbaum MA: Meningiomas: Knowledge base, treatment outcomes, and uncertainties. A RANO review. *J Neurosurg* 122: 4-23, 2015.
- 25 Perry A, Louis DN, Budka H, von Deimling A, Sahm F, Rushing EJ, Mawrin C, Claus EB, Loeffler J and Sadetzki S: Meningioma. *In: WHO Classification of Tumours of the Central Nervous System*. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Ellison DW, Figarella-Branger D, Perry A, Reifenberger G and Von Deimling A (eds.). Lyon: International Agency for Research on Cancer (IARC), pp. 232-245, 2016.
- 26 Buttrick S, Shah AH, Komotar RJ and Ivan ME: Management of atypical and anaplastic meningiomas. *Neurosurg Clin N Am* 27: 239-247, 2016.
- 27 Messerer M, Richoz B, Cossu G, Dhermain F, Hottinger AF, Parker F, Levivier M and Daniel RT: Recent advances in the management of atypical meningiomas. *Neurochirurgie* 62: 213-222, 2016.
- 28 Paldor I, Awad M, Sufaro YZ, Kaye AH and Shoshan Y: Review of controversies in management of non-benign meningioma. *J Clin Neurosci* 31: 37-46, 2016.

- 29 Chamberlain MC, Tsao-Wei DD and Groshen S: Temozolomide for treatment-resistant recurrent meningioma. *Neurology* 62: 1210-1212, 2004.
- 30 Bello MJ, Aminoso C, Lopez-Marin I, Arjona D, Gonzalez-Gomez P, Alonso ME, Lomas J, de Campos JM, Kusak ME, Vaquero J, Isla A, Gutierrez M, Sarasa JL and Rey JA: DNA methylation of multiple promoter-associated CpG islands in meningiomas: Relationship with the allelic status at 1p and 22q. *Acta Neuropathol* 108: 413-421, 2004.
- 31 Liu Y, Pang JC, Dong S, Mao B, Poon WS and Ng HK: Aberrant CpG island hypermethylation profile is associated with atypical and anaplastic meningiomas. *Hum Pathol* 36: 416-425, 2005.
- 32 de Robles P, McIntyre J, Kalra S, Roldan G, Cairncross G, Forsyth P, Magliocco T, Hamilton M and Easaw J: Methylation status of *MGMT* gene promoter in meningiomas. *Cancer Genet Cytogenet* 187: 25-27, 2008.
- 33 Brokinkel B, Fischer BR, Peetz-Dienhart S, Ebel H, Sepehrnia A, Rama B, Albert FK, Stummer W, Paulus W and Hasselblatt M: *MGMT* promoter methylation status in anaplastic meningiomas. *J Neurooncol* 100: 489-490, 2010.
- 34 Aydemir F, Yurtcu E, Balci TB, Sahin FI, Gulsen S and Altinors N: Identification of promoter region methylation patterns of *MGMT*, *CDKN2A*, *GSTP1*, and *THBS1* genes in intracranial meningioma patients. *Genet Test Mol Biomarkers* 16: 335-340, 2012.
- 35 Jabini R, Moradi A, Afsharnezhad S, Ayatollahi H, Behravan J, Raziee HR and Mosaffa F: Pathodiagnostic parameters and evaluation of O(6)-methyl guanine methyl transferase gene promoter methylation in meningiomas. *Gene* 538: 348-353, 2014.
- 36 Larijani L, Madjd Z, Samadikuchaksaraei A, Younespour S, Zham H, Rakhshan A, Mohammadi F, Rahbari A and Moradi A: Methylation of O<sup>6</sup>-methyl guanine methyltransferase gene promoter in meningiomas—comparison between tumor grades I, II, and III. *Asian Pac J Cancer Prev* 15: 33-38, 2014.
- 37 Bujko M and Kober P: Targeted bisulfite sequencing-based analysis of *MGMT* promoter methylation in meningiomas. *Asian Pac J Cancer Prev* 17: 2727-2728, 2016.
- 38 Beier D, Rohrl S, Pillai DR, Schwarz S, Kunz-Schughart LA, Leukel P, Proescholdt M, Brawanski A, Bogdahn U, Trampe-Kieslich A, Giebel B, Wischhusen J, Reifenberger G, Hau P and Beier CP: Temozolomide preferentially depletes cancer stem cells in glioblastoma. *Cancer Res* 68: 5706-5715, 2008.
- 39 Bienkowski M, Berghoff AS, Marosi C, Wohrer A, Heinzl H, Hainfellner JA and Preusser M: Clinical Neuropathology practice guide 5-2015: *MGMT* methylation pyrosequencing in glioblastoma: Unresolved issues and open questions. *Clin Neuropathol* 34: 250-257, 2015.
- 40 Preusser M, Berghoff AS, Manzl C, Filipits M, Weinhausel A, Pulverer W, Dieckmann K, Widhalm G, Wohrer A, Knosp E, Marosi C and Hainfellner JA: Clinical Neuropathology practice news 1-2014: Pyrosequencing meets clinical and analytical performance criteria for routine testing of *MGMT* promoter methylation status in glioblastoma. *Clin Neuropathol* 33: 6-14, 2014.
- 41 Quillien V, Lavenu A, Ducray F, Meyronet D, Chinot O, Fina F, Sanson M, Carpentier C, Karayan-Tapon L, Rivet P, Entz-Werle N, Legrain M, Zalcman EL, Levallet G, Escande F, Ramirez C, Chiforeanu D, Vauleon E and Figarella-Branger D: Clinical validation of the CE-IVD marked Therascreen *MGMT* kit in a cohort of glioblastoma patients. *Cancer Biomark* 20: 435-441, 2017.
- 42 Mikeska T, Bock C, El-Maarri O, Hubner A, Ehrentraut D, Schramm J, Felsberg J, Kahl P, Buttner R, Pietsch T and Waha A: Optimization of quantitative *MGMT* promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J Mol Diagn* 9: 368-381, 2007.
- 43 Preusser M, Elezi L and Hainfellner JA: Reliability and reproducibility of PCR-based testing of O<sup>6</sup>-methylguanine-DNA methyltransferase gene (*MGMT*) promoter methylation status in formalin-fixed and paraffin-embedded neurosurgical biopsy specimens. *Clin Neuropathol* 27: 388-390, 2008.
- 44 Shaw RJ, Akufo-Tetteh EK, Risk JM, Field JK and Liloglou T: Methylation enrichment pyrosequencing: combining the specificity of MSP with validation by pyrosequencing. *Nucleic Acids Res* 34: e78, 2006.
- 45 Warnecke PM, Stirzaker C, Song J, Grunau C, Melki JR and Clark SJ: Identification and resolution of artifacts in bisulfite sequencing. *Methods* 27: 101-107, 2002.
- 46 Genereux DP, Johnson WC, Burden AF, Stoger R and Laird CD: Errors in the bisulfite conversion of DNA: Modulating inappropriate-and failed-conversion frequencies. *Nucleic Acids Res* 36: e150, 2008.
- 47 Piperi C, Farmaki E, Vlastos F, Papavassiliou AG and Martinet N: DNA methylation signature analysis: How easy is it to perform? *J Biomol Tech* 19: 281-284, 2008.

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