




# A novel analytical model of *MGMT* methylation pyrosequencing offers improved predictive performance in patients with gliomas

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## Abstract

The methylation status of the promoter of *MGMT* gene is a crucial factor influencing clinical decision-making in patients with gliomas. *MGMT* pyrosequencing results are often dichotomized by a cut-off value based on an average of several tested CpGs. However, this method frequently results in a “gray zone”, representing a dilemma for physicians. We therefore propose a novel analytical model for *MGMT* methylation pyrosequencing. *MGMT* CpG heterogeneity was investigated in 213 glioma patients in two tested cohorts: cohort A in which CpGs 75–82 were tested and cohort B in which CpGs 72–78 were tested. The predictive performances of the novel and traditional averaging models were compared in 135 patients who received temozolomide using receiver operating characteristic curves and Kaplan–Meier curves, and in patients stratified according to isocitrate dehydrogenase gene mutation status. The results were validated in an independent cohort of 65 consecutive patients with high-grade gliomas from the Chinese Glioma Genome Atlas database. Heterogeneity of *MGMT* promoter CpG methylation level was observed in most gliomas. The optimal cut-off value for each individual CpG varied from 4–16%. The current analysis defined *MGMT* promoter methylation as occurring when at least three CpGs exceeded their respective cut-off values. This novel analysis could accurately predict the prognosis of patients in the methylation “gray zone” according to the standard averaging method, and improved the area under the curves from 0.67, 0.76, and 0.67 to 0.70, 0.84, and 0.72 in cohorts A, B, and the validation cohort, respectively, demonstrating superiority of this analytical method in all three cohorts. Furthermore, the advantages of the novel analysis were retained regardless of WHO grade and isocitrate dehydrogenase gene mutation status. In conclusion, this novel analytical model offers an improved clinical predictive performance for *MGMT* pyrosequencing results and is suitable for clinical use in patients with gliomas.

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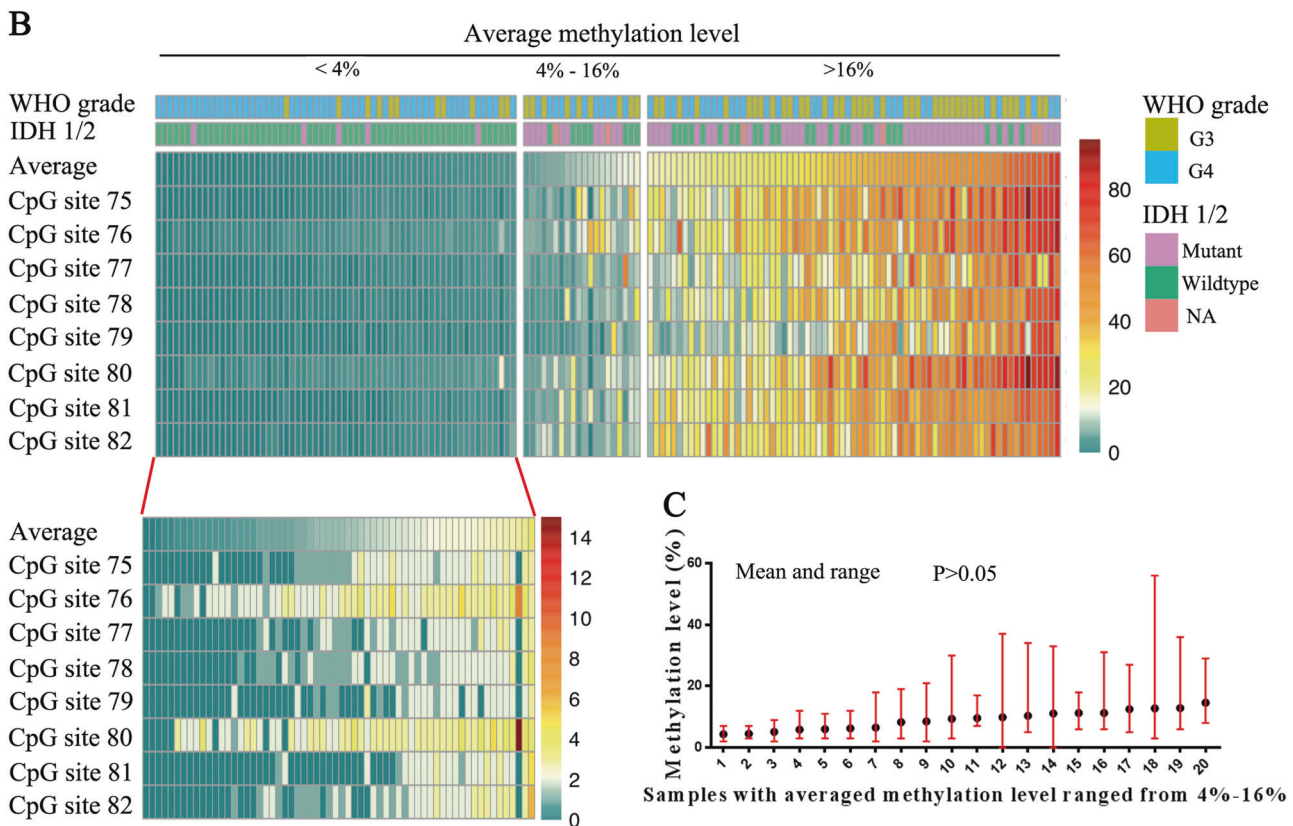
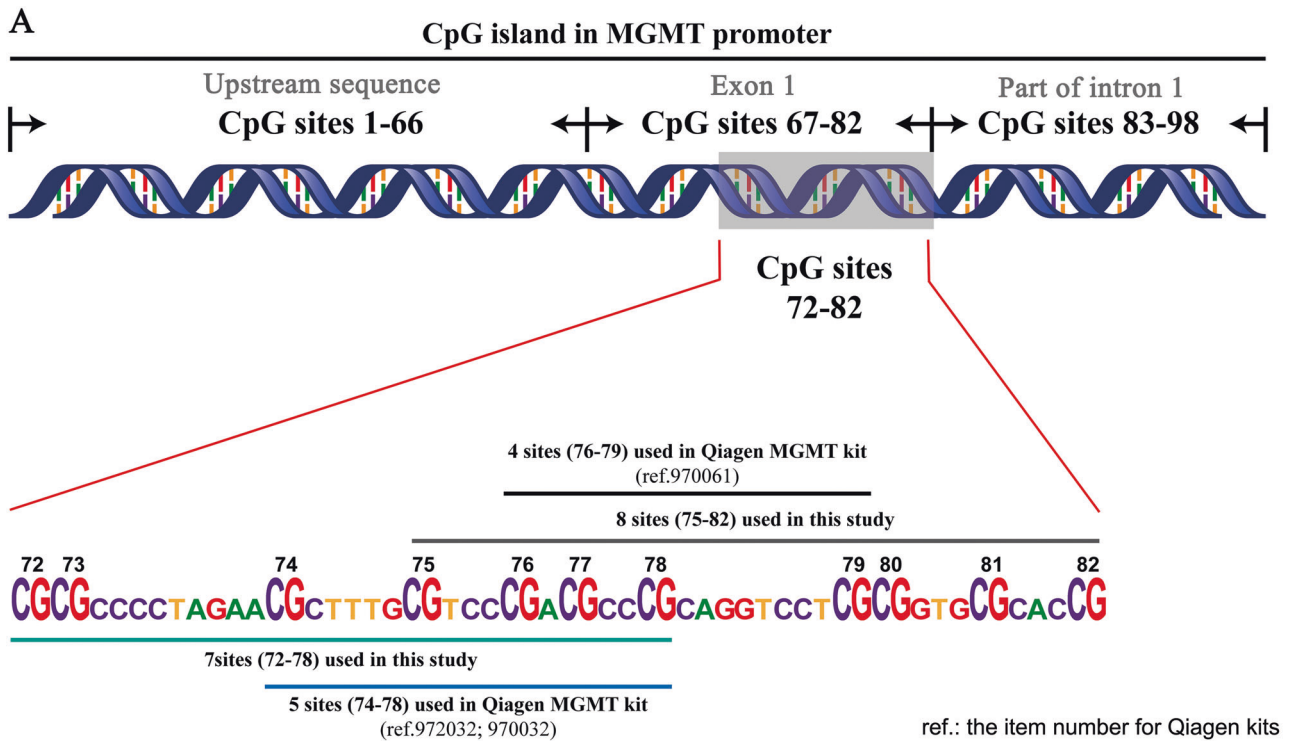
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## Introduction

O<sub>6</sub>-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation is a useful predictive biomarker in relation to temozolomide chemotherapy, as demonstrated in a series of studies and clinical trials [1–5]. *MGMT* methylation testing has been established as a routine molecular pathological technique for patients with glioma. Pyrosequencing is a robust technique for quantifying the *MGMT* CpG methylation level compared with methylation-specific PCR, which has been used in most clinical trials [2, 6–10]. Pyrosequencing has been regarded as the “gold standard” for *MGMT* promoter methylation testing [9, 11–17], and is recommended by clinical glioma guidelines [18].

The *MGMT* promoter contains 98 individual CpGs surrounding the transcription start site (Fig. 1). The methylation statuses of CpGs 72–90, localized in exon 1 and subsequent intron 1, have been shown to have a major



impact on *MGMT* expression and therefore on predicting the response to temozolomide treatment [11, 19, 20]. CpGs 72–82, which are localized in exon 1, are therefore the most

commonly selected CpGs for pyrosequencing testing [11, 16], while CpGs 74–78 and 76–79 have also been used in commercial kits (Fig. 1).

◀ **Fig. 1** CpG islands and heterogeneity of methylation statuses for each individual CpG site in the *MGMT* promoter region. **a** Map of the distribution of CpGs in the *MGMT* 5' CpG island. CpGs 72–82, which are most commonly used in pyrosequencing, are shown in the lower panel. The eight and seven CpGs used in this study are indicated by gray and light blue lines, respectively. CpGs 74–78 and 76–79 are indicated by black and dark blue lines, respectively. **b** Methylation levels of each CpG were depicted in a heatmap for cohort A. Average refers to the average methylation level of CpGs 75–82. A part of the heatmap (with average methylation level < 4%) was displayed again in another heatmap (in the lower left corner) with a more discriminative color bar. **c** Methylation levels in patients with average methylation levels of 4–16% were compared. The mean and range were indicated by the black point and red line, respectively

The arithmetic average method, which simply averages the percentage methylation of several consecutive CpGs using a threshold value, has been widely used to interpret pyrosequencing testing results [11, 12, 15–17]. However, this method neglects the potentially large deviations from the average methylation level for individual CpGs, thus reducing the apparent heterogeneity in CpG methylation levels. In our practice, 10–20% of patients had methylation levels within  $\pm 5\%$  of the threshold value. Several investigators have noted the existence of a possible “gray zone” resulting from the averaging method, leading to an ambiguous predictive performance [15, 21–23]. The performance of this arithmetic average approach is thus inadequate in clinical practice, especially in the case of methylation levels around the set threshold value. There is therefore a need for a more comprehensive analytical approach to interpreting pyrosequencing results.

In the present study, we determined the heterogeneity of CpG methylation levels in exon 1 of the *MGMT* gene in two independent cohorts including 213 patients with WHO grade III/IV gliomas. In view of the results, we applied a novel analysis that considered a separate cut-off value for each individual CpG and confirmed that this approach provided a more useful interpretation of pyrosequencing results for *MGMT* promoter methylation in gliomas. The superiority of the novel analysis was also validated in an independent cohort of 65 glioma patients.

## Materials and methods

### Patients and samples

A total of 213 patients with WHO grade III/IV gliomas were enrolled from the Chinese Glioma Genome Atlas. *MGMT* pyrosequencing data for these patients was based on tests of two batches of freshly frozen samples: cohort A (CpGs 75–82;  $n = 153$  patients) and cohort B (CpGs 72–78;  $n = 60$  patients). The clinical information for these 213 patients is summarized in Supplementary Table S1. The

heterogeneity of different individual *MGMT* CpG methylation levels was analyzed for all 213 patients.

To assess the predictive value of the *MGMT* promoter methylation status on the efficacy of the temozolomide chemotherapy, in total 200 patients who underwent temozolomide-based chemotherapy were included into the survival analysis. Among the 200 patients (Table 1), 135 were from the previous 213 patients, and the other 65 were from an additional independent cohort of 65 patients with WHO grade III/IV gliomas of which formalin-fixed paraffin-embedded samples were available.

The chemotherapy protocols for the patients were following the suggestion from the National Comprehensive Cancer Network Clinical Practice Guidelines and the Chinese Glioma Cooperative Group Clinical Practice Guidelines for the management of adult diffuse gliomas [18]. In detail, most of the patients (77.0%) received temozolomide treatment following the standard Stupp protocol, during the radiotherapy for 6 weeks, 75 mg temozolomide per square meter of body-surface area per day, 7 days per week; and during the 6 temozolomide treatment cycles, 150–200 mg temozolomide per square meter of body-surface area per day, 5 days during each 28-day cycle. Some of the patients (18%) received temozolomide between 3 and 5 cycles, while some of the patients (4%) received temozolomide more than 6 cycles. A few of patients (2%) received only temozolomide cycles without radiotherapy. The detailed treatment information of patients with gliomas in different subgroups were summarized (Table 2), and the information in each cohort was also listed (Supplementary Table S2).

All specimens were re-evaluated by two independent pathologists and samples with >80% tumor cells were used to determine *MGMT* promoter methylation. The 1p/19q status of these specimens were determined using dual-color fluorescence in situ hybridization, and the *IDH1* R132H and *IDH2* R172K/M mutations were determined by the DNA pyrosequencing. The characteristics of the test and validated cohorts are summarized in Table 1.

### DNA isolation and bisulfite modification

Genomic DNA was extracted from freshly frozen tumor tissues and formalin-fixed paraffin-embedded samples using a QIAamp DNA Mini Kit (Qiagen, Germany) and QIAamp DNA FFPE Tissue Kit (Qiagen), respectively. The DNA concentration and quantity were tested using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA), and 100 ng DNA was then used for bisulfite conversion using an Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol.

**Table 1** Clinicopathological characteristics for patients who received temozolomide

		Frozen tissue			FFPE samples
		Total (200)	The cohort A (86)	The cohort B (49)	The validation cohort (65)
Age (years)	Median (range)	44 (17–79)	43 (19–72)	46 (24–79)	45 (17–69)
	Age ≥ 45	100 (50%)	41 (48%)	26 (53%)	33 (51%)
	Age < 45	100 (50%)	45 (52%)	23 (47%)	32 (49%)
Gender	Male	126 (63%)	54 (63%)	33 (67%)	39 (60%)
	Female	74 (37%)	32 (37%)	16 (33%)	26 (40%)
KPS score	KPS ≥ 80	103 (52%)	44 (51%)	26 (53%)	33 (51%)
	KPS < 80	87 (44%)	37 (43%)	21 (43%)	29 (45%)
	N/A	10 (5%)	5 (6%)	2 (4%)	3 (5%)
WHO grade	III	81 (41%)	35 (41%)	24 (49%)	22 (34%)
	IV	119 (60%)	51 (59%)	25 (51%)	43 (66%)
WHO 2016 classification	Anaplastic Oligo, IDH-mutant, 1p/19q codeletion	8 (4%)	3 (3%)	3 (6%)	2 (3%)
	Anaplastic Astro, IDH-mutant	34 (17%)	21 (24%)	8 (16%)	5 (8%)
	Anaplastic Astro, IDH-wildtype	30 (15%)	11 (13%)	13 (27%)	6 (9%)
	Glioblastoma, IDH-mutant	28 (14%)	11 (13%)	6 (12%)	11 (17%)
	Glioblastoma, IDH-wildtype	84 (42%)	36 (42%)	18 (37%)	30 (46%)
	Anaplastic Astro, NOS <sup>a</sup>	9 (5%)	2 (2%)	0 (0%)	7 (0%)
	Glioblastoma, NOS <sup>a</sup>	7 (4%)	2 (2%)	1 (2%)	4 (17%)
1p19q codeletion	Codel	8 (4%)	3 (3%)	3 (6%)	2 (3%)
	Non-codel	192 (96%)	83 (97%)	46 (94%)	63 (97%)
IDH1/2 mutation	Mutation	70 (35%)	35 (41%)	17 (35%)	18 (28%)
	Wildtype	114 (57%)	47 (55%)	31 (63%)	36 (55%)
	N/A	16 (8%)	4 (5%)	1 (2%)	11 (17%)

*Oligo* oligodendroglioma, *Astro* astrocytoma

<sup>a</sup>NOS refers to the *IDH* status is lacking. And the 1p/19q of these gliomas are non-codeletion in this study

## Pyrosequencing of *MGMT* promoter

The *MGMT* pyrosequencing template was prepared as described previously [12, 24]. Briefly, bisulfite-treated DNA was amplified using the forward primer 5'-GTT TYG GAT ATG TTG GGA TAG TT-3' and biotinylated reverse primer 5'-biotin-ACR ACC CAA ACA CTC ACC AA-3'. Two independent assays were performed in different samples with two pyrosequencing sequencing primers: 5'-GAT ATG TTG GGA TAG T-3' (for CpGs 72–78) and 5'-GTT TTT AGA AYG TTT TG-3' (for CpGs 75–82). The analyzed sequences for CpGs 72–78 and 75–82 were TYG YGT TTT TAG AAY GTT TTG YGT TTY GAY GTT YGT AGG T and YGT TTT GYG TTT YGA YGT TYG TAG GTT TTY GYG GTG YGT A, respectively. Pyrosequencing was performed using a PyroMarker Q96 instrument and the results were analyzed with PyroMarker Q96 software (Qiagen). The formalin-fixed paraffin-embedded samples from the 65 patients in the validation

cohort were tested using a PyroMarker Q24 instrument with pyrosequencing primers: 5'-GAT ATG TTG GGA TAG TT-3' (for CpGs 72–78), and the analyzed sequence was YGY GTT TTT AGA AYG TTT TGY GTT TYG AYG TTY GT.

## Methylation-specific PCR

Bisulfite-treated DNA was amplified with SYBR<sup>®</sup> Select Master Mix (Thermo Fisher, Waltham, MA, USA). The sequences of the methylated primers were 5'-TTT CGACGTTTCGTAGGTTTTTCGC-3' (forward primer) and 5'-GCACTCTTCCGAAAACGAAACG-3' (reverse primer), and the sequences of the unmethylated primers were 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (forward primer) and 5'-AACTCCACACTCTTCCAAAACA AAACA-3' (reverse primer). The annealing temperature was 59°C. The results were analyzed with ABI 7500 software (Thermo Fisher). Unmethylated DNA and commercial

**Table 2** The detailed information on the therapeutic protocol used in patients

	Total	Anaplastic Oligo, IDH-mutant, 1p/19q codeletion	Anaplastic Astro, IDH-mutant	Anaplastic Astro, IDH-wildtype	Glioblastoma, IDH-mutant	Glioblastoma, IDH-wildtype	Anaplastic Astro, NOS <sup>a</sup>	Glioblastoma, NOS <sup>a</sup>
TMZ <sup>b</sup> cycles	200	8 (4%)	34 (17%)	30 (15%)	28 (14%)	84 (42%)	9 (5%)	7 (4%)
RT + TMZ <sup>c</sup> →TMZ cycles	36 (18%)	1	6	4	7	14	3	1
	154 (77%)	6	25	25	21	66	5	6
Only TMZ	7–12	–	3	–	–	4	–	–
	3–5	–	–	1	–	–	1	–
	6	1 (1%)	–	–	–	–	–	–
	7–12	–	–	–	–	–	–	–

RT: focal radiotherapy 60 Gy in 6 weeks to tumor volume + 2–3 cm margin  
TMZ temozolomide

<sup>a</sup>NOS refers to the IDH status is lacking. And the 1p/19q of these gliomas are non-codeletion in this study

<sup>b</sup>150–200 mg/m<sup>2</sup> daily x 5 days per 28 days (1 cycle)

<sup>c</sup>75 mg/m<sup>2</sup> daily during RT

methylated DNA (Qiagen) were used as controls in each set of reactions. In addition to analyzing the methylation status by comparing the melting curves and Ct values with the controls, the reaction products were also checked by 2.5% agarose gel electrophoresis to verify the products with lengths 81 bp (methylated) and 93 bp (unmethylated), respectively.

## Statistical analysis

The analytical strategy of the present study is shown in Supplementary Figure S1. The patients were stratified based on the median overall survival (17 months) according to receiver operating characteristic curve analysis. The calculated area under the curve, *P*-value, sensitivity, specificity, positive likelihood ratio (sensitivity / 1–specificity), and Youden's factor (sensitivity + specificity–1) were used to evaluate the predictive value of *MGMT* methylation according to the different analytical models based on the methylation levels of individual and consecutive tested CpGs.

Consecutive CpG weighted average methylation levels were calculated using the following formula [25]:

$$\text{Weighted average} = \frac{\sum_i \beta_i * \text{site}_i}{\sum_i \beta_i}, \text{ where the coefficient } \beta \text{ for}$$

each individual CpG methylation level was calculated using univariate Cox analysis [26].

Univariate and multivariate survival analyses were performed using the Cox regression model, and survival curves were estimated by Kaplan–Meier curves and compared using the log-rank test. Methylation levels were compared among different individual CpGs or different patients by one-way analysis of variance with the Kruskal–Wallis test. Multiple comparisons were performed using Dunn's multiple comparison tests. All statistical analyses were performed using SPSS software (IBM, NY, USA) and GraphPad Prism 7 (GraphPad Software, CA, USA).

## Results

### Heterogeneity among CpG methylation levels in *MGMT* promoter

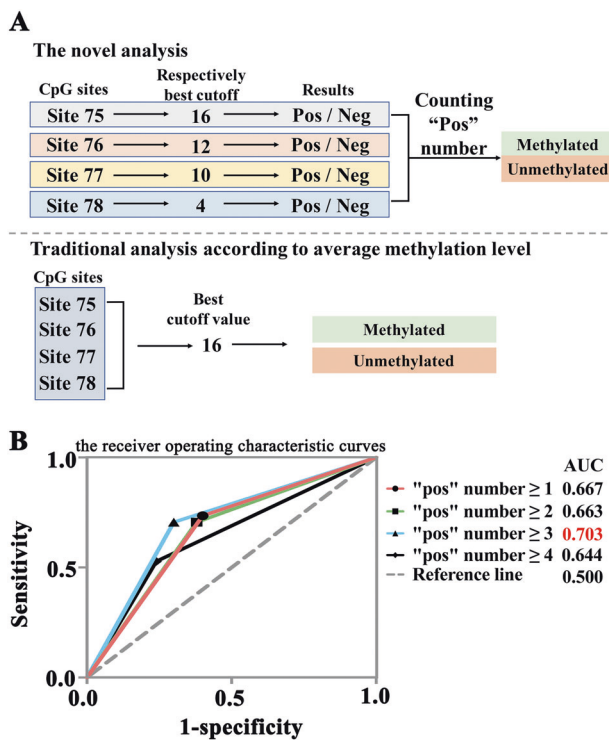
Some of the 98 individual CpGs in the *MGMT* gene promoter are shown in Fig. 1a. CpGs 72–82, localized in exon 1, are the most commonly used sites for pyrosequencing testing, while CpGs 74–78 and 76–79 have also been used in commercial kits. We tested CpGs 72–78 and 75–82 in cohorts A and B, respectively (Fig. 1a), and CpGs 72–78 in the independent validation set.



**Table 3** Optimal cut-off value for each individual CpG

CpG sites	Cut-off value	AUC	Standard error	P	95% confidence interval		Sensitivity	Specificity
					Lower	Upper		
75	16	0.693	0.060	0.002	0.576	0.809	71%	68%
78	4	0.682	0.060	0.004	0.566	0.799	76%	60%
81	11	0.678	0.060	0.006	0.560	0.797	68%	68%
82	15	0.674	0.061	0.007	0.554	0.793	65%	70%
76	12	0.673	0.060	0.007	0.555	0.791	71%	64%
77	10	0.659	0.062	0.013	0.538	0.781	56%	76%
80	15	0.659	0.062	0.014	0.538	0.780	62%	70%
79	5	0.644	0.062	0.026	0.522	0.765	65%	64%

AUC area under the curve of the receiver operating characteristic curve analysis



**Fig. 2** Comparison of the novel analytical and traditional arithmetic average methods. **a** Novel analytical and traditional average methods for CpGs 75–78. The respective optimal cut-off values are calculated in Table 2. The cut-off for the novel model was determined by the positive (Pos) and negative (Neg) numbers of selected CpGs. **b** The best cut-off for the novel analytical model was calculated by the receiver operating characteristic curve analysis. AUC, area under the curve

To gather a general idea of the methylation levels of each individual CpG among the gliomas, we presented the methylation levels for each individual CpG in cohort A (Fig. 1b) and cohort B (Supplementary Figure S2A) as heatmaps. Most previous studies used cut-off values of 4–16% to determine the final methylation status [11–17], and we therefore separated the heatmaps into three parts

according to average methylation levels of 4% and 16%. High degrees of heterogeneity were observed among *MGMT* promoter CpGs in all three parts, and individual CpG methylation levels showed high standard deviations in most samples. There was consequently no significant difference in CpG methylation levels between any two samples with average methylation levels of 4–16% in either tested cohort (Fig. 1c, Supplementary Figure S2B).

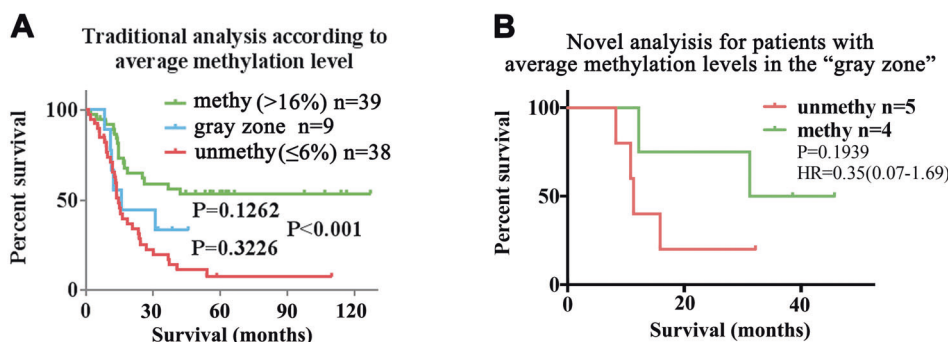
### Distinct predictive effect of each individual CpG methylation level

The heterogeneity among different individual CpGs indicated their potentially different predictive effects. This hypothesis was confirmed by the receiver operating characteristic curve analysis for each individual CpG of CpGs 75–82 in cohort A. All the individual CpGs could predict survival, but the optimal cut-off values for each individual CpG varied from 4%–16% (Table 3). Similar results were observed for CpGs 72–78 in cohort B (data not shown).

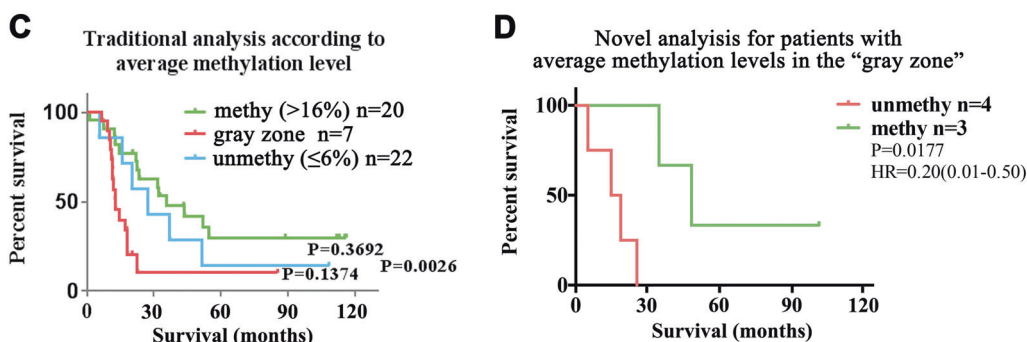
### Advantages of the novel analytical model for *MGMT* pyrosequencing

The high methylation heterogeneity of *MGMT* promoter CpGs suggested that the arithmetic average method maybe not be ideal for determining the final *MGMT* methylation status. We therefore developed a novel analytical model that defined the final *MGMT* methylation status with reference to the methylation statuses of each individual CpG, with their own cut-off values. For convenience and to improve on the traditional average method with minimal change, our novel analysis also utilized groups of several consecutive CpGs. The analytical processes for the novel and traditional average methods were demonstrated using CpGs 75–78 in cohort A (Fig. 2a). CpGs 75–78 showed the best predictive accuracy among all four-consecutive CpGs in cohort A

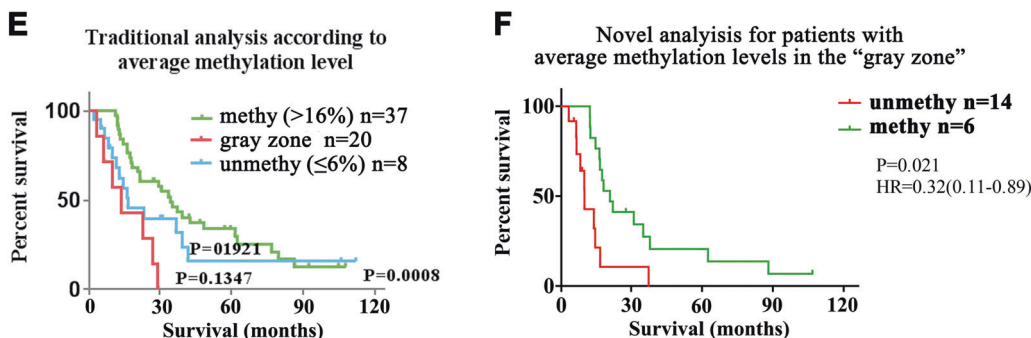
## The cohort A



## The cohort B



## The validation cohort



**Fig. 3** Practicability of the novel analytical model in patients with methylation levels in the “gray zone” according to the traditional average approach. **a–f** Kaplan–Meier survival curves for patients

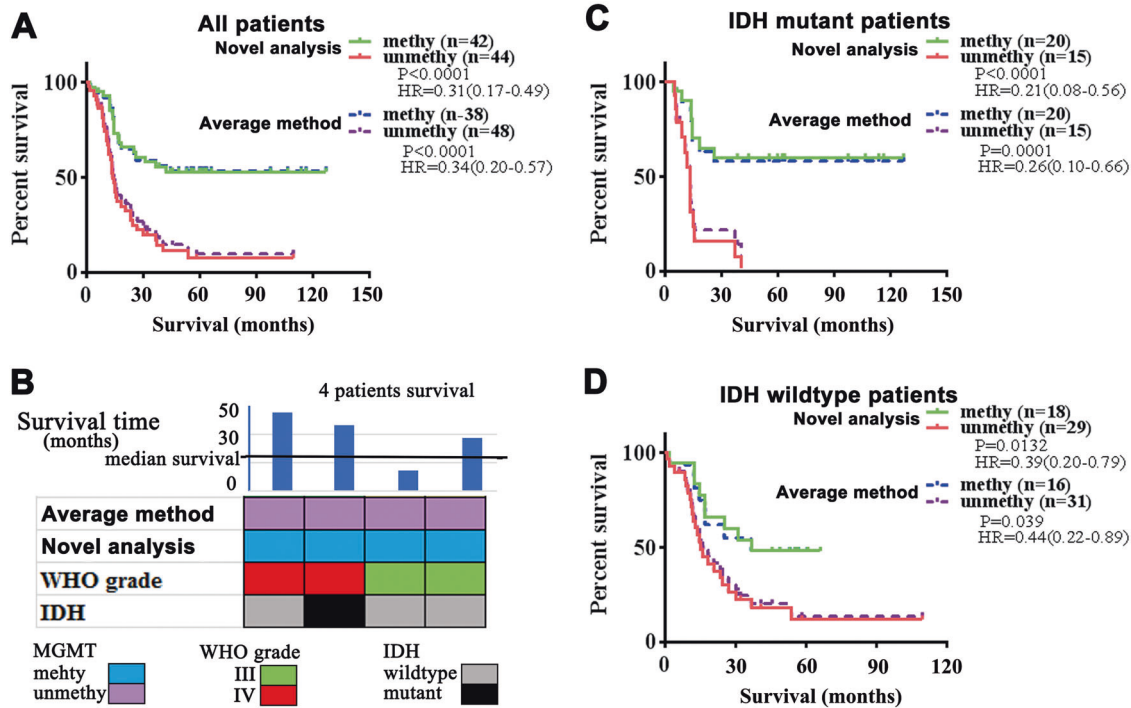
according to the traditional average method (Supplementary Table S3). The optimal number of “positive methylation counts” for determining the methylation status in the novel analytical model was determined by receiver operating characteristic curve analysis, and the best predictive effect occurred using a positive methylation count  $\geq 3$  as the judgment criterion (Fig. 2b). According to this criterion, the novel model could distinguish the prognosis of patients with “gray zone” methylation status (average methylation levels of 6–16%) according to the traditional analysis in all three cohorts (Figs. 3a–f).

In addition to comparing the novel analysis with the traditional average method, we also compared its predictive

accuracy with the weighted average analytical approach, which weighted each CpG with a factor calculated by univariate Cox regression analysis (Supplementary Table S4). The predictive accuracy of the novel analytical model was superior according to the area under the curves of the receiver operating characteristic curve analysis. We therefore focused on comparing the novel and traditional average methods in the following investigations.

We compared the predictive values of the novel and traditional average analytical methods in cohort A (Fig. 4). Although the difference was marginal, the novel approach showed a better predictive performance in terms of overall survival in Kaplan–Meier curves (Fig. 4a). Of note, four

## The cohort A



**Fig. 4** Superiority of the novel analytical model in cohort A. **a** Kaplan–Meier curves for patients stratified according to the novel and average methods for CpGs 75–78. **b** Clinicopathological features of patients with discrepant results between the novel and average

approaches. **c, d** Kaplan–Meier curves for patients with *IDH* mutant (**c**) and *IDH* wild-type statuses (**d**) stratified according to the novel and average methods for CpGs 75–78. *HR* hazard ratio

patients, three of who survived longer than the median overall survival (17 months), were regarded as unmethylated according to traditional analysis but as methylated according to the novel analysis (Fig. 4b). Given the potential influences of *IDH* status on evaluating the therapeutic prognostic value of *MGMT* methylation [27, 28], we also evaluated the novel analytical model in patients stratified by *IDH* status. The predictive performance of the novel approach was better than that of the traditional analysis in patients with either mutant (Fig. 4c) or wild-type *IDH* (Fig. 4d). Considering the differences among gliomas in different subgroups according to the WHO 2016 Classification [29], we evaluated the novel analytical model in patients with glioblastoma, *IDH*-wildtype subgroup, and the predictive performance of the novel approach was also better than that of the traditional analysis (Supplementary Figure S3A). But we were unable to compare the methods in other subgroups because of the limited patient numbers. Meanwhile, we also observed that the methylation status of *MGMT* promoter by this novel analysis could not predict the prognosis in patients not receiving alkylating chemotherapy in the glioblastomas or all high-grade gliomas (Supplementary Figure S4).

We also evaluated the usability of the novel analytical model for CpGs 75–78 in cohort B (Supplementary

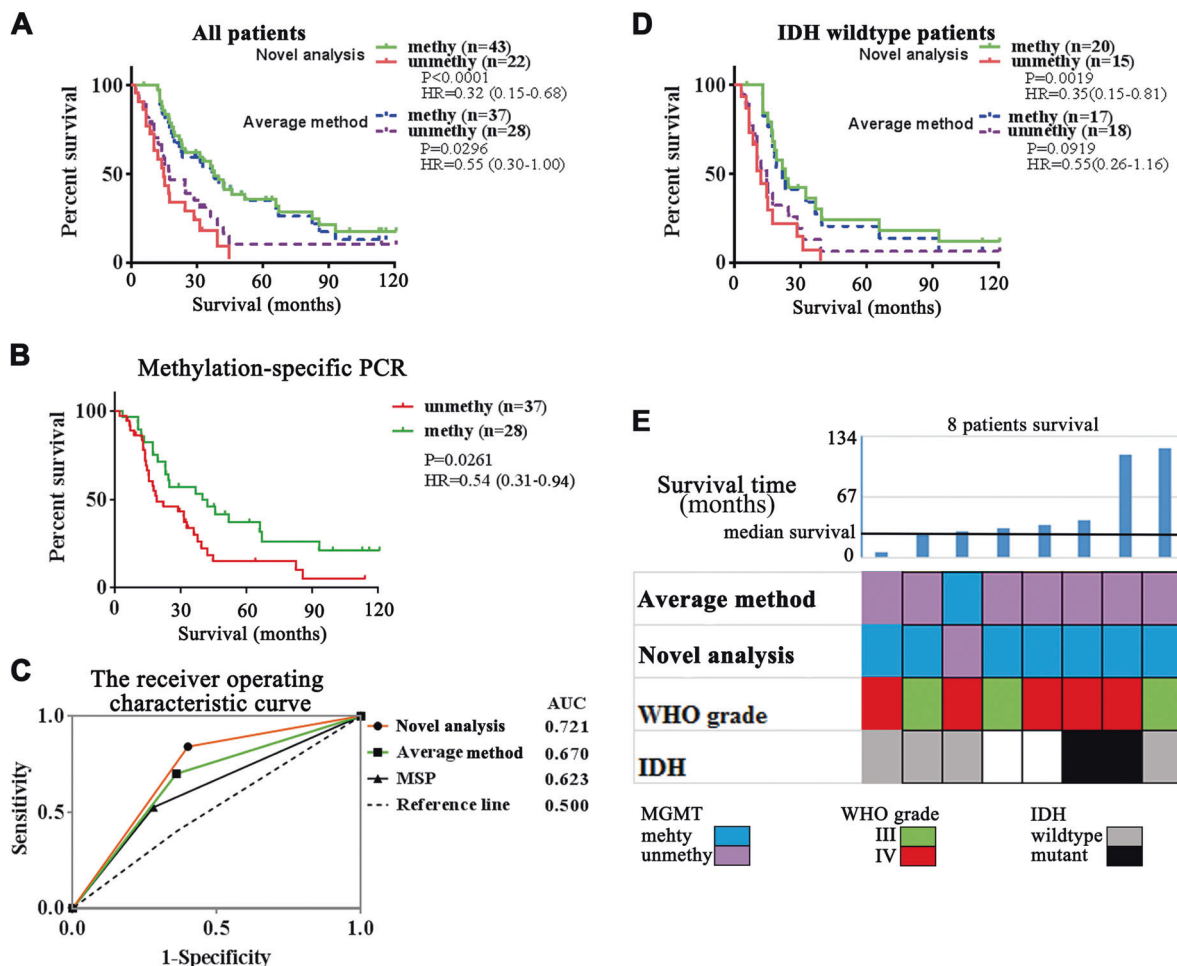
Figure S5), using the same criteria as in cohort A. We calculated the area under the curves of the receiver operating characteristic curve analysis for both methods. The area under the curve for the novel analysis (hazard ratio, 0.836; 95% confidence interval 0.715–0.958) was greater than that for the average method (hazard ratio, 0.761; 95% confidence interval 0.617–0.905) (Supplementary Figure S5A), as confirmed by Kaplan–Meier analysis (Supplementary Figure S5B). In the four patients with discrepant methylation statuses noted above, the methylation status determined by the novel approach was better correlated to the patient's prognosis (Supplementary Figure S5C). The novel analytical model was also superior to the average method in *IDH* wild-type patients (Supplementary Figure S5D).

Finally, we performed univariate and multivariate Cox regression analyses. In univariate analysis, WHO grade ( $P < 0.001$ ), *MGMT* status determined by the novel analytical model ( $P = 0.002$ ), and *IDH* status ( $P = 0.003$ ) were significantly correlated with prognosis, while *MGMT* status determined by the novel approach remained an independent prognostic factor in multivariate analysis ( $P = 0.009$ ) (Supplementary Table S5).

Overall, *MGMT* status defined using the novel analytical approach provided a more accurate predictive value compared with the traditional arithmetic average approach.



## The validation cohort



**Fig. 5** Validation of the advantages of the novel analytical model in the validation cohort. **a, b** Survival analysis of 65 patients stratified according to the novel analytical model for CpGs 75–78 (**a**), average method for CpGs 75–78 (**a**), and Methylation-specific PCR detection results (**b**). **c** Predictive accuracies of the novel and average methods,

and Methylation-specific PCR testing (**d**) were evaluated by the receiver operating characteristic curve analysis. *AUC* area under the curve. **e** Survival and pathological characteristics of the patients with discrepant results between the novel and average analytical methods. HR, hazard ratio

### Validation of the novel analytical model in an independent cohort

We further confirmed the advantages of the novel analytical model for *MGMT* pyrosequencing by comparing the predictive accuracies of the different methods in formalin-fixed paraffin-embedded samples from 65 patients with WHO III/IV gliomas, and also with methylation-specific PCR testing. Kaplan–Meier curves showed that the predictive performance of the novel method (hazard ratio, 0.32; 95% confidence interval, 0.150–0.680) was better than that of the average method (hazard ratio, 0.55; 95% confidence interval, 0.300–1.000) and of methylation-specific PCR testing (hazard ratio, 0.54; 95% confidence interval 0.310–0.940) (Figs. 5a, b). The area under the curve of the receiver operating characteristic curve analysis for the novel analysis (0.721, 95% confidence interval 0.588–0.853) was greater

than that for the average method (0.670, 95% confidence interval 0.533–0.807) and for methylation-specific PCR testing (0.623, 95% confidence interval 0.483–0.762) (Fig. 5c). Moreover, we also validated the superiority of the novel analysis in *IDH* wild-type patients (Fig. 5d) and patients with glioblastoma, *IDH*-wildtype subgroup (Supplementary Figure S3B). Methylation status determined by the novel approach was also better correlated with prognosis in eight patients with discrepant results between the two methods (Fig. 5e)

### Performance of the novel analytical model in commercial kits

CpGs 76–79 (named “Kit 1”) and CpGs 74–78 (“Kit 2”) have been used in Qiagen commercial kits (Fig. 1). Kit 1 was included in the cohort A CpGs (75–82) and Kit 2 was

included in the cohort B CpGs (72–78). In Kit 1, the area under the curve for the novel model (0.685, 95% confidence interval 0.570–0.780) was greater than that for the arithmetic average approach (0.668, 95% confidence interval 0.550–0.786). The hazard ratio according to Kaplan–Meier for the novel analysis (0.330, 95% confidence interval 0.193–0.563) was also better than that for the average approach (0.361, 95% confidence interval 0.184–0.555). Similar results were observed for Kit 2 (Supplementary Figure S6).

## Discussion

Quantitative *MGMT* pyrosequencing results are often dichotomized based on the arithmetic average of several tested CpGs and a threshold value [12, 16, 30, 31]. However, this traditional averaging method involves some uncertainty when the average methylation value is close to the threshold value (gray zone), which results from neglecting the high heterogeneity among individual CpGs [3, 22]. In the present study, we applied a novel analytical model to comprehensively evaluate the methylation status of each selected CpG according to its own cut-off value. This novel approach defined *MGMT* methylation as occurring when at least three CpGs exceeded the respective threshold value determined from the receiver operating characteristic curve analysis. Our results demonstrated that this novel analytical model of *MGMT* pyrosequencing could predict the therapeutic efficacy of patients with glioma more accurately than the traditional average method, and was also superior to methylation-specific PCR testing. The advantage of the novel model was particularly evident in patients with ‘gray zone’ results according to the traditional average approach.

Currently, several methods for *MGMT* promoter methylation testing have been reported, including gel-based methylation-specific PCR [32], methylation-specific quantitative PCR [33], methylation-specific quantitative PCR plus specific probe, MethyLight quantitative PCR [7], Methylation-sensitive high resolution melting [34], pyrosequencing [9], Methylation-specific, multiplex ligation-dependent probe amplification [35] and microarray chips, e.g. Illumina methylation array with HM-450 K or HM-27K chips [36–38]. Among these methods, Methylation-specific, multiplex ligation-dependent probe amplification is the only method not requiring bisulfite treatment, but usually it can only detect the methylation level of one CpG site, which is also limited to the restriction sites [23]. Methylation-specific PCR is the first method that has been repeatedly shown to be of predictive or prognostic value in clinical trials [1, 39]. Methylation-specific PCR is also the basis of other PCR-based methods, including methylation-specific quantitative

PCR, MethyLight quantitative PCR and Methylation-sensitive high-resolution melting. Though Methylation-specific PCR and Methylation-specific PCR-derived methods are easy to perform, they can only recognize fully methylated or unmethylated sequences with specially designed methylation-specific primers or probes, and each primer or probe typically interrogates a series of 3 to 5 CpG sites [9, 32, 33]. Given the heterogeneity of the methylation levels of CpG sites in the *MGMT* promoter [11], Methylation-specific PCR based methods have the drawbacks to reflect the heterogeneity of CpG sites [23]. Moreover, the results of these methods critically depend on the employed PCR protocols [15], and thus lacking stability [40]. The method with microarray chips is usually utilized in large-scale analyses of the methylome of gliomas, it is inconvenient to be used in clinical testing, due to the high cost and complicated data analysis process [11, 38].

Pyrosequencing is a robust technique, which could assess the methylation percentage of all individual CpGs measured by quantification of each added nucleotide during sequencing, and has the ability to evaluate the bisulfite conversion efficiency through internal control [11, 23]. In this study, we confirmed the predictive performance of pyrosequencing is better than Methylation-specific PCR. The superiority of pyrosequencing had also been demonstrated by two studies comparing others methods in glioblastoma patients [9, 41]. Thus, pyrosequencing, which could offer valid, reliable and quick evaluation of the *MGMT* promoter methylation status from both formalin fixed and paraffin-embedded specimens, was thought as the “gold standard” for *MGMT* promoter methylation testing [15, 17, 42].

The CpGs selection and cut-off value determination are the two critical factors that determine the final methylation result of pyrosequencing testing [11, 23]. In a recent study, we confirmed that combinations of four or more CpGs methylation within CpGs 72–82 in *MGMT* promoter present equivalent predictive value for *MGMT* expression and temozolomide therapeutic outcomes in gliomas [42]. However, the cut-off value determination is still a pending critical issue for pyrosequencing testing with traditional average analysis method [21, 22]. Here, the novel analytical model could provide improved solution for the cut-off value determination in pyrosequencing testing. Importantly, there was a clear tendency for the novel analysis to show greater predictive accuracy in both the tested cohorts with fresh frozen tissues and the independent validation cohort with formalin-fixed paraffin-embedded samples. Overall, the proposed novel analytical model of *MGMT* pyrosequencing offered an optimal predictive performance in patients with WHO grade III/IV gliomas.

A retrospective analysis of the NOA-04 trial revealed that the prognostic and predictive values of *MGMT* methylation depended on *IDH* status. It also indicated that

*MGMT* was a prognostic factor in patients with *IDH1/2* mutant gliomas, and predicted the benefit of alkylating chemotherapy in patients with *IDH1/2* wild-type tumors [27]. Given that the clinical course, prognosis, and mechanism of *MGMT* methylation may differ between *IDH*-mutated and wild-type gliomas [28], we evaluated the performances of the novel and traditional average methods in patients according to specific *IDH* status, and demonstrated superiority of the novel approach irrespective of *IDH* mutation status.

Loss of one copy of the chromosomal region containing the *MGMT* gene (10q26) is quite common in glioblastoma (60–90%) [37, 43]. It has been reported that there is a negative correlation between *MGMT* promoter methylation and *MGMT* expression in glioblastoma with chromosome 10 / 10q deletion, and there is no significant interaction between *MGMT* promoter methylation and chromosome 10 / 10q deletion [37, 41]. Therefore, we did not involve copy number alteration for the chromosome 10 / 10q in this study. However, it is still an important issue in future study that whether chromosome 10 / 10q copy number alterations might be with prognostic or predictive values concerning response to TMZ treatment through affecting *MGMT* expression in GBM. Because it has been reported that patients with glioblastoma harboring chromosome 10 / 10q deletion could benefit more from temozolomide treatment than other glioblastoma patients [38, 43, 44]. Although chromosome 10 / 10q deletion does not significantly affect *MGMT* expression of glioblastoma in the TCGA dataset [37].

This study was limited by its retrospective nature and the relatively small population size, and the optimal cut-off value for each CpG may thus need to be adjusted when applying this novel analytical model. Nevertheless, this study confirmed the superiority of the novel approach to analyzing *MGMT* pyrosequencing results for predicting the prognosis of glioma patients using either fresh frozen or formalin-fixed paraffin-embedded tissue samples.

In conclusion, the novel analytical approach for pyrosequencing testing has three advantages over the traditional arithmetic average method: it accounts for heterogeneity among individual CpGs in the *MGMT* promoter region, it provides more accurate results in cases where the methylation level according to the average method is in the ‘gray zone’, and it shows a better clinical predictive performance for alkylating agent chemotherapy.

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## Compliance with ethical standards

**Ethics approval and consent to participate** This study was approved by the Beijing Tiantan Hospital institutional review board, and informed consent was obtained from each patient involved in our research.

**Conflict of interest** The authors declare that they have no conflict of interest.

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