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ORIGINAL ARTICLE

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Combinations of four or more CpGs methylation present equivalent predictive value for *MGMT* expression and temozolomide therapeutic prognosis in gliomas

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Abstract

Aims: The pyrosequencing (PSQ) has been regarded as the gold standard for *MGMT* promoter methylation testing in gliomas. However, various CpG combinations are currently used in clinical practice. We aimed to clarify how and how many CpGs combined is robust enough to predict MGMT mRNA expression and therapeutic prognosis of patients.

Methods: Total 223 patients with WHO III/IV gliomas were enrolled from Chinese Glioma Genome Atlas, including two independent cohorts, the eight-site cohort (with CpGs 75-82 tested) and the seven-site cohort (with CpGs 72-78 tested). Spearman's correlation and ROC curves were employed to investigate the value of different CpG combinations on predicting *MGMT* mRNA expression. The ROC curves and Kaplan-Meier steps were performed to compare the TMZ therapeutic prognostic values of different CpG combinations.

Results: The methylation level of all individual CpG and CpG combinations for the eleven CpGs (CpGs 72-82), significantly correlated to MGMT mRNA expression (Spearman, all $P < 0.0001$), could effectively predict the mRNA expression (AUC, 0.86-0.91 in the eight-site cohort, 0.83-0.90 in the seven-site cohort). Moreover, the correlation coefficients and the predictive values presented equivalent when four or more CpGs combinedly used (AUC, 0.88-0.90 in the eight-site cohort, 0.87-0.88 in the seven-site cohort). Finally, similar results were also observed when using selected CpG combinations to predict therapeutic prognosis of patients.

Conclusions: Four-CpG combinations of pyrosequencing are sufficient for evaluating the methylation status of MGMT and predicting therapeutic prognosis in gliomas.

KEYWORDS

CpGs, glioma, *MGMT*, pyrosequencing, temozolomide

1 | INTRODUCTION

O^6-methylguanine-DNA methyltransferase (*MGMT*), a ubiquitous DNA repair enzyme, can rapidly reverse alkylation at the O^6 position of guanine by transferring the alkyl group to its cysteinyl residue.[1,2] Temozolomide (TMZ), a typical alkylating agent, is the first-line chemotherapy drug for glioma. And the expression deficiency of *MGMT* in glioma has been acknowledged as a predictive marker for TMZ sensitivity.[2] The cysteine-phosphate-guanine (CpG) island (CGI) in the promoter region of *MGMT* is susceptible to DNA methylation,

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which is highly related to the *MGMT* transcription suppression.[2,3] Thus, *MGMT* promoter methylation implies a TMZ sensitivity status of glioma patients, confirmed in several subsequent studies and clinical trials.[4–10]

Given the difficulty of detecting *MGMT* mRNA or protein expression directly in glioma,[11] *MGMT* promoter methylation testing is now wildly employed in clinical practice. Total 98 CpGs situated in the *MGMT* CGI,[12] named CpGs 1-98 in this study according to whose location in the 762 bps (chr10: 131264949-131265710) from the 5′-end to the 3′-end. In early clinical trials, the methylation-specific PCR(MSP) was mainly used to determine the methylation status, and the primers of which were designed specifically to CpGs 76-80 and CpGs 84-87 fully methylated sequences, respectively.[4,5] However, along with the high heterogeneity of the CpGs methylation gradually identified,[13,14] MSP-based methods were unable to reflect such heterogeneity.[12,15,16] Currently, pyrosequencing (PSQ) has been developed to be a stable technique, offering a valid, reliable and quick evaluation of both fresh frozen and formalin fixed paraffin embedded (FFPE) specimens.[11,13,14,17–22]

Similar to the MSP method, PSQ determines the *MGMT* promoter methylation by the mean methylation level of several selected consecutive CpGs.[11,15,23] The methylation statuses of CpGs 25-50 and 72-90 are proved highly correlated to *MGMT* transcription, and CpGs 72-90 seems to play a more critical role.[12,24] However, the high heterogeneity of different CpGs' methylation level is also recognized,[12,16,24] how many CpGs in the *MGMT* CGI is robust enough to reflect the transcription status is still a controversial issue for *MGMT* methylation PSQ testing.[14,15]

Various number of CpGs from four to eighteen has been used as CpG combinations in reported studies (Figure 1).[11,13–15,17,20,25,26] Among which, the commonly adopted combinations are CpGs 76-79 and 74-78, which are currently used in Qiagen commercial kits. Thus, it is critical to clarify the following issues for *MGMT* methylation PSQ testing. (a) whether four CpGs is robust enough in the *MGMT* promoter methylation PSQ testing; (b) whether different CpG combinations can provide equivalent predictive value on *MGMT* transcription; (c) for the commonly utilized combinations of CpGs 76-79 and 74-78 should be analyzed separately or in combination?

The aim of this study was to clarify the issues mentioned above with patients from two independent cohorts, the eight-site cohort with CpGs 75-82 tested and the seven-site cohort with CpGs 72-78 tested. We used Spearman's correlation analysis and ROC curve to compare the predictive values of individual CpG and different CpG combinations on the mRNA expression of *MGMT*. Moreover, we also compared the therapeutic prognosis value of several selected CpG combinations, including CpGs 76-79 and CpGs 74-78, in patients received TMZ treatment. Finally, our study indicated that combinations including four CpGs were robust enough in the *MGMT* methylation PSQ testing.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

To study the relationship between different *MGMT* promoter CpGs methylation status and *MGMT* mRNA expression level, 159 cases

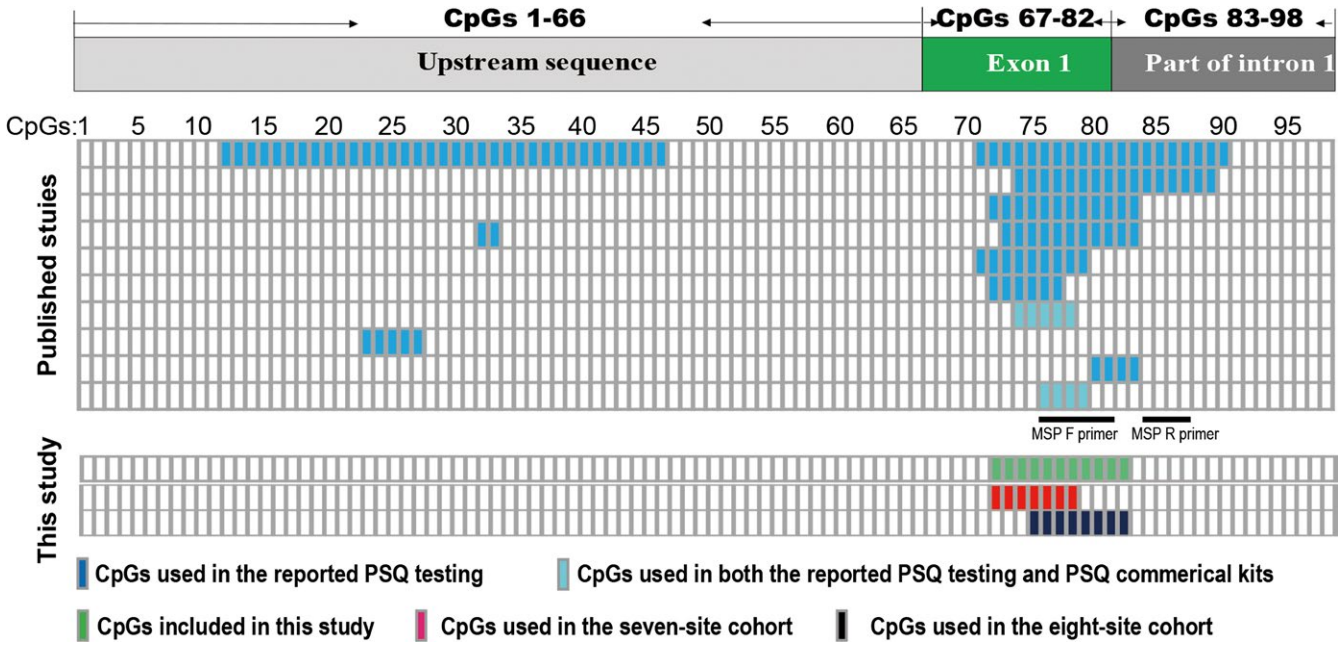


FIGURE 1 Schematic diagram of CpG studied in previous and current studies. The distribution of CpG in the MGMT 5′ CpG island is shown in the upper panel. CpGs that had been used in pyrosequencing (PSQ) testing of the published literature are shown in the middle panel. The CpGs tested in the current study are shown in the lower panel

were totally enrolled according to the following criteria: (a) diagnosed with WHO grade III or IV glioma; (b) containing *MGMT* promoter methylation PSQ testing data in detail; (c) including exact *MGMT* mRNA sequencing data. Within the 159 cases, of 82 contain the CpGs 75-82 methylation information, and the other 77 contain the CpGs 72-78 methylation information (Supporting Information Table S1).

To compare the therapeutic prognostic value of several selected CpG combinations, 86 cases with CpGs 75-82 methylation information and 48 cases with CpGs 72-78 methylation information were included. More than detailed CpGs methylation information, the inclusion criteria include the following: (a) diagnosed with WHO grade III or IV glioma; (b) having received radiotherapy (RT) + temozolomide (TMZ) treatment; (c) containing overall survival (OS) information (Table 1).

The pathological grade was determined by two independent pathologists, and the freshly frozen samples with >80% tumor cells were used to determine the *MGMT* promotor methylation status. All the cases included in this study were assigned to the eight-site (CpGs 75-82) or the seven-site (CpGs 72-78) cohort according to the CpG sites tested. All the case information included was collected from the Chinese Glioma Genome Atlas (CGGA) database. This study was approved by the institutional review board of Beijing Tiantan Hospital.

2.2 | DNA isolation and bisulfite modification

Genomic DNA was extracted from freshly frozen tumor tissue with the QIAamp DNA Mini Kit (Qiagen, Stockach, Germany). The DNA concentration and quality were determined by a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). And then the bisulfite conversion used 100 ng DNA and an Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol.

2.3 | PSQ testing

The template used in the MGMT PSQ testing was prepared as previously described.[17,27] Briefly, bisulfite-treated DNA was preamplified with the primers (a) F-primer 5′-GTT TYG GAT ATG TTG GGA TAG TT-3′; (b) biotinylated R-primer 5′-biotin-ACR ACC CAA ACA CTC ACC AA-3′. Different samples were analyzed with two independent assays, and the PSQ primers were (a) 5′-GAT ATG TTG GGA TAG T-3′ (for CpGs 72-78); (b) 5′-GTT TTT AGA AYG TTT TG-3′ (for CpGs 75-82). The sequence of CpGs 72-78 and CpGs 75-82 for analysis is 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. PSQ testing was performed on a PyroMarker Q96 instrument, and the results were analyzed with PyroMarker Q96 software (Qiagen).

Mutations of the isocitrate dehydrogenase (*IDH*) 1/2 genes were also determined by PSQ, and the templates were prepared with following primers (a) F-primer for *IDH1*, 5′-GCT TGT GAG TGG ATG GGT AAA AC-3′; (b) biotinylated R-primer for *IDH1*, 5′-biotin-TTG CCA ACA TGA CTT ACT TGA TC-3′; (c) F-primer for *IDH2*, 5′-ATC CTG GGG ACT GTC TT-3′; and (d) biotinylated R-primer for *IDH2*, 5′-biotin-CTC TCC ACC CTG GCC TAC CT-3′. PSQ testing was performed with the primers 5′-TGG ATG GGT AAA ACC T-3′ for *IDH1* and 5′-AGC CCA TCA CCA TTG-3′ for *IDH2*.

2.4 | RNA sequencing (RNA-seq), quality control, and mRNA expression calculation

RNA sequencing library was constructed as previously published study.[28] Briefly, RNA was extracted from the frozen tissue sample, and the RNA-seq library was constructed and subsequently sequenced on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) using 101-bp pair-end sequencing strategy. Image data

TABLE 1 Characteristics of patients received Radiotherapy (RT) and Temozolomide (TMZ) treatment

		Eight-site cohort			Seven-site cohort		
WHO grade		Total	IV	III	Total	IV	III
Total		86	51	35	48	24	24
Gender	Male	52 (60.5%)	33 (64.7%)	19 (54.3%)	32 (66.7%)	14 (58.3%)	18 (75.0%)
	Female	34 (39.5%)	18 (35.3%)	16 (45.7%)	16 (33.3%)	10 (41.7%)	6 (25.0%)
Age	Median	43 (19-72)	44 (19-72)	42 (25-62)	48 (24-79)	55 (29-79)	43 (24-65)
KPS	<80	28 (32.6%)	16 (31.4%)	12 (34.3%)	16 (33.3%)	11 (45.8%)	5 (20.8%)
	≥80	35 (40.7%)	21 (41.2%)	14 (40.0%)	20 (41.7%)	9 (37.5%)	11 (45.8%)
	NA	26 (30.2%)	14 (27.4%)	12 (34.3%)	12 (25.0%)	4 (16.7)	8 (33.3%)
Resection	Total	58 (67.4%)	36 (70.6%)	22 (62.9%)	23 (47.9%)	12 (50.0%)	11 (45.8%)
	Sub	27 (31.4%)	15 (29.4%)	12 (34.3%)	24 (50.0%)	12 (50.0%)	12 (50.0%)
	NA	1 (1.2%)	0 (0.0%)	1 (2.9%)	1 (2.1%)	0 (0.0%)	1 (4.2%)
IDH status	Mutant	36 (41.9%)	12 (23.5%)	24 (68.6%)	15 (31.3%)	5 (20.8%)	10 (41.7%)
	Wild type	46 (53.5%)	36 (70.6%)	10 (28.6%)	32 (66.7%)	18 (75.0%)	14 (58.3%)
	NA	4 (4.6%)	3 (5.9%)	1 (2.9%)	1 (2.0%)	1 (4.2%)	0 (0.0%)

were converted into sequence data using base calling software (Illumina pipeline CASAVA v1.8.2, CA, USA) and then further estimated by standard quality control criteria.

Sequencing reads were excluded when any one of the following parameters fitted: (a) the read is aligned to adaptor or primer with no more than two mismatches; (b) the read contains over 10% unknown bases;

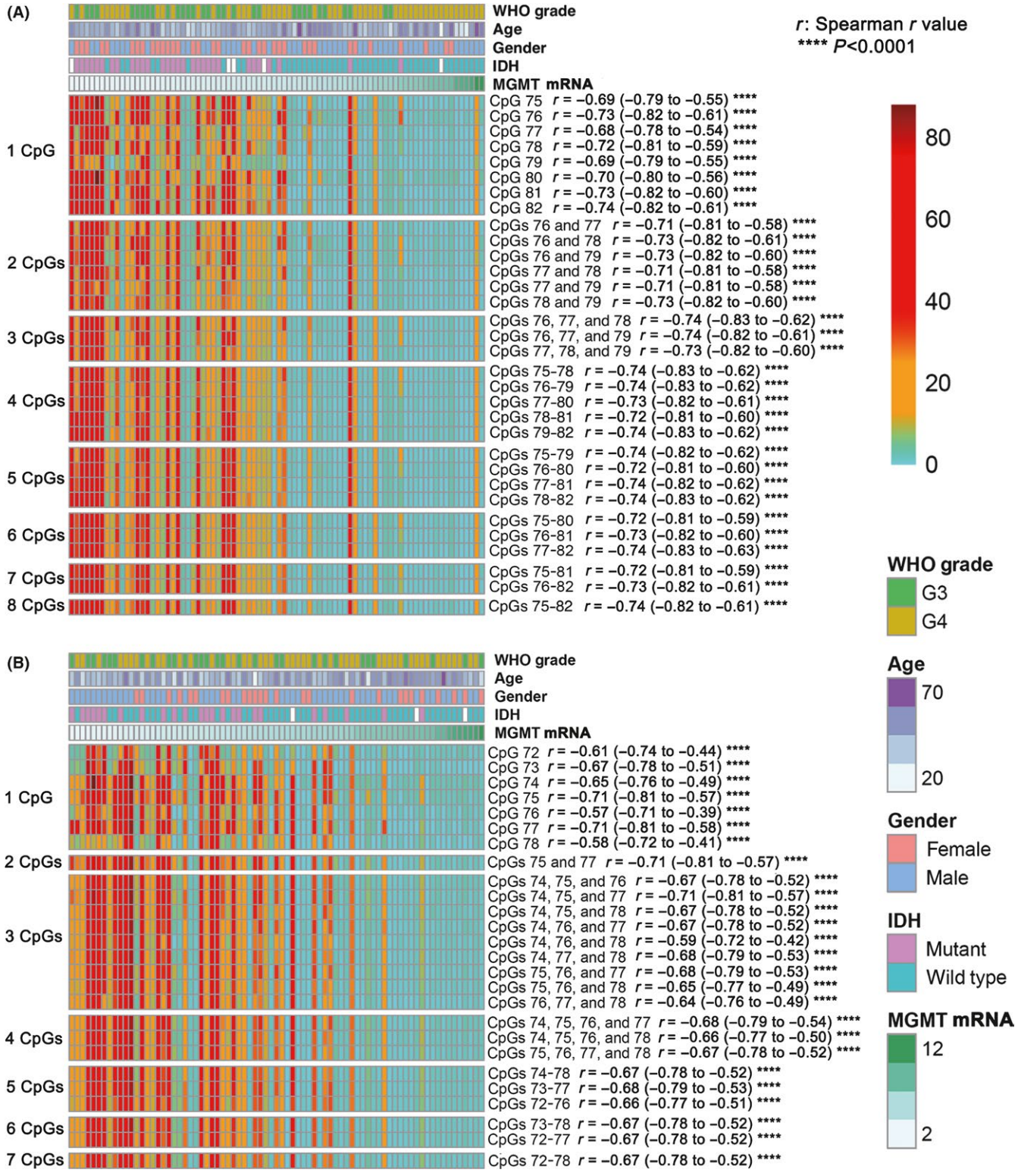


FIGURE 2 Correlation of *MGMT* mRNA expression to the methylation level of *MGMT* promoter individual CpG or CpG combinations. A and B, The methylation level of individual CpG or CpG combinations is applied to *heatmap* in the eight-site cohort (A) and the seven-site cohort (B). The methylation level of a CpG combination is the average methylation level of all CpGs included in this combination. "r" is the Spearman r value, and the 95% confidence interval of the "r" is shown in the following bracket, respectively. ****$P < 0.0001$

and (c) the read includes more than 50% of low-quality bases (quality value ≤5). The detailed information of the quality control for the whole RNA sequencing database had been described in our previous study.[29]

The *MGMT* mRNA expression level was calculated following the listed procedures: (a) mapping the reads to the Ref Seq-RNA reference sequence set Hg 19 (RNA sequences, GRCh37, available online: https://genome.ucsc.edu); (b) evaluating mRNA expression level according to the number of reads per kilobase transcriptome per million; and (c) normalizing of the estimated gene expression between different samples.

2.5 | Statistical analysis

Statistical analysis was performed with R (https://www.r-project.org/, v3.4.1), and GraphPad Prism 7 (GraphPad Software, the state of California, USA). 0.05 was chosen to be the significance cutoff for *P*-value. The relationship between methylation level of CpGs and *MGMT* mRNA expression was analyzed by Spearman's correlation analysis. The patients were stratified into two groups according to their *MGMT* mRNA expression (using the median expression as the cutoff), and the receiver operating characteristic (ROC) analysis was employed to evaluate the predictive value of *MGMT* promoter methylation status on the mRNA expression level by calculating the area under the curve (AUC).

The ROC analysis was also used to evaluate the predictive value of *MGMT* promotor methylation status for patients' survival. The median OS was used to stratified the patients. Kaplan-Meier step was used to estimating the survival of subgrouped patients, and the value between groups was compared by the log-rank test.

3 | RESULTS

3.1 | Strategy for CpG combinations selection, and correlation of *MGMT* mRNA expression with single CpG or selected CpG combinations

In the eight-site cohort, CpGs 75-82 were tested, and the CpGs 76-79 combination is currently used in the commercial kit (Qiagen, 970061). We aimed to (a) compare the predictive values of each individual CpG and CpG combinations, which are composed by a different number of consecutive CpGs, on *MGMT* mRNA expression; (b) study whether total or part of CpGs within CpGs 76-79 should be analyzed. Thus, all the individual CpG, all the two or three CpGs composed CpG combinations within CpGs 76-79, and all consecutive four, five, seven, and eight CpG combinations were selected for the analysis in this study (Figure 2A and Supporting Information Table S2).

In the seven-site cohort, CpGs 72-78 were tested, and the CpGs 74-78 combination is currently used in the commercial kit (Qiagen, 972032, and 970032). Based on the similar strategy used in the eight-site cohort, all individual CpG, representative two-CpG combination (CpGs 75 and 77), all three or four CpG combinations within CpGs 74-78 combination, and all consecutive five, six, and seven CpG combinations were finally selected for investigation (Figure 2B and Supporting Information Table S2).

We presented the methylation level of each individual CpG and selected CpG combinations of patients included as heatmaps, in ascending order of MGMT mRNA expression (Figure 2). In the eight-site cohort, although the methylation levels of all individual CpG and selected CpG combinations were significantly associated with the *MGMT* mRNA expression ($P < 0.0001$), the heterogeneity among individual CpG and selected CpG combinations methylation level could also be observed, and the Spearman r value deviated from -0.68 to -0.74. Meanwhile, we noticed that there was a tendency that the heterogeneity decreases among CpG combinations which included four or more CpGs, and the Spearman r values also converged to be around -0.73 (from -0.72 to -0.74) when the including four or more CpGs (Figure 2A). Similarly, consistent phenomena were observed in the seven-site cohort (Figure 2B). Moreover, the combinations with only part of CpGs within CpGs 76-79 or CpGs 74-78 showed worse correlation with *MGMT* mRNA expression than that of total combination, respectively. These findings indicated that the CpG combinations with four or more than four CpGs are enough to eliminate the influence of heterogeneity among individual CpG within CpGs 72-82. Thus, we inferred that CpG combinations with more four or more CpGs have similar predictive value on *MGMT* mRNA expression.

3.2 | Predictive value of each individual CpG and selected CpG combinations methylation level for *MGMT* mRNA expression

To verify our inference, we further compared the predictive value of each individual CpG and selected CpG combinations methylation level for the *MGMT* mRNA expression with ROC curve (Supporting Information Table S2). The calculated AUC of each individual CpG and selected CpG combinations to range from 0.86 to 0.91 but converge to around 0.89 (from 0.88 to 0.90) when three or more CpGs combined in the eight-site cohort (Figure 3A). Similar results could also be observed in the seven-site cohort, and the AUCs ranging from 0.83 to 0.90 converged to 0.87 or 0.88 when four or more CpGs combined (Figure 3B). This finding confirmed our inference that CpG combinations with four or more CpGs have similar predictive value on the *MGMT* mRNA expression.

3.3 | Therapeutic prognostic effects of the selected CpG combinations in glioblastoma

Based on the above findings, we further compared the therapeutic prognostic effect of the *MGMT* promoter methylation status determined by the several-CpG combinations mean methylation level, and the selected CpG combinations were CpGs 75-82, 76-79, and 75-78 from the eight-site cohort and CpGs 72-78, 74-78, and 75-78 from the seven-site cohort. In the eight-site cohort, the therapeutic prognostic effects of CpGs 75-82 (Figure 4A), 76-79 (Figure 4B), and 75-78 (Figure 4C) were evaluated by ROC curve within 51 glioblastoma patients. And the respective AUCs were 0.7624, 0.7609, and 0.7741, which indicated that the three CpG combinations had similar therapeutic prognostic effects for

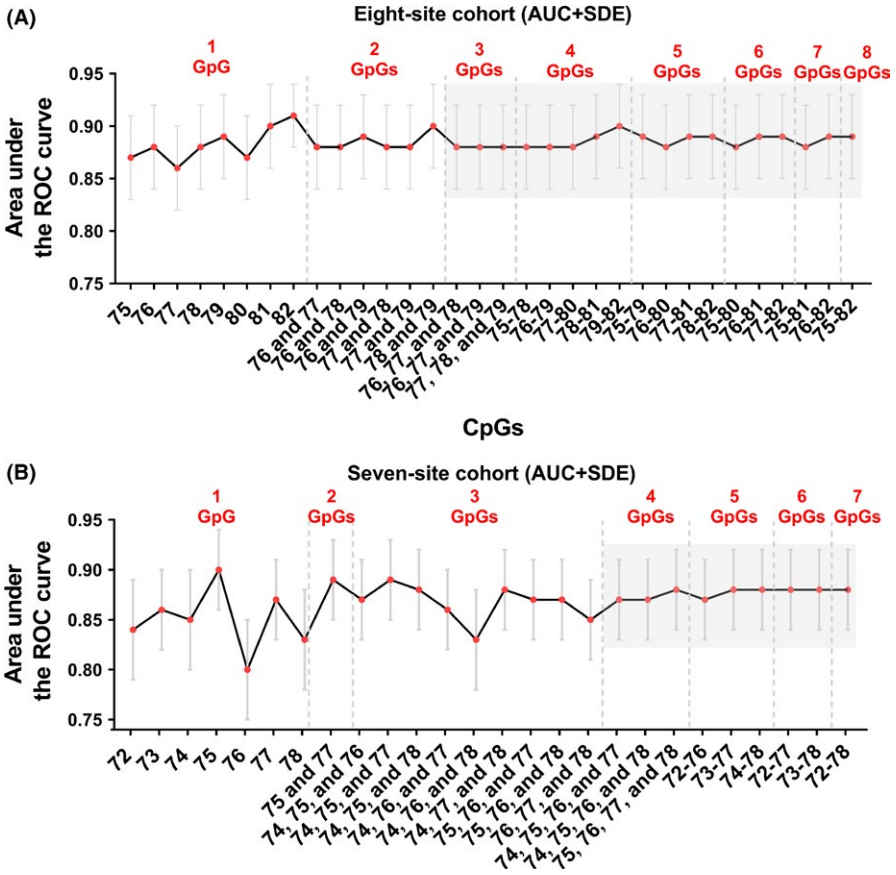


FIGURE 3 Predictive value of different individual CpG or selected CpG combinations methylation levels for *MGMT* mRNA expression. A and B, The area under the ROC (AUC) and corresponding Standard error (SDE) of different individual CpG or selected CpG combinations in the eight-site cohort (A), and the seven-site cohort (B) is arranged along with the CpG numbers contained in each combination. The CpG combinations with convergent AUCs are highlighted with the gray background

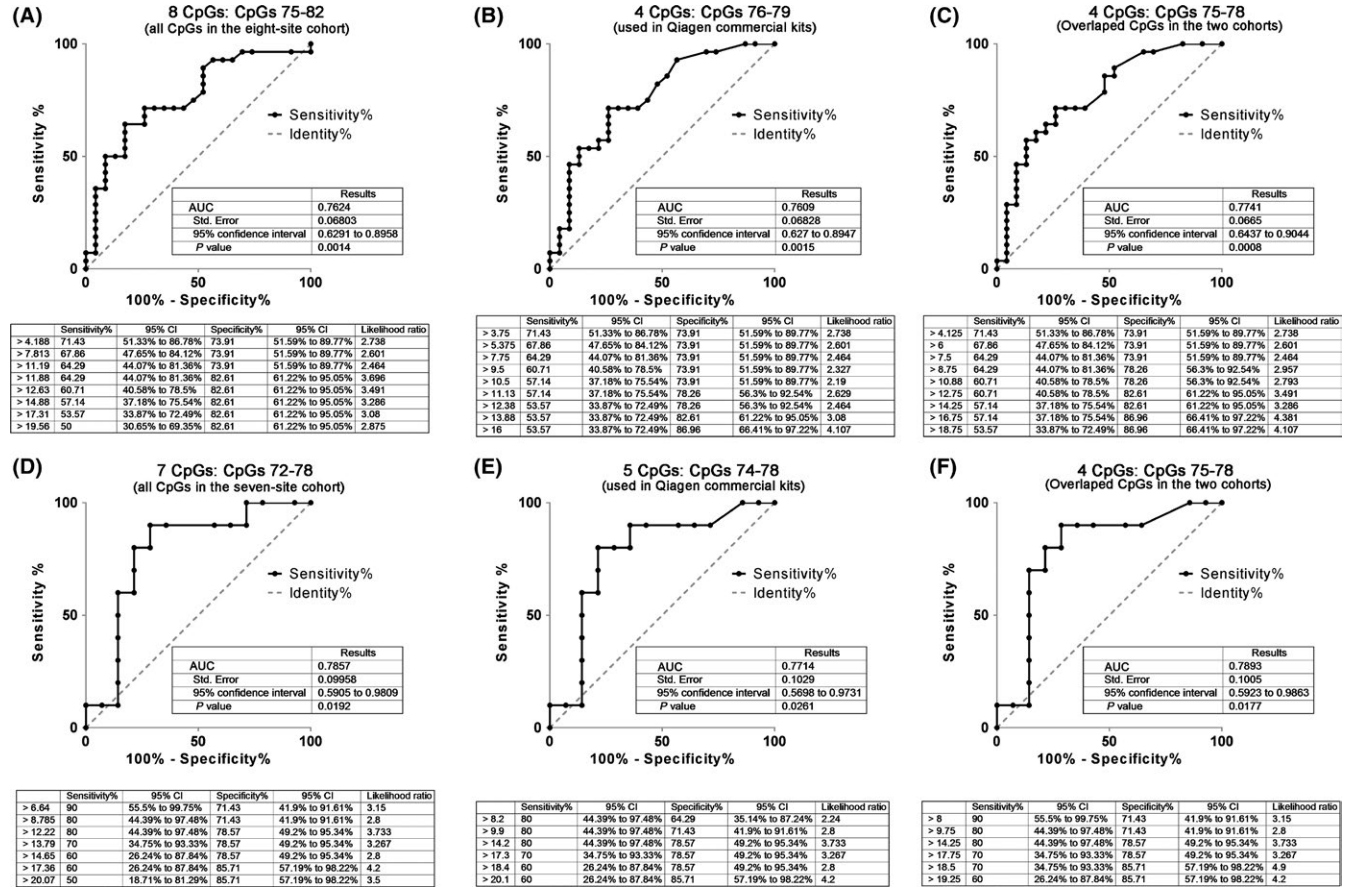
glioblastoma patients (Figure 4A-C). The similar result was observed in the 24 glioblastoma patients from the seven-site cohort, and the AUCs of CpGs 72-78, 74-78, and 75-78 were 0.7851, 0.7714, and 0.7893, respectively (Figure 4D-F).

The Kaplan-Meier steps showed that the OS of patients is significantly different, separated by the calculated cutoff of mean methylation level of CpGs 75-82 (Figure 5A), 76-79 (Figure 5B), and 75-78 (Figure 5C). The results also showed that the methylation statuses of different CpG combinations had similar stratification ability for patient survival. In the seven-site cohort, the methylation statuses determined by the mean methylation level of CpGs 72-78 (Figure 5D), 74-78 (Figure 5E), and 75-78 (Figure 5F) also showed approximative predictive values. Then we compared the stratification ability of these CpG combinations in WHO grade III glioma patients by Kaplan-Meier steps (Supporting Information Figure S1). For the predictive and prognostic roles of *MGMT* methylation in WHO grade III glioma patients have been reported,[9] the results indicated that all the selected CpG combinations in the eight-site and the seven-site cohort had similar predictive value.

4 | DISCUSSION

The PSQ testing has been regarded as the gold standard for*MGMT* promoter methylation testing and wildly used in reported studies and clinical practice.[12,16,17,21,30,31] However, which CpGs should be included in the *MGMT* methylation PSQ testing and analysis is still a controversial issue. In this study, we demonstrated that CpG combinations with four CpGs were robust enough to be adopted in the *MGMT* methylation PSQ testing, for the CpG combinations with four or more consecutive CpGs had the similar correlation with the *MGMT* mRNA expression, and they also showed alike predictive value on the *MGMT* mRNA expression. We also found that the predictive value of CpGs 75-78 was close to that of CpGs 76-79 and 74-78 in the eight-site cohort and seven-site cohort, respectively, indicating that the commercial kits utilized CpGs combinations, CpGs 76-79 and 74-78, had almost equal predictive value for the MGMT mRNA expression and survival of patients received TMZ.

Several studies have tried to compare the predictive values of individual CpG and CpG combinations on *MGMT* mRNA expression and survival of TMZ treated patients, but the result is still controversial. Watts et al identified that three distinct regions of *MGMT* CGI were highly related to the *MGMT* expression level.[3] Among the three regions, two overlap the CpGs 25-50 and CpGs 73-90, which were proved to be crucial for *MGMT* promoter activity.[12] In glioblastoma tissues, individual CpG 27, 32, 73, 75, 79 and 80 were reported to be significantly correlated to the *MGMT* mRNA expression, and combinations of CpGs 32-33 and CpGs 72-83 were concordant with *MGMT* mRNA expression.[24] But, there was no consensus on which individual CpG or CpG combinations should be used in *MGMT* methylation PSQ testing, and the combinations of CpGs 72-83, 72-80, 72-77, 74-78, 74-89, 76-79, and 80-83 were used in distinct



	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 4.188	71.43	51.33% to 86.78%	73.91	51.59% to 89.77%	2.738
> 7.813	67.86	47.65% to 84.12%	73.91	51.59% to 89.77%	2.601
> 11.19	64.29	44.07% to 81.36%	73.91	51.59% to 89.77%	2.464
> 11.88	64.29	44.07% to 81.36%	82.61	61.22% to 95.05%	3.696
> 12.63	60.71	40.58% to 78.5%	82.61	61.22% to 95.05%	3.491
> 14.88	57.14	37.18% to 75.54%	82.61	61.22% to 95.05%	3.286
> 17.31	53.57	33.87% to 72.49%	82.61	61.22% to 95.05%	3.08
> 19.56	50	30.65% to 69.35%	82.61	61.22% to 95.05%	2.875

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 3.75	71.43	51.33% to 86.78%	73.91	51.59% to 89.77%	2.738
> 5.375	67.86	47.65% to 84.12%	73.91	51.59% to 89.77%	2.601
> 7.75	64.29	44.07% to 81.36%	73.91	51.59% to 89.77%	2.464
> 9.5	60.71	40.58% to 78.5%	73.91	51.59% to 89.77%	2.327
> 10.5	57.14	37.18% to 75.54%	73.91	51.59% to 89.77%	2.19
> 11.13	57.14	37.18% to 75.54%	78.26	56.3% to 92.54%	2.629
> 12.38	53.57	33.87% to 72.49%	78.26	56.3% to 92.54%	2.464
> 13.88	53.57	33.87% to 72.49%	82.61	61.22% to 95.05%	3.08
> 16	53.57	33.87% to 72.49%	86.96	66.41% to 97.22%	4.107

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 4.125	71.43	51.33% to 86.78%	73.91	51.59% to 89.77%	2.738
> 6	67.86	47.65% to 84.12%	73.91	51.59% to 89.77%	2.601
> 7.5	64.29	44.07% to 81.36%	73.91	51.59% to 89.77%	2.464
> 8.75	64.29	44.07% to 81.36%	78.26	56.3% to 92.54%	2.957
> 10.88	60.71	40.58% to 78.5%	78.26	56.3% to 92.54%	2.793
> 12.75	60.71	40.58% to 78.5%	82.61	61.22% to 95.05%	3.491
> 14.25	57.14	37.18% to 75.54%	82.61	61.22% to 95.05%	3.286
> 16.75	57.14	37.18% to 75.54%	86.96	66.41% to 97.22%	4.381
> 18.75	53.57	33.87% to 72.49%	86.96	66.41% to 97.22%	4.107

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 6.64	90	55.5% to 99.75%	71.43	41.9% to 91.61%	3.15
> 8.785	80	44.39% to 97.48%	71.43	41.9% to 91.61%	2.8
> 12.22	80	44.39% to 97.48%	78.57	49.2% to 95.34%	3.733
> 13.79	70	34.75% to 93.33%	78.57	49.2% to 95.34%	3.267
> 14.65	60	26.24% to 87.84%	78.57	49.2% to 95.34%	2.8
> 17.36	60	26.24% to 87.84%	85.71	57.19% to 98.22%	4.2
> 20.07	50	18.71% to 81.29%	85.71	57.19% to 98.22%	3.5

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 8.2	80	44.39% to 97.48%	64.29	35.14% to 87.24%	2.24
> 9.9	80	44.39% to 97.48%	71.43	41.9% to 91.61%	2.8
> 14.2	80	44.39% to 97.48%	78.57	49.2% to 95.34%	3.733
> 17.3	70	34.75% to 93.33%	78.57	49.2% to 95.34%	3.267
> 18.4	60	26.24% to 87.84%	78.57	49.2% to 95.34%	2.8
> 20.1	60	26.24% to 87.84%	85.71	57.19% to 98.22%	4.2

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 8	90	55.5% to 99.75%	71.43	41.9% to 91.61%	3.15
> 9.75	80	44.39% to 97.48%	71.43	41.9% to 91.61%	2.8
> 14.25	80	44.39% to 97.48%	78.57	49.2% to 95.34%	3.733
> 17.75	70	34.75% to 93.33%	78.57	49.2% to 95.34%	3.267
> 18.5	70	34.75% to 93.33%	85.71	57.19% to 98.22%	4.9
> 19.25	60	26.24% to 87.84%	85.71	57.19% to 98.22%	4.2

FIGURE 4 Comparison of the therapeutic prognostic value of different CpG combinations for glioblastoma patient survival. A-C, Therapeutic prognostic effects of the *MGMT* methylation statuses determined by CpGs 75-82 (A), CpGs 76-79 (B), and CpGs 75-78(C) for glioblastoma patient OS were evaluated by ROC in the eight-site cohort. D-F,Therapeutic prognostic effects of the *MGMT* methylation statuses determined by CpGs 72-78 (D), CpGs 74-79 (E), and CpGs 75-78(F) for glioblastoma patient OS were evaluated by ROC in the seven-site cohort

studies.[12,14–17,21,23,26,32–34] Here, we systematically compared the predictive value of all combinations within the CpGs 72-82 on the MGMT mRNA expression through analyzing paired samples with both the MGMT methylation PSQ testing and mRNA expression data. We indicated that the predictive value differences among combinations with four or more CpGs within CpGs 72-82 were marginal, which may explain why controversial results got from different studies.

A study had compared the prognostic value of different CpGs on patients clinical outcome and showed that CpG 89, 84 and the combination of CpGs 84-88 was the optimal predictive models within CpGs 74-89 for the survival of glioblastoma patients, but the predictive values of which were merely improved, compared with the CpGs 74-78 combination.[13] In this study, we directly evaluated the predictive value of different CpG combinations and individual CpG for *MGMT* mRNA expression and verified the findings with the survival of TMZ treated glioblastoma patients as well. Besides, the strategy for CpG combination selection in our study is more comprehensive and targeted to the frequently used combinations in commercial kits, such as CpGs 76-79 and 74-78. Our results suggested that combinations with part of CpGs within CpGs 76-79 or CpGs 74-78 presented poorer predictive value than totally included, respectively. Given the convenience of standardization between different laboratories, it is a proper choice to analyze results with included CpGs in commercial kits totally.

The cutoff value is another important issue for the*MGMT* PSQ testing. Here, we found that the optimal cutoff for the mean methylation level of different CpG combinations varied obviously. This is consistent with previous studies, where the cutoff varied from 2.68% to 30% for different CpG combinations.[14,17,23–26] Some studies even suggested that there may be a “gray zone” between the true methylated status and true un-methylated status, and the cutoff value around 10% used in most studies was overestimated.[25,26] We also noticed that the cutoff of the mean methylation level cannot be easily determined, and the different cutoff for the same CpG combination was used in different studies.[13,14,17,33] Recently, a study indicated that the optimal cutoff should not only be determined by the ROC likelihood value but also by the sensitivity and specificity.[17] Thus, we determined the cutoff in this study by similar strategy, comprehensively considering the ROC likelihood value, sensitivity, specificity, and cutoffs used in reported studies. However, a more comprehensive and targeted study is still required for the optimal cutoff determination for *MGMT* methylation PSQ testing.

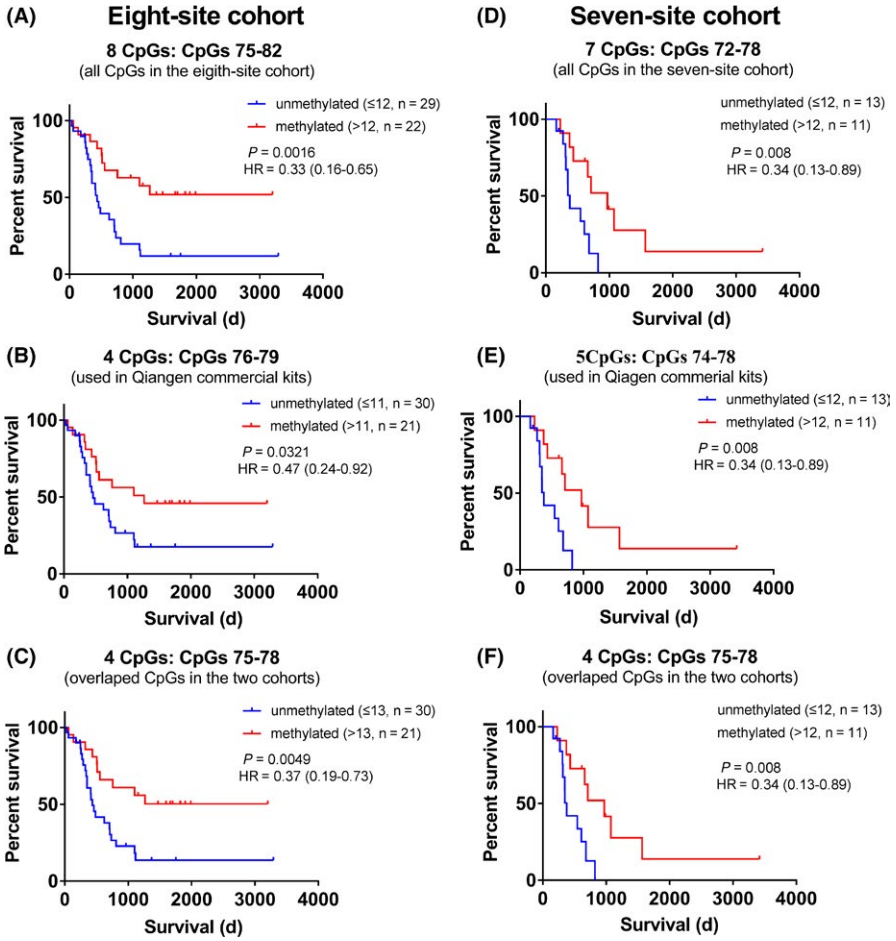


FIGURE 5 Kaplan-Meier steps for the OS of glioblastoma patients stratified by the methylation level of different CpG combinations. A-C, Kaplan-Meier step for the OS of glioblastoma patients in the eight-site cohort. The *MGMT* methylation statuses determined by CpGs 75-82 (A), CpGs 76-79 (B), and CpGs 75-78(C) were used as the stratification reference, respectively. D-F, Kaplan-Meier curves for the OS of glioblastoma patients in the eight-site cohort. The *MGMT* methylation statuses determined by CpGs 72-78 (D), CpGs 74-79 (E), and CpGs 75-78(F) were used as the stratification reference, respectively

5 | CONCLUSIONS

In summary, our study demonstrates that (a) combinations of four CpGs within CpGs 72-82 are robust enough in *MGMT* promoter methylation PSQ testing; (b) CpG combinations with four or more consecutive CpGs within CpGs 72-82, including the combinations of CpGs 76-79 and CpGs 74-78 used in commercial kits, are equally effective to predict the *MGMT* mRNA expression and the survival of TMZ treated glioma patients; (c) for the commonly used combinations of CpGs 76-79 and CpGs 74-78, it is proper to analyze the final methylation status of *MGMT* promoter with included CpGs entirely. All above-mentioned indicated that four-CpG combinations are sufficient for MGMT methylation testing by the PSQ approach.

CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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