“Hyper-Hypermethylated Status” of the MGMT Gene Promoter Confers Additional Survival Benefits in IDH-Wild-Type Glioblastoma Patients

Moonsik Kim (teiroa83@yuhs.ac)
Severance Hospital https://orcid.org/0000-0002-5804-8790
Jihwan Yoo
Severance Hospital
Yun Ho Roh
Severance Hospital
Jong Hee Chang
Severance Hospital
Se Hoon Kim
Severance Hospital

Clinical Study

Keywords: MGMT, methylation status, hyper-hypermethylation, glioblastoma, temozolomide chemotherapy, IDH wild-type GBM

Posted Date: February 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-178009/v1

Abstract

Introduction

Methylation status of the O(6)-Methylguanine-DNA methyltransferase (*MGMT*) promoter plays key role in glioblastoma (GBM) with respect to the patient's responses to temozolomide chemotherapy and disease prognosis. Although the cut-off value of *MGMT* methylation (≥10%) is currently widely used to dichotomize the *MGMT* status as "methylated" or "unmethylated" in pyrosequencing (PSQ) analysis, it is still unclear whether it reflects the actual *MGMT* methylation status of the patients. Thus, we investigated if there is a so called "hyper-hypermethylation cut-off value" that confers additional survival benefits in *IDH*-wild-type GBM patients.

Methods

We retrospectively analyzed a cohort of 110 isocitrate dehydrogenase (*IDH)*-wild-type GBM patients who underwent gross total resection followed by the standard treatment. Predictive "hyper-hypermethylated" cutoff values that yielded maximal differences in survival were investigated.

Results

The estimated hyper-hypermethylated cutoff value was 40%. The median OS values for unmethylated, low-methylated, and hyper-hypermethylated groups were 16, 17, and 23 months, respectively. With regard to disease progression, the median progression-free survival (PFS) values were 11, 14, and 16 months, respectively. The hyper-hypermethylated *MGMT* cutoff values were correlated with significantly improved overall survival (OS), compared to the low-methylated (p=0.039, HR=3.86, CI=1.1 to 13.8) or unmethylated groups (p=0.023, HR=3.92, CI=1.21 to 12.6).

Conclusion

Thus, in addition to the standard *MGMT* cut-off point of 10%, we suggest that a so-called "hyper-hypermethylated" *MGMT* cut-off point may confer additional survival benefits in *IDH*-wild-type GBM patients.

Introduction

Glioblastoma (GBM) is the most commonly occurring malignant brain tumor in adults that manifests hostile clinical behavior [1]. Despite the aggressive therapy, its prognosis continues to be dismal, with an overall survival (OS) of 15–17 months [2]. Stupp protocol is the standard of care for GBM treatment; it consists of neurosurgical resection followed by concomitant chemoradiation and adjuvant chemotherapy with temozolomide (TMZ) [3, 4]. TMZ is a cytotoxic alkylating agent, which causes double-strand DNA breaks in the cells. Chemosensitivity to TMZ varies greatly, owing to the methylation status of O(6)-Methylguanine-DNA methyltransferase (*MGMT*) promoter [3-6]. Previous randomized trials have shown that *MGMT* promoter methylation is significantly associated with higher survival rates in TMZ-treated

GBM patients. The DIRECTOR trial indicated TMZ rechallenge as a treatment possibility for recurrent GBM with methylated *MGMT* promoter [7]. Based on an NOA-09 trial, it was suggested that more intense first-line treatment therapy with combined lomustine/TMZ can improve prognosis compared to the conventional TMZ therapy [5]. All these clinical trials emphasized the importance of *MGMT* promoter methylation status for prognosis. Thus, precise measurement of the *MGMT* promoter methylation status is essential for clinical planning in GBM patients. Pyrosequencing (PSQ) is a widely used technique for determining the *MGMT* promoter methylation status [7-10]. PSQ quantitatively assesses this status, thereby rendering advantages over methylation-specific polymerase chain reaction (MSP) [11-13].

Currently, whether the currently widely accepted *MGMT* methylation cut-off value (≥ 10%) used to dichotomize the *MGMT* promoter methylation status as “methylated” or “unmethylated” can reliably reflect the actual *MGMT* methylation status of GBM patients remains unclear [14-18]. Several studies have suggested that there exists a “gray zone” where the methylation level is close to the cut-off value, its actual methylation status remains unclear [9, 11, 16, 19-21]. Thus, the further subclassification of the *MGMT* methylation status may be needed to confer survival benefits in glioma patients. In addition, although the association of *MGMT* promoter methylation status with GBM prognosis has been established, only a few studies have focused particularly on isocitrate dehydrogenase (*IDH)*-wild-type GBM [22].

In this study, we investigated if there is a “hyper-hypermethylated” *MGMT* status that could further benefit GBM patients in terms of the survival and progression of *IDH*-wild-type GBM.

Materials/subjects And Methods

Patient selection and clinicopathologic evaluation

We retrieved 317 GBM cases from computerized files of surgical pathology diagnosis. These cases underwent surgically resections from May 2016 to December 2019. Of these cases, 110 consecutive cases of *IDH*-wild-type GBM that: 1) had undergone gross total tumor resection and 2) completed the Stupp protocol with 3) Karnofsky performance status ≥70 were selected. Postoperative MRI was obtained within 48 h after surgery and gross total resection was defined when no enhancing lesion was observed. The decision of gross total resection was carried out by two neuro-radiologists. GBM diagnosis was determined according to the 2016 WHO classification of CNS tumors. All cases were reviewed and confirmed by two specialized neuro-oncopathologists (authors, MK and SHK). 1p/19q status of the cases was evaluated using fluorescence in situ hybridization. *IDH* mutational status was assessed using *IDH1* R132H immunohistochemistry and Sanger sequencing of *IDH1* and *IDH2* for all cases. Clinicopathologic data including age at diagnosis, sex, Karnofsky performance status, extent of tumor resection and residual tumor volume, types and duration of adjuvant therapy, follow-up time, progression-free survival, and OS were thoroughly investigated from the electronic medical records of the hospital. The study plan was reviewed and accepted by the Institutional Review Board of Severance Hospital (4-2020-0547).

DNA extraction and bisulfate modification

Areas with > 80% tumor cells were chosen for *MGMT* promoter methylation analysis. Genomic DNA was extracted from freshly frozen tumor tissues and formalin-fixed paraffin-embedded samples using QIAamp DNA Mini and QIAamp DNA FFPE Tissue Kits (Qiagen, Hilden, Germany), respectively. DNA concentration was quantitated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). Further, 100 ng DNA was subjected to bisulfite conversion using Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol.

MGMT promoter pyrosequencing and 'Hyper-hypermethylated' cut-off value determination

Pyrosequencing was performed using Therascreen MGMT Pyro Kit (Qiagen) per the manufacturer's instructions. The average percentage of methylated alleles was determined by the mean value of the individual methylation percentage. For every PSQ run, standardized positive and negative controls were included as well. Among GBM cases with an *MGMT* methylation cut-off value ≥10, we analyzed another *MGMT* promoter methylation PSQ cut-off value that could maximize differences in the OS of GBM patients ("hyper-hyper methylation" cut-off value) using the Contal and O'Quigley method.

Statistical analysis

OS and PFS was evaluated using Kaplan–Meier method. Statistical differences in survival times were determined using the log-rank test. Cox proportional hazards regression analysis was applied to examine predictive factors among significantly correlated variables. *p*-value of < 0.05 was considered statistically significant. All statistical analyses were conducted using R package (version 3.4.3, http://www.R-project.org).

Results

Patient cohort

Clinicopathologic characteristics of the 110 *IDH*-wild-type GBM patients are summarized in Table 1. The detailed information of the patient cohorts is listed in supplementary Table 1. The median age was 57 years; the men slightly outnumbered the women. The median progression-free survival (PFS) and median overall survival (OS) were 11 months and 16 months, respectively. Among the 110 patients, 38 (34.5%) were in the *MGMT* methylated subgroup. The median OS and PFS of the *MGMT* methylated subgroup were 18.5 months and 14 months, respectively, whereas the median OS and PFS of the *MGMT* unmethylated subgroup were 15.5 months and 11 months, respectively.

"Hyper-hypermethylation" cut-off points in *MGMT* promoter methylation status

We then investigated the additional cut-off points of the *MGMT* promoter methylation status that can maximize the difference in OS among the methylated groups. The estimated hyper-hypermethylation *MGMT* cut-off value was ≥40%.

Survival and disease progression according to *MGMT* methylation status

We then categorized *IDH*-wild-type GBM as unmethylated (n=72), low-methylated (n=24), and hyper-hypermethylated (n=14) according to the *MGMT* methylation status. The median OS of the hyper-hypermethylated group was 23 months; the median OS for the low-methylated group was 17 months. The median PFS was 16 months for the hyper-hypermethylated subgroup, and 14 months for the low-methylation subgroup. Kaplan–Meier curves were generated according to the *MGMT* methylation status. With regard to the OS, the Kaplan–Meier curves demonstrated significant differences between the hyper-hypermethylated vs low-methylated groups (p = 0.039, HR = 3.86, CI = 1.1 to 13.8) and hyper-hypermethylated vs unmethylated groups (p = 0.023, HR = 3.92, CI = 1.21 to 12.6). There was no significant difference in the OS between the unmethylated vs. low-methylated groups (Fig. 1). The PFS between the unmethylated vs low-methylated groups (p = 0.012, HR = 2.24, CI = 1.2 to 4.2) and the unmethylated vs hyper-hypermethylated groups (p = 0.028, HR = 2.41, CI = 1.1 to 5.32) showed a significant difference. However, there was no significant difference in PFS between the low-methylated and hyper-hypermethylated groups (Fig 2).

Discussion

In this well-established *IDH*-wild-type GBM cohort, we investigated the association between the *MGMT* promoter methylation status and GBM prognosis. Out of all the reports on *MGMT* promoter methylation status, only a few have strictly focused on patient cohorts limited to *IDH*-wild-type GBM [15]. Currently, PSQ is the accepted 'gold standard' to evaluate *MGMT* promoter methylation status [12, 23, 24]. In addition, the cutoff value of ≥10% is widely used to determine *MGMT* promoter status as "methylated" and "unmethylated" in PSQ [12, 19, 21]. Although previous studies have shown that *MGMT* promoter methylation confers survival benefit in glioma patients [25-28], there were some issues that needed to be clarified. Studies have reported the presence of a "gray zone" in the methylation status and questioned whether the current dichotomizing method could reflect the actual methylation status of GBM patients [14, 15, 24]. In addition, some studies have suggested the possibility of a "hypermethylated" *MGMT* methylation status, which may provide additional prognostic benefits to GBM patients [15, 26, 29].

In this study, we demonstrated that *MGMT* "hyper-hypermethylation" can confer additional survival benefits to GBM patients showing *MGMT* methylation. Because individual CpG sites show methylation heterogeneity and may have different impacts on the prognostic value [24], low-methylated GBMs could be partly methylated tumors that cannot fall into methylated or unmethylated category. The extent of *MGMT* promoter methylation or protein expression level may have affected the distinct biologic behavior between hyper-hypermethylated group and unmethylated group [26]. In the meanwhile, the hyper-hypermethylation status did not demonstrate significant differences in PFS compared with the low-methylated group in the present study. Taken together, we suggest that even low levels of *MGMT* methylation can benefit the patient in terms of disease progression; a hyper-hypermethylated status is more likely to affect the additional survival benefits of the patient; however, studies using larger cohorts will be needed to confirm the results of this study.

Although the present study has major findings, there are some associated limitations as well. Clinicopathologic characteristics [30-32], such as age, mutational status of *TERT* promoter or *TP53*, and gliomatosis, may have affected the results of this study. In addition, this study strictly focused on *IDH*-wild-type GBM. Further studies thus will be needed to confirm that the hypermethylation status will also have prognostic value in other types of gliomas.

In conclusion, we suggest that further dividing the *MGMT* methylation status according to the hype-hyperrmethylated status can have prognostic implications for *IDH*-wild-type GBM patients. Subsequent studies in larger patient cohorts should be used to validate the findings of our study.

Declarations

Acknowledgments

The authors would like to gratefully thank Won Young Park and Yi Rang Kim for their dedicated effort in *MGMT* promoter pyrosequencing.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

S.H.K. was supported by grants from the Brain

Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (Grant No. 2016M3C7A1913844). The funding source had no role in

the design, practice, or analysis of this study.

Ethics approval

The study plan was reviewed and accepted by the Institutional Review Board of Severance Hospital (4-2020-0547).

Availability of data and material

Not applicable

Code availability

Not applicable

Author's contributions

Se Hoon Kim and Jong Hee Chang provided the conception and design of the manuscript. Moonsik Kim and Jihwan Yoo drafted the manuscript. Yun Ho Ro analyzed and interpreted the clinical data. Se Hoon Kim and Jong Hee Chang carefully reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Clinicopathologic characteristics of the patient cohort

Study cohort n=110		n	%
Age (years)	Mean	57.8	
	Median	59	
	Range	31–81	
Sex	Male	67	60.9 %
	Female	43	39.1 %
MGMT	Methylated (mean ≥10)	38	34.5 %
	Unmethylated (mean <10)	72	65.5 %
PFS (months) (total)	Mean	13.3	
	Median	11	
	Range	2–43	
OS (months) (total)	Mean	18.9	
	Median	16	
	Range	3–49	
PFS (months) (Methylated)	Mean	15.7	
	Median	14	
	Range	4-42	
OS (months) (Methylated)	Mean	20.6	
	Median	18.5	
	Range	6-43	
PFS (months) (Unmethylated)	Mean	12.1	
	Median	11	
	Range	2-43	
OS (months) (Unmethylated)	Mean	18.1	
	Median	15.5	
	Range	3-49	

Figures

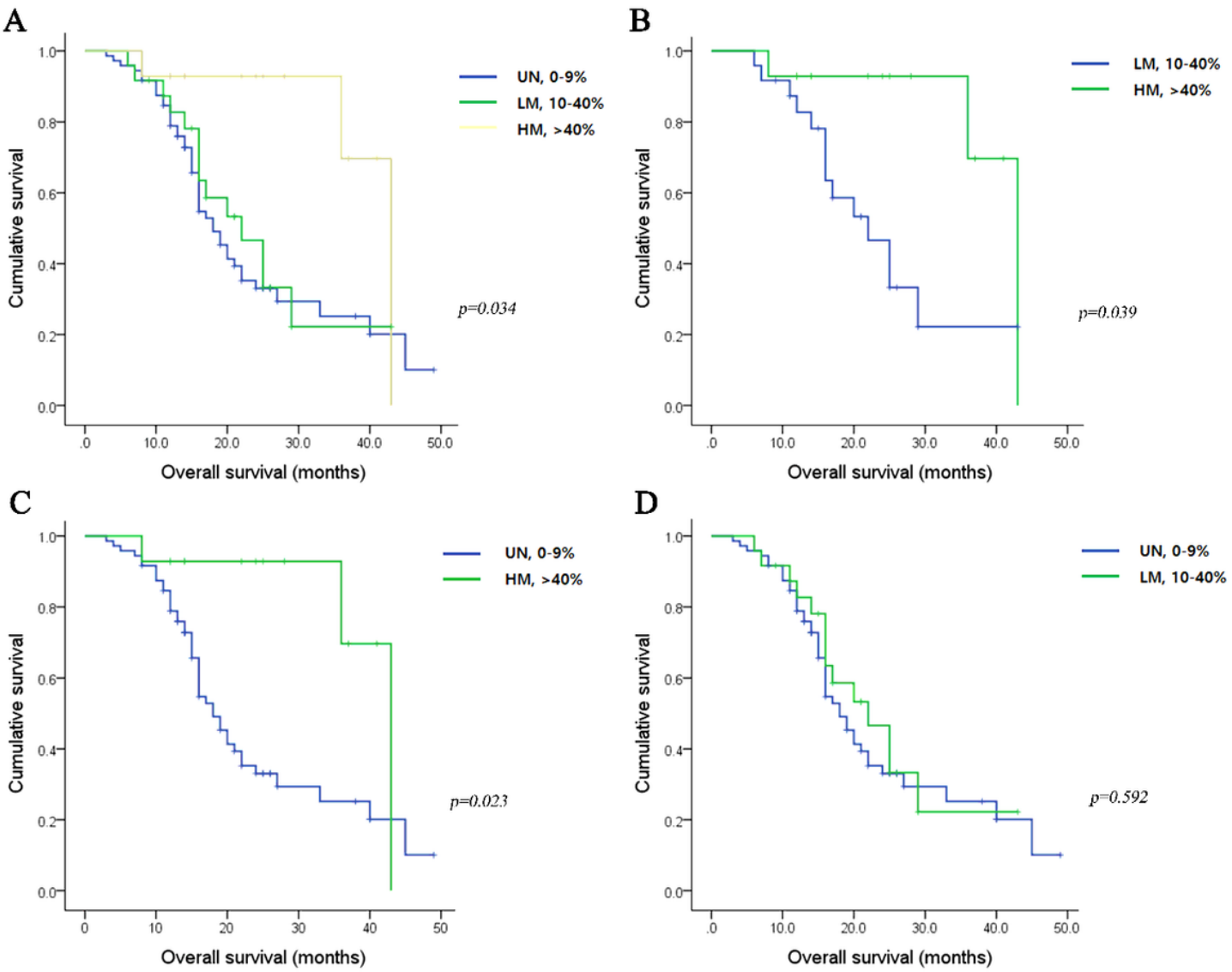

Figure 1

Overall survival according to the MGMT methylation status. A: UN vs LM vs HM, B: LM vs HM, C: UN vs HM, D: UN vs LM. UN, unmethylated; LM; low-methylated; HM, hyper-hypermethylated

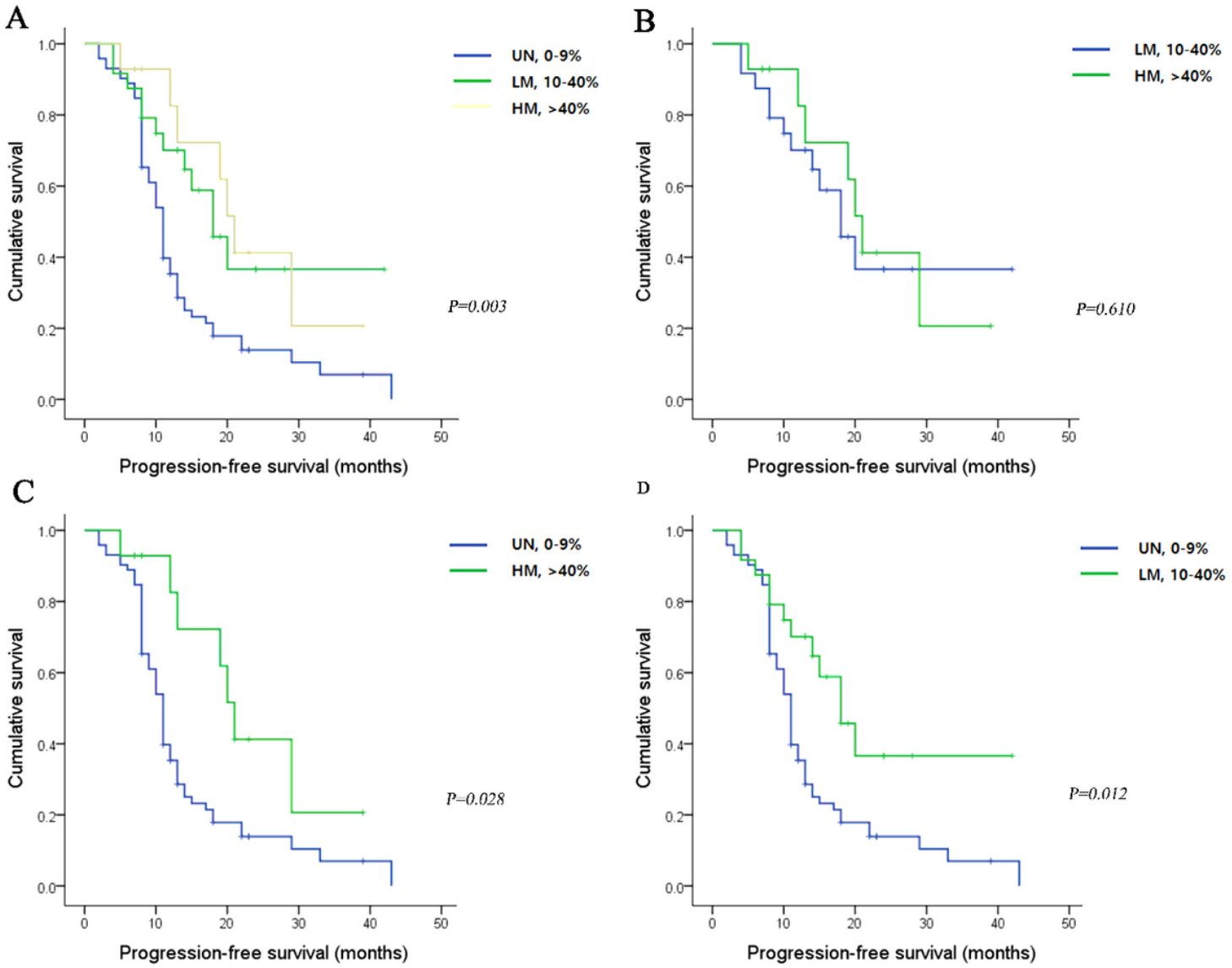

Figure 2

Progression-free survival according to the MGMT methylation status. A: UN vs LM vs HM, B: LM vs HM, C: UN vs HM, D: UN vs LM. UN, unmethylated; LM; low-methylated; HM, hyper-hypermethylated

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx