Chaperone protein P4HB predicts temozolomide response and prognosis in malignant glioma

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Abstract. Prolyl 4-hydroxylase beta polypeptide (P4HB) is a chaperone protein associated with temozolomide (TMZ) resistance through the unfolded protein response. Cancer cells with constitutive activation of endoplasmic reticulum stress and upregulation of P4HB have been observed to show resistance against chemotherapies. The present study focused on the evaluation of the prognostic value of P4HB in subtypes of glioma with or without O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation. P4HB expression was assessed by immunohistochemical staining in 73 grade I-IV gliomas and its association with the clinicopathological data was determined. It was indicated that P4HB expression was significantly associated with several parameters, including age, tumour grade and the number of TMZ treatment cycles received. In the Kaplan-Meier analysis, P4HB expression was positively associated with risk of mortality and disease progression. In patients treated with TMZ, high P4HB expression was significantly associated with poor overall survival (OS) and progression-free survival (PFS). The association between MGMT promoter methylation and P4HB expression was also assessed. Patients with $MGMT^{Meth}P4HB^{Low}$ tumours had the most favourable PFS (48 months) among cases with various combinations of MGMT methylation status and P4HB expression. Multivariate analysis revealed that P4HB may be used as an independent prognostic indicator of OS, particularly in high-grade gliomas. The present study uncovered the potential role of P4HB in a nuanced pathological stratification during clinical decision-making with respect to MGMT promoter methylation status and TMZ treatment.

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Introduction

Malignant glioma is the most common subtype of primary brain tumour in adults, with an overall survival (OS) time of ~18 months (1). Temozolomide (TMZ), an oral DNA alkylating agent, has been the current standard of care. In the standard regimen after maximal surgical resection, patients receive concomitant chemoradiotherapy with TMZ (75 mg/m^2), followed by adjuvant TMZ (150 to 200 mg/m^2) for five days every 28 days for six cycles after radiotherapy (2). Despite promising results in terms of improving patient survival, drug resistance and tumour relapse are almost inevitable. The expression of DNA damage repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) in glioma cells may also protect cells from alkylating drugs including TMZ (3), whereas a methylated MGMT gene promoter may inactivate MGMT expression, leading to a greater therapeutic response to TMZ. The MGMT methylation status has thus been adopted as a useful prognostic and predictive biomarker for better patient management.

The endoplasmic reticulum (ER) stress response and the associated unfolded protein response (UPR) are one major mechanism in the development of TMZ resistance in glioblastoma (4). In cancer cells, adaptive ER stress response due to prolonged ER stress (e.g. caused by hypoxia and glucose deprivation) is a common feature and chronic ER stress imposed by long-term chemotherapeutic treatments may protect against further insults by tipping the balance in favour of a pro-survival UPR (5). Cancer cells with constitutive activation of ER stress response and upregulation of ER chaperone have been indicated to be associated with therapeutic resistance (4,6). One of the ER chaperone proteins, prolyl 4-hydroxylase, beta polypeptide (P4HB), has been reported to have critical roles in TMZ resistance and contribute to glioma recurrence driven by ER stress response. Significant upregulation of P4HB expression was observed in patients with recurrent glioma, as well as in TMZ-resistant xenografts (7,8). P4HB gene silencing also enhanced cellular apoptosis in TMZ-resistant glioma cells (4). Furthermore, Xipell *et al* (9) demonstrated the adjuvant effect for TMZ treatment through targeting ER stress, where MGMT expression was reduced following a combinatorial treatment

regimen, suggesting a dual effect in sensitizing tumours to TMZ by mediating ER stress-induced apoptosis, as well as limiting the DNA damage repair by MGMT.

ER chaperones have emerged as predictive markers for treatment response. Given that P4HB is associated with glioma malignancy and TMZ resistance, the present study evaluated whether assessment of P4HB expression may provide valuable clinical information for predicting response to TMZ and patient survival, irrespective of the MGMT promoter methylation status. The potential application of P4HB as a novel biomarker to further stratify glioma into more clinically relevant entities was also discussed and highlighted.

Materials and methods

Study population. The present study was a retrospective analysis of 73 patients (age range, 6-75 years; mean age, 47.3±14.9 years; 61.6% males and 38.4% females) with gliomas [World Health Organization (WHO) grade I, 4.1%; WHO grade II, 17.8%; WHO grade III, 19.2% and WHO grade IV, 58.9%]. All patients recruited in the study received the standard of care, i.e. maximal surgical resection followed by adjuvant radiation with concurrent and adjuvant TMZ, except those labelled as 'patients without TMZ treatment', who had undergone maximal surgical resection and were treated with radiotherapy without TMZ from the same cohort. Clinical specimens were collected from Queen Mary Hospital, Hong Kong, between February 2001 and February 2012. Clinical data were retrieved for statistical analysis, including patients' demographic data, tumour characteristics (i.e. lesion sites, pathological classification, WHO grade), treatment approaches after surgical resection (radiotherapy and/or chemotherapy), number of TMZ cycles received (≤6 or >6) and MGMT promoter methylation status, as well as OS and progression-free survival (PFS) duration. OS is defined as the time from initial pathological diagnosis to death or to the last contact if the patient was alive or the last day of the study period. PFS is defined as the time from initiation of treatment to the occurrence of disease progression or death. All patients had their diagnosis confirmed by a pathology specialist. The study was approved by the Institutional Review Board of The University of Hong Kong, Hong Kong west cluster and all tissues were collected with written informed consent from the patients.

P4HB immunohistochemical analysis. Immunohistochemical staining procedures were performed according to the protocol described in a previous study by our group (8). Paraffin sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Heat-induced antigen retrieval was performed with citrate buffer (pH 6.0; MilliporeSigma) for 10 min in a microwave at high power and endogenous tissue peroxidase in the sections was quenched with 3% hydrogen peroxide for 20 min at room temperature. After blocking with 5% normal goat serum (Dako; Agilent Technologies, Inc.) for 30 min at room temperature, sections were immunostained with rabbit monoclonal anti-P4HB (cat. no. 3501; 1:1,000 dilution; Cell Signaling Technology Inc.) at 4˚C overnight. After incubation with HRP-conjugated antibody (Zymed; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, signals were detected using an EnVision™+ Kit (Dako; Agilent Technologies, Inc.). P4HB expression was assessed semi-quantitatively by two experienced pathologists. A total of 10 random high-power fields were examined under a light microscope (Olympus Corporation). The staining intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The proportional of stained tumour cells was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). Multiplication of these two variables was performed to calculate the final score. Samples were considered positive if the score exceeded the median value.

Statistical analysis. SPSS 18.0 (SPSS, Inc.) was used for statistical analysis. The difference between two sets of data was analyzed by the χ^2 test, Mann-Whitney U-test (non-parametric test) or Fisher's exact test. The Kruskal-Wallis test was used to compare differences among more than two groups of data. In the Kaplan-Meier survival analysis, patients were dichotomized into two groups and survival differences between groups were assessed by the log-rank test. Risk factors for mortality after treatment were identified by univariate and multivariate analysis using the Cox proportional hazards regression model. All statistical tests were two-sided and $P<0.05$ was considered to indicate statistical significance.

Results

P4HB levels in glioma. P4HB has previously been identified by our group to be dysregulated in high-grade glioma (8). In the present study, its association with other clinicopathological parameters was further analyzed in 73 patients with glioma with complete clinical follow-up data using immunohistochemical staining. Consistent with the previous findings by our group, P4HB expression was significantly associated with the WHO grade (P=0.002; Table I). WHO grade IV glioma had the highest expression of P4HB among all WHO grades of glioma (Fig. 1A). The χ^2 test revealed that high P4HB expression levels were significantly associated with a patient age of >55 years (P=0.035). Fisher's exact test further indicated that in patients who received radiotherapy (P=0.015) and chemotherapy (P=0.050), significant differences between $P4HB^{Low}$ and $P4HB^{High}$ groups were present (Table I).

Prognostic value of P4HB in glioma. To explore the prognostic value of P4HB in glioma, patients were stratified into two groups by transforming the continuous variable of P4HB immunohistochemical scoring into a categorical variable [$P4HB^{Low}$ (score values ranging from 0 to <1) and $P4HB^{High}$ groups (score values ranging from ≥1-12)], based on the median value of the study samples. According to Kaplan-Meier analysis, patients of the two groups differed significantly in terms of OS duration; the median survival of patients with a high level of P4HB was ~16 months, significantly shorter than that of patients with a lower level of P4HB (131 months; log-rank P=0.015; Fig. 1B). To avoid potential bias due to relatively low P4HB expression in less malignant gliomas and to identify a meaningful subgroup for the clinical predictive value of P4HB, only WHO grades III and IV were included in the subsequent analysis (n=57). Similar

Table I. Association of P4HB expression with the clinical and molecular characteristics in high-grade glioma (n=57).

Characteristic	Cases	P4HB		P-value
		Low	High	
Sex				0.162
Female	18 (31.6)	9 (50.0)	9 (50.0)	
Male	39 (68.4)	12 (30.8)	27 (69.2)	
Age at diagnosis, years				0.035
≤55	36 (63.2)	16 (44.4)	20 (55.6)	
>55	21 (36.8)	5 (23.8)	16 (76.2)	
Tumour location				0.352
Frontal	24 (42.1)	9 (37.5)	15 (62.5)	
Temporal	13 (22.8)	3 (23.1)	10 (76.9)	
Parietal	4 (7.0)	3 (75.0)	1 (25.0)	
Occipital	2 (3.5)	0 (0.0)	2 (100.0)	
Multiple	14 (24.6)	6 (42.9)	8 (57.1)	
WHO grade				0.002
III	14 (24.6)	10 (71.4)	4 (28.6)	
IV	43 (75.4)	11 (25.6)	32 (74.4)	
Nature of glioma				0.179
Primary	51 (89.5)	17 (33.3)	34 (66.7)	
Recurrent	6 (10.5)	4 (66.7)	2 (33.3)	
Radiotherapy				0.015
Yes	53 (93.0)	17 (32.1)	36 (67.9)	
No	4 (7.0)	4 (19.0)	0 (0.0)	
Chemotherapy				0.050
Yes	44 (77.2)	13 (29.5)	31 (70.5)	
No	13 (22.8)	8 (61.5)	5 (38.5)	
MGMT expression status				0.596
Negative	20 (39.2)	5 (25.0)	15 (75.0)	
Positive	23 (45.1)	9 (39.1)	14 (60.9)	
Undetermined	8 (15.7)	3 (37.5)	5 (62.5)	
TMZ cycles				0.053
≤6	8 (18.2)	0 (0.0)	8 (100.0)	
>6	36 (81.8)	13 (36.1)	23 (63.9)	

Values are expressed as n (%). P4HB, prolyl 4-hydroxylase beta polypeptide; MGMT, O6-methylguanine-DNA methyltransferase; TMZ, temozolomide; WHO, World Health Organization.

results were obtained, suggesting that high P4HB predicted unfavorable survival and *vice versa* ($P4HB^{Low}$, 20 months; $P4HB^{High}$, 13 months; log-rank P=0.014; Fig. 1C).

To further investigate the prognostic value of P4HB in the prediction of PFS, Kaplan-Meier survival analysis was performed in the same dataset of WHO III and IV gliomas. The results demonstrated that the subgroup with low P4HB expression had a longer time to progression, whereas that with high P4HB had a shorter time to progression ($P4HB^{Low}$, 12 months; $P4HB^{High}$, 6 months; log-rank P=0.026; Fig. 1D).

P4HB predicts survival in TMZ-treated population. Whilst several chemotherapeutic agents are available for the treatment of glioma (10), TMZ is the most widely used and is a Food and Drug Administration-approved first-line chemotherapeutic (11,12). In the present dataset, patients who received TMZ exhibited longer OS (20 months) than those who did not (11 months; P=0.001; Fig. 2A). Patients who were on TMZ treatment expressed a significant level of P4HB compared to those who did not receive TMZ (P=0.045; Fig. 2B). The patients were then stratified into those who had received TMZ treatment and those who had not in order to determine the prognostic value of P4HB with regard to TMZ treatment. Among the patients who had received TMZ, a high level of P4HB expression was observed to be significantly associated with shorter OS as compared with a lower level of P4HB expression (P=0.014; Fig. 2C), whereas no such association was found in patients

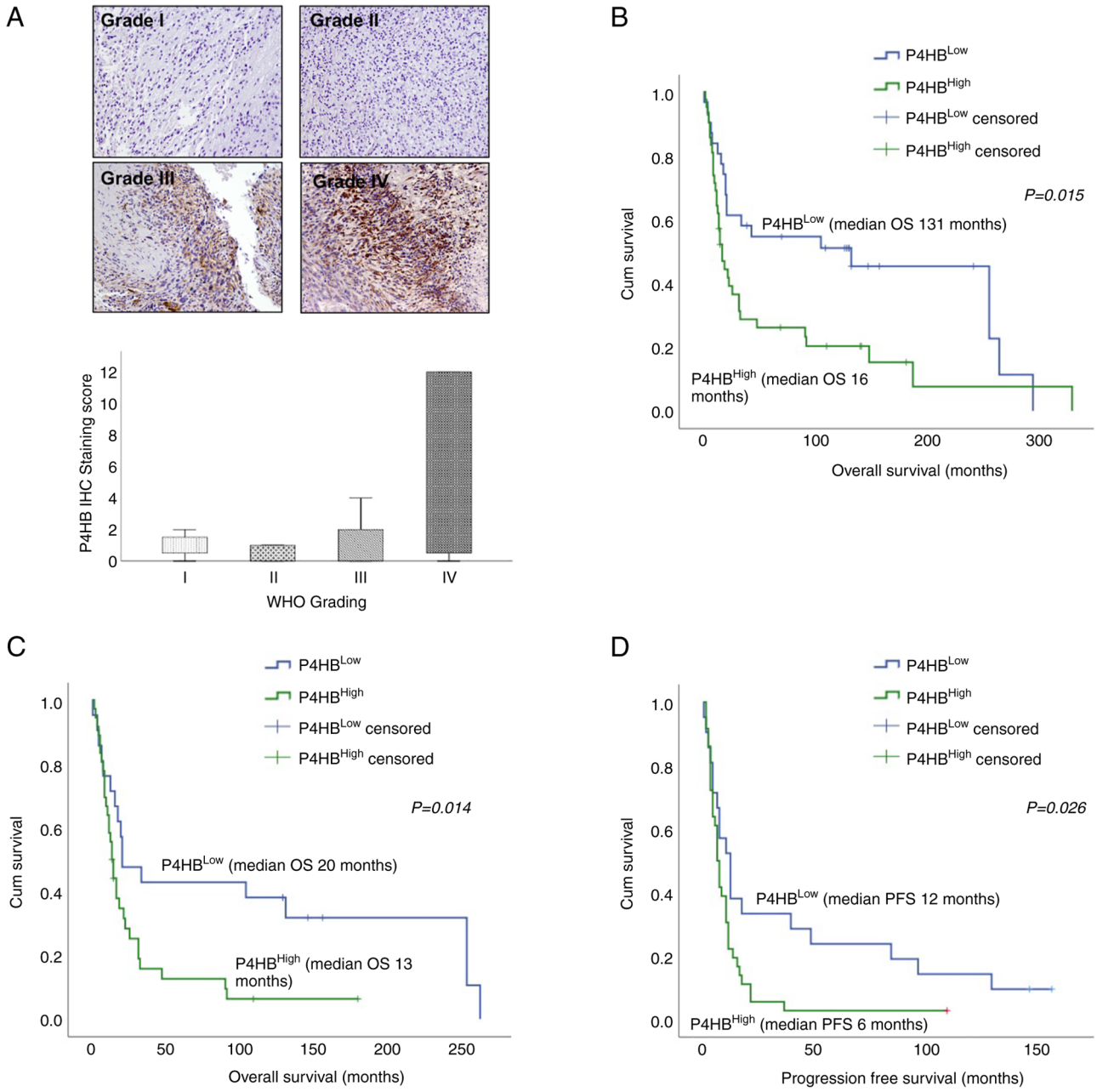

Figure 1. High P4HB expression is associated with poor outcomes for patients with glioma in a clinical cohort. (A) Representative images of P4HB immunohistochemical staining in grade I to IV gliomas (original magnification, x200), and quantification of staining intensity from 73 samples. It was indicated that P4HB expression was significantly upregulated with the ascending tumour grade. Values are expressed as the median (interquartile range) and error bars indicate the 95% confidence intervals for the median with the percentile method. The Kruskal-Wallis test was used to compare medians between groups (P=0.0001). (B-D) Kaplan-Meier survival analysis after classifying patients into $P4HB^{Low}$ and $P4HB^{High}$ groups based on immunohistochemical staining scores. Overall survival prediction in (B) All WHO grade gliomas (n=73) and (C) WHO grade III and IV gliomas (n=57). (D) Progression-free survival prediction in WHO grade III and IV gliomas (n=57). The log-rank test was performed to determine the P-value indicated on the graphs. P4HB, prolyl 4-hydroxylase beta polypeptide; WHO, World Health Organization; cum, cumulative; OS, overall survival; PFS, progression-free survival.

who received no TMZ therapy, suggesting a potential relationship between TMZ chemotherapy response and P4HB expression (Fig. 2D). The same was observed with regard to PFS: High P4HB expression was associated with shorter PFS (8 months) compared to low P4HB expression in patients treated with TMZ (39 months; P=0.027), but not in those who did not receive TMZ therapy (Fig. 2E and F). These results are suggestive of a prominent predictive impact of P4HB in a subset of patients with high-grade glioma who received TMZ therapy.

P4HB predicts survival in patients with methylated MGMT. MGMT is a DNA repair enzyme that is able to effectively reverse DNA damage induced by TMZ, and the MGMT promoter methylation status has a significant impact on survival of patients with glioma. A high level of MGMT activity may render tumours resistant to alkylating agents and may therefore serve as both a predictive and prognostic molecular marker in high-grade glioma (3). A significant difference in OS was determined between patients with tumours exhibiting MGMT methylation and those without,

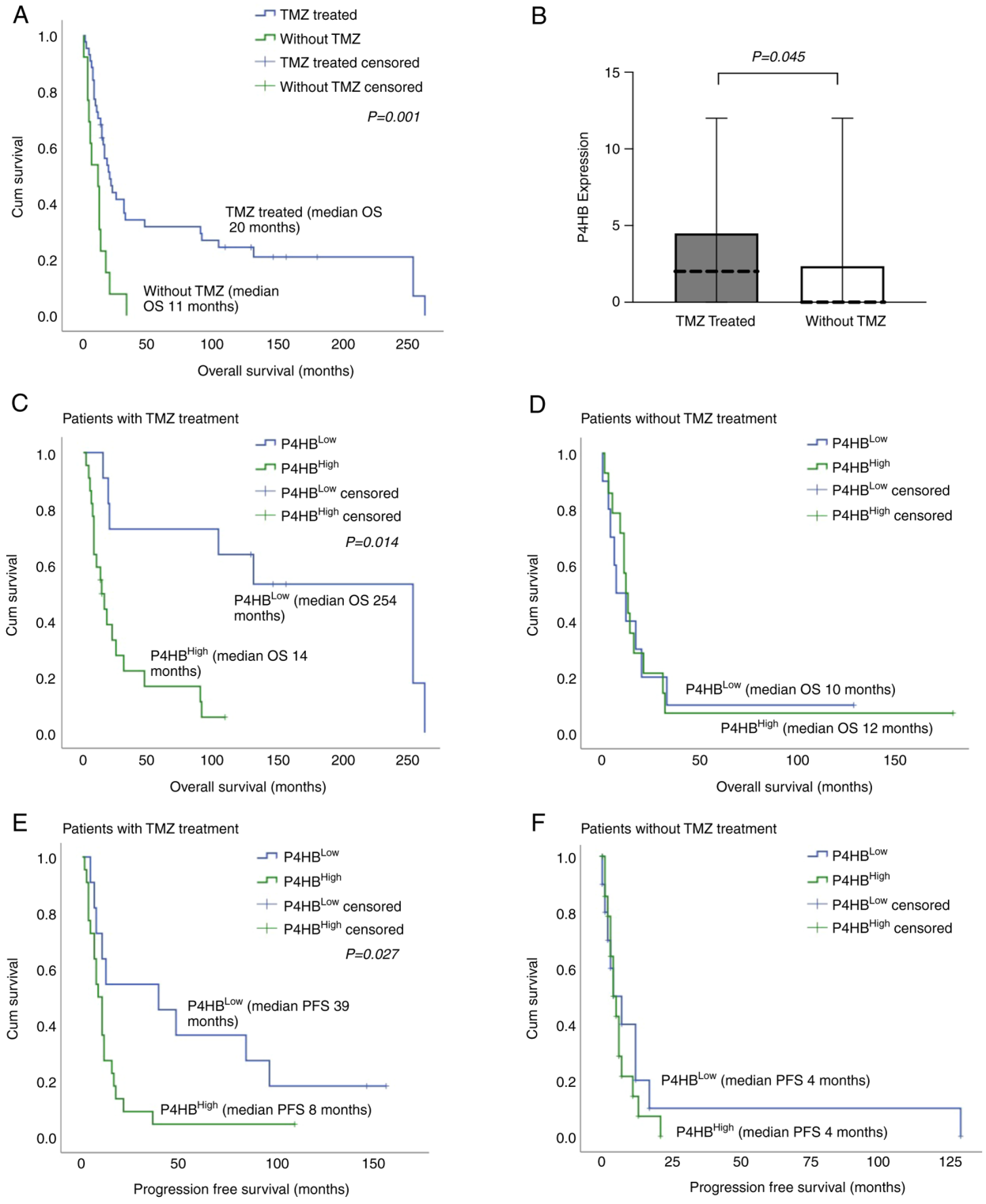

Figure 2. P4HB predicts survival in a TMZ-treated population. (A) Kaplan-Meier survival curves indicated that patients who received TMZ chemotherapy had better OS than patients who did not receive TMZ (P=0.001). (B) The use of TMZ chemotherapy was significantly associated with higher P4HB expression (P=0.045). Values are expressed as the mean and range, with the median value indicated by the dashed line. (C) Kaplan-Meier curves for a TMZ-treated population indicated that the OS outcome in the $P4HB^{Low}$ group was better than that in the $P4HB^{High}$ group. (D) In patients without TMZ treatment, no significant difference in OS was observed. Regarding PFS, it was indicated that (E) among patients who received TMZ treatment, those in the $P4HB^{Low}$ group had better PFS than those in the $P4HB^{High}$ group, (P=0.027), whereas (F) no significant difference in PFS was demonstrated between the $P4HB^{Low}$ and $P4HB^{High}$ groups in a non-TMZ treated population. P4HB, prolyl 4-hydroxylase beta polypeptide; TMZ, temozolomide; cum, cumulative; OS, overall survival; PFS, progression-free survival; $P4HB^{Low}$, low P4HB expression.

regardless of treatment received in this subset of patients with high-grade glioma ($MGMT^{Meth}$, 47 months; $MGMT^{Unmeth}$, 16 months; log-rank P=0.002; Fig. 3A). The association between the MGMT methylation status and P4HB expression was then investigated. A significant difference in OS was observed between the $P4HB^{Low}$ (254 months) and $P4HB^{High}$

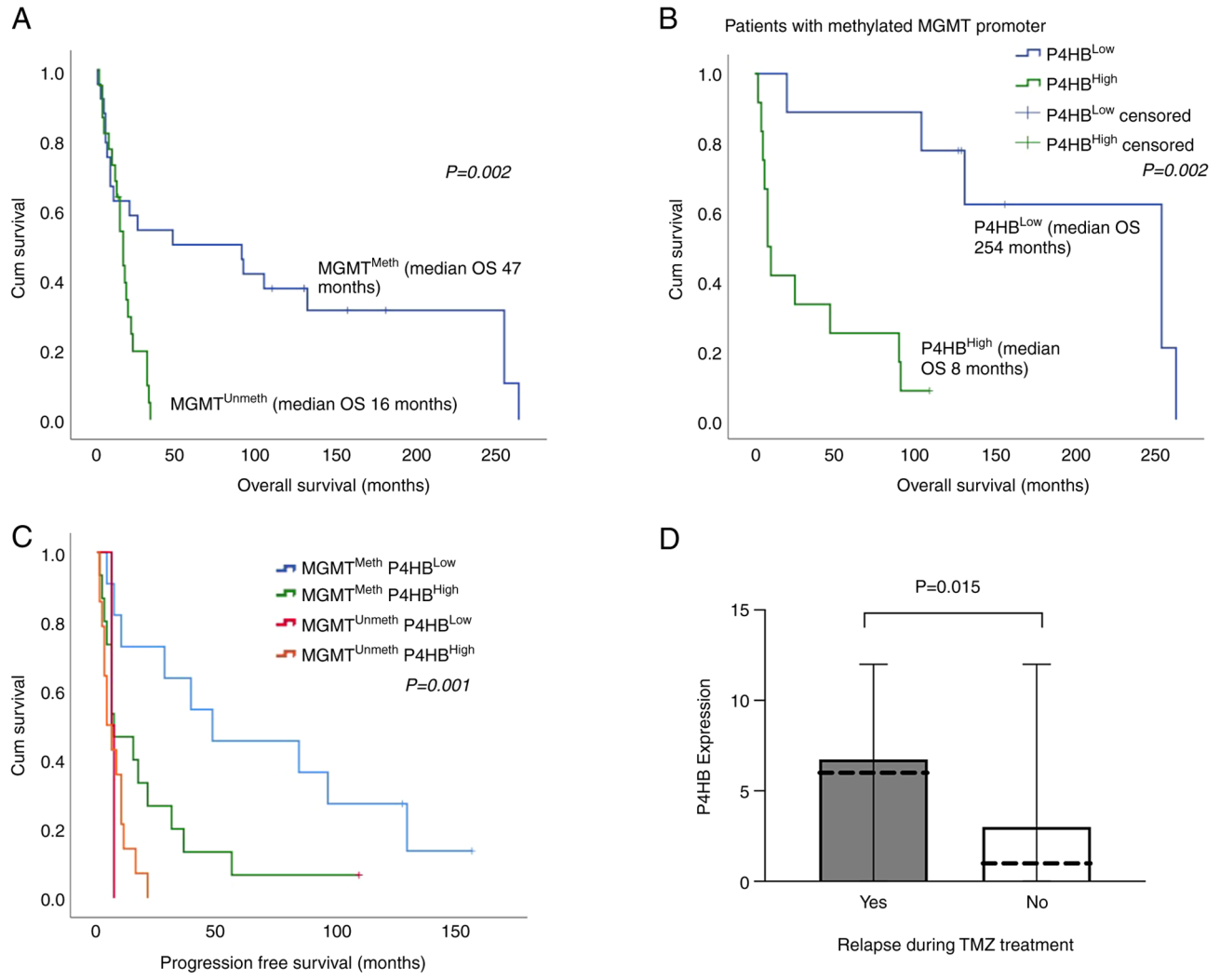

Figure 3. P4HB predicts TMZ treatment response in patients with methylated MGMT. (A) Kaplan-Meier survival curves indicated that patients who exhibited methylated MGMT in their tumours had better OS outcomes than patients who had unmethylated MGMT in their glioma lesions (P=0.002). (B) In patients with methylated MGMT, $P4HB^{Low}$ predicted better OS outcomes compared to the $P4HB^{High}$ group (P=0.002). (C) Progression-free survival analysis suggested that an $MGMT^{Meth}$ $P4HB^{Low}$ status was associated with the best survival outcome among the different combinations of MGMT methylation status and P4HB expression (P=0.001). (D) High P4HB expression was significantly associated with an increased number of patients relapsed during TMZ treatment (P=0.015). Values are expressed as the mean and range with the median value indicated by the dashed line. P4HB, prolyl 4-hydroxylase beta polypeptide; TMZ, temozolomide; MGMT, O6-methylguanine-DNA methyltransferase; OS, overall survival; $P4HB^{Low}$, low P4HB expression.

groups (8 months) of patients with methylated MGMT tumours (P=0.002; Fig. 3B), whereas patients with unmethylated MGMT tumours exhibited no significant difference in OS between P4HB subgroups (data not shown). Univariate analysis using the Cox proportional hazards model indicated that P4HB expression had a significant impact on OS of patients with methylated MGMT tumours [hazard ratio (HR)=4.261, 95% CI=1.312-13.846, P=0.016] but not in those with unmethylated MGMT lesions (HR=1.038, 95% CI=0.372-2.892, P=0.944; Table SI).

MGMT is a well-established biomarker for the prediction of TMZ treatment response (3) and high P4HB expression has previously been identified by our group to be associated with TMZ resistance both *in vitro* and *in vivo* (4). In the present study, the interrelationship between MGMT promoter methylation and P4HB expression was further investigated by Kaplan-Meier analysis of PFS. Patients were divided into four groups according to the expression of the two markers. Among the 42 TMZ-treated patients with high-grade glioma, $MGMT^{Meth}P4HB^{Low}$ was associated with the longest PFS (n=11; 48 months), followed by $MGMT^{Meth}P4HB^{High}$ (n=15; 7 months), $MGMT^{Unmeth}P4HB^{Low}$ (n=2; 6 months) and $MGMT^{Unmeth}P4HB^{High}$ (n=14; 4 months; Fig. 3C). Results were statistically significant (P=0.001), suggesting that P4HB may be used to assist in the survival prediction of patients with methylated MGMT and by identifying certain patients who may respond poorly to TMZ.

P4HB in the prediction of response to TMZ in high-grade glioma. Since it has been observed that P4HB expression was significantly associated with both OS and PFS exclusively in patients treated with TMZ and that P4HB may have important roles in affecting glioma malignancy and TMZ resistance both *in vitro* and *in vivo* (8), the clinical significance of P4HB in predicting TMZ treatment response was further investigated.

Among the 57 patients with high-grade glioma, 37 patients had been treated with adjuvant TMZ, and 19 patients with high P4HB expression were indicated to have relapse during TMZ treatment. The latter group exhibited a mean P4HB expression score of 6.74, which was almost double that in those who exhibited favourable responses to TMZ (P4HB mean expression score, 3.25). High expression of P4HB was significantly associated with relapse during TMZ treatment (P=0.015; Fig. 3D).

P4HB is an independent prognostic factor for high-grade glioma. Clinicopathological factors affecting OS and PFS in patients with high-grade glioma were examined (Table II). Univariate analysis indicated that age at diagnosis (P=0.005), WHO grade (P=0.000), chemotherapy (i.e., TMZ) (P=0.001), MGMT methylation status (P=0.004) and P4HB expression level (P=0.018) were significantly associated with OS. Multivariate analysis using the Cox regression model demonstrated that not only WHO grade (P=0.002) and chemotherapy (TMZ) (P=0.001), but also P4HB expression levels were independent prognostic markers for OS (P=0.048). Patients with high P4HB expression also had less favorable PFS outcomes compared with those with low P4HB expression. On univariate analysis, WHO grade (P=0.007), MGMT (P=0.010), the number of TMZ cycles (P=0.020) and P4HB expression (P=0.035) were significant prognostic factors by PFS. Multivariate analysis indicated that among all prognostic factors, whilst P4HB expression did not reach statistical significance, it was the only factor with a marginal association with PFS, suggestive of its potential as an independent prognostic factor in terms of PFS (Table II).

Discussion

TMZ has been widely used for treating primary and recurrent high-grade gliomas. However, the efficacy of TMZ is frequently limited by the development of chemoresistance. Intertumoral heterogeneity among patients with glioma, such as epigenetic silencing of MGMT, is the most studied mechanism and is a promising predictive marker for TMZ response (13). Whilst the prognosis of patients with high-grade malignant glioma is generally poor (with median OS <16 months in the present cohort), it was observed that it is possible to further identify subgroups of patients with differing prognoses according to P4HB expression, on top of MGMT promoter methylation status. As a key member of the protein disulfide isomerases (PDI) family, P4HB acts as a chaperone mediator in the UPR and modulates ER stress response similar to glucose-regulated protein 78, which is a master regulator of UPR (14). Activation of UPR via inhibition of chaperone proteins has previously been identified to be associated with a reduction in DNA repair capacity (15) and may create vulnerabilities that sensitize aggressive tumours to cytotoxic drugs and prevent cancer progression and/or recurrence (16,17).

P4HB was previously identified by our group to possess oncogenic (pro-survival) properties in malignant glioma (7). PDI has recently been reported to be a promising target for survival prediction and tumour progression in glioma (18). However, the reported findings were limited to bioinformatics modeling of gene expression using datasets from The Cancer Genome Atlas and Chinese Glioma Genome Atlas databases; no clinical specimens were directly used in that study. In the present study, a glioma cohort comprising 73 patients was included to evaluate the prognostic significance of P4HB. The results were consistent with the literature that reported upregulation of P4HB in high-grade gliomas when compared to low-grade lesions. Furthermore, overexpression of P4HB was significantly associated with several clinical parameters, including older age and prolonged use of TMZ, which may be indicative of a more aggressive tumour that required extended treatment.

The present study indicated that P4HB expression may be used to predict OS and PFS in patients with malignant glioma treated with TMZ. The predictive value of P4HB was particularly prominent in subgroups of TMZ-treated malignant glioma populations as compared with those who did not receive TMZ. This may be explained by the poor pre-existing neurological or general condition of the patients who were not fit for chemotherapy, as the median OS in both $P4HB^{Low}$ and $P4HB^{High}$ patients in the non-TMZ-treated group were only 10 and 12 months, respectively. It is important to note that the median OS in $P4HB^{Low}$ patients was significantly longer (254 months) than in $P4HB^{High}$ patients (14 months), suggesting that tumours with low P4HB may be more responsive to TMZ treatment compared to tumours with P4HB upregulation. In other words, overexpression of P4HB may confer resistance to chemotherapies secondary to increased ER stress and UPR, which is in line with previous findings by our group. Under treatment with TMZ, the UPR signaling cascade is initiated upon activation of protein kinase RNA-like ER kinase, an ER stress sensor. Such perturbation on ER activates upregulation of different target genes, including ER chaperones and folding enzymes, in order to restore ER homeostasis (4). Indeed, aberrant expression of P4HB was previously reported to be associated with TMZ-resistant D54 and U87 glioma cell lines (7).

Furthermore, inhibition of P4HB was reported to be able to attenuate TMZ resistance (4), whereas overexpression promoted malignancy via MAPK signaling (8). Chronic TMZ treatment may also exacerbate drug resistance by further upregulating P4HB expression (4).

Of note, P4HB expression levels enabled the stratification of patients with methylated MGMT to guide the treatment by predicting the response to TMZ. Whilst the MGMT promoter methylation status retained significance in predicting treatment response, P4HB expression was observed to have additional value, when used alone or in combination with the MGMT methylation status, for identifying patients at risk of adverse health outcomes (19). $P4HB^{Low}$ in conjunction with $MGMT^{Meth}$ was associated with the most favorable prognosis regarding PFS in TMZ-treated patients with malignant glioma, whereas $P4HB^{High}$ $MGMT^{Unmeth}$ tumours were more likely to have suboptimal responses to TMZ. It is noteworthy that the MGMT promoter methylation status may be altered upon tumour recurrence. Kohsaka *et al* (20) demonstrated upregulation of MGMT protein expression during the acquisition of TMZ resistance in U87 glioma cells. Whilst it remains elusive whether this was the result of MGMT promoter unmethylation, the increase in MGMT expression during the course of TMZ treatment appeared to further reduce TMZ responsiveness.

Table II. Univariate and multivariate Cox regression analyses of OS and PFS in patients with high-grade glioma (n=57).

	OS				PFS			
	Univariate analysis	Multivariate analysis			Univariate analysis	Multivariate analysis		
Variable	P-value	HR	95% CI	P-value	P-value	HR	95% CI	P-value
Sex (female vs. male)	0.805	-	-	-	0.881	-	-	-
Age at diagnosis (≤55 vs. >55 years)	0.005	1.093	0.528-2.260	0.811	0.084	-	-	-
WHO grade (III vs. IV)	0.000	7.881	2.179-28.504	0.002	0.007	1.657	0.659-4.165	0.283
Radiotherapy (yes vs. no)	0.216	-	-	-	0.185	-	-	-
Chemotherapy (TMZ; yes vs. no)	0.001	5.688	2.132-15.174	0.001	0.089	-	-	-
MGMT expression (negative vs. positive)	0.004	1.282	0.562-2.923	0.555	0.010	1.497	0.626-3.579	0.365
TMZ cycles (≤6 vs. >6)	0.688	-	-	-	0.020	0.579	0.215-1.563	0.281
P4HB expression (low vs. high)	0.018	2.377	1.008-5.605	0.048	0.035	2.511	0.926-6.809	0.070

P4HB, prolyl 4-hydroxylase beta polypeptide; MGMT, O6-methylguanine-DNA methyltransferase; TMZ, temozolomide; WHO, World Health Organization; OS, overall survival; PFS, progression-free survival; HR, hazard ratio.

It is a common observation that initially, TMZ-sensitive malignant glioma may eventually become resistant, partly due to the restoration of MGMT activity (21). The present findings suggest that P4HB expression may be used to identify tumours likely to benefit from prolonged TMZ treatment even before recurrence. For recurrent diseases, rechallenge with TMZ is a commonly adopted strategy but no reliable predictive factors have been identified so far (22). It may be surmised that P4HB expression may be used to identify recurrent tumours that may still respond favourably to TMZ rechallenge. The present findings also have the potential for informing future treatment paradigms. While the stratification of patient subgroups by P4HB was primarily based on the MGMT methylation status, the clinical significance of P4HB may be limited by the small cohort size and missing clinical parameters, such as extent of resection and other prognostic markers, including isocitrate dehydrogenase mutation status. Future studies may also include molecular investigations to determine drug response upon P4HB inhibition in glioma.

In conclusion, P4HB is a component of UPR that has important roles in mediating glioma survival, therapeutic resistance and tumour progression. In this context, P4HB expression has been indicated to be significantly associated with PFS and OS in patients with malignant glioma and may be used as an independent prognostic marker. P4HB may be used on its own or in combination with MGMT to stratify patients who are good responders to glioma therapeutics. Furthermore, P4HB expression may also inform a more nuanced approach to the use of an extended TMZ regimen as well as TMZ rechallenge. Future research may be conducted in a larger and standardized cohort, and evaluate the association between P4HB expression and other important biomarkers, which eventually give rise to fruitful clinical translations.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SS collected the data, performed statistical analysis and wrote the manuscript. KK participated in the study design and wrote the manuscript. GL conceived the study and contributed to the critical revision. SS, KK and GL confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Human tissue specimens were obtained with informed consent from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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