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ORIGINAL ARTICLE

Clinical and Pathological Significance of ER Stress Marker (BiP/GRP78 and PERK) Expression in Malignant Melanoma

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Abstract Glucose-regulated protein of 78 kD (GRP78) also referred to as immunoglobulin heavy chain binding protein (BiP/GRP78) plays an important role in the endoplasmic reticulum (ER) stress. The level of BiP/GRP78 is highly elevated in various human cancers. The purpose of this study is to examine the prognostic significance of BiP/GRP78 expression in patients with malignant melanoma. A total of 133 malignant melanoma patients were analyzed, and tumor specimens were stained by immunohistochemistry for BiP/GRP78, PKR-like endoplasmic reticulum kinase (PERK), Ki-67, p53 and microvessel density (MVD) determined by CD34. BiP/GRP78 and PERK were highly expressed in 40 % (53/133) and 78 % (104/133), respectively. BiP/GRP78 disclosed a significant relationship with PERK expression, thickness, T factor, N factor, disease staging, cell proliferation (Ki-67) and MVD (CD34). By multivariate analysis, the high expression of BiP/GRP78 was identified as an independent prognostic factor for predicting poor survival against malignant melanoma. The increased BiP/GRP78 expression was clarified as an independent prognostic marker for predicting worse outcome. ER stress marker, BiP/GRP78 could be a powerful molecular target for the treatment of malignant melanoma.

Keywords ER stress · BiP/GRP78 · PERK · Prognosis · Malignant melanoma · Skin cancer · Immunohistochemistry

Introduction

Malignant melanoma is a most deadly disease of skin cancer with a steadily rising incidence and poor outcome in the advanced stage [1]. There is a critical need to clarify clinicopathological biomarker to predict the outcome of those patients. Several clinicopathological variables such as increased Breslow thickness, Clark level, presence of ulceration and increased number of mitoses, have been identified as prognostic significance in malignant melanoma [2]. However, the assessment of molecular biomarkers which influence the tumor progression and metastases of melanoma, could be useful to identify the patients with poor survival and could improve clinical management of patients with malignant melanoma.

The glucose-regulated protein GRP78, a 78-kDa protein, also referred to as immunoglobulin heavy chain binding protein (BiP/GRP78), is a major molecular chaperone in the endoplasmic reticulum (ER) [3]. BiP/GRP78 is involved in the folding and assembly of newly synthesized proteins in the ER and increases resistance to ER-stress-induced apoptosis [3–5].

The level of BiP/GRP78 is highly elevated in many cancer cells and human cancers, and also closely associated with metastases and resistance to chemotherapy [4, 5]. There have been only a few studies about the prognostic significance of BiP/GRP78 for various patients with breast cancer, lung cancer, gastric cancer, hepatocellular cancer or prostate cancer [5].

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Recently, several researchers had described that the expression level of BiP/GRP78 is closely associated with tumor aggressiveness and survival in patients with malignant melanoma [6–9]. In two reports, the increasing expression of BiP/GRP78 correlated with tumor progression and poor survival in patients with malignant melanoma [6, 7]. However, little is known about the relationship between BiP/GRP78 expression and progression markers such as angiogenesis, tumor cell proliferation and cell cycle, and it remains unclear whether a high expression of BiP/GRP78 could be an independent factor to predict poor outcome in patients with malignant melanoma. In in vitro study, Dong et al. showed that BiP/GRP78 is a significant mediator of angiogenesis by regulating cell proliferation, survival and metastasis [8].

PKR-like endoplasmic reticulum kinase (PERK) is considered to be sensors of ER stress [10]. It has been reported that PERK induces apoptosis via CCAAT/enhancer-binding protein homologous protein (CHOP) accumulation under irremediable ER stress [11]. Vamdexynckel et al. documented that the PERK pathway was activated during tumor progression and proapoptotic target CHOP was upregulated, and a small molecule inhibitor of PERK could be a promising target for cancer therapy [10]. In human tissues, however, it remains unclear about the relationship between BiP/GRP78 and PERK.

Based on these backgrounds, we conducted the clinicopathological study to clarify the prognostic significance of BiP/GRP78 expression, in terms of PERK expression, angiogenesis and tumor proliferation.

Materials and Methods

Patients

We analyzed 156 consecutive patients with malignant melanoma who underwent surgical resection at Gunma University Hospital between September 1989 and October 2011. Twenty three patients were excluded, because the information of the patients was not available. In total, 133 patients were analyzed in the study. Clinical stages were defined according to the 2009 guidelines of the American Joint Committee on Cancer (AJCC). This study was approved by the institutional review board of Gunma University Hospital (ethical committee for clinical studies-Gunma University faculty of Medicine). The authors' approach to the evaluation and resection of these tumors has been described previously [12].

Immunohistochemical Staining

BiP/GRP78 and PERK were detected using rabbit monoclonal antibodies (both Cell Signaling Technology, Danvers, MA, 1:100 dilution) as described previously. The BiP/GRP78 and PERK expression scores, on a scale from 1 to 5, were assessed as follows: 1, ≤ 10 % of tumor area stained; 2, 11–25 % stained; 3, 26–50 % stained; 4, 51–75 % stained; and 5, ≥76 % stained. Tumors scoring 3, 4 or 5 were defined as high-expression tumors.

For CD34, Ki-67 and p53, immunohistochemical staining was performed according to the procedures described in previous reports [12, 13]. The following antibodies were used: mouse monoclonal antibodies against CD34 (Nichirei, Tokyo, Japan, 1:800 dilution), Ki-67 (Dako, Glostrup, Denmark, 1:40 dilution), and p53 (D07; Dako, 1:50 dilution). The number of CD34-positive vessels was counted in four selected hot spots in a × 400 field (0.26 mm^2 field area). Microvessel density (MVD) was defined as the mean count of microvessels per 0.26 mm^2 field area. The median number of CD34-positive vessels was evaluated, and the tumours in which stained tumour cells made up more than each median value were defined as high expression. For p53, microscopic examination for the nuclear reaction product was performed and scored, and p53 expression in greater than 10 % of tumour cells was defined as positive expression. For, Ki-67, a highly cellular area of the immunostained sections was evaluated. All epithelial cells with nuclear staining of any intensity were defined as high expression. Approximately 1000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median value of the Ki-67 labeling index was evaluated, and the tumour cells with greater than the median value were defined as high expression. The sections were assessed using a light microscopy in a blinded fashion by at least two of the authors.

Statistical Analysis

Probability values of <0.05 indicated a statistically significant difference. The significance of difference was determined by Fisher's exact test. The correlation between different variables was analyzed using the nonparametric Spearman's rank test. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Overall survival (OS) was determined as the time from tumour resection to death from any cause. Progression-free survival (PFS) was defined as the time between tumour resection and the first disease progression or death. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using GraphPad Prism 4 software (Graph Pad Software, San Diego, CA, USA) and JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patient's Demographics

One hundred thirty-three patients with malignant melanoma were analyzed. Clinicopathologic results stratified by BiP/GRP78 expression are listed in Table 1. The age of the patients ranged from 42 to 86 years, and the median age was 71 years. Most tumors (n = 126, 90.6 %) were pathological stages I to III. Eighty-eight patients had received postoperative adjuvant chemotherapy. The day of surgery was considered the starting day for measuring postoperative survival. A median follow-up duration for all patients was 1725 days (range, 30 to 7404 days).

Immunohistochemical Assessment

The immunohistochemical staining was done on the 133 primary lesions with malignant melanoma. Figure 1 represents the immunohistochemical staining of BiP/GRP78 in malignant melanoma. The BiP/GRP78 immunostaining was detected in melanoma cells in tumor tissues and localized predominantly on their cytoplasmic and plasma membrane. BiP/GRP78 and PERK were highly expressed in 40 % (53/133) and 78 % (104/133), respectively. We previously reported the results of immunohistochemical staining of Ki-67, CD34 and p53 in malignant melanoma [12]. The cutoff points for high CD34 expression and high Ki-67 labeling index were defined as follows. The median number of CD34-positive vessels was 4 (range, 0–90), and the value of 4 was chosen as a cutoff point. The median value of the Ki-67 labeling index was 10 % (range, 0–47), and the value of 10 % was chosen as cutoff point. Positive expression of p53 was recognized in 73 % (97/133).

Table 1 shows patient's demographics according to BiP/GRP78 expression status. The expression of BiP/GRP78 was significantly associated with tumor thickness, T factor, N factor, disease stage, the expression of PERK, cell proliferation (Ki-67) and MVD (CD34).

Correlation between BiP/GRP78 and Different Variables

Spearman's rank correlation revealed that BiP/GRP78 expression was significantly correlated with PERK (r = 0.273, p = 0.001), Ki-67(r = 0.414, p = 0.001), CD34(r = 0.553, p < 0.001) and tumor size (r = 0.235, p = 0.006). (Table 2).

Survival Analysis According to BiP/GRP78 Expression

The five-year survival rates of OS and PFS for all patients were 75 % and 65 %, respectively. Of 133 patients, 36 were died and 50 had a recurrence after initial surgery. By univariate analysis, age, tumor thickness, ulceration, disease stage, BiP/GRP78, Ki-67 and CD34 had a significant relationship with overall and progression-free survival (Table 3). Multivariate analysis confirmed that BiP/GRP78 was an independent prognostic factor for predicting worse OS and PFS after surgery in patients with malignant melanoma. Figure 2 shows the Kaplan-Meier survival curve in patients with high and low expression for BiP/GRP78.

Discussion

This is a clinicopathological investigation to assess the prognostic significance of BiP/GRP78 expression in malignant

Table 1 Patient's demographics according to BiP/GRP78 expression

	Variables	Total	BiP/GRP78		
		(n = 133)	High (n = 53)	Low (n = 80)	p-value
Age	≤65 / > 65 yr	67/66	23/30	44/36	0.217
Sex	Male / female	67/66	30/23	37/43	0.289
Thickness, mm	≤2.00 / > 2.00	70/63	16/37	54/26	**<0.001**
Ulceration	Yes / No	21/112	11/42	10/70	0.229
T factor	T1–2 / T3–4	71/62	15/38	56/24	**<0.001**
N factor	No / N1–2	100/33	33/20	67/13	**0.007**
Disease stage	I or II / III or IV	93/40	31/22	62/18	**0.022**
Anatomic site	Axial / Extremity	36/97	11/42	25/55	>0.999
Tumor size, mm	≤20 / > 20	72/61	25/28	47/33	0.215
PERK	High/ Low	104/29	48/5	56/24	**0.005**
Ki-67	High / Low	62/71	35/18	27/53	**<0.001**
CD34	High / Low	65/68	42/11	23/57	**<0.001**
p53	High / Low	97/36	43/10	54/26	0.111

Bold character showing statistical significance

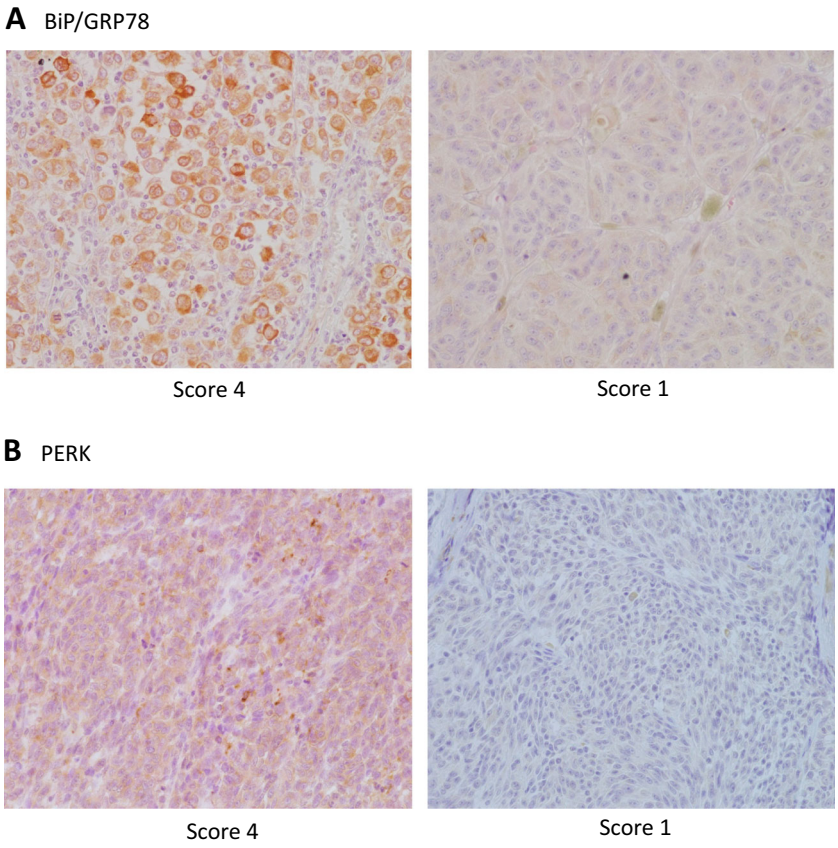

Fig. 1 Immunohistochemical staining of BiP/GRP78 in malignant melanoma. BiP/GRP78 and PERK expressions in malignant melanoma with a reactivity score of grade 4 and 1 are shown in (**a**) and (**b**), respectively. Positive staining of BiP/GRP78 (**a**) and PERK (**b**) expression observed in the cytoplasmic and plasma membrane of tumor cells

melanoma. The increased expression of BiP/GRP78 was elucidated to be an independent prognostic factor for predicting poor survival after surgery, and yielded a significant association with tumor aggressiveness, cell proliferation and angiogenesis. Although the expression of PERK was not identified as a novel prognostic factor in melanoma patients, there was a close correlation between BiP/GRP78 and PERK within tumor tissues. In previous reports [6, 7], however, the expression level of BiP/GRP78 did not correlate with tumor progression and survival of melanoma, and other biomarker such as Ki67, MVD and PERK. The discrepancy may be explained by a small sample size <100 patients and/or different assessment of BiP/GRP78 expression. Further study is warranted to confirm the results of our study by a large-scale study.

Table 2 Correlation with BiP/GRP78 expression

	Spearman r	95 % CI	*p*-value
PERK	0.273	0.102 to 0.428	**0.002**
Ki-67	0.414	0.256 to 0.551	**<0.001**
CD34	0.553	0.417 to 0.664	**<0.001**
Tumor size	0.235	0.062 to 0.394	**0.006**

Bold character showing statistical significance

Abbreviation: 95 % *CI*, 95 % confidence interval

Recently, it has been described that BiP/GRP78 is antiapoptotic and plays an important cytoprotective role in oncogenesis [5]. There is a contraversial discussion about the prognostic significance of BiP/GRP78 expression in various human neoplasms [5]. A high BiP/GRP78 expression in patients with hepatocellular, gastric, prostate and renal cell carcinoma was a worse prognostic factor, while a low BiP/GRP78 expression yielded an unfavorable survival in esophageal and lung cancer [5]. In in vitro studies, it has been described that BiP/GRP78 is required for the tumor progression and highly metastatic cancer cell lines revealed high expression level of BiP/GRP78 [14].

In an experimental study using melanoma model, BiP/GRP78 has been shown to be an important mediator for endothelial cell proliferation, survival and migration and be required in the microenvironment during tumor angiogenesis by MVD and cell proliferation [8]. These results suggest that a novel therapeutic agent against BiP/GRP78 provide a promising strategy to suppress cancer initiation, metastasis and progression [8]. In our study, we found that the expression of BiP/GRP78 was closely associated with tumor cell proliferation and angiogenesis by MVD in human malignant melanoma tissues, being consistent with those of previous experimental studies.

The expression of BiP/GRP78 was significantly associated with PERK expression in our study. Although PERK may play an important role in tumor progression to protect

Table 3 Univariate and multivariate survival analysis in all patients

Variables		Overall survival					Progression-free survival				
		Univariate		Multivariate			Univariate		Multivariate		
		5-yrs rate (%)	*p*-value	HR	95 % CI	*p*-value	5-yrs rate (%)	*p*-value	HR	95 % CI	*p*-value
Age	≤65 / > 65 yr	81 / 64	**0.026**	1.650	0.828–3.424	0.156	75 / 54	**0.023**	1.536	0.857–2.823	0.15
Sex	Male / female	71 / 58	0.475	1.087	0.548–2.121	0.807	62 / 68	0.649	1.178	0.665–2.075	0.57
Thickness, mm	≤2.00 / > 2.00	94 / 52	**<0.001**				88 / 39	**<0.001**			
Ulceration	Yes / No	27 / 81	**<0.001**				28 / 71	**<0.001**			
Disease stage	I or II / III or IV	85 / 52	**<0.001**	2.629	1.300–5.352	**0.007**	77 / 38	**<0.001**	2.784	1.541–5.055	**<0.001**
Anatomic site	Axial / Extremity	78 / 73	0.905				65 / 64	0.829			
Tumor size, mm	≤20 / > 20	82 / 66	0.123				71 / 57	0.194			
BiP/GRP78	High/ Low	61 / 84	**<0.001**	2.528	1.254–5.248	**0.009**	49 / 73	**<0.001**	2.094	1.160–3.822	**0.014**
PERK	High / Low	75 / 77	0.192				62 / 76	0.065			
Ki-67	High / Low	68 / 83	**0.02**				54 / 75	**0.006**			
CD34	High / Low	58 / 92	**<0.001**				40 / 91	**<0.001**			
p53	High / Low	75 / 76	0.275				66 / 63	0.514			

Bold character showing statistical significance

Abbreviation: *HR* hazard ratio; 95 % *CI*, 95 % confidence interval

cells from ER stress, it remains unclear whether PERK expression could be prognostic factor in patients with malignant melanoma.

The relationship between BiP/GRP78 expression and drug resistance, recurrence and survival has been reviewed previously [5]. In locally advanced rectal cancer, high expression of

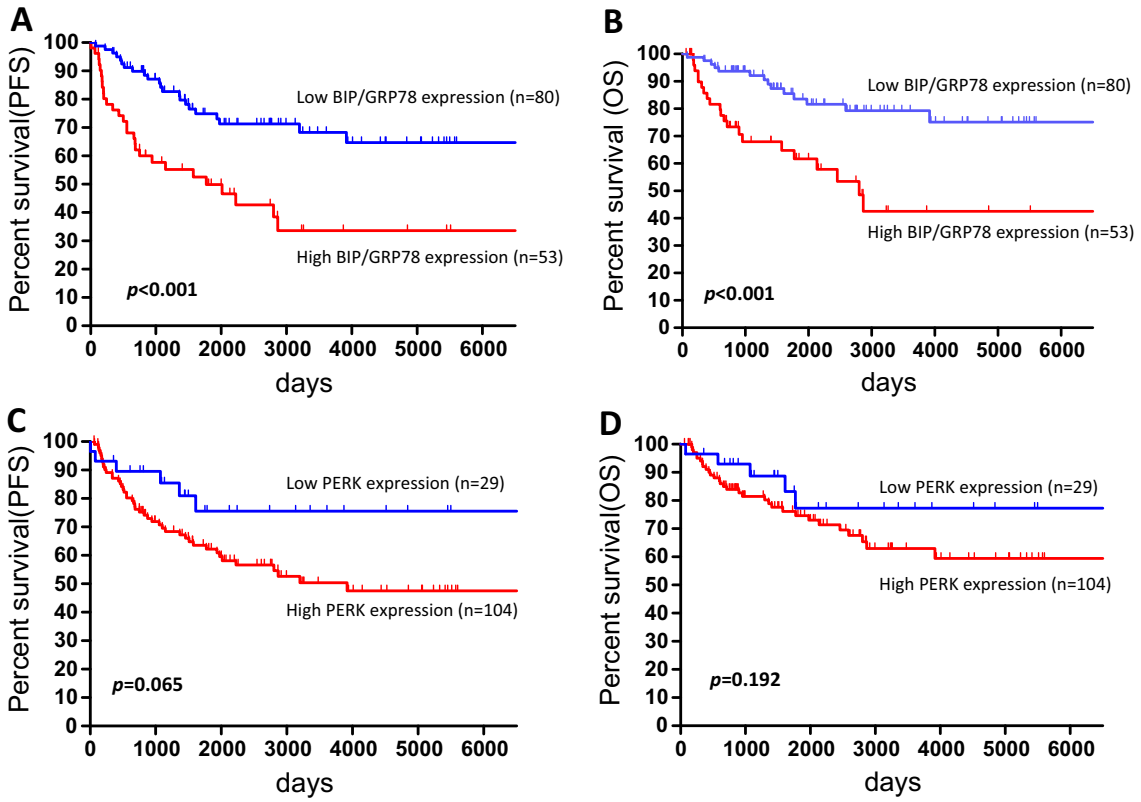

Fig. 2 Outcomes after surgical resection shown by Kaplan-Meier analysis of progression-free survival (PFS) and overall survival (OS) according to BiP/GRP78 expression. A statistically significant difference in PFS (**a**) and OS (**b**) was observed between patients with high and low BiP/GRP78 expression No statistically significant difference in PFS (**c**) and OS (**d**) was observed between patients with high and low PERK expression

BiP/GRP78 is related to poor responses to treatment and poor prognosis, suggesting a correlation between BiP/GRP78 and chemoresistance and radioresistance [15]. The down-regulation or inhibition of BiP/GRP78 activity may be a potential molecular target for the treatment of cancers.

Limitations of the current study must be addressed. One limitation is that the sample size in the current study was small, which may bias our results. Another limitation is that there is no optimal cut-off value to dichotomize the expression level of BiP/GRP78. Since the assessment of BiP/GRP78 protein expression has varied in each study, it remains unclear whether or not the semi-quantitative technique of our study is better compared to that of previous studies. Further study is warranted to find an optimal assessment of the expression of ER stress marker and evaluate a novel prognostic factor to predicting outcome in malignant melanoma using a large sample size.

In conclusion, BiP/GRP78 was highly expressed in patients with malignant melanoma, and fabricated a significant relationship with PERK expression, tumor thickness, disease stage, lymph node metastases, cell proliferation and angiogenesis. By multivariate analysis, the high expression of BiP/GRP78 was identified as an independent prognostic factor for predicting worse survival after surgery. ER stress marker, BiP/GRP78 could be a molecular target for the treatment of malignant melanoma.

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Compliance with Ethical Standards

Conflicts of Interest We (all authors) have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

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