SPANDIDOS PUBLICATIONS

Overexpression of papillomavirus binding factor in Ewing's sarcoma family of tumors conferring poor prognosis

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Abstract. Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults. Despite intensive treatments for patients with ESFT, disease which presents with metastatic spread or relapses after primary treatment remains incurable in the majority of cases, indicating the importance of efforts to develop new treatment modalities, including immunotherapy. The present study was designed to examine the expression profile of papillomavirus binding factor (PBF), which we previously defined as an osteosarcoma-associated antigen, and its prognostic significance for patients with ESFT. Biopsy specimens from 20 ESFT were stained with an anti-PBF antibody. Survival was estimated using Kaplan-Meier plots and the prognostic significance of several variables, including the expression status of PBF, on disease-free and overall survival was determined by univariate analysis using the log-rank test. Of 20 specimens, 18 (90%) reacted positively to the anti-PBF antibody. Fifteen specimens (75%) were graded as PBF overexpression. Of the 11 variables analyzed, stage III disease, inadequate surgical margins and PBF overexpression were significantly associated with decreased disease-free and overall survival. None of the other variables, including age, gender, origin of tumor, tumor site or levels of LDH, ALP, CRP and ESR, showed any significant association. These findings indicate that the overexpression of PBF is a factor indicative of poor prognosis in ESFT. PBF may also serve as a putative target antigen in immunotherapy for patients with ESFT that have a poor prognosis and PBF overexpression.

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Introduction

Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults (1). Although systemic adjuvant chemotherapy has significantly improved the prognosis for patients with ESFT, disease which presents metastatic spread or relapses after primary treatment remains incurable in the majority of cases (2,3).

After a nearly 30-year interval from the initial immunotherapeutic trials for osteosarcoma (4,5), immunotherapy has recently re-emerged as a targeted therapy for bone and soft tissue sarcomas, including ESFT (6,7). In a clinical trial with ESFT patients, peptides derived from the junction regions of EWS-FLI1 fusion genes were used as ESFT-specific vaccines (6). However, clinical response was seen in only 1 of the 12 patients enrolled, suggesting the importance of finding further efficacious antigens and of developing antigenic peptide vaccines (8).

Recently, papillomavirus binding factor (PBF) was identified as an autologous cytotoxic T-lymphocyte-defined osteosarcoma antigen (9). It was originally defined as a transcriptional regulator of genomic DNA of human papillomavirus type 8 (10). The antigenic and oncologic roles of PBF in ESFT remain uncertain. In this study, with the aim of gaining basic information on these aspects of PBF, we examined its expression in 20 cases of ESFT and analyzed its prognostic significance.

Materials and methods

This study was approved according to institutional guidelines for the use of human subjects in research. Patient specimens were analyzed after informed written consent was obtained from the patients or their families.

Patients and samples. Between 1979 and 2005, 20 consecutive patients with ESFT were treated at Keio University hospital. The clinical picture is summarized in Table I. There were 12 male and 8 female patients, with an average age at diagnosis of 23.9 years (range, 1-63 years). Twelve tumors arose from bone and the remaining 8 from soft tissue. Eight tumors were located in the trunk and 13 in the extremities. Fusion genes, including

Table I. Clinical characteristics and expression status of PBF in patients with ESFT.

Patient	Age (years)	Gender	Location	Stage[a]	Fusion gene	Treatment	PBF status	Event-free survival (mos)	Overall survival (mos)	Prognosis
Bone tumor										
1	1	M	Right tibia	IIB	ND	Chx (VAC) + Amp + Rx	++	179	179	CDF
2	36	F	Right femur	IIB	ND	Chx (A-VAC) + Amp+ Rx	-	174	174	CDF
3	16	M	Right humerus	IIB	ND	Chx (A-VAC) + WE + Rx	+	250	250	CDF
4	13	M	Left tibia	IIB	ND	Chx (CYVADIC) + WE + Rx	+++	166	166	CDF
5	17	M	Thoracic spine	IIB	EWS/ERG	Chx (T11) + ME + Rx	+++	8	45	DOD
6	16	M	Sacrum	IIB	ND	Chx (T11) + WE + Rx	++	99	99	CDF
7	16	F	Left tibia	IIIB	EWS/FLI-1	Chx (KS-1) + WE	+++	0	16	DOD
8	22	F	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE	+++	108	108	CDF
9	18	M	Left 5th rib	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	105	105	CDF
10	18	M	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	7	21	DOD
11	24	M	Left tibia	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	-	15	15	CDF
12	23	M	Pelvis	IIIB	ND	Chx (KS-1) +Rx	+++	0	17	DOD
Soft tissue tumor										
13	29	M	Right thigh	IIIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	0	12	DOD
14	63	F	Paraspine	IIB	ND	Chx (KS-1) + ILE +Rx	+++	0	71	DOD
15	20	F	Right tibia	IIB	EWS/FLI-1	Chx (KS-1) + ME +Rx	+++	9	14	DOD
16	56	F	Right forearm	IIIB	ND	Chx (CYVADIC + KS-1) + WE	+++	0	11	DOD
17	7	M	Paraspinal cord	IIB	Not detected	Chx (KS-1) + ME	+++	14	16	DOD
18	11	F	Paraspinal cord	IIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	17	22	DOD
19	35	M	Left femur	IIB	ND	Chx (KS-1) + WE	+++	126	126	CDF
20	36	F	Right upper arm	IIB	Not detected	Chx (KS-1) + WE	+++	101	101	CDF

[a]Stage was determined according to Enneking's surgical staging system. ND, not determined; Chx, chemotherapy; Rx, radiotherapy; ILE, intralesional resection; ME, marginal excision; WE, wide excision; CDF, continuous disease free; DOD, death of the disease.

EWS/FLI-1, EWS/ERG, EWS/ETV1 and EWS/E1AF, were determined by RT-PCR (11) in 9 of the 11 cases for which frozen biopsy specimens were available. According to Enneking's surgical stage (12), 16 patients were in stage IIB and 4 in IIIB. Treatment consisted of chemotherapy and surgery for 6, chemotherapy, surgery, and radiotherapy for 13, and chemotherapy and radiotherapy for 1. The chemotherapy protocols used were VAC (13), A-VAC (14), CYVADIC (15), T11 (16) and KS1, which is a modified protocol of New A3 (17). Radiotherapy (50-60 Gy) was instituted postoperatively. The average follow-up period after diagnosis was 78.4 months (range, 11-250 months).

Immunohistochemistry. Polyclonal antibody against PBF was generated previously (9). Formalin-fixed paraffin-embedded sections of biopsy specimens were boiled for 20 min in a microwave oven for antigen retrieval. Sections were blocked with 1% non-fat dry milk and stained with streptavidin-biotin-complex (Nichirei), followed by hematoxylin staining as previously described (9). The reactivity of the anti-PBF polyclonal antibody was determined by staining the nuclei. The expression status of PBF was estimated based on the number of tumor cells according to Ahmed *et al* (18) (Fig. 1): the presence of ≤5% of positively-stained tumor cells was represented by a minus (-), 6-25% by a plus (+) and 26-60% by ++. Overexpression, a number of positive tumor cells >60%, was represented by +++.

Clinicopathological analysis. Survival was estimated using Kaplan-Meier plots. Univariate analysis with the log-rank test

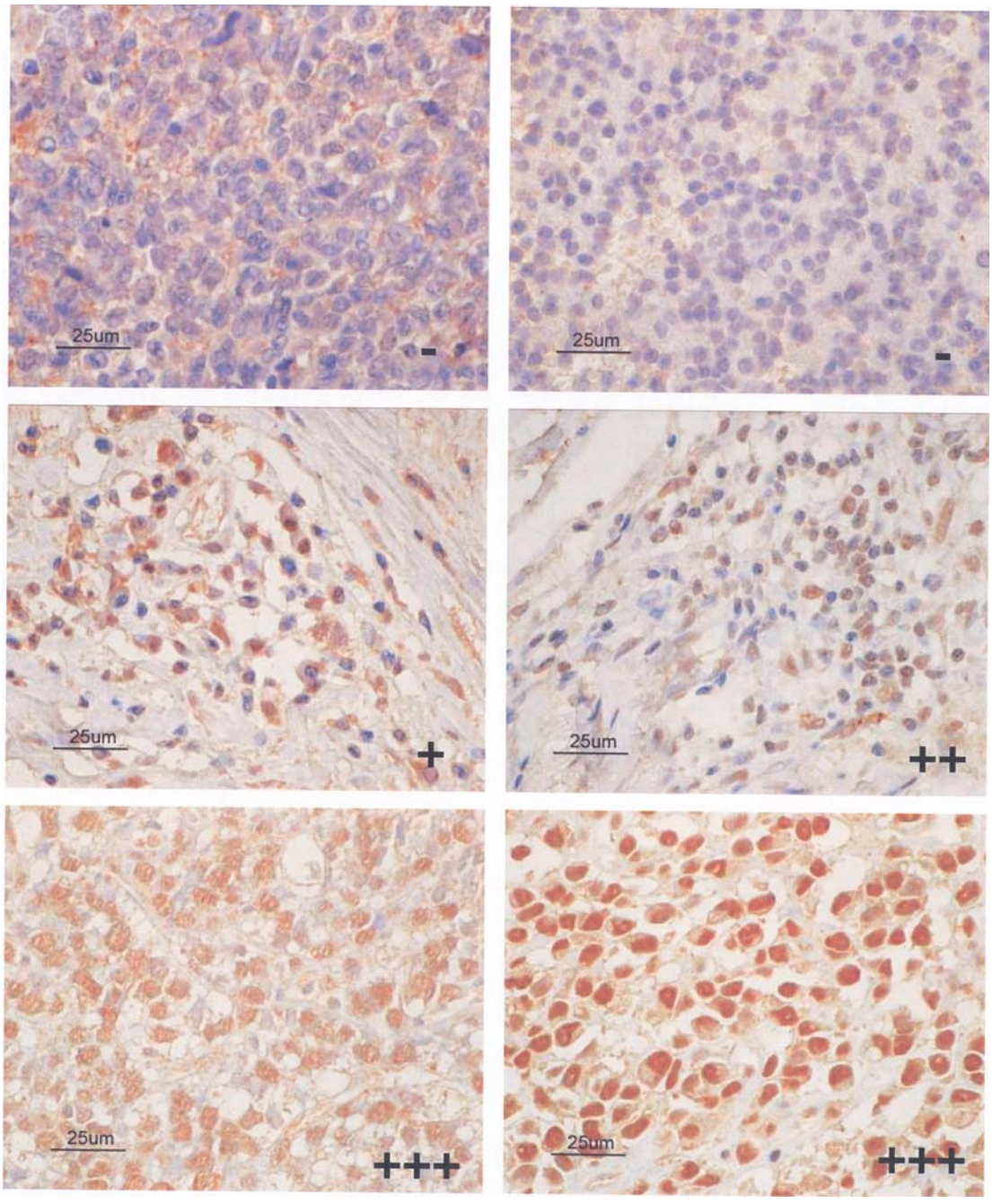

Figure 1. Immunohistochemical grading of PBF expression. Representative sections of ESFT specimens stained with anti-PBF antibody. Tumor cells showing positive reactivity in the nuclei were counted: -, ≤5% positive cells; +, 6-25% positive cells; ++, 26-60% positive cells; +++, >60% positive cells.

(19) was used to determine the prognostic significance of the following variables for disease-free and overall survival: age (≥30 or <30), gender (female or male), tumor site (trunk or limb), origin of tumor (bone or soft tissue), stage (I, II or III), laboratory parameters (within or higher than normal range), surgical margin (adequate or inadequate) and PBF expression status (-, +, ++ or +++). Laboratory parameters included LDH, alkaline phosphatase (ALP), C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) at 1 h. Analysis of the surgical margins was performed in 16 stage IIB patients. Wide excision and amputation were regarded as adequate margins, whereas intralesional and marginal excision were regarded as inadequate. A probability of <0.05 was considered to be statistically significant.

Results

PBF expression in ESFT. To determine the expression profiles of PBF in ESFT, we stained 20 ESFT biopsy specimens with anti-PBF antibody. Of these, 18 (90%) reacted positively to the anti-PBF antibody where the nuclei of tumor cells were stained (Fig. 1 and Table I). Two specimens were grade ++ and 1 was +. Fifteen specimens (75%) were graded as +++, indicating PBF overexpression.

Prognostic significance of PBF expression in ESFT. We then analyzed the prognostic significance of several variables, including the overexpression of PBF (grade +++). Overall survival rates of the 20 patients with ESFT were 53.1 and

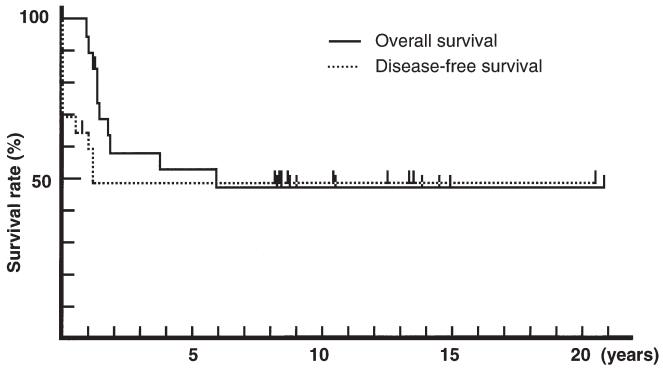

Figure 2. Overall survival of 20 patients with ESFT. Overall survival was estimated using Kaplan-Meier plots. The date of histological diagnosis was used as time 0.

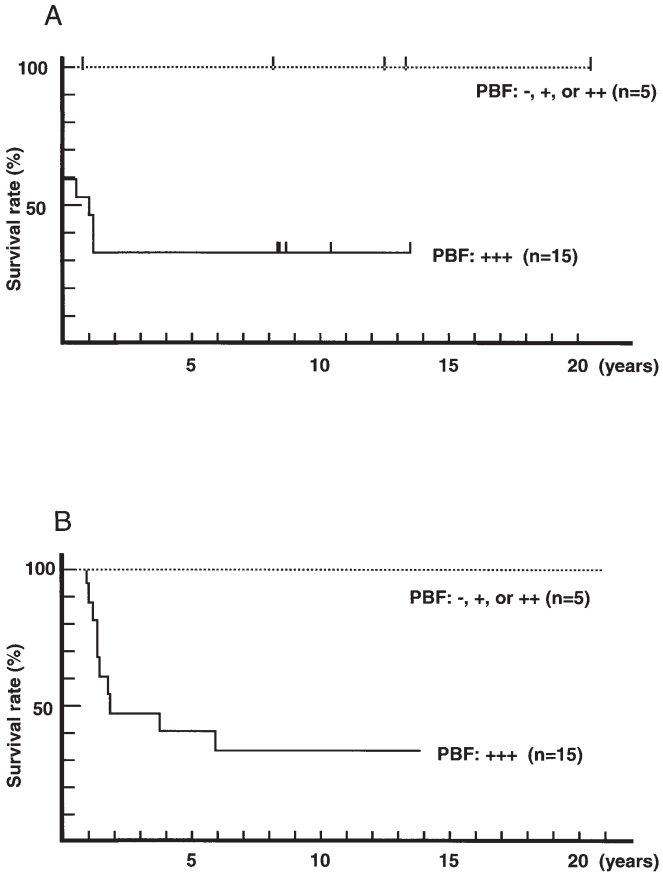

Figure 3. Survival curves of 20 patients with ESFT stratified by PBF expression status. (A) Disease-free survival curve. (B) Overall survival curve. Patients were divided according to PBF expression status into 2 groups (PBF expression of -, +, and ++, n=5; PBF expression of +++, n=15). Survival was estimated using Kaplan-Meier plots.

47.8% at 5 and 10 years, respectively (Fig. 2). Of the 11 variables analyzed, stage III, an inadequate surgical margin and PBF overexpression of were significantly associated with decreased disease-free and overall survival (Fig. 3 and Table II). Of note, the overall survival of the 15 patients with PBF overexpression was 33.3% at 10 years, whereas 5 patients remained continuously disease free during the entire follow-up period. None of the other variables, including age, gender, origin of tumor, tumor site and levels of LDH, ALP, CRP and ESR, showed a significant association to disease-free or overall survival.

Table II. Univariate analysis of potential unfavorable prognostic factors.

Factor	No. of patients	P-value	
		Disease-free survival	Overall survival
Age (≥30 years)	5	0.71	0.54
Male sex	12	0.31	0.45
Soft tissue tumor	8	0.09	0.06
Trunk tumor	7	0.24	0.47
Stage III	4	0.006	P<0.001
High ALP	7	0.61	0.56
High LDH	8	0.34	0.51
High CRP	8	0.27	0.35
High ESR	8	0.30	0.46
Inadequate surgical margin[a]	6	P<0.001	P<0.001
PBF +++	15	0.04	0.04

[a]Marginal excision and intralesional excision were regarded as inadequate surgical margin and 16 stage IIB patients having undergone surgical treatment were analyzed.

Discussion

By staining 20 biopsy specimens of ESFT treated at a single institute with an antibody against PBF we found i) that PBF was expressed in 18 ESFT specimens (90%), including 15 specimens (75%) with grade +++ overexpression, and ii) that PBF overexpression was significantly associated with the decreased disease-free and overall survival of patients. These findings indicate that the overexpression of PBF is a factor of poor prognosis for ESFT. PBF, which was originally defined as an osteosarcoma-associated antigen (9), may also serve as a putative target antigen in immunotherapy for patients with ESFT and PBF overexpression, which confers a poor prognosis.

Compared to malignant melanoma and epithelial cancers, there is a marked delay in the identification of tumor-associated antigens in bone and soft tissue sarcomas (7,8). In ESFT, antigens proven to have specific T cell responses have been limited to EWS-FLI1 fusion gene products (6,20,21). More recently, cancer-testis antigens (also termed cancer-germline genes) were defined in 11 of 18 ESFT specimens and included MAGE-A3, A4, A6, A10, A12, C2 and GAGE-1, -2 and -8 (22). However, their expression levels were lower than those of other sarcomas, including osteosarcoma and rhabdomyosarcoma (22).

Among the tumor-associated antigens identified in malignant melanoma, some were later found in solid tumors in a significant association with poor prognosis (Table III). These include cancer-testis antigens MAGE-3 (23,24), MAGE-4

tumor-associated antigens showing prognostic significance nors.

Antigen	Tumor	No. of samples (% positive)	Detection procedure	Refs.
Cancer testis				
MAGE-A3	NSCLC	523 (55.2)	RT-PCR	23
	Pancreatic cancer	57 (44.0)	qRT-PCR	24
MAGE-A4	NSCLC[a]	19 (36.8)	RT-PCR	25
	Squamous cell lung carcinoma	153 (56.9)	IHC	26
	Ovarian carcinoma	53 (57.0)	IHC	27
	Bladder carcinoma	908 (4.0)[b]	IHC	28
NY-ESO-1	NSCLC	523 (26.6)	RT-PCR	23
Overexpression				
PRAME	Neuroblastoma	95 (33.7)[b]	qRT-PCR	29
WT1	Soft tissue sarcomas	52 (32.7)[b]	qRT-PCR	30
	Osteosarcoma	37 (27.0)[b]	IHC	31
PBF	ESFT	20 (75.0)[b]	IHC	Present study

NSCLC, non-small cell lung carcinoma; qRT-PCR, quantitative real-time RT-PCR; IHC, immunohistochemistry. [a]Advanced stage cancers. [b]Percentage of samples with overexpression.

(25-28) and NY-ESO-1 (23), and an overexpression antigen, PRAME (29). Apart from melanoma-derived antigens, it has been reported that the overexpression of WT1 is associated with poor prognosis in bone and soft tissue sarcomas (30,31) (Table III). Though WT1 was originally defined as the tumor-suppressor gene responsible for Wilms' tumor, antigenic peptides derived from it have recently been used as vaccines for hematopoietic malignancies and solid cancers (32). PBF is classified as an overexpression antigen as it is detected in some normal tissues by RT-PCR (9). In addition to ESFT, expression of PBF was found to be significantly associated with poor prognosis in patients with osteosarcoma, with statistical significance (Tsukahara *et al*, unpublished data).

The antigenic role of PBF in patients with ESFT remains to be defined by T cell responses specific to PBF-derived peptides. To this end, we recently developed a limiting dilution/mixed lymphocyte peptide culture/tetramer/cytotoxicity assay by which the frequency and anti-tumor cytotoxicity of peripheral T lymphocytes directed against PBF were determined in patients with osteosarcoma (Tsukahara *et al*, unpublished data). This approach is also applicable to patients with ESFT. Another limitation of the present study is the small number of samples used, due mainly to the rare occurrence of ESFT in the Japanese population. It is, however, based on a consecutive series of patients treated at a single institute for more than 25 years.

In conclusion, the present analysis serves as a pilot study showing the prognostic significance of PBF for patients with ESFT. Large-scale analyses need to be conducted to verify the present findings if PBF-targeted immunotherapy for patients with ESFT is to be developed.

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