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PRAME Expression as a Potential Biomarker for Hematogenous Recurrence of Esophageal Squamous Cell Carcinoma

HAYATO BABA[1,2], MITSURO KANDA[2], KOICHI SAWAKI[2], DAI SHIMIZU[2], SHINICHI UMEDA[2], MASAHIKO KOIKE[2], YASUHIRO KODERA[2] and TSUTOMU FUJII[1]

[1]*Department of Surgery and Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan;*
[2]*Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*

Abstract. *Background/Aim: To investigate the function of preferentially expressed antigen of melanoma (PRAME) in esophageal squamous cell carcinoma (ESCC). Materials and Methods: mRNA expression levels of PRAME were analyzed in resected esophageal tissues of 150 ESCC patients and correlated with clinicopathological parameters. We also investigated the potential function of PRAME by analyzing coordinately expressed genes in 13 ESCC cell lines. Results: RT-qPCR analysis of clinical samples revealed aberrantly high PRAME expression in tumors compared with normal esophageal tissues. High PRAME expression was significantly associated with shorter disease-specific survival and hematogenous recurrence, but not with overall recurrence. The cumulative incidence of hematogenous recurrence was significantly greater for patients with high compared to those with low PRAME expression. In vitro, PCR array analysis revealed that PRAME was coordinately expressed with EGFR, ITGB, and TCF3. Conclusion: PRAME is overexpressed in ESCC tissues and may serve as a novel biomarker for predicting hematogenous recurrence.*

Esophageal carcinoma is the sixth leading cause of cancer-related mortality worldwide (1). Histologically, esophageal carcinoma is classified as two main subtypes; adenocarcinoma and esophageal squamous cell carcinoma (ESCC), the latter being the predominant subtype of esophageal carcinoma in Asia and Africa (2). While an optimal multimodal therapeutic strategy for ESCC has yet to be established, the disease is generally associated with poor prognosis, largely due to its propensity to metastasize to various organs. The 5-year overall survival rate for ESCC patients is less than 40%, even for those undergoing radical treatment (3). Hematogenous metastasis, for which there is no effective treatment, has an especially poor clinical outcome, whereas locoregional or lymph node metastasis can sometimes be effectively treated with surgical resection (4-6). Specific biomarkers that can predict the recurrence pattern in ESCC, particularly for hematogenous recurrence after radical treatment, may thus be helpful in improving the clinical outcome. However, no predictive biomarkers are currently available, highlighting the need for further research in this area.

Previous studies have demonstrated an association between the prognosis of ESCC patients and expression of several members of the melanoma-associated antigen (MAGE) gene family, including *MAGE-A9* (7), *MAGE-A11* (8), and *MAGE-D4* (9). Another member of this family, preferentially expressed antigen of melanoma (*PRAME*), has been reported to play a role in the progression of various malignant tumors, including head and neck squamous cell carcinoma (10-14). Although global gene profiling identified *PRAME* as a candidate diagnostic biomarker for esophageal cancer, its expression and function in ESCC has not been previously reported (15).

In the present study, we aimed to assess whether *PRAME* could have utility as a predictive biomarker for the prognosis of ESCC patients. To this end, we quantified *PRAME* mRNA in ESCC clinical samples and evaluated its association with disease recurrence patterns and survival. In addition, we performed an *in vitro* analysis to investigate the potential function of *PRAME* in ESCC.

Correspondence to: Mitsuro Kanda, MD, Ph.D., FACS, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81 527442249, Fax: +81 527442252, e-mail: m-kanda@med.nagoya-u.ac.jp

Key Words: Esophageal squamous cell carcinoma, preferentially expressed antigen of melanoma (*PRAME*), hematogenous recurrence.

Materials and Methods

Ethics. This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki–Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of Nagoya University, Japan. Written informed consent for the use of clinical samples and data, as required by the institutional review board, was obtained from all patients.

Clinical samples. A total of 150 primary ESCC tissues and adjacent normal tissues were collected from patients who underwent radical esophageal resection at Nagoya University Hospital between October 2001 and January 2016. Radical resection was performed on patients pathologically diagnosed with stage I-III disease. Tissue samples were immediately frozen and stored at −80˚C upon resection, and specimens were confirmed to be ESCC by histological classification according to the 8th edition of the Union for International Cancer Control (UICC) staging system for esophageal cancer (16). Postoperative follow-up included physical examination, measurement of serum tumor markers every 3 months, and enhanced computed tomography of the chest and abdominal cavity every 6 months. Adjuvant chemotherapy was administered to selected patients according to their condition and at the discretion of the physician.

For external validation of our data, we analyzed a freely available genomic dataset from 96 ESCC patients from The Cancer Genome Atlas (TCGA) (17).

Analysis of PRAME mRNA levels. *PRAME* mRNA expression was analyzed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) as described previously (18). Total RNA (10 μg per sample) was reverse transcribed to cDNA, which was amplified with primers specific for *PRAME*. qPCR was performed using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green reagents. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA served as an internal standard and was used to calculate the relative PRAME mRNA levels in each sample.

Assessment of the clinical significance of PRAME expression. Patients were stratified into two groups using as a cutoff the median *PRAME* mRNA expression level in tumor tissues from all analyzed patients. High and low *PRAME* expression were considered >median or ≤median values, respectively. Correlations between high/low *PRAME* mRNA expression, clinicopathological parameters, and outcome analyses, including disease-specific survival (DSS), disease-free survival (DFS), and recurrence pattern-specific survival, were evaluated.

Cell lines. The human ESCC cell lines TE1, TE2, TE3, TT, and TTn, and a non-tumorigenic epithelial cell line Het-1A were obtained from the American Type Culture Collection (Manassas, VA, USA). KYSE510, KYSE590, KYSE890, KYSE1170, KYSE1260, and KYSE1440 cell lines were obtained from the Japanese Collection of Research Bio Resources Cell Bank (Osaka, Japan). NUEC2 and WSSC cell lines were established at Nagoya University (19). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and maintained in a 5% CO_2 atmosphere at 37˚C.

PCR array analysis. To identify genes coordinately expressed with *PRAME* in ESCC cell lines, we used the Human Epithelial to Mesenchymal Transition (EMT) RT2 Profiler PCR Array (Qiagen, Hilden, Germany). This array includes 84 key genes that encode proteins with the following functions: transcription factors, extracellular matrix proteins, and proteins involved in EMT, cell differentiation, morphogenesis, growth, proliferation, migration, cytoskeleton, and signaling pathways (20).

Statistical analysis. Differences in relative *PRAME* mRNA levels between two groups were analyzed using the Mann–Whitney *U*-test. Correlations between two variables were assessed using Spearman's rank correlation coefficient. The χ^2 test was used to analyze associations between gene expression and clinicopathological parameters. DSS, DFS, and recurrence pattern-specific survival rates were calculated using the Kaplan–Meier method and analyzed using a Cox proportional hazards model. Univariate regression analysis of prognostic factors was performed using a Cox proportional hazards model, and variables with $p<0.05$ were included in the final multivariate model (21). All statistical analyses were performed using JMP 14 software (SAS Institute Inc., Cary, NC, USA). A value of $p<0.05$ was considered statistically significant.

Results

Study population and expression of PRAME in ESCC tissues. Of the total cohort of 150 patients, 118 were men and the median age was 66 years (range=44-84 years). The majority of patients (129) were diagnosed with differentiated ESCC and the remainder (21) with undifferentiated ESCC. The number of patients with pathological stage I, II, and III disease (8th edition UICC classification) was 37, 51, and 71, respectively. Neoadjuvant and adjuvant chemotherapy were administered to 70 and 35 patients (47% and 35%), respectively. The median duration of follow-up was 45.2 months, during which 55 patients (37%) experienced recurrence and 39 patients (26%) succumbed to the disease.

PRAME mRNA expression in the 150 paired samples of primary ESCC tissues and adjacent normal tissues was assessed by qRT-PCR. *PRAME* mRNA levels were higher in ESCC tissue than adjacent normal esophageal tissue in 131/150 (87%) patients. The level of *PRAME* mRNA was significantly higher in ESCC tissues than in normal adjacent tissues ($p<0.001$, Figure 1A). Consistent results were obtained when the TCGA extra-validation cohort of 96 patients with ESCC was analyzed ($p<0.001$, Figure 1A).

Prognostic impact of PRAME mRNA level. To assess the potential prognostic utility of *PRAME* mRNA expression, the patients were dichotomized using the median *PRAME* mRNA levels as the cutoff value. Analysis of correlations between *PRAME* expression and clinicopathological factors revealed that high tumor *PRAME* mRNA expression was significantly associated only with lymphatic involvement (Table I). Survival analyses demonstrated a significantly lower 5-year DSS rate for patients with high *vs*. low *PRAME*

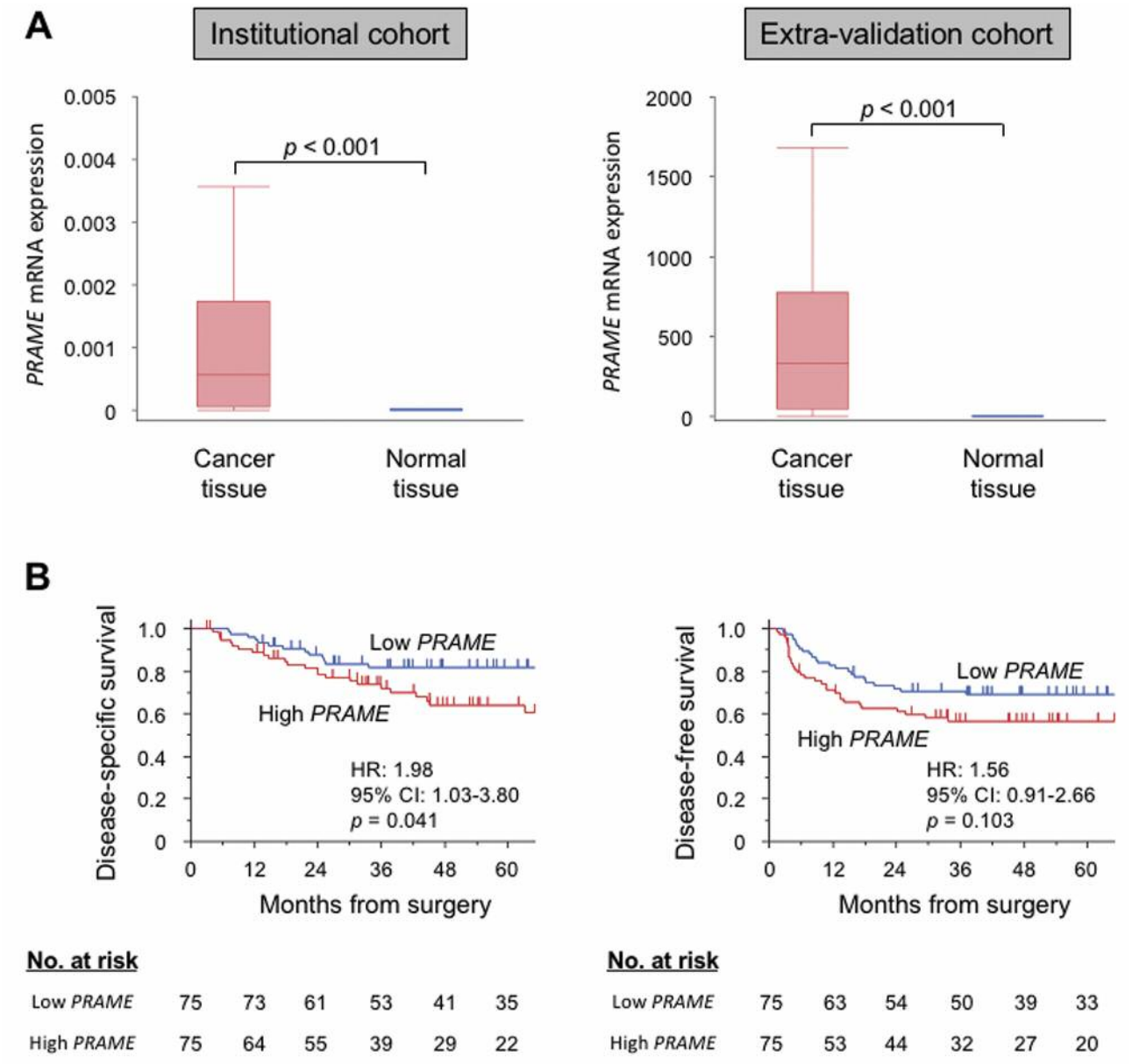

Figure 1. *PRAME mRNA expression in ESCC tissues and its prognostic implications. A) Left: qRT-PCR analysis of PRAME mRNA expression in ESCC tissues and paired normal adjacent esophageal tissues from 150 patients. Right: PRAME mRNA expression in an extra-validation cohort of 96 ESCC patients from The Cancer Genome Atlas dataset. B) Kaplan–Meyer analysis of disease-specific and disease-free survival of 150 patients who underwent radical resection for stage I-III ESCC.*

mRNA level [64% *vs*. 82%, hazard ratio (HR)=1.98, 95% confidence interval (CI)=1.03-3.80, p=0.041; Figure 1B]. The 5-year DFS rate also tended to be lower for patients with high tumor expression of *PRAME*, but the difference did not reach a level of statistical significance (p=0.103, Figure 1B). The discrepancy between DSS and DFS rates prompted us to determine whether *PRAME* expression correlated with recurrence patterns. Of the 150 patients, 55 (37%) experienced postoperative recurrence at a total of 68 initial recurrence sites. High *PRAME* mRNA was significantly associated with hematogenous recurrence (p=0.002, Figure 2A), but not with overall, lymph node, local, or other recurrence patterns. The cumulative incidence of hematogenous recurrence was significantly higher for patients with high *PRAME* expression compared to those with low expression (HR=4.40, 95%CI=1.61-11.8, p=0.003;

Table I. *Association between tumor PRAME mRNA expression levels and clinicopathological parameters in 150 patients with ESCC.*

Variables	High PRAME mRNA in GC tissue (n)	Low PRAME mRNA in GC Tissue (n)	p-Value
Age (years)			0.249
<65	29	36	
≥65	46	39	
Gender			0.690
Male	60	58	
Female	15	17	
Smoking history			0.583
Present	56	58	
Absent	18	15	
Double cancer			0.094
Present	18	10	
Absent	57	65	
Tumor location			0.167
Ce	3	1	
Ut	9	2	
Mt	34	41	
Lt	28	29	
Ae	1	2	
Tumor multiplicity			1.00
Present	8	8	
Absent	67	67	
Tumor size (mm)		4	0.366
<50	48	42	
≥50	27	32	
CEA (ng/ml)			1.00
≤5	68	68	
>5	7	7	
SCC (IU/ml)			0.596
≤1.5	49	52	
>1.5	25	22	
pT			0.742
T1 or T2	32	34	
T3 ot T4	43	41	
Lymph node metastasis			0.618
Present	43	46	
Absent	32	29	
Differentiation			0.480
Differentiated	63	66	
Undifferentiated	12	9	
Lymphatic involvement			0.019
Present	60	47	
Absent	15	28	
Vascular invasion			0.496
Present	29	25	
Absent	46	50	
Intraepithelial progress			0.816
Present	16	22	
Absent	16	19	
Pathological UICC stage			0.124
I	20	17	
II-III	55	58	
Neoadjuvant chemotherapy			0.570
Present	38	32	
Absent	37	43	
Postoperative adjuvant chemotherapy			0.177
Present	14	21	
Absent	61	54	

CEA: Carcinoembryonic antigen; SCC: squamous cell carcinoma-related antigen; CI: confidence interval; PRAME: preferentially expressed antigen of melanoma.

Figure 2B). Multivariable Cox proportional hazards analysis revealed that high *PRAME* expression in ESCC tissues was an independent predictive factor for hematogenous recurrence (HR=3.73, 95%CI=1.39-10.1, p=0.009; Table II).

Subgroup analyses of the predictive value of PRAME expression. Next, we performed subgroup analyses to determine the predictive value of *PRAME* expression for hematogenous recurrence. However, we found no significant interaction with any of the subgroups tested (Figure 3), although the small number of patients in some subgroups (*e.g.*, women and patients with UICC stage I ESCC) may have limited the predictive effects.

Expression of PRAME and cancer-related genes in ESCC cell lines. To validate the overexpression of *PRAME* mRNA in ESCC tissues and to investigate its potential function, we examined 13 human ESCC cell lines for *PRAME* mRNA expression. Although the absolute levels differed, *PRAME* mRNA was present at higher levels in 10 of the ESCC cell lines than in the control non-tumorigenic epithelial cell line (Figure 4A). There were no significant differences in *PRAME* mRNA levels between ESCC cell lines derived from metastases (TT, TTn, KYSE1170, and KYSE1260) and primary tumors (p=0.705) or between lines with different degrees of differentiation (p=0.511). Next, we performed PCR array analysis to identify cancer-related genes expressed coordinately with *PRAME* in the ESCC cell lines. Our findings revealed that the mRNA expression levels of *EGFR*, *ITGB1*, and *TCF3* correlated significantly with those of *PRAME* (Figure 4B), suggesting that they may have similar and/or interdependent functions.

Discussion

In the present study, we investigated the expression of the MAGE family member *PRAME* in ESCC tissues and showed that it was overexpressed in ESCC compared with adjacent normal esophageal tissues. High tumor *PRAME* expression was significantly associated with poorer DSS, but not DFS. In addition, *PRAME* overexpression was not associated with an increase in overall recurrence, but it was strongly associated with, and an independent predictive factor for, hematogenous recurrence. Our investigation of the potential function of *PRAME* revealed that it was coordinately expressed with several cancer-related genes in ESCC cell lines.

PRAME is located on human chromosome 22q11.22 and encodes a 509-amino acid protein (22). PRAME protein was first identified as a tumor antigen in cells isolated from a melanoma (23). Aberrantly high expression levels have since been detected in various tumors, including breast cancer, head and neck, and lung cancers, lymphoma, and several types of soft tissue neoplasm, in which it acts as a tumor

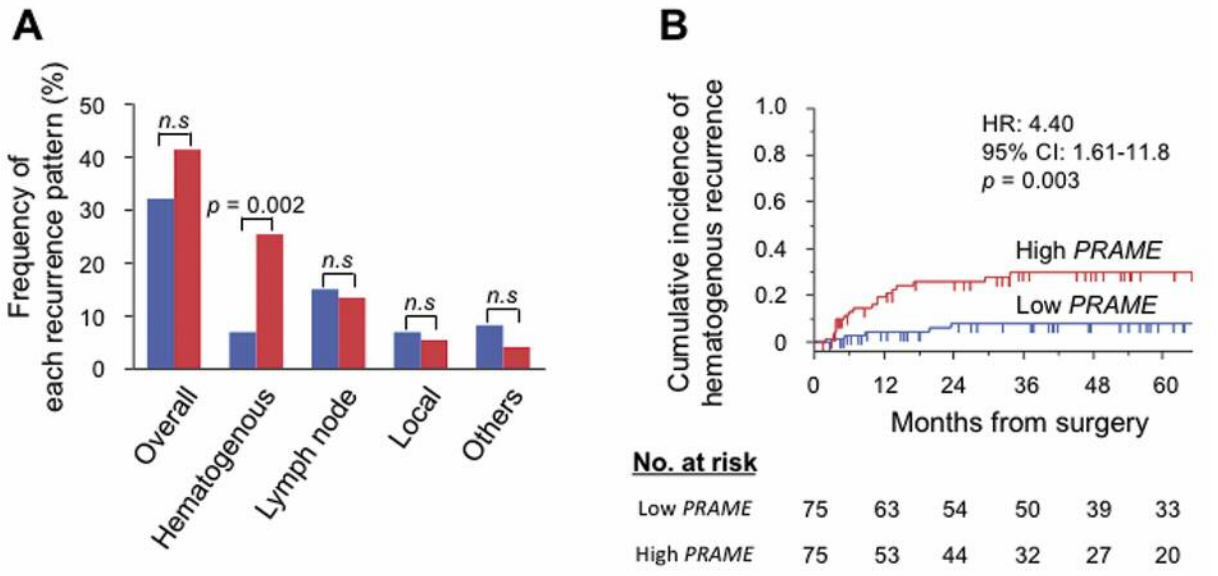

Figure 2. *Correlation between PRAME mRNA expression and ESCC recurrence patterns after radical resection. A) Frequencies of initial recurrence sites in 150 patients stratified by high (red bar) and low (blue bars) tumor PRAME mRNA level. B) Cumulative incidence of hematogenous recurrence in patients stratified as described for (A).*

Table II. *Risk factors for hematogenous recurrence.*

	Univariate			Multivariate		
	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age (≥65 years)	1.40	0.61-3.19	0.424			
Gender (male)	1.52	0.52-4.55	0.443			
Smoking	0.44	0.19-1.01	0.052			
Tumor location (lower)	0.79	0.45-1.38	0.415			
Double cancer	1.17	0.44-3.13	0.757			
Tumor multiplicity	0.37	0.05-2.73	0.329			
Tumor size (≥50 mm)	0.61	0.25-1.48	0.275			
CEA (>5 ng/ml)	0.83	0.20-3.53	0.802			
SCC (>1.5 IU/ml)	1.87	0.82-4.27	0.138			
Tumor depth (pT3-4)	2.25	0.93-5.43	0.071			
Lymph node metastasis	2.45	0.97-6.19	0.057			
Tumor differentiation (undifferentiated)	1.72	0.64-5.00	0.283			
Lymphatic involvement	5.72	1.34-24.4	0.018	4.62	1.08-19.80	0.039
Vascular invasion	1.77	0.79-3.97	0.162			
Intraepithelial progress	0.42	0.13-1.36	0.149			
Neoadjuvant chemotherapy	1.42	0.64-3.18	0.387			
Postoperative adjuvant chemotherapy	1.06	0.42-2.66	0.907			
High PRAME expression	4.40	1.64-11.8	0.001	3.73	1.39-10.1	0.009

CEA: Carcinoembryonic antigen; SCC: squamous cell carcinoma-related antigen; CI: confidence interval; PRAME: preferentially expressed antigen of melanoma.

biomarker (10-14). In contrast, little is known about the expression or function of *PRAME* in ESCC. To our knowledge, only one study of the relationship between esophageal cancer and *PRAME* mRNA expression has been reported. In that report, Warnecke-Eberz *et al*. (15) performed transcriptomic analysis of esophageal cancer samples (ESCC and adenocarcinomas) and identified *PRAME* as one of 19 possible diagnostic markers. In the

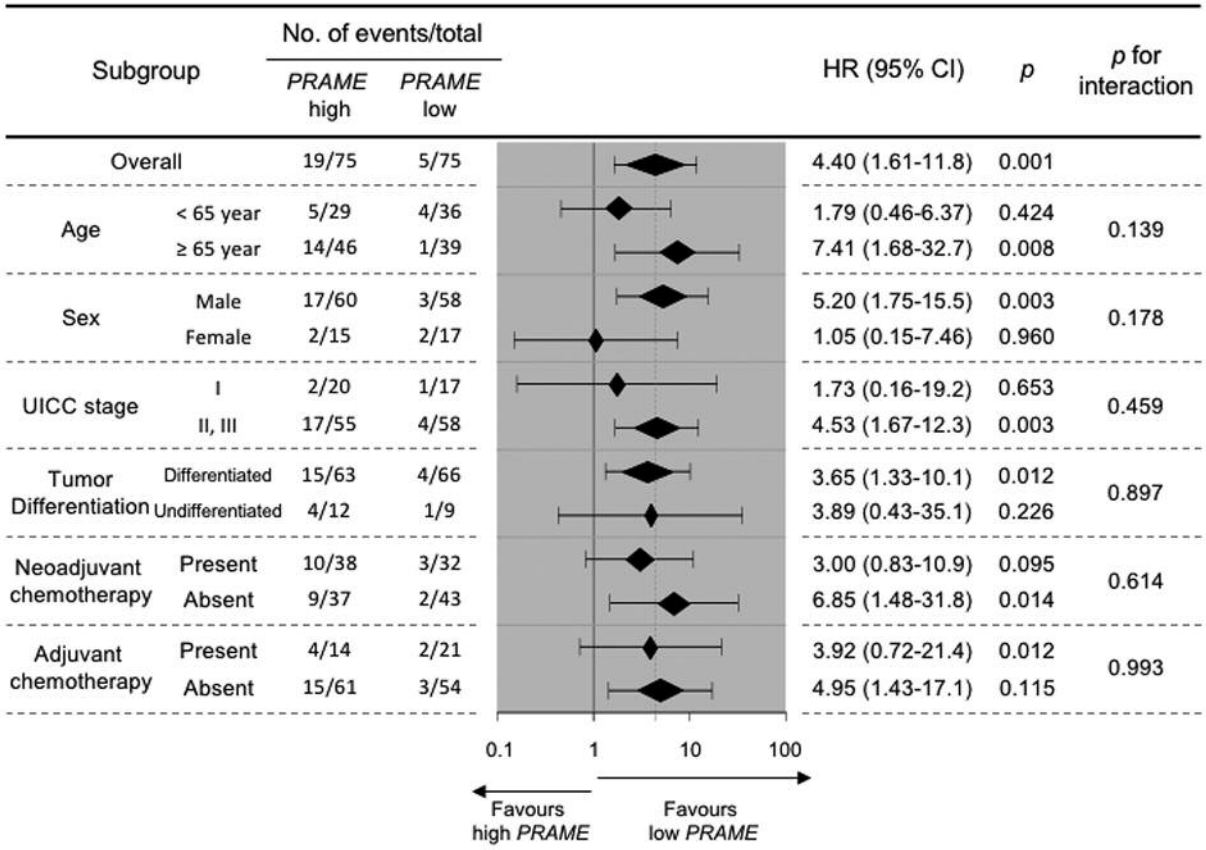

Figure 3. *Subgroup analysis of the predictive value of PRAME mRNA expression for hematogenous recurrence after radical surgery. Forest plot of the impact of PRAME mRNA expression levels on hematogenous recurrence in 150 patients. HR: Hazard ratio; CI: confidence interval.*

present study, we focused on assessing the clinical significance of *PRAME* expression in ESCC, and revealed the potential utility of *PRAME* predictive biomarker for hematogenous recurrence after radical esophagectomy.

The EMT plays a role in the hematogenous dissemination of carcinoma (24). A recent study showed that PRAME can promote EMT in several malignancies, resulting in increased metastasis (25). Overexpression of the *EGFR, ITGB1*, and *TCF3* genes, which were coordinately expressed with *PRAME* in our mRNA array analysis of ESCC, has also been reportedly involved in the EMT, and to be associated with increased hematogenous metastasis in various cancers (26-28). Therefore, *PRAME* may act in concert with other genes in the EMT cascade to promote hematogenous dissemination in ESCC. In the present study, we did not detect any correlations between high *PRAME* expression and well-known risk factors for hematogenous recurrence, such as pathological vascular invasion and lymph node metastasis (29), although a significant correlation with lymphatic involvement was found. This finding suggests that *PRAME* expression is independent of established risk factors in ESCC, highlighting its significance as a biomarker specifically for hematogenous recurrence.

Our results may have clinical application in two areas. First, patients with high tumor levels of *PRAME* mRNA may benefit from postoperative surveillance with a focus on early detection of hematogenous recurrence. Contrast-enhanced computerized tomography, which is the most common imaging modality in the postoperative follow-up, may not detect early metastasis to the liver and bone, indicating that multimodal imaging surveillance, including Gd-EOB-DTPA-enhanced magnetic resonance imaging and positron emission tomography, may be required to detect early hematogenous recurrence. Second, *PRAME* mRNA expression in biopsy samples or surgical specimens may serve as a reference measure in the design of perioperative therapy. However, our subgroup analyses showed no beneficial effect of neoadjuvant or adjuvant chemotherapy in preventing hematogenous recurrence in patients with high *PRAME* mRNA levels. Neoadjuvant chemotherapy has been reported to reduce hematogenous recurrence after radical surgery compared with surgery alone in 418 patients (30), suggesting

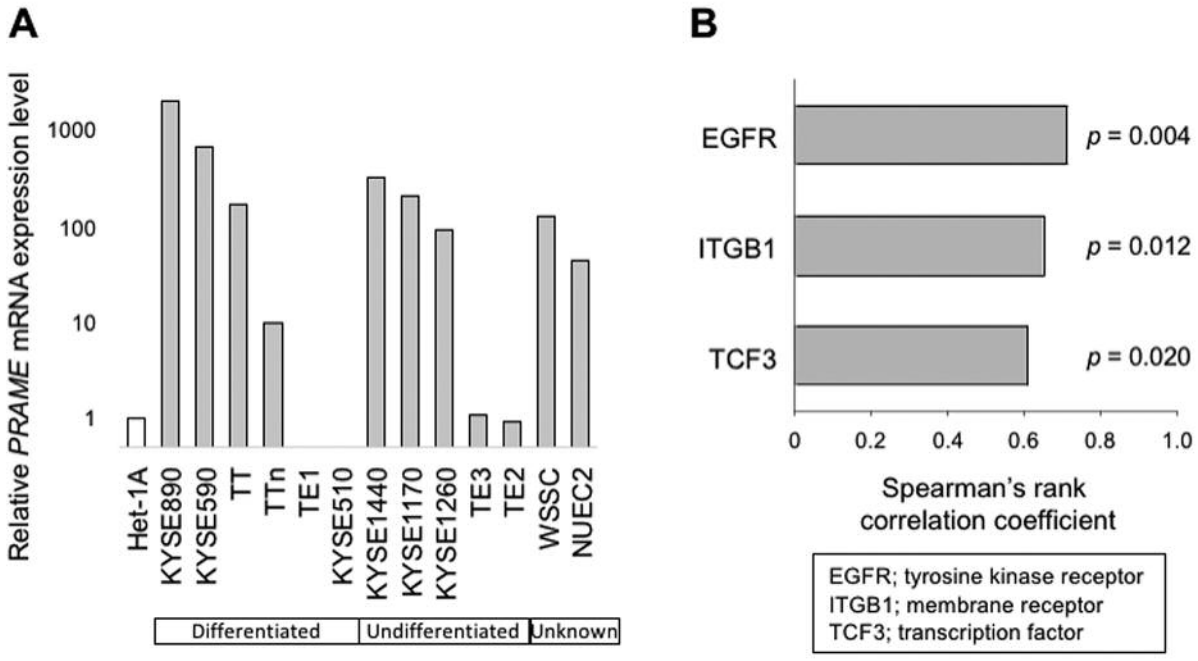

Figure 4. *Expression of PRAME mRNA in human ESCC cell lines. A) qRT-PCR analysis of PRAME mRNA expression in 13 ESCC cell lines and a normal epithelial cell line (Het-1A). B) Correlation coefficients of three cancer-related genes coordinately expressed with PRAME derived from PCR array analysis of ESCC cell lines.*

that active neoadjuvant chemotherapy may improve the prognosis of patients with high *PRAME* expression. While the application of adjuvant chemotherapy is still controversial, it is considered to be beneficial for specific groups of ESCC patients (31). Therefore, measurement of *PRAME* expression in clinical samples may assist clinicians in deciding whether to administer adjuvant chemotherapy. Chemotherapeutic strategies based on recurrence patterns are currently being established for many cancers (32, 33), but they are not yet available for ESCC. In the future, tumor PRAME expression levels may provide useful information in guiding treatment selection when such strategies do become available for esophageal cancer.

There are several limitations to the present study. First, the study was retrospective in nature. Second, we used the median value as the cutoff for stratification of patients based on high/low *PRAME* mRNA expression level, and an optimal cutoff value derived from larger-scale studies will be required for clinical application. Third, interpretation of the results of the subgroup analyses may have been affected by the sample size. Thus, the efficacy of neoadjuvant or adjuvant chemotherapy will require further examination in a larger patient cohort.

In conclusion, our results showed that *PRAME* mRNA was overexpressed in ESCC tissues compared with normal tissue and may serve as a biomarker for predicting hematogenous recurrence after radical surgery.

Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

Authors' Contributions

HB and MK conceived the study concept and design, analyzed data and wrote the manuscript. MK, KS, DS, SU, MK, YK and TF contributed to data acquisition and interpretation. SU contributed to statistical analysis. KS, DS, SU, MK, YK, and TF revised the draft. All Authors have read and approved the final version of the manuscript.

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