Overexpression of heat-shock factor 1 is associated with a poor prognosis in esophageal squamous cell carcinoma

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Abstract. Heat-shock factor 1 (HSF1) is the primary regulator of the response to various stressors. A previous study showed that HSF1 expression is associated with a poor prognosis in breast cancer and hepatocellular carcinoma; however, the prognostic significance of HSF1 in esophageal squamous cell carcinoma (ESCC) is unknown. Therefore, the present study investigated the association between HSF1 expression and the clinicopathological parameters of patients, as well as the association between HSF1 expression, and heat shock protein (Hsp)27, Hsp70 and Hsp90 expression induced by HSF1, by cDNA microarray and immunohistochemistry analyses. HSF1 protein and mRNA expression were assessed in resected specimens from 270 patients with ESCC in two independent cohorts. Hsp27, Hsp70 and Hsp90 expression were also assessed in 55/270 patients. Patients with high HSF1 expression had a significantly worse OS than those with low HSF1 expression in both cohorts. In multivariate analyses, pathological T stage [hazard ratio (HR), 2.21; 95% confidence interval (CI), 1.38-3.65; P=0.0008], pathological N stage (HR, 1.73; 95% CI, 1.04-3.02; P=0.03) and HSF1 expression (HR, 2.29; 95% CI, 1.48-3.64; P=0.0002) were statistically significant independent prognostic factors. Furthermore, Hsp27 and Hsp90 expression were significantly correlated with HSF1 expression (P<0.0001), but Hsp70 expression was not (P=0.38). These results indicate that HSF1 is a prognostic factor for patients with ESCC, and that Hsp27 and Hsp90, but not Hsp70, may be the downstream targets of HSF1 in ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer in East Asian countries, and is the eighth most common malignancy, and the sixth leading cause of cancer death worldwide (1). Despite improvements in surgical procedures, multidisciplinary therapies and perioperative management, the prognosis of patients with ESCC remains unsatisfactory (2). A staging system for ESCC based on clinicopathological features that can provide an accurate prediction of survival in patients aids clinicians in selecting the best treatment approach. Currently, the TNM classification is widely used to stage patients and to select treatment strategies (3). However, the assessment of prognosis using this system remains inadequate due to the considerable biological heterogeneity within the same stage of ESCC. Therefore, novel biological markers are required that provide a more accurate prognosis of patients and that facilitate in selecting the appropriate treatment strategy for individual patients.

Heat-shock transcription factor 1 (HSF1) is a transcription factor that induces the expression of heat shock proteins (Hsp) in response to various stressors (4,5). These Hsps act as molecular chaperones to maintain cellular homeostasis by restoring protein folding and stability. HSF1 facilitates survival in response stressors by regulating various cellular processes, such as cell-cycle control, protein translation and glucose metabolism (6,7). The expression of HSF1 is reportedly upregulated in cancer cell lines and cancer tissues of hepatocellular carcinoma, and high expression of HSF1 is associated with a poor prognosis in breast cancer and hepatocellular carcinoma (8,9). However, the role of HSF1 as a prognostic biomarker in ESCC has not been well investigated. Therefore, the present study analyzed HSF1 mRNA and protein expression in resected ESCC tissues to determine if the expression of HSF1 could be used as a prognostic biomarker in ESCC.

Materials and methods

Sample collection. Samples of resected ESCC and normal squamous epithelium were obtained from two independent cohorts,

and assayed for HSF1 mRNA and protein expression. Cohort 1 was composed of 58 ESCC samples, which were collected during subtotal esophagectomy between February 2005 and March 2009 after obtaining written informed consent. All patients underwent subtotal esophagectomy at one of five hospitals (Juntendo University Hospital, Tokyo, Japan; National Cancer Center Hospital, Tokyo, Japan; Kurume University Hospital, Fukuoka, Japan; Saitama Cancer Center, Saitama, Japan; Kagoshima University Hospital, Kagoshima, Japan). The clinicopathological characteristics of patients are indicated in Table I. All patients underwent curative resection and no patients received neoadjuvant therapy. The tumor samples were submitted for cDNA microarray analysis.

Cohort 2 consisted of 212 ECC samples collected during subtotal esophagectomy at Osaka University Hospital (Osaka, Japan) between June 2000 and February 2013, following receipt of written informed consent. Patient characteristics, including the number of patients that underwent neoadjuvant therapy, are indicated in Table II. These specimens were submitted for immunohistochemical analysis. The present study was approved by the Ethics Committee of Osaka University Hospital (Osaka, Japan) and the written consent of all the patients was obtained. Pathological tumor stage was evaluated using the seventh edition of the TNM classification established by the Union for International Cancer Control (3).

Extraction of RNA and cDNA microarray analysis. Resected tumor tissues in cohort 1 were immediately cut (5 mm thickness), and embedded in Tissue-Tek OCT medium (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen and maintained at -80˚C until RNA extraction. Following isolation of RNA and DNA using the QIAamp DNA Micro kit (Qiagen, Valencia, CA, USA) and the RNeasy Micro kit (Qiagen), cDNA was synthesized from 8.0 μg of total RNA (10) using the commercially available Whole Human Genome Oligo DNA Microarray kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Cyanine-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification kit manual (Agilent Technologies, Inc.). Labeled cRNA was then fragmented (with fragmentation buffer from the Agilent Low RNA Input Fluorescent Amplification kit, according to the manufacturer's protocol; Agilent Technologies, Inc.) and hybridized to an oligonucleotide microarray (Whole Human Genome Microarray kit, 4x44K; product no. G4112F; Agilent Technologies, Inc.). Fluorescence intensities were determined with a DNA microarray scanner (G2565BA/DB; Agilent Technologies, Inc.) and analyzed using G2567AA Feature Extraction Software (version A7.5.1; Agilent Technologies, Inc.), which used the locally weighted linear regression curve fit normalization method (11). This microarray study followed Minimum Information About a Microarray Experiment guidelines issued by the Microarray Gene Expression Data group (12). Further analyses were performed using GeneSpring (version 7.3; Agilent Technologies, Inc.). mRNA expression level in cancer tissue by microarray was categorised by the median expression level into high and low expression groups.

Immunohistochemistry. Tumor specimens in cohort 2 were fixed in 10% formalin (Wako Pure Chemical Industries,

Table I. Correlation between HSF1 mRNA expression and clinicopathological parameters in cohort 1.

Parameter	Low (n=29)	High (n=29)	P-value
Age, years[a]	66 (52-82)	66 (52-79)	0.72
Gender, n			
Male	24	18	0.08
Female	5	1	
Location, n			
Ut	3	8	0.24
Mt	16	13	
Lt	10	8	
Histological grade, n			
G1	12	8	0.49
G2	14	16	
G3	3	5	
pT stage, n			
1	4	4	0.69
2	3	5	
3	19	15	
4	3	5	
pN stage, n			
0	15	9	0.61
1	5	6	
2	6	5	
3	3	9	

[a]Data are presented as median (range). HSF1, heat-shock factor 1; pT/N, pathological T/N; Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; Lt, lower thoracic esophagus.

Ltd., Osaka, Japan) and embedded in paraffin (Wako Pure Chemical Industries, Ltd.). The expression of proteins was evaluated by immunohistochemistry in 4-μm-thick sections following deparaffinization with xylene (Wako Pure Chemical Industries, Ltd.) and dehydration in graded ethanol solutions. For antigen retrieval, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 115˚C for 20 min. After blocking with hydrogen peroxide (Wako Pure Chemical Industries, Ltd.), the sections were incubated with specific antibodies overnight at 4˚C in a moist chamber. Rat monoclonal antibody against HSF1 (10H8; Enzo Life Sciences, Inc., Farmingdale, NY, USA), mouse monoclonal antibody against Hsp27 (G3.1; Enzo Life Sciences, Inc.), mouse monoclonal antibody against Hsp70 (C92F3A-5; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal antibody against Hsp90 (ADI-SPA-830-D; Enzo Life Sciences, Inc.), were used at dilutions of 1:100, 1:1,000, 1:500 and 1:300, respectively. Sites of antibody binding were visualized with the ABC peroxidase detection system according to the manufacturer's protocol (catalog nos., PK-4004 and PK-4010; Vector Laboratories, Inc., Burlingame, CA, USA) using a fluorescence microscope (BZ-X700; Keyence Corporation, Osaka, Japan). The intensity of antibody-stained nuclei in cancer cells was evaluated by two

Table II. Correlation between HSF1 protein expression and clinicopathological parameters in cohort 2.

Parameter	Low expression (n=103)	High expression (n=109)	P-value
Age, years[a]	64 (47-79)	65 (39-80)	0.43
Gender, n			
Male	89	95	0.87
Female	14	14	
Histological grade, n			
G1	27	22	0.14
G2	43	62	
G3	24	16	
Gx	9	9	
Location, n			
Ut	11	18	0.03
Mt	65	47	
Lt	27	44	
pT stage, n			
T1	33	24	0.41
T2	16	19	
T3	51	61	
T4	3	5	
pN stage, n			
N0	41	31	0.12
N1	37	35	
N2	16	29	
N3	9	13	
pStage, n			
I	22	18	0.10
II	34	24	
III	31	49	
IV	16	18	
Neoadjuvant therapy			
None	39	46	0.52
Chemotherapy	64	63	

[a]Data are presented as median (range). HSF1, heat-shock factor 1; pT/N/Stage, pathological T/N/Stage; Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; Lt, lower thoracic esophagus.

independent pathologists, who were blinded to the clinicopathological parameters. The intensity of HSF1, Hsp27, Hsp70 and Hsp90 immunoreactions in the cells were scored as follows: 0, not stained; 1+, weak staining; 2+, moderate staining; and 3+, intense staining. Finally, the expression of these proteins in ESCC specimens was classified into a HSF1 low expression group (0 or 1+) and a HSF1 high expression group (2+ or 3+).

Statistical analysis. The association between HSF1 protein expression and various clinicopathological factors was analyzed using the χ^2 test and Fisher's exact probability test. The overall survival (OS) was assessed by performing the Kaplan-Meier method and compared using the log-rank test. OS was defined as the period from the date of surgery to the date of death from any cause. All parameters with a P<0.10 in univariate analysis by the Cox proportional hazard model
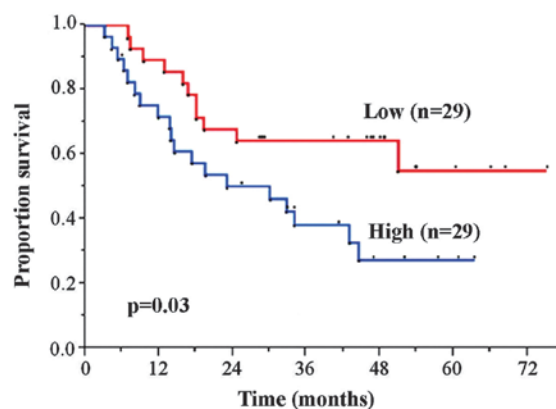

Figure 1. Kaplan-Meier overall survival curves based on the heat-shock factor 1 mRNA expression status of patients with esophageal squamous cell carcinoma in cohort 1.

were entered into multivariate survival analysis. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed with JMP Pro software (version 10.0.2; SAS Institute, Cary, NC, USA). Each test was repeated six times.

Results

Correlation between HSF1 mRNA expression and clinico-pathological characteristics in cohort 1. The 58 ESCC cases were classified into two groups using the median HSF1 mRNA expression level in cancer tissue as determined by microarray. Table I shows the correlation between HSF1 expression and various clinicopathological parameters. No significant association was observed with age, gender, tumor location, histological grade, pathological T (pT) stage or pathological N (pN) stage (P<0.05).

Correlation between HSF1 mRNA expression and OS in cohort 1. The 5-year OS rates in the HSF1 high and low expression groups was 27.3 and 55.1%, respectively. The HSF1 high expression group exhibited significantly worse OS rate compared with the low expression group (P=0.03) (Fig. 1).

HSF1 protein expression in cohort 2. A total of 212 cancerous lesion samples were evaluated for HSF1 protein expression by immunohistochemical analysis. Of these, 109 (51.4%) exhibited high HSF1 expression (Table II), predominantly in the nuclei of the tumor cells, with faint cytoplasmic staining (Fig. 2A), while the remaining 103 (48.6%) samples showed low HSF1 expression (Fig. 2B). The staining was almost homogenous among different areas of the cancerous lesions, including the surface, central and deep areas. None of the normal squamous epithelium samples showed significant levels of HSF1 expression, although faint immunostaining was observed in the nuclei of a small number of basal cells (Fig. 2C). Scoring of the immunostained sections was almost identical by the two pathologists, with interobserver variation of <5%.

Correlation between HSF1 protein expression and clinico-pathological parameters in cohort 2. Table II indicates the correlations between HSF1 protein expression and various clinicopathological parameters. In comparison to primary tumors with low HSF1 expression, primary tumors with high HSF1 expression were significantly more likely to be in the upper location (P=0.03). All other parameters, including age, gender, pT, pN, pathological stage and preoperative therapy, did not significantly correlate with HSF1 protein expression.

Correlation between HSF1 protein expression and clinical outcome in cohort 2. The 5-year OS rates for patients with low and high HSF1 expression tumors were 69.4 and 37.8%, respectively (Fig. 3). The high HSF1 expression group exhibited significantly poorer OS than those in the low HSF1 expression group (P<0.0001). Furthermore, univariate analysis revealed that the OS significantly correlated with age, pT, pN and HSF1 protein expression (Table III). Multivariate analysis using the five parameters with P<0.10 on univariate analysis identified high HSF1 expression [hazard ratio (HR), 2.29; 95% confidence interval (CI), 1.48-3.64; P=0.0002] as
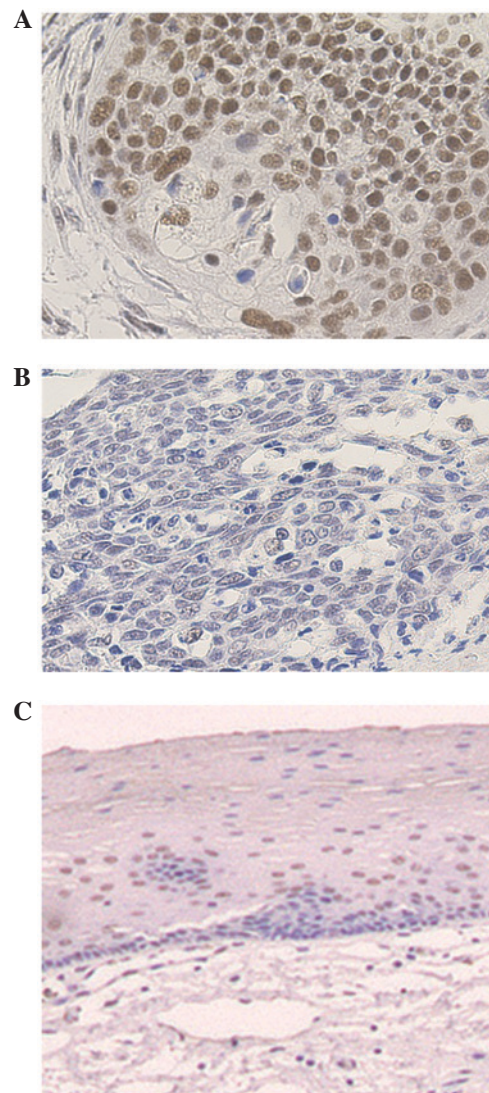

Figure 2. Immunohistochemical staining showing the expression of heat-shock factor 1 (HSF1) in esophageal squamous cell carcinoma (ESCC) tumor samples from cohort 2. Representative images of (A) high HSF1 expression in ESCC, showing staining predominantly in the nuclei of tumor cells (magnification, x200); (B) low HSF1 expression ESCC, showing almost no appreciable staining of the tumor cells (magnification, x200); and (C) normal squamous epithelium, which is negative for HSF1 expression except for a few basal cells (magnification, x100).
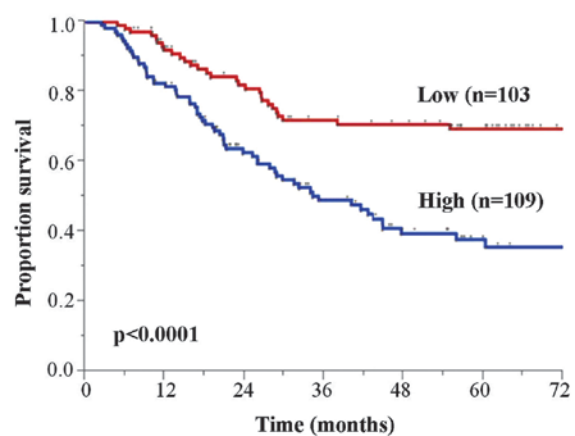

Figure 3. Kaplan-Meier overall survival curves based on the heat-shock factor 1 protein expression status of esophageal squamous cell carcinoma patients in cohort 2.

Table III. Univariate and multivariate survival analyses of overall survival rate by Cox's proportional hazard model.

Parameter	Patients, n	Univariate			Multivariate		
		HR	95% CI	P-value	HR	95% CI	P-value
Age, years (65≤/≤64)	106/106	1.64	1.08-2.50	0.02	1.51	0.99-2.32	0.06
Gender (male/female)	184/28	1.36	0.74-2.8	0.34			
Histological grade (G1/G2, G3, Gx)	49/163	0.81	0.51-1.32	0.39			
Location (Ut, Mt/Lt)	141/71	0.66	0.44-1.02	0.06	1.36	0.88-2.07	0.16
pT (T3, T4/T1, T2)	92/120	2.66	1.69-4.34	<0.0001	2.22	1.39-3.65	0.0007
pN (N1, N2, N3/N0)	72/140	2.42	1.49-4.14	0.0002	1.76	1.06-3.06	0.03
Neoadjuvant therapy (none/chemotherapy)	85/127	0.91	0.59-1.38	0.65			
HSF1 expression (high/low)	109/103	2.53	1.64-4.0	<0.0001	2.24	1.44-3.56	0.0003

HR, hazard ratio; CI, confidence interval; pT/N, pathological T/N; HSF1, heat-shock factor 1; Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; Lt, lower thoracic esophagus.

Table IV. Correlation between HSF1, and Hsp27, Hsp70 and Hsp90 expression.

Expression	HSF1		Total	P-value
	+	-		
Hsp27				
+	24	6	30	<0.0001
-	7	18	25	
Hsp70				
+	7	8	15	0.38
-	24	16	40	
Hsp90				
+	28	7	35	<0.0001
-	3	17	20	
Total	31	24	55	

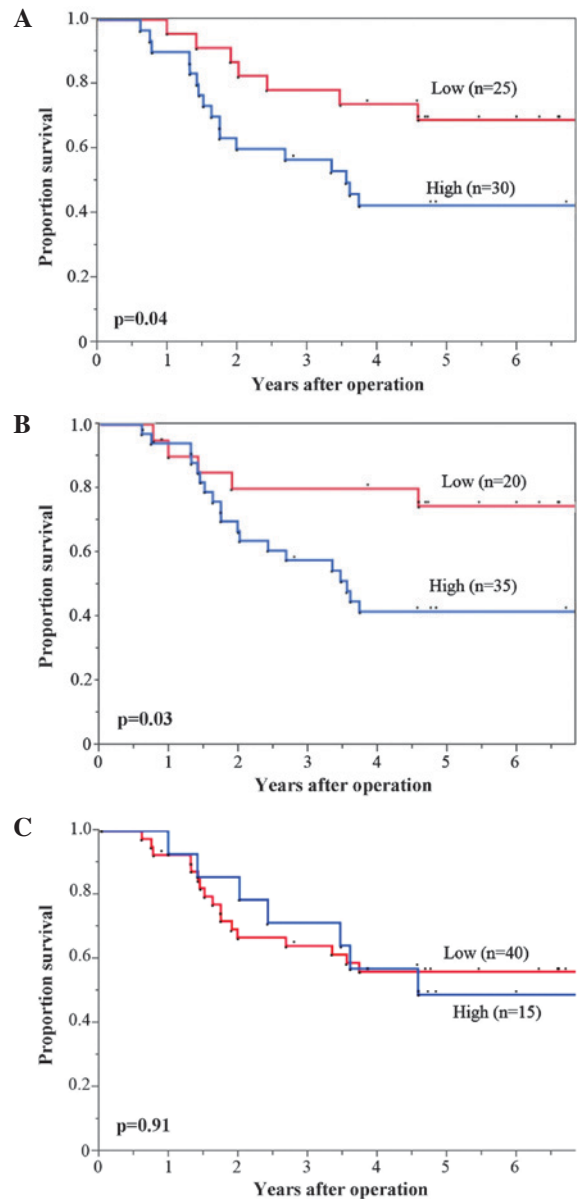HSF1, heat-shock factor 1; Hsp, heat-shock protein.

Figure 4. Overall survival curves based on (A) heat-shock protein 27 (Hsp27), (B) Hsp90 and (C) Hsp70 expression status of patients with esophageal squamous cell carcinoma.

an independent OS prognostic factor, in addition to pT (HR, 2.21; 95% CI, 1.38-3.65; P=0.0008) and pN (HR, 1.73; 95% CI, 1.04-3.02; P=0.03).

Correlation between HSF1 protein expression and expression of Hsps in cohort 2. To determine whether the expression of Hsps correlated with HSF1 tumor expression in ESCC tumor specimens, 55/212 ESCC randomly selected samples in cohort 2 were immunohistochemically evaluated for Hsp27, Hsp70 and Hsp90 expression. High expression of HSF1 and Hsp27 was observed in 24 ESCC samples, 18 samples showed low expression of HSF1 and Hsp27, 7 samples exhibited high expression of HSF1 but low expression of Hsp27, and 6 samples exhibited low HSF1 expression but high Hsp27 expression (Table IV). The results indicate that HSF1 expression is significantly correlated with Hsp27 expression (P<0.0001). Hsp90 expression was also significantly correlated with HSF1 expression (P<0.0001), but Hsp70 expression was not (P=0.38).

These results indicate that the expression of HSF1 is closely correlated with poor prognosis in ESCC. The results also indicate that Hsp27 and Hsp90 expression, but not Hsp70 expression, are possible downstream targets of HSF1 in ESCC.

Thus, the correlation between the Hsp27, Hsp70 and Hsp90 expression, and OS were investigated. The high Hsp27 and Hsp90 expression groups showed significantly poorer OS compared with those in the low Hsp27 and Hsp90 expression groups (P=0.04 and P=0.03, respectively) (Fig. 4A and B, respectively), while there were no significant differences in OS between high and low Hsp70 expression (P=0.91) (Fig. 4C).

Discussion

Hsps are overexpressed in numerous types of cancer (including hepatocellular carcinoma, colorectal carcinoma, bladder cancer and neuroblastoma), and are correlated with tumor proliferation, invasion and metastasis (13,14). Various studies report that Hsp levels are useful biomarkers of carcinogenesis and aggressiveness in specific types of cancer, including hepatocellular carcinoma, colorectal carcinoma, breast cancer, primary oral squamous cell carcinoma and gastric adenocarcinoma (15-18). Hsp expression is regulated by HSF1, which can bind to the 5' promoter regions of all Hsp genes, and trigger their immediate and massive transcription (19,20). HSF1 expression itself is also involved in numerous crucial steps of carcinogenesis and tumor development (7). Its expression is closely correlated with invasion, metastasis and angiogenesis (21,22), and has been identified as a prognostic factor in breast cancer and hepatocellular carcinoma.

The present study demonstrated that high HSF1 expression is associated with poor prognosis in two independent patient cohorts, and appears to be an independent prognostic factor for patients undergoing curative resection for ESCC. The prediction of prognosis following curative surgical resection will enable clinicians to determine the necessity for intensive follow-up and adjuvant therapy (23,24). Furthermore, if HSF1 expression can be measured in pretreated biopsy samples it may yield useful information for determining the treatment strategy for individual patients.

Additionally, the present study revealed that the expression of Hsp27 and Hsp90 are closely correlated with HSF1 expression in ESCC tumor specimens. This suggests that the HSF1/Hsp27 and HSF1/Hsp90 pathways may be involved in the progression of ESCC. A number of studies previously reported that Hsp27 and Hsp90 were associated with survival and could be potential molecular targets in various types of cancer (23,25-29).

Recently, Hsp90 inhibitors have been developed, and used as preclinical and clinical anticancer therapies (30,31). The present study indicates that Hsp27 and Hsp90 may be potential therapeutic targets in patients with ESCC, as HSF1 is a regulator of Hsps. Therefore, inhibiting HSF leads to inhibition of not only Hsp27, but also Hsp90. However, the results suggest that HSF1, which regulates the transcription of Hsp27 and Hsp90, may be a more effective and stronger therapeutic target. HSF1 may, therefore, be a valid prognostic marker in ESCC and a candidate for novel approaches to ESCC treatment.

Prospective studies are required to further clarify the significance of HSF1 in the clinical setting. Additionally, it will be important to investigate the molecular mechanisms underlying the effect of HSF1 expression on prognosis. The present findings may mark a new step for the exploration of appropriate treatment strategies and the development of novel therapeutic approaches for ESCC.

In conclusion, the results of the present study indicate that HSF1 is a prognostic factor for patients with ESCC, and that Hsp27 and Hsp90, but not Hsp70, may be the downstream targets of HSF1 in ESCC.

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