Tumor Immune Systems in Esophageal Cancer with Special Reference to Heat-shock Protein 70 and Humoral Immunity

MASANOBU NAKAJIMA, HIROYUKI KATO, TATSUYA MIYAZAKI, MINORU FUKUCHI, NORIHIRO MASUDA, YASUYUKI FUKAI, MAKOTO SOHDA, FARIED AHMAD and HIROYUKI KUWANO

Department of General Surgical Science, Gunma University, Graduate School of Medicine, Gunma, 371-8511, Japan

Abstract. *The objective of this study was to clarify the participation of heat-shock protein 70 (HSP70) and the humoral immune system in antitumor immunity in esophageal cancer. Patients and Methods: Immunohistochemical staining for HSP 70, and CD4$^+$ T-, CD8$^+$ T-, B- and plasma cells was performed on surgical specimens obtained from 125 patients with esophageal cancer. An enzyme-linked immunosorbent assay (ELISA) was then performed to measure serum anti-HSP70 antibodies in the azygos vein. Results: The expression of HSP 70 correlated inversely with depth of invasion ($p<0.0001$), pathological stage ($p<0.0001$) and blood vessel invasion ($p<0.001$), and there was a positive correlation between HSP70 and CD4$^+$ T-, CD8$^+$ T-, B- and plasma cells. Of these, the B- and plasma cells had the strongest correlation to HSP70 expression. Serum anti-HSP70 antibody levels in the azygos vein correlated with HSP70 expression, and B and plasma cell infiltration. Patients positive for HSP70, and B- and plasma cell infiltration had good prognosis compared to other cases. According to multivariate analyses, simultaneous occurrence of HSP70 expression, and B- and plasma cell infiltration is a stronger prognostic factor than simultaneous occurrence of HSP70 expression, and CD4$^+$ T- and CD8$^+$ T-cell infiltration. Conclusion: It is suggested that the HSP70-humoral immune cell system might play an important role in antitumor effects in patients with esophageal cancer.*

Correspondence to: Masanobu Nakajima, MD, Ph.D., Department of General Surgical Science (Surgery I), Gunma University, Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi, Gunma, 371-8511, Japan. Tel: +81 272208224, Fax: +81 272208230, e-mail: mnakajim@med.gunma-u.ac.jp

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Heat-shock proteins (HSPs) are a set of highly evolutionarily conserved proteins found in nearly all organisms. Under physiological conditions, these proteins act as molecular chaperones, facilitating protein transport and polypeptide assembly (1). However, under nonphysiological conditions, such as exposure to heat stress, infection, and malignant transformation, the synthesis of HSPs is accentuated (2). Expression levels of HSPs are reportedly altered, either increasing or decreasing, during malignant transformation (3, 4). Among the families of HSPs, HSP70 has been shown to have a strong association with various carcinomas (5-12). We also previously reported the association of HSP70 with esophageal squamous cell carcinomas (13). The HSP70 family proteins include both cognate members, which are found within major intracellular compartments, and highly inducible isoforms, which appear to be predominantly cytoplasmic or nuclear in distribution (14). Inducible HSP70 is typically regarded as an intracellular protein, however, it has also been shown to play a role as a tumor-specific antigen expressed on the cell membrane (15, 16). Moreover, in esophageal carcinomas, it has been suggested that HSP70 might play an important role in the presentation of tumor-specific antigens (17).

On the other hand, studies have shown the presence of soluble HSP70 and anti-HSP70 antibodies in the peripheral circulation of healthy individuals (18, 19) and in various diseases (20-25). With regards to neoplasms, there are also reports that suggest HSP70 and anti-HSP70 antibodies might have significance in gynecological malignancies (26).

We previously reported the significance of HSP27 and 70 expressions in esophageal squamous cell carcinomas and, in particular, reported that expressions of these proteins are related to the presence of tumor-infiltrating lymphocytes (TILs) (13). Although this report suggested the importance of the immune system, which recognizes HSPs as tumor-specific antigens, it could not reveal the mechanism.

TILs are considered a manifestation of the antitumor immune response (27), and cellular immunity plays a central role in this response. In particular, inclusion of CD8$^+$

Table I. *Panel of primary antibodies for immunohistochemistry.*

Antibody	Clone	Dilution	Antigen retrieval	Source, city, nation
HSP70	G3.1	1:100	Microwaved in 20 mM citric acid buffer (pH 6.0) at 90˚C for 10 min	StressGen Biotechnologies Corp, Victoria, BC, Canada
CD4	1F6	1:100	Autoclaved in 20 mM citric acid buffer (pH 6.0) at 90˚C for 3 min	Nichirei Corp., Tokyo, Japan
CD8 C8/144B	C8/144B	1:60	Microwaved in 20 mM citric acid buffer (pH 6.0) at 90˚C for 10 min	Nichirei Corp., Tokyo, Japan
L26	L26	1:400	Autoclaved in 20 mM citric acid buffer (pH 6.0) at 90˚C for 3 min	Nichirei Corp., Tokyo, Japan
CD38	SPC32	1:400	Autoclaved in 20 mM citric acid buffer (pH 6.0) at 90˚C for 3 min	Novocastra Laboratories Ltd., Newcastle, UK

T- and/or $CD4^+$ T-cells among the TIL population is important (28-31). Similar reports have also been presented for esophageal squamous cell carcinoma (32, 33). However, there are many B-cell infiltrations with follicle formation, which are remarkable in early esophageal carcinomas (34). In addition, the existence of HSP antibodies in some malignancies suggests an association between the humoral immune response and carcinoma.

In this study, we hypothesized that humoral immunity might take part in an immune system besides cellular immunity. If so, anti-HSP70 antibodies to HSP70 as a tumor-specific antigen might be produced by the humoral immune system. Consequently, esophageal cancer patients at low risk from tumor development should have an increased level of serum anti-HSP70 antibodies in venous blood obtained from the azygos vein, because it should reflect local antitumor immune responses. The azygos vein is mainly responsible for venous return from the esophagus (35-38) and its level of serum anti-HSP70 antibody might reflect tumor progression and conditions directly and accurately. Therefore, we examined the immunohistochemical expression of HSP70 and TILs using lymphocyte surface markers, namely, CD4 (regulatory T), CD8 (cytotoxic T), L26 (B) and CD38 (plasma) cells, and the serum anti-HSP70 antibody level in the azygos vein during surgery. We then analyzed the correlation between the results and clinicopathologic features in patients with esophageal cancer who underwent potentially curative surgery.

Patients and Methods

Before collecting human samples, all patients signed informed consent forms according to our institutional guidelines. Tumor stage and disease grade were classified according to the fifth edition of the TNM classification of the International Union Against Cancer (UICC). All the distant metastatic lesions were in lymph nodes.

Surgical specimens. Surgical specimens were obtained from 125 patients with esophageal cancer who underwent potentially curative surgery at the Department of General Surgical Science (Surgery I), Gunma University, Graduate School of Medicine, between 1983 and 2002. The age of the patients ranged from 40 to 79 years, with a mean of 62.1 years. None of the patients had received irradiation or chemotherapy prior to surgery, nor did any of them have hematogenic metastases at the time of surgery. Patients who underwent noncurative surgery and/or inadequate follow-up were not included in this study. Postoperative chemotherapy and/or radiation therapy were not performed until recurrence of the tumor was confirmed by radiological or endoscopic examinations.

Antibodies for immunohistochemistry and antigen retrieval. Five kinds of monoclonal antibodies were used for immunohistochemistry. Their dilution, antigen retrieval abilities and sources are summarized in Table I.

Immunohistochemistry for HSP70, CD4, CD8, L26 and CD38. Resected specimens were fixed with 10% natural buffered formalin and embedded in paraffin blocks. Sections, 4-μm thick, were deparaffinized with xylene, rehydrated, and incubated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, each tissue section was treated for antigen retrieval (Table I) and then cooled to 30˚C. After incubation with normal rabbit serum (Histofine SAB-PO (M) kit; Nichirei, Tokyo, Japan) for 30 min, the sections were incubated with each primary monoclonal antibody at each dilution (Table I) in phosphate buffered saline (PBS) containing 1% bovine serum albumin at 4˚C overnight, washed in PBS, and incubated with secondary antibody for 30 min at room temperature. Immunohisto-chemistry was performed using the SAB-PO (M) kit. The chromogen, diaminobenzidine (Dojindo, Kumamoto, Japan), was applied as a 0.02% solution containing 0.0055% H_2O_2 in 50 mM ammonium acetate-citric acid buffer, pH 6.0. The sections were lightly counterstained with hematoxylin. Negative controls were prepared by substituting normal mouse serum for each primary antibody, and no detectable staining was evident.

Evaluation of immunohistochemistry. The immunostained sections for HSP70 were evaluated according to our previous report (13) with some modifications. When 40% or more of the tumor cells in a given specimen were positively stained with a normal epithelium, the sample was graded HSP70 (+); it was graded HSP70 (–) when fewer than 40% of the tumor cells were stained.

Regarding $CD4^+$ and $CD8^+$ T-cells, evaluation was conducted according to a previous report (33). The degree of T-cell infiltration was assessed in more than 10 independent high-power (×200) microscopic fields for each tissue sample, and the five areas with the most abundant distribution were selected. The number of T-cells was counted both in the mesenchymal stroma and within the cancer cell nest.

B-cells, which are immunostained specifically by L26 monoclonal antibody, were distributed with various forms. There were many follicle formations distributed at the invasive front, in the tumor stroma, or within the tumor nest. The B-cell distribution was therefore evaluated and scored as follows. Concerning follicle formation, if there was no follicle around the tumor nest, a score of 0 points was given; if there was one follicle around the tumor, a score of 1; two follicles, a score of 2; and three or more follicles, a score of 3. Concerning B-cell infiltration at the invasive front, if there were no B cells, a score of 0 was given; if there were scanty infiltrations, a score of 1; moderate infiltrations, a score of 2; and abundant infiltrations, a score of 3. Concerning lymphocyte infiltration in the tumor stroma, if there were no B-cells, a score of 0 was given; if there were scanty infiltrations, a score of 1; moderate infiltrations, a score of 2; and abundant infiltrations, a score of 3. Finally, concerning B-cell infiltration within the tumor cell nest; if there were no B-cells, a score of 0; if there were scanty infiltrations, a score of 1; and moderate infiltration, a score of 2. There were no cases of abundant infiltration. The total scores were then counted and evaluated collectively.

Regarding plasma cells, infiltration was assessed in more than 10 high-power (×400) fields. Although they were immunostained with CD38 antibody, CD38 is expressed in wide range of cells. Therefore, plasma cells were identified as those cells with strong CD38 expression, and with a wheel-like nucleus and clear cytoplasm on serial hematoxylin-eosin stained sections. The number of plasma cells was counted in the mesenchymal stroma around the tumor cell nest.

Serum samples. Forty-six serum samples were obtained intraoperatively from the azygos vein of esophageal carcinoma patients (39 males and 7 females) who underwent potentially curative surgery without preoperative therapy at the Department of General Surgical Science (Surgery I), Gunma University, Graduate School of Medicine, between 1999 and 2002. The age of the patients ranged from 41 to 79 years, with a mean age of 61.2 years. Venous blood samples from the azygos vein were drawn using a syringe during surgery, as soon as possible after thoracotomy. Azygos veins were resected during dissection for curative surgery in all patients.

Serum anti-HSP70 antibody quantification. With the enzyme-linked immunosorbent assay (ELISA) used to measure serum anti-HSP70 antibody, the blood samples were stored at 4˚C after collection, and the serum was obtained by centrifugation at 2000 ×g for 20 min. The plasma samples were kept frozen at –80˚C until assayed. For quantitative measurements of serum anti-HSP70 antibody levels, a quantitative sandwich enzyme immunoassay was used (StressXpressTM Anti-Human Hsp70 (IgG/A/M) ELISA Kit; StressGen Biotechnologies Corporation, Victoria, BC, Canada). All samples were assayed in duplicate in a blinded fashion; the mean was used for data analysis.

Statistical analysis. The Chi-square test and Fisher's exact test were used as appropriate. Survival curves of the patients were calculated using the Kaplan-Meier method, and analysis was performed using the log rank test. The Cox proportional hazards model for risk ratio was used for univariate and multivariate analyses to assess the contribution of each factor to overall survival. A p-value of <0.05 was considered statistically significant in all analyses.

Results

Immunohistochemistry for HSP70 and immune cells. Immunoreactivity for HSP70 was positive in normal stratified squamous epithelium of the esophagus, and was localized in the cytoplasm or partially in the nucleus. Several staining patterns were observed for the expression of HSP70 in tumor tissues. Some tumors showed a diffuse decrease in HSP70 expression, while others showed strong expression. Although $CD4^+$ and $CD8^+$ T-cells, B-cells and plasma cells were detected within the cancer cell nests or stroma, regarding B-cells, some tumors formed follicles in the subepithelial region (Figure 1).

Relationship between HSP70 expression and clinicopathological findings and overall survival. We investigated the correlation between the clinicopathological characteristics of patients with esophageal cancer and expression of HSP70 in their tumors (Table II). As reported previously (13), there was a significant inverse correlation between HSP70 expression and depth of invasion ($p<0.0001$) and pathological stage ($p<0.0001$). In addition, there was a significant inverse correlation between HSP70 expression and blood vessel invasion ($p=0.0054$) and tumor location ($p=0.0148$). However, there was no significant association with patient age, gender, histological grade, lymph node metastasis, distant metastasis, infiltrative growth pattern, intraepithelial spread, lymphatic invasion, or blood vessel invasion. We then investigated the relationship between HSP70 expression and overall survival. The results revealed that HSP70 expression leads to good prognosis (Figure 2).

Relationship between the expression level of HSP70 and TILs. The level of each immune cell infiltration was compared to the expression level of HSP70 (Figure 3). Although all of the four kinds of immune cells had a significant positive correlation with HSP70 expression, the B-cells and plasma cells had the most significant relationship. Therefore, we suspected the participation of humoral immunity against tumor-presenting antigens.

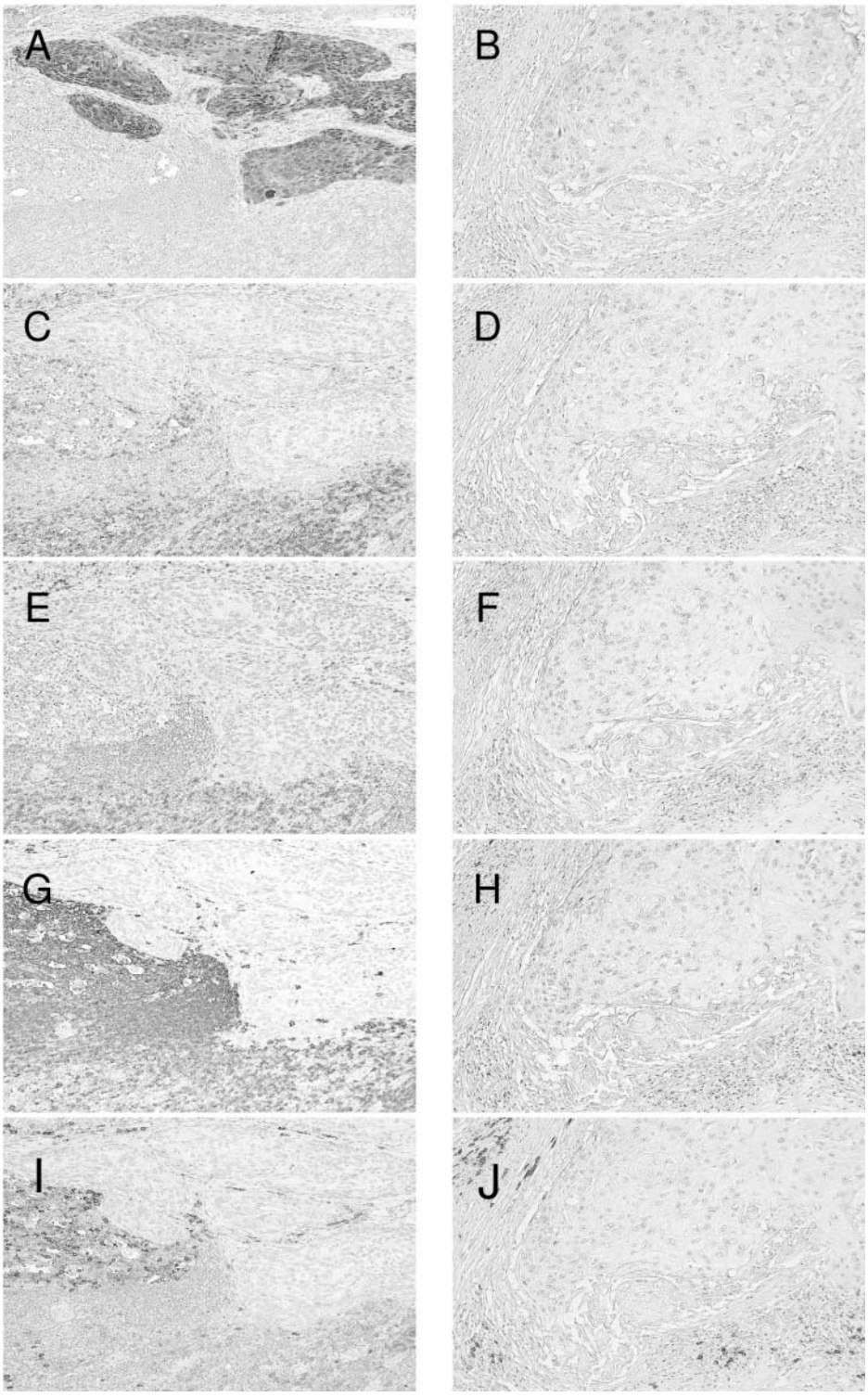

Figure 1. *Photographs of tissue sections immunostained for HSP70, CD4, CD8, and CD38 (×100). A, C, E, G, and I represent one series of sections, and B, D, F, H and J represent another series of sections. A, Strong HSP70 expression within the cytoplasm and nucleus of tumor cells: HSP70^{+}. B, No HSP70 expression within the tumor cells: HSP70^{-}. C, Abundant CD4^{+} T-cell infiltration within cancer stroma: CD4^{+}. D, No CD4^{+} T-cell infiltration: CD4^{-}. E, CD8^{+}. F, CD8^{-}. G, L26(CD20) staining is demonstrated. Abundant B-cell infiltration recognized as L26-positive cells were seen around the tumor nest containing lymphoid follicles. H, Scanty B-cell infiltration around the tumor nest. I, J, CD38 staining is demonstrated. The strongly CD38-positive cells are plasma cells. They were confirmed by serial hematoxylin-eosin section.*

Table II. *The correlation between clinicopathological characteristics and HSP70 expression.*

Parameters	n	HSP70 expression		*p*-Value
		Negative (n=61)	Positive (n=64)	
Age (mean±SD, years)		60.9±9.1	63.2±8.1	0.1318
Gender				
Male	108	53	55	
Female	17	8	9	0.8772
Location				
Upper thoracic	17	6	11	
Mid-thoracic	77	33	44	
Lower thoracic	31	22	9	0.0148*
Grade				
Well-differentiated SCC	29	16	13	
Moderately differentiated SCC	54	23	31	
Poorly differentiated SCC	33	18	15	
Other				
TNM classification	9	4	5	0.6131
T				
T1	52	8	44	
T2	16	12	4	
T3	50	35	15	
T4	7	6	1	<0.0001*
N				
N0	56	22	34	
N1	69	39	30	0.0821
M				
M0	105	47	58	
M1a	4	2	2	
M1b	16	12	4	0.0787
Stage				
I	37	7	30	
II	40	22	18	
III	28	18	10	
IV	20	14	6	<0.0001*
Infiltrative growth pattern				
α	28	11	17	
β	84	43	41	
γ	13	7	6	0.5120
Blood vessel invasion				
(–)	61	22	39	
(+)	64	39	25	0.0054*
Lymphatic invasion				
(–)	39	14	25	
(+)	86	47	39	0.0567

SD: Standard deviation; *significant; SCC: squamous cell carcinoma.

Prognostic significance of immune cell infiltrations. The mean total B-cell score was 5.02. Therefore, we classified patients into the following two groups: patients with a total score above 5, B-cell$^+$, and patients with a total score below 5, B-cell$^-$. The mean plasma cell score was 59.0, so we classified as follows: patients with more than 59 cells, plasma cell$^+$, and patients with less than 59, plasma cell$^-$. We then analyzed the patient prognoses. Both B-cell$^+$ and plasma cell$^+$ groups had a significantly better prognosis than the negative groups. Regarding the anti-HSP70 antibody, the high level group had a significantly better prognosis than the low level group (Figure 4A, B). In addition, we analyzed the participation of cellular immunity and found that the mean number of counted CD4$^+$ T-cells was 68.2, so we classified as follows: patients with more than 68 cells, CD4$^+$ T-cell$^+$, and patients with less than 68, CD4$^+$ T-cell$^-$. The mean number of counted CD8$^+$ T-cells was 27.8, so we classified as follows: patients with more than 28 cells, CD8$^+$ T-cell$^+$, and patients with less than 28 cells, CD8$^+$ T-cell$^-$. Although there was a positive correlation between CD8$^+$ T-cell infiltration and good prognosis, there was no significant correlation between CD4$^+$ T-cell infiltration and prognosis (Figure 4C, D).

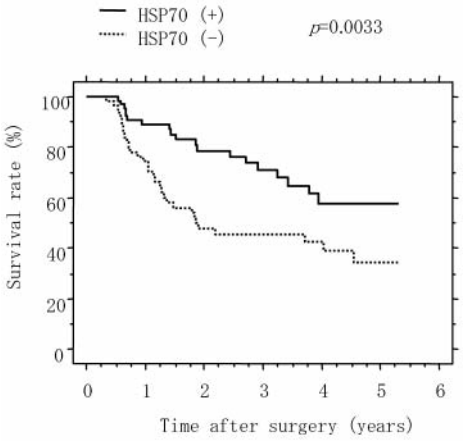

Figure 2. *The overall survival curve. Patients with HSP70$^+$ had significantly better prognosis than patients with HSP70$^-$ (p=0.0033).*

Prognostic significance of coexpression of HSP70-humoral immune cells and coexpression of HSP70-cellular immune cells. To confirm the importance of the HSP70-humoral immune cell system, overall survival curves were constructed by the Kaplan-Meier method. In total, 125 patients were divided into 8 groups according to HSP70, B-cell, and plasma cell status. The HSP70/B-cell/plasma cell$^{+/+/+}$ group had better prognosis than the other 7 groups (Figure 5A). In addition, the HSP70-cellular immune cell system was also analyzed for overall survival (Figure 5B). As above, the results revealed that the HSP70/CD4/CD8$^{+/+/+}$ group had better prognosis than the other 7 groups.

Univariate and multivariate analyses. To clarify whether the HSP70-humoral immune cell system was a significant prognostic marker for patients with esophageal cancer, univariate and multivariate survival analyses were performed. In univariate analyses using the Cox model, HSP70/B-

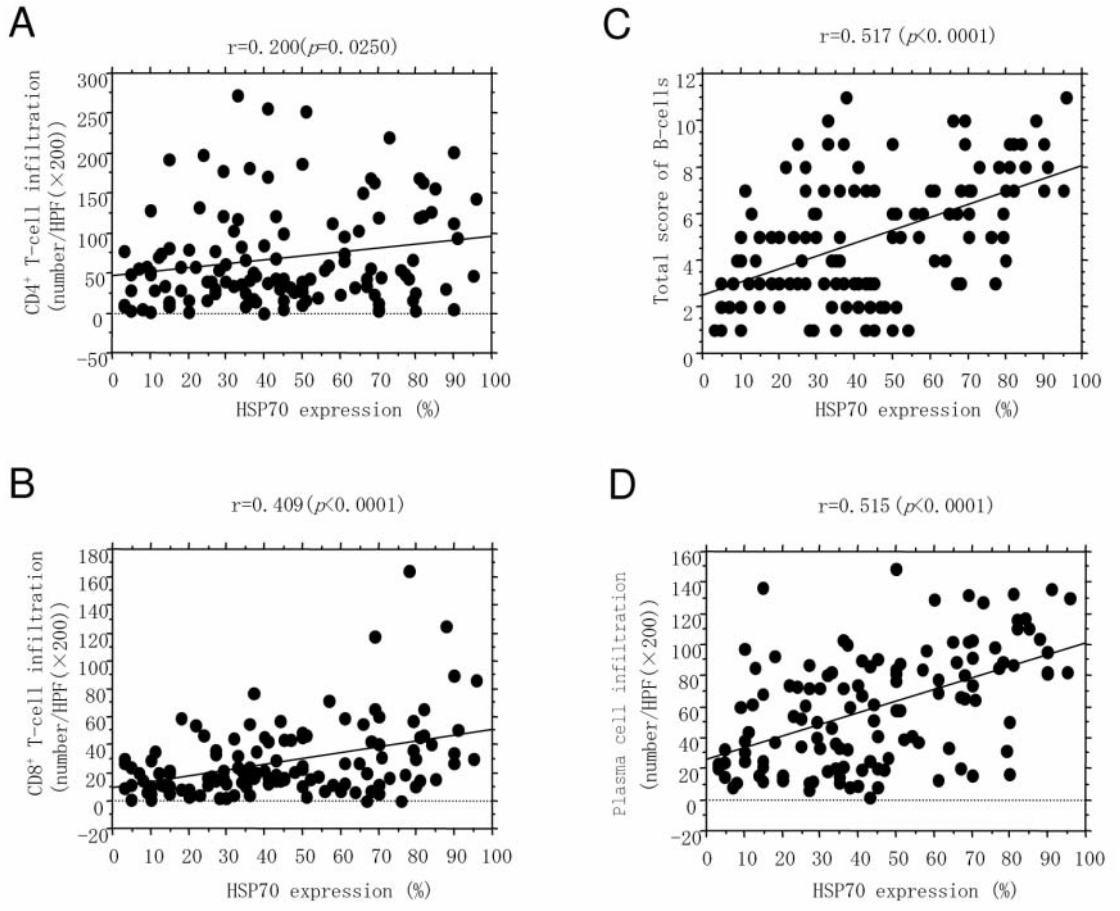

Figure 3. *Relationship between HSP70 expression and immune cell infiltrations. There were significant relationships between HSP70 expression and CD4+ (A) and CD8+ T-cell (B), B-cell (C), and plasma cell (D) infiltration. Of these, B-cells (r=0.517, p<0.0001) and plasma cells (r=0.515, p<0.0001) had the strongest correlation.*

cell/plasma cell$^{+/+/+}$, HSP70/CD4/CD8$^{+/+/+}$, and pT, pN, and pM classification were identified as predictors. In multivariate analyses, pN classification was recognized as an independent prognostic factor. Although HSP70/B-cell/plasma cell$^{+/+/+}$ was not recognized as an independent prognostic factor, it was the second most important prognostic factor (Table III). Therefore, we believed there to be a close relationship between esophageal cancer and the HSP70-humoral immune cell system, and measured serum anti-HSP70 antibody levels in the azygos vein, which is considered a drainage vein of the esophagus, to clarify the effect of the humoral immune system.

Serum anti-HSP70 antibody levels in the azygos vein of esophageal cancer patients. The mean serum anti-HSP70 antibody level in the azygos vein of 46 esophageal cancer patients was 131.62 ± 16.94 μg/ml (mean ± SEM). Because the median concentration of anti-HSP70 antibody in the azygos vein was 130 μg/ml, we performed statistical analysis using this value as the cut-off.

Correlations among the clinicopathological characteristics of patients with esophageal cancer and serum anti-HSP70 antibody levels in the azygos vein in patients were analyzed. There was no significant correlation between the anti-HSP70 antibody levels and any of the factors (data not shown). We thought it natural that total protein levels of anti-HSP70 antibody increase as tumor volume increases, therefore, we corrected the value of anti-HSP70 by tumor volume (longest diameter × shortest diameter × depth); the mean corrected value was 77.24±20.57 μg/ml (mean±SEM). The median value was 9.25 μg/ml, so we re-analyzed using this value as the cut-off. There were significant correlations between the level of anti-HSP70 antibody and depth of invasion (p=0.0007), lymph node status (p=0.0145), and pathological stage (p=0.0004); however, anti-HSP70 antibody levels in the azygos vein did not correlate with gender, age, tumor location, histological type, or distant metastasis (Table IV).

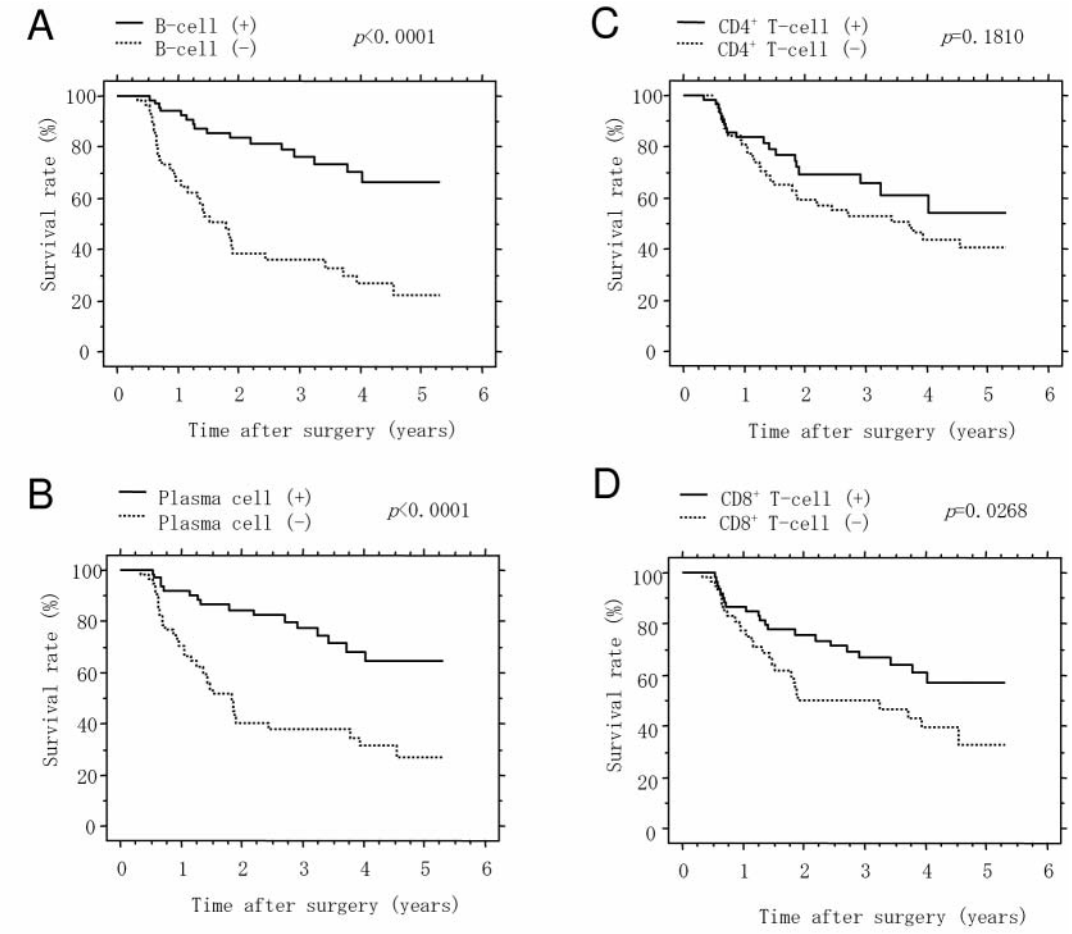

Figure 4. *Overall survival curves of each immune cell infiltration level. A, The B-cell$^+$ infiltration group had better prognosis than the B-cell$^-$ infiltration group. B, Regarding plasma cell infiltration, the same relationship of B-cell infiltration to survival was revealed. C, There was no significant relationship between CD4$^+$ T-cell infiltration levels and prognosis. D, The CD8$^+$ T-cell$^+$ group had better prognosis than the CD8$^+$ T-cell$^-$ group.*

Table III. *Univariate and multivariate analyses.*

Factors	Hazard ratio (95% CI)	*p*-Value
Univariate		
Age (60/60 years)	0.842 (0.483-1.467)	0.5434
Gender (male/female)	0.763 (0.325-1.793)	0.5355
Grade (3, other/1, 2)	1.583 (0.897-2.792)	0.1131
pT classification (3, 4/1, 2)	6.370 (3.391-11.965)	<0.0001*
pN classification (1/0)	11.112 (4.400-28.066)	<0.0001*
pM classification (1/0)	4.667 (2.430-8.965)	<0.0001*
p stage (III, IV/I, II)	9.624 (5.063-18.296)	<0.0001*
HSP70/B-cell/plasma cell [HSP70/B-cell/plasma cell (+/+/+)/other]	6.808 (2.448-18.934)	0.0002*
HSP70/CD4/CD8 [HSP70/CD4/CD8 (+/+/+)/other]	3.682 (1.145-11.840)	0.0287*
Multivariate		
pT classification (3, 4/1, 2)	2.619 (0.826-8.296)	0.1018
pN classification (1/0)	6.988 (2.097-23.289)	0.0015*
pM classification (1/0)	1.726 (0.841-3.546)	0.1369
p stage (III, IV/I, II)	0.959 (0.239-3.857)	0.9533
HSP70/B cell/plasma cell [HSP70/B cell/plasma cell (+/+/+)/other]	3.023 (0.751-12.176)	0.1195
HSP70/CD4/CD8 [HSP70/CD4/CD8 (+/+/+)/other]	0.818 (0.194-3.451)	0.7839

CI: Confidence interval, *significant.

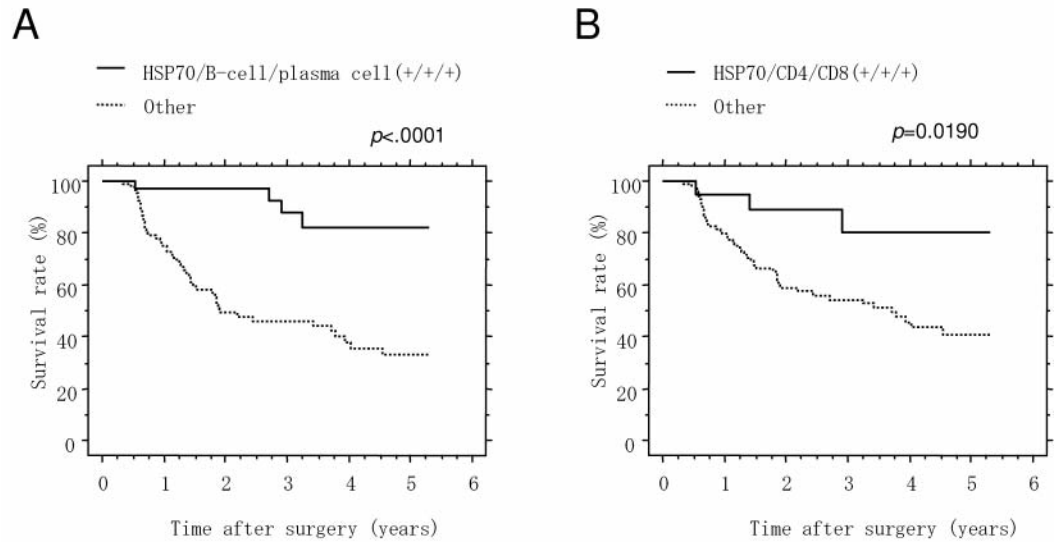

Figure 5. *Relationship between HSP70–immune cell status and the survival curve. A, Simultaneous occurrence of HSP70 expression, and B-cell and plasma cell infiltration led to good prognosis. Other comprises are HSP70/B cell/plasma cell (–/+/+), (+/–/+), (+/+/–), (+/–/–), (–/–/+), (–/+/–), and (–/–/–). B, Simultaneous occurrence of HSP70 expression, and CD4+ and CD8+ T-cell infiltration led to good prognosis as in (A). Other comprises HSP70/CD4/CD8 (–/+/+), (+/–/+), (+/+/–), (+/–/–), (–/–/+), (–/+/–), and (–/–/–).*

Relationship between HSP70 expression and anti-HSP70 antibody levels. The relationship between HSP70 expression in the tumor tissue and serum anti-HSP70 antibody levels in the azygos vein was analyzed. The result revealed a positive correlation (Figure 6).

Relationship between B-cell and plasma cell infiltration and anti-HSP70 antibody levels. To clarify the participation of the host humoral immune system in the production of anti-HSP70 antibodies, the relationship between B cell and plasma cell infiltration and anti-HSP70 antibody levels were analyzed. There were positive correlations between them (Figure 7).

Prognostic significance of anti-HSP70 antibodies. The relationship between the level of anti-HSP70 antibodies and prognosis was analyzed. The high-level anti-HSP70 antibody group had significantly better prognosis than the low-level group (Figure 8). This result also confirmed the importance of humoral immunity in patients with esophageal cancer.

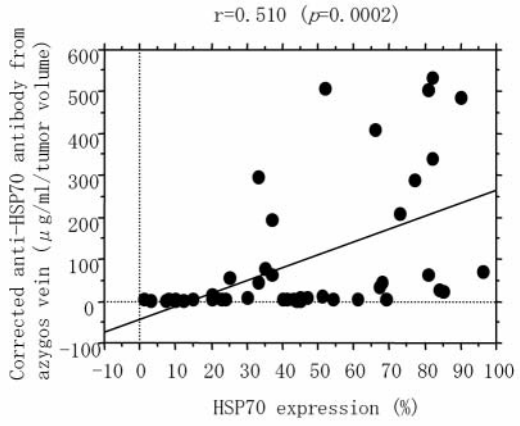

Figure 6. *Relationship between HSP70 expression and anti-HSP70 antibody levels in the azygos vein corrected by tumor volume. There was a significant correlation (r=0.510, p=0.0002).*

Discussion

In this study, immunohistochemical examination of TILs revealed a significant correlation between humoral immunity and HSP70 expression. In addition, serum anti-HSP70 antibody levels obtained from the azygos vein correlated with B-cell and plasma cell infiltration.

There have been many reports regarding TILs (28-31) and HSP70, and such reports have also been published for esophageal carcinomas (32, 33). However, these studies were discussed from the viewpoint of cellular immunity. To our knowledge, the current study is the first study to refer to humoral immunity against gastrointestinal tumor.

First, we investigated immunohistochemical HSP70 expression. The results confirmed those of previous reports including a previous study by the authors (13, 39). Briefly, the degree of HSP70 expression was stronger in early stage tumors than advanced tumors, and was significantly correlated with prognosis.

Next, we analyzed the relationship between HSP70 expression and TILs. According to our current results, B- and plasma cell

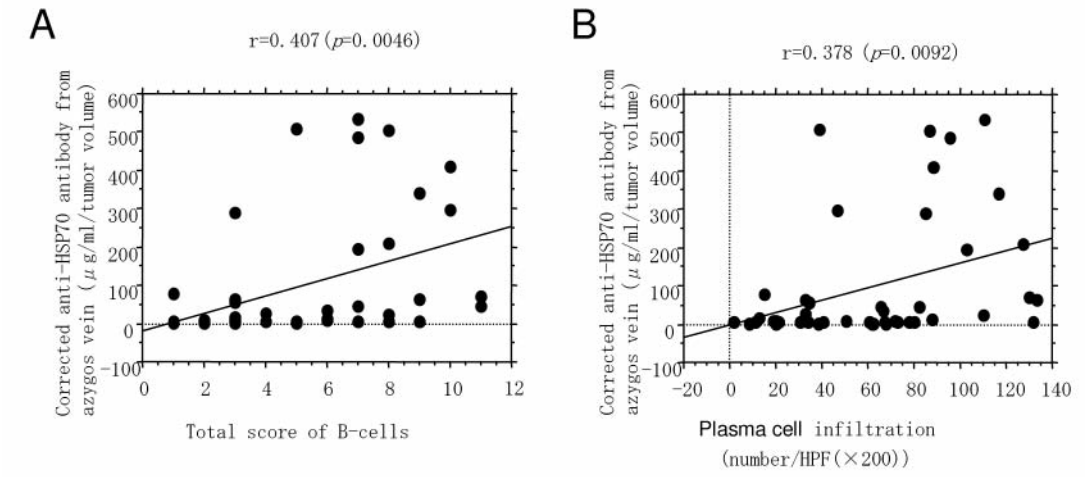

Figure 7. *Relationship between humoral cell infiltration and anti-HSP70 antibody levels in the azygos vein corrected by tumor volume. A, The relationship between total B-cell scores and anti-HSP70 antibody values was significant (r=0.407, p=0.0046). B, There was also a significant correlation between plasma cell infiltration and anti-HSP70 antibody values (r=0.378, p=0.0092).*

infiltration had a stronger relationship with HSP70 expression than $CD4^+$ and $CD8^+$ T-cell infiltration. HSP70 is known to be involved in class I major histocompatibility complex (MHC)-linked tumor-specific antigen presentation (40). A number of peptides that bind to HSP70 are recognized as HSP-peptide complexes by specific cytotoxic T lymphocytes (41). Our results also confirmed participation of cellular immunity. However, B-cell infiltration was most significantly correlated with HSP70 expression among the immune cells studied. Subepithelial lymphoid follicle formation by B-cells is a frequent phenomenon in esophageal cancer (34, 42), therefore, to clarify the participation of humoral immunity in tumor-antigen presentation by HSP70, we investigated the existence of anti-HSP70 antibodies in serum from the azygos vein.

There are many reports regarding anti-HSP70 antibodies, and their expression has been detected in peripheral circulation of normal individuals (18), and at relatively higher levels in elderly individuals (19), smokers (20), and patients with uveitis (21), arteriosclerosis (22), schizophrenia (43) and neoplastic disease. Therefore, there is some doubt as to whether anti-HSP70 antibody levels should be measured in peripheral blood. The azygos vein is chiefly responsible for venous return from the esophagus (36-38). We previously reported the significance of measuring protein TGF-β levels in esophageal cancer patients (44). Therefore we supposed that the serum anti-HSP70 antibody level in the azygos vein might reflect tumor progression and conditions more directly and accurately than the levels in other veins. First, we analyzed the relationship between the original value of anti-HSP70 antibody and clinicopathological factors, but there was no significant correlation. We thought that the volume of tumor tissue might have had an effect on the anti-HSP70 antibody value; it is natural that total protein levels of anti-

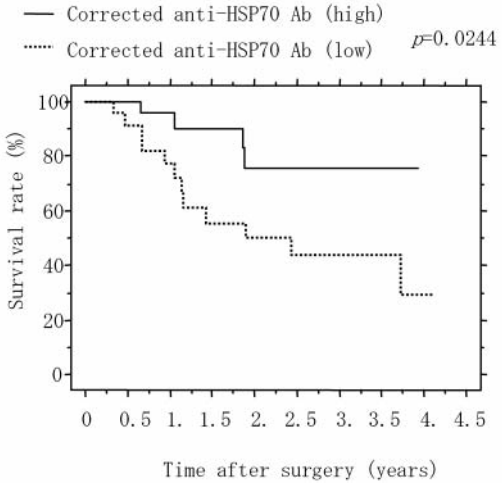

Figure 8. *Relationship between anti-HSP70 antibody levels and the survival curve. Patients with a high corrected anti-HSP70 antibody level within the serum from the azygos vein had better prognosis than the low-level group.*

HSP70 antibody increase as tumor volume increases because infiltrating antibody-producing cells also increase. Therefore, we corrected the value of anti-HSP70 by tumor volume. Thus, we observed a significant correlation between the anti-HSP70 antibody level measured in the azygos vein and B cell and plasma cell infiltration and intratumoral HSP70 expression. Previously the antibody to HSP 70 was analyzed in gynecological malignancies (26), nasopharyngeal carcinoma (45) and esophageal squamous cell carcinoma (46), and HSP90, which has the same function as HSP70, has been detected in the sera of patients with breast cancer (47)

Table IV. *The correlation between characteristics and anti-HSP70 antibody.*

Parameters	n	Anti-HSP70 antibody from Azygos Vein		*p*-Value
		Low (n=23)	High (n=23)	
Age(mean±SD, yrs)		62.3±8.1	60.2±8.7	0.4028
Gender				0.1006
Male	39	17	22	
Female	7	6	1	
Location				0.5863
Upper thoracic	6	4	2	
Mid-thoracic	29	13	16	
Lower thoracic	11	6	5	
Histological type				0.2818
Well differentiated SCC	11	7	4	
Moderately differentiated SCC	15	6	9	
Poorly differentiated SCC	15	9	6	
Others	5	1	4	
TNM classification				
T				0.0007*
T1	19	3	16	
T2	4	2	2	
T3	20	15	5	
T4	3	3	0	
N				0.0145*
N0	17	4	13	
N1	29	19	10	
M				0.4575
M0	39	18	21	
M1	7	5	2	
Stage				
I	13	1	12	
II	12	5	7	
III	14	12	2	
IV	7	5	2	0.0004*

SD: standard deviation; SCC: squamous cell carcinoma; *significant.

and osteosarcoma (48); however, the current study is the first to investigate the relationship between anti-HSP70 antibody levels and humoral immune system cells.

Although the significant correlation between immunohistochemical expression of intratumoral HSP70, B-cell and plasma cell infiltration around the tumor tissues, and serological expression of anti-HSP70 antibodies suggests that the host humoral immune system recognizes HSP70 in tumor cells and produces autoantibody, it is impossible to explain why the humoral immune system has a direct antitumor effect. As described above, the leading effector of tumor-specific antigen presentation by HSP70 is the cellular immune system. Concomitant with this, the expressed HPS70 on the surface of tumor cells might be recognized as self-antigens, resulting in detectable HSP70 autoantibodies in the serum. However, the immune system is very complicated, and another pathway that involves MHC class I molecule presentation of intrinsic antigens was indicated. It is possible that anti-HSP70 antibodies themselves might have a direct effect on esophageal cancer cells. The simultaneous occurrence of HSP70 expression and B-cell and plasma cell infiltration is a stronger prognostic factor than simultaneous occurrence of HSP70 expression and $CD4^+$ T- and $CD8^+$ T-cell infiltration according to univariate and multivariate analyses is an important result. If obvious evidence of antitumoral effects of humoral immunity were demonstrated, it might be possible to develop antibody therapy against esophageal cancer.

In conclusion, this study suggests analysis of HSP70 and TILs, including B-cell and plasma cells, are useful in predicting the prognosis of patients with esophageal cancer. Furthermore, they suggest that there might be an unknown antitumor humoral immune system besides cellular immunity.

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