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Expression of heat-shock protein Hsp60 correlated with the apoptotic index and patient prognosis in human oesophageal squamous cell carcinoma

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Abstract

Cellular stress response and apoptosis are two highly conserved mechanisms for maintaining homeostasis. Hsp60 and Hsp90 have been shown to play pro- and anti-apoptotic roles, respectively. Our present study examined whether there is a correlation between the expression of Hsp60 and Hsp90, clinical parameters, the apoptotic index (AI), and the prognosis of patients with oesophageal squamous cell carcinoma (ESCC). We immunohistochemically stained cells for Hsp60, Hsp90, and single-stranded DNA (ssDNA), which acts as an apoptotic marker. In normal oesophageal epithelium tissue, Hsp60 and Hsp90 were expressed in the cytoplasm and membrane from the basal cell layer to the supra-basal cell layers. Hsp60 and Hsp90 positive stainings (+) were found in 63 of 123 cases (51%) and 62 of 123 cases (50%), respectively. There was no correlation between Hsp60 and Hsp90 expression levels and any of the clinical parameters examined. The five-year survival rate for ESCC patients with Hsp60 (+) expression was significantly higher than for those patients with Hsp60 (−) expression ($P = 0.0371$). Five-year survival rates of patients with Hsp60 (+) and (−) were 49% and 33%, respectively. By contrast, Hsp90 expression failed to predict patient prognosis ($P = 0.7965$). The high-AI group did not have a significantly better prognosis than the low-AI group ($P = 0.2218$). Statistical analysis showed a significant correlation between the expression of Hsp60 and AI in ESCC patients ($P = 0.008$). Thus, the five-year survival rate for the high-AI/Hsp60 (+) group was statistically significantly better than for the other groups ($P = 0.0281$). The results obtained in this study indicate that positive Hsp60 expression is a good prognostic indicator. This may be due to its role as a chaperone in contributing to the induction of apoptosis. These data suggest that Hsp60 expression correlates with the AI and patient prognosis in human ESCC.

Keywords: Hsp60; Hsp90; Oesophageal squamous cell carcinoma; Immunohistochemistry; Apoptotic index; Prognosis

1. Introduction

The cellular response to stress is represented at the molecular level by the induced synthesis of the heat-shock proteins (Hsp) as an essential defense mechanism for the protection of the cell from many harmful conditions, such as heat shock, alcohol, heavy metal, oxidative stress, fever or inflammation [1,2]. During carcinogenesis, Hsp has been reported to show alteration of its expression level, either increasing or decreasing [3,4]. Hsp has been classified into six major families according to the size of the molecule: small heat-shock protein, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100.

Hsp60 is abundant in most mammalian cells under normal conditions [5]. It has major roles in protein

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chaperoning and protein folding [6]. Hsp60 is a mitochondrial protein that is involved in the activation of apoptosis [7]. Its overexpression has been reported in exo-cervix [8] and colorectal carcinogenesis [9], suggesting that it may be involved in early carcino-genesis.

Hsp90 is associated with the folding of signal-transduction proteins, such as steroid hormone receptors and protein kinases. Hsp90 is an essential cytosolic protein; its expression in a wide variety of malignant tumours makes Hsp90 a candidate for pharmacological intervention [10]. Recently, several mechanisms by which Hsp90 acts as an anti-apoptotic factor have been reported [11]. Overexpression of Hsp90 in breast tumour, lung cancer, leukaemia, Hodgkin's disease, pancreatic carcinoma, and gastric cancer [12–17] has been observed.

In oesophageal cancer, the major risk factors for oesophageal squamous cell carcinoma (ESCC) are diet, tobacco use, and alcohol consumption [18,19]. Hsp expression has been reported to correlate with prognosis and lymphocyte infiltration in ESCC [20,21]. Based on these considerations, we performed an immunohistochemical study on Hsp60 and Hsp90, and used single-stranded DNA (ssDNA) as a marker for apoptosis. In this study, we examined whether there is a correlation between Hsp60 and Hsp90 expression levels, clinical parameters, apoptotic index (AI), and prognosis in patients with ESCC.

2. Materials and methods

2.1. Patients

Surgical specimens were obtained from 123 patients (106 males and 17 females) who had ESCC and underwent potentially curative surgery at the Department of General Surgical Science, Gunma University, between 1983 and 2002. The patients' age ranged from 40 to 79 years, with a mean of 61.2 years. Tumour stage and disease grade were classified according to the 5th edition of the TNM classification of the International Union against Cancer (UICC) [22]. The evaluation of tumour differentiation was based on histological criteria by the Japanese Society for Esophageal Diseases [23]. None of the patients had received irradiation or chemotherapy prior to surgery, nor did any of them have haematogenic metastases at the time of surgery. Patients who underwent non-curative surgery and/or who had received inadequate follow-up were excluded from this study. Post-operative chemotherapy and/or radiation therapy were not performed until recurrence of the tumour was confirmed by a radiological or endoscopic examination.

2.2. Immunohistochemical staining for Hsp60 and Hsp90

Resected specimens were fixed with 10% natural buffer formalin and embedded in a paraffin block. Immunohistochemical staining of the section for Hsp60 and Hsp90 were performed using the streptoavidin–biotin method. Sections (4-μm thick) were deparaffinised with xylene, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, the specimen was washed in phosphate-buffered saline (PBS). After a blocking treatment with 5% skimmed milk for 30 min, the specimens were removed by blotting. The specimens were then incubated with the primary anti-Hsp60 monoclonal antibody (SPA-829; StressGen) and anti-Hsp90 monoclonal antibody (SPA-830; StressGen) at a dilution of 1:400 and 1:200, respectively in PBS containing 1% bovine serum albumin (BSA) at 4 °C overnight. They were then washed with PBS and incubated in secondary antibody for 30 min at room temperature. Immunohistochemistry was performed using a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). The chromogen was a 3.3–0.02% solution containing 0.0055% H_2O_2 in a 50 mM ammonium acetate–citric acid buffer, pH 6.0. The specimens were lightly counterstained with haematoxylin. Negative controls were prepared by substituting normal mouse serum for each primary antibody: no detectable staining was evident. Hsp expression was calculated as the percentage of cytoplasmic and membranous staining of the cells at the central layers of cancer cell nest in three consecutive high-powered fields. The staining evaluation was performed by two independent observers who did not have any knowledge of the clinical outcome. The means of the Hsp60 and Hsp90 expression rate in 123 primary tumours were almost 60% and 40%. Therefore, when more than 60% and 40% of the tumour cells were positively-stained, the sample were classified as positive for Hsp60 and Hsp90 expression (+). When less than 60% and 40% staining was observed, the samples were classified as negative for Hsp60 and Hsp90 expression (−).

2.3. Immunohistochemical detection of apoptotic cells and bodies

The ssDNA immunohistochemistry procedure was carried out by the standard avidin-biotin peroxidase complex methods on the paraffin sections as described above, anti-ssDNA polyclonal antibody (DakoCytomation, Kyoto, Japan) at a dilution 1:100 was used. Slides of ESCC, which were already known to contain many apoptotic cells were used as positive controls. Tumour cells with positive staining were morphologically identified using standard apoptotic criteria: chromatin condensation, nuclear disintegration, and formation of crescentic caps at the nuclear periphery [24]. Single and roundish

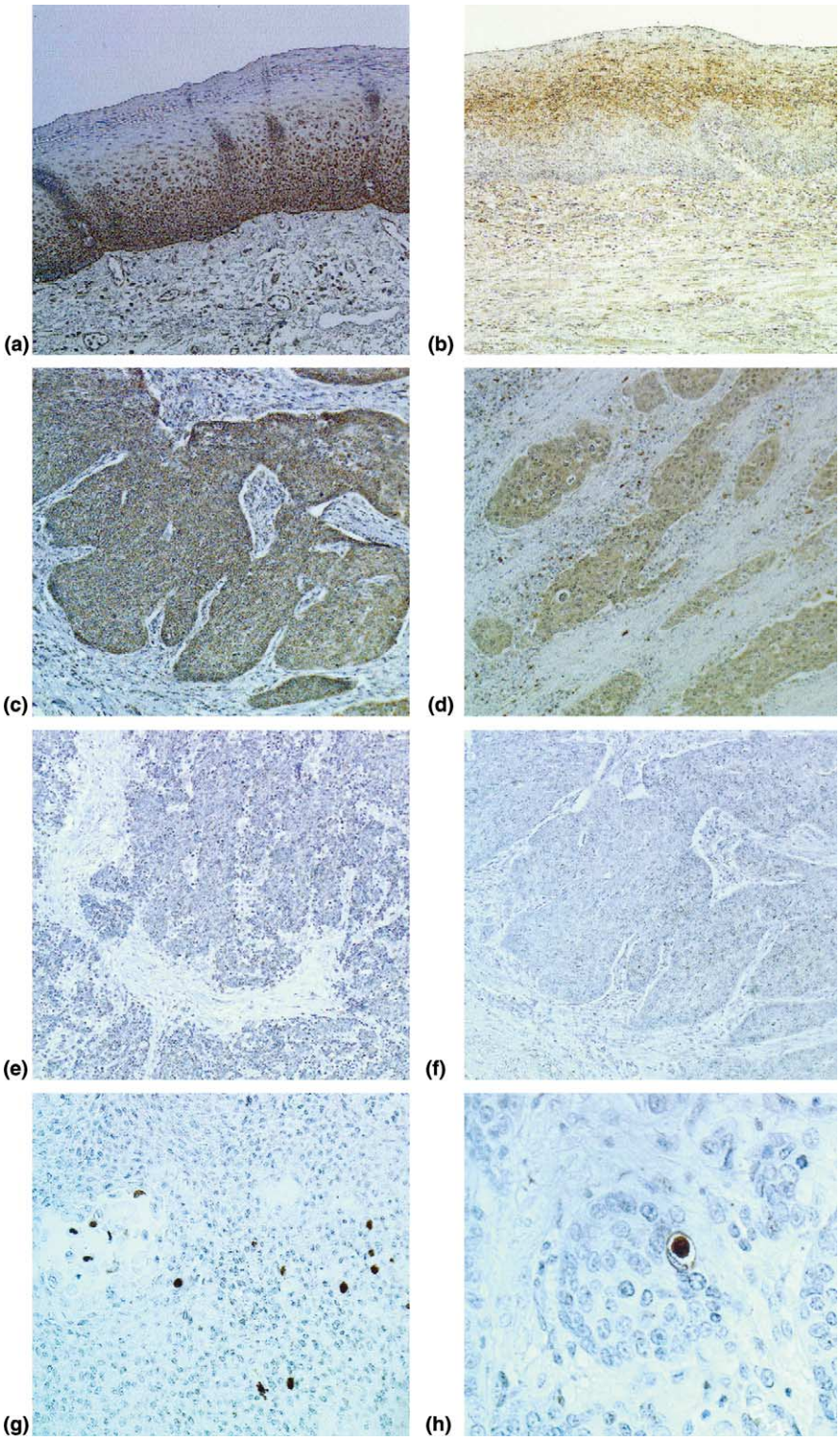

Fig. 1. Representative photomicrographs of tissue sections. Immunostained for Hsp60, Hsp90, and ssDNA. (a) Hsp60 protein was detected in the cytoplasm and membrane in normal oesophageal squamous epithelium (100×). (b) Hsp90 protein was detected in the cytoplasm and membrane in normal oesophageal squamous epithelium (100×). (c) Primary oesophageal cancer with Hsp60 (+) was detected in the central layers of cancer cell nest (100×). (d) Primary oesophageal cancer with Hsp90 (+) was detected in the central layers of cancer cell nest (100×). (e) Primary oesophageal cancer with Hsp60 (−) in the cancer cell nest (100×). (f) Primary oesophageal cancer with Hsp90 (−) in the cancer cell nest (100×). (g) Primary oesophageal cancer stained with ssDNA polyclonal antibody, nuclei of apoptotic cancer cells and bodies were strongly stained brown (200×). (h) The apoptotic cell in the cancer cell nest (400×).

nuclear residues existing in intra-tumoural cells with intensive staining were identified as apoptotic bodies [24]. The AI was obtained as the ratio of the number of ssDNA-positively stained tumour cells to the total number of tumour cells counted per section. At least 1000 cells were counted under 400-fold magnification.

2.4. Statistical analysis

Statistical analysis was performed using the Stat View software program (Version 5, SAS Institute, NC, USA). The unpaired two-group *t*-test was used for age, Hsp60 expression, Hsp90 expression, and AI. A Chi-squared test was used for gender, differentiation, TNM clinical classification, stage, location, lymphatic invasion, and infiltrative growth pattern. 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 the risk ratio was used to assess the simultaneous contribution of levels of Hsp60 and Hsp90 and AI to the survival rate. Significant differences were noted when $P < 0.05$.

3. Results

3.1. Hsp60, Hsp90, and ssDNA expression

In normal oesophageal epithelium tissue, Hsp60 and Hsp90 were expressed in the cytoplasm and membrane

Table 1
Clinicopathological findings and Hsp expression

Parameters	Total $n = 123$	Hsp60 (−) $n = 60$	Hsp60(+) $n = 63$	*P*-value	Hsp90 (−) $n = 61$	Hsp90(+) $n = 62$	*P*-value
Age (mean ± SD years)	61.2 ± 8.6	62.7 ± 8.1	61.2 ± 9.0	0.3266	62.5 ± 8.2	61.3 ± 8.9	0.4275
Gender							
Male	106	52	54		51	55	
Female	17	8	9	0.8784	10	7	0.4123
Differentiation							
Well	28	15	13		15	13	
Moderate	54	24	30		27	27	
Poorly	33	17	16		14	19	
Other	8	4	4	0.8573	5	3	0.7073
Location							
Cervical	2	0	2		0	2	
Upper	15	6	9		6	9	
Middle	74	38	36		37	37	
Lower	32	16	16	0.4606	18	14	0.3776
TNM clinical classification							
T							
Tl	51	24	27		30	21	
T2	16	6	10		7	9	
T3	49	26	23		21	28	
T4	7	4	3	0.6984	3	4	0.3958
N							
N0	55	28	27		33	22	
Nl	68	32	36	0.8066	28	40	0.0579
M							
M0	103	48	55		51	52	
Ml	20	12	8	0.2727	10	10	0.9683
Stage							
I	36	20	16		23	13	
II	40	13	27		17	23	
III	27	15	12		11	16	
IV	20	12	8	0.0933	10	10	0.2039
Lymphatic invasion							
Negative	38	18	20		21	17	
Positive	85	42	43	0.8341	40	45	0.4004
Infiltration growth pattern							
α	27	15	12		14	13	
β	83	38	45		41	42	
γ	13	7	6	0.6288	6	7	0.9428

SD, standard deviation.

from the basal cell layer to the supra-basal cell layers (Figs. 1(a) and (b)). Immunostaining of Hsp60 and Hsp90 were seen in the cytoplasm and membrane of the cancer cells. Hsp60 expression (+) was observed as a diffuse staining of tumour cell cytoplasm and membrane as coarse granules (Fig. 1(c)). Hsp90 expression (+) was evident as diffuse cytoplasm staining (Fig. 1(d)). Positive staining for Hsp60 and Hsp90 were found in 63 of the 123 cases (51%) and 62 of the 123 cases (50%), respectively. Sixty (49%) and sixty-one (50%) of 123 cases were then classified as Hsp60 and Hsp90 expression (−), respectively (Figs. 1(e) and (f)). Apoptotic cancer cells and bodies were identified by brown nuclear staining with ssDNA (Figs. 1(g) and (h)).

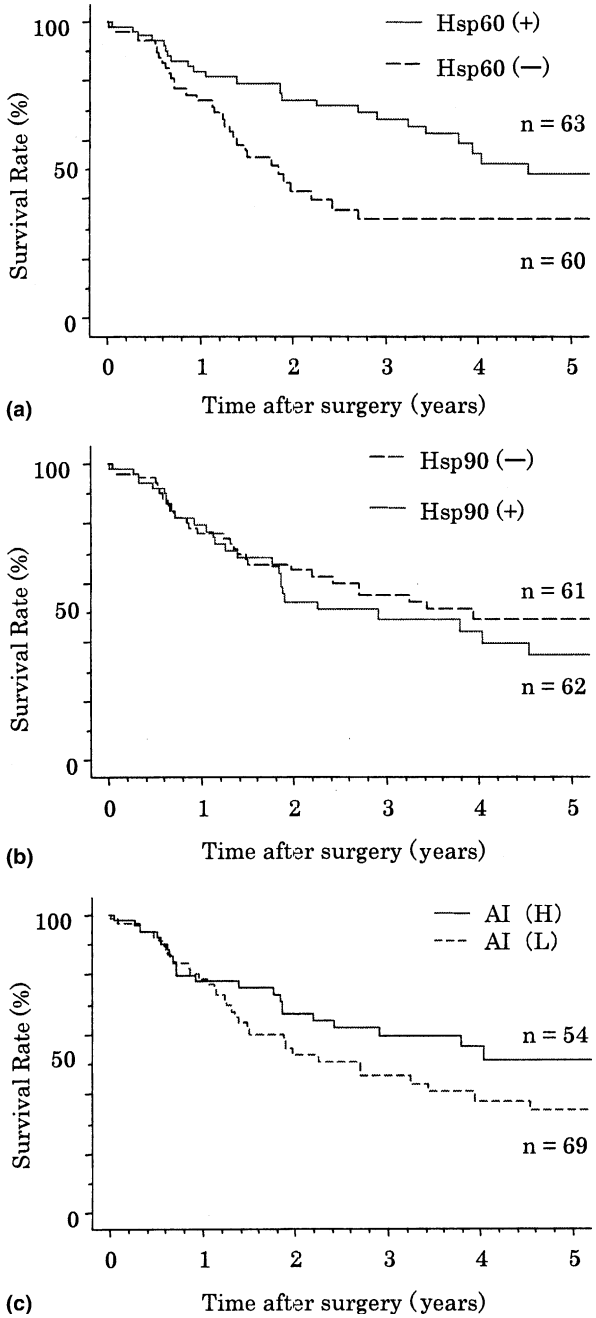

Fig. 2. The post-operative overall survival rate is shown according to expression of Hsp60, Hsp90 and AI. (a) Patient survival according to Hsp60 expression. (b) Patient survival rates according to Hsp90 expression. (c) Patient survival according to AI; 56% for high-AI (H) patients ($n = 54$) and 37% for low-AI (L) patients ($n = 69$).

3.2. Correlation between Hsp60, Hsp90 expression and clinical parameters

We examined the correlation between clinical parameters in 123 ESCC patients with Hsp60 and Hsp90 (Table 1). There was no correlation between Hsp60 and Hsp90 expression with any of the clinical parameters.

3.3. Correlations between expression of Hsp60 and Hsp90, AI, and survival rate

There was no significant correlation between Hsp60 and Hsp90 expression ($P = 0.2809$; Table 2). The mean AI was 0.895 ± 0.845 (range 0–2.6%). The patients were divided into two groups according to their AI; low-AI (AI < mean) and high-AI (AI ⩾ mean). Table 3 summarises the correlation between Hsp60 or Hsp90 expression and AI. The AI in tumours with Hsp60 (+) was significantly higher than that of those with Hsp60 (−) ($P = 0.008$). Next, we examined the correlation between Hsp60 and Hsp90 with the five-year survival rate of ESCC patients. The five-year survival rate for ESCC patients with Hsp60 (+) was significantly higher than that for patients with Hsp60 expression (−) ($P = 0.0371$; Fig. 2(a)). The five-year survival rates of patients with

Table 2
Correlation between HspGO and Hsp90 expression

	Total $n = 123$	Hsp90 expression		P-value
		(−)	(+)	
Hsp60 expression				
(−)	60	33	27	
(+)	63	28	35	0.2809

Hsp, heat-shock protein; (−), negative; (+), positive.

Table 3
Apoptotic index (AI) and Hsp expression in ESCC

	Total $n = 123$	Apoptotic index (mean ± SD[a])	P-value
Hsp60			
Positive	63	1.07 ± 0.80	
Negative	60	0.66 ± 0.85	0.008[b]
Hsp90			
Positive	62	0.88 ± 0.86	
Negative	61	0.86 ± 0.84	0.9084

ESCC, oesophageal squamous cell carcinoma.
[a] Standard deviation.
[b] Significant.

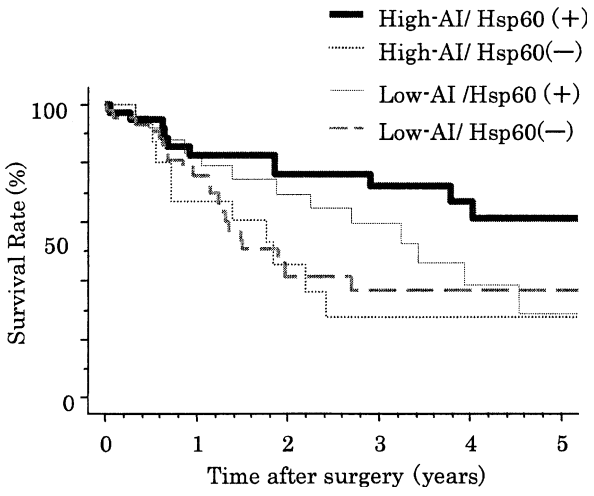

Fig. 3. The post-operative overall survival rate is shown according to Hsp60 expression and AI.

Hsp60 (+) and (−) were 49% and 33%, respectively. Multivariate analysis showed that Hsp60 expression was not a prognostic factor by itself (data not shown). By contrast, Hsp90 expression failed to predict prognosis ($P = 0.7965$; Fig. 2(b)). Five-year survival rates were 36% (Hsp90 (+)) and 49% (Hsp90 (−)). The five-year survival rate was 56% for patients with a high-AI ($n = 54$) and 37% for patients with a low-AI ($n = 69$). The high-AI group did not have a significantly better prognosis than the low-AI group ($P = 0.2218$; Fig. 2(c)). The five-year survival rate was 61% for patients with a high-AI/Hsp60 (+) ($n = 38$) and 28% for patients with a high-AI/Hsp60 (−) ($n = 16$), respectively. The five-year survival rate was 29% for patients with a low-AI/Hsp60 (+) ($n = 25$) and 37% for patients with a low-AI/Hsp60 (−) ($n = 44$). The high-AI/Hsp60 (+) group had a statistically significant better prognosis than the other groups ($P = 0.0281$) (see Fig. 3).

4. Discussion

Heat-shock proteins can aid or inhibit the apoptotic machinery through their role as chaperones by affecting protein assembly, folding, and the ubiquitin degradation pathways. Hsp60 is a protein that primarily localises in the matrix of mitochondria. Kawanishi and colleagues [20] observed heterogenous/mosaic reduction of Hsp expression in a colony among cells that could not be distinguished morphologically. This may imply that their alteration is not due to genomic changes. One possibility for this mechanism is aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. The active role of the aberrant methylation in transcriptional silencing of the genes involves a synergy between the methylation and histone deacetylase (HDAC) [25]. Johnson and colleagues [26] showed that Hsp60 is associated with the HDAC complex, providing evidence that the stimulatory effect of adenosine triphosphate (ATP) on HDAC catalytic activity operates through these proteins. Two independent groups reported a role for Hsp60 in helping caspase-3 maturation [27,28], suggesting that the chaperone function of Hsp60 is involved in the apoptotic pathway. By contrast, Hsp90 has been shown to play an important anti-apoptotic role. Recently, the mechanisms whereby Hsp90 acts as an anti-apoptotic factor have been reported. Hsp90 forms a cytosolic complex with Apaf-1 and inhibits Cyt *c*-mediated oligomerisation of Apaf-1 and the activation of procaspase-9 [29]. Therefore, we studied the correlation between the expression of Hsp60 and Hsp90 with AI in ESCC patients. The apoptotic activity of a tumour with Hsp60 (+) was higher (1.07 ± 0.80) than that with Hsp60 expression (−) (0.66 ± 0.85), a statistically significant result ($P = 0.008$). Thus, the five-year survival rate for the high-AI/Hsp60 (+) group was statistically significantly better than for the other groups ($P = 0.0281$). By contrast, for Hsp90 expression, there was no correlation with the AI. These data suggest that the expression of Hsp60 (+) may be related to the apoptotic activity of human ESCC. This result agrees with a previous study that reported a role for Hsp60 in helping to induce apoptosis by acting as a chaperone to procaspase-3 and aiding in its maturation into active caspase-3 [27,28]. Our study suggests that the expression of Hsp60 (+) is a predictive factor for ESCC patients: loss of its expression indicates a poor prognosis. However, multivariate statistical analysis showed that Hsp60 expression was not a prognostic factor by itself. The correlation of Hsp60 expression with a better prognosis has been described for patients with ovarian tumours [30].

Two key pathways for the induction of apoptosis are well known. They are the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. In the mitochondrial pathway, a cell death signal induces the release of cytochrome *c* (Cyt *c*), which then binds to the apoptotic protease activating factor-1 (Apaf-1) and results in the eventual recruitment of procaspase-9. Activation of this complex can trigger the caspase pathway by activating the downstream caspase-3 [31,32]. The ssDNA immunohistochemical method was applied for the first time in ESCC in the present study. It is based on the detection of ssDNA regions in apoptotic cells by a polyclonal antibody working on a paraffin section. This antibody recognises DNA fragmentation caused by a single-stranded break in nuclei during programmed cell death. This method has been demonstrated to be very valuable in the identification of cells undergoing apoptosis during embryogenesis and tumourigenesis [33,34]. The ssDNA antibody may be more specific (no staining of necrotic cell) and sensitive (staining indicat-

ing early apoptotic effects in the absence of internucleosomal DNA fragmentation) for the detection of apoptotic cells than the Terminal deoxynucleotidyl transferase (TdT)-Mediated incorporation of biotinylated nucleotides (uTP) onto the 3′-exposed hydroxyl groups (nick end labeling) nuclear deoxyribonucleic acid (TUNEL) methods [35]. The rate of apoptotic activity in tumour cells has been reported to be useful as a prognostic indicator in colorectal carcinoma [36] and ESCC [37], but not in ovarian [38] or laryngeal carcinomas [39]. Shibata and colleagues [37] found that AI is an independent prognostic indicator in ESCC. However, the present study showed that there was no significant correlation between AI and prognosis in human ESCC. In addition, there was no significant correlation between AI and any clinical parameter (data not shown). This controversial result concerning AI and prognosis possibly occurred for the following reasons: (1) A different method was used to detect apoptotic activity. (2) The number of cases involved differed in each study. Further study is needed to elucidate the role of AI in evaluating patient prognosis in ESCC.

Although these results indicate a potential role for Hsp60 in the induction of apoptosis, its role in this process has not yet been determined. Transfection experiments and measurements of the effects on apoptosis are needed, to further explore this role.

In summary, there was no correlation between Hsp60 and Hsp90 expression and any of the clinical parameter examined. Hsp60 expression may be a good prognostic indicator. By contrast, Hsp90 expression levels failed to predict ESCC patient prognosis. Hsp60 expression correlated with the apoptotic index (AI) and prognosis in human ESCC.

Conflict of interest statement

None declared.

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