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Expression and Roles of Heat Shock Proteins in Human Breast Cancer

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Heat shock proteins (hsps) are thought to play important roles in the cell cycle and various processes of carcinogenesis. Therefore, we evaluated the expression of hsps, mainly hsp90 and hsp70, in human breast cancer tissues. Hsp90α mRNA was expressed at much higher levels in the cancerous tissue than in the non-cancerous tissue. In addition, a close correlation between hsp90α mRNA expression and the proliferating-cell-nuclear-antigen labeling index (PCNA LI) was observed for the cancerous tissue. These findings suggest that increased expression of the hsp90α isoform may play a role in cell proliferation. On the other hand, hsp90β mRNA expression was significantly higher in poorly differentiated carcinomas than in well differentiated carcinomas of the breast. The intracellular localization of hsp70 was consistent with that of ubiquitin. In specimens showing hsp70 in the nucleus, the PCNA LI was significantly high. Hsc73 mRNA, a member of the hsp70 family, was also expressed at higher levels in cancerous tissues associated with a high PCNA LI than in non-cancerous tissues. These results suggest that hsp90α may play a role in cancer cell proliferation and that hsp90β may contribute to cell differentiation and structural constitution. In addition, hsp70, especially hsc73, is related to ubiquitin and seems to be a marker for cancer proliferation.

Key words: Human breast cancer — Heat shock protein — Proliferation — Differentiation — Carcinogenesis

The synthesis of heat shock proteins (hsps) in cells is induced by various environmental factors, pathological conditions, and physiological stresses.[1)] Hsps are classified by molecular weight into groups showing specific localizations and various functions. Recent studies have suggested that some hsps are involved in cell cycle regulation and the metabolism of gene products such as *p53*. These hsps play important roles in the processes of carcinogenesis. In particular, hsp90,[2)] hsp70,[3)] and ubiquitin (Ub)[4,5)] contribute to the regulation of the cell cycle. Hsc 73, a member of the hsp70 family, is primarily localized in the nucleus and forms complexes with mutant p53 protein.[6)] Hsp90 binds to steroid receptors, including estrogen receptors (ERs), and contributes to the regulation of their function.[7)] It forms complexes with many proteins such as src family proteins,[8,9)] eIF2 kinase,[10)] casein kinase II,[11)] actin,[12)] and tubulin.[13)] However, the *in situ* expression and localization of hsps in human breast cancer in relation to tumor growth and differentiation has not been clarified. We examined the relationships between hsps and ERs, c-*erb*B2 localization, and proliferating-cell-nuclear-antigen labeling index (PCNA LI) in human breast cancer.

MATERIALS AND METHODS

Breast cancer tissues were obtained by surgery at the First Hospital of the Nippon Medical School and at an affiliated hospital (Kohsei Hospital) between June 1989 and April 1994. Only primary lesions of invasive cancer that had not been treated by preoperative adjuvant therapy were used. In accordance with the General Rules for Breast Cancer Study (Japanese Society of Breast Cancer), the lesions were histologically classified into 47 papillotubular carcinomas (pap), 19 solid-tubular carcinomas (sol), 49 scirrhous carcinomas (sci) and 16 others. In addition, other mastopathical lesions were evaluated, and non-cancerous tissues around the cancer nest and benign mammary lesions were studied as benign controls.

The specimens were fixed and stored by the following methods: (a) fixed in 10% formalin and embedded in paraffin, (b) fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) solution (4°C, 6 h), permeated with 30% sucrose in PBS (4°C, 18 h, stepwise from 10% to 30%), frozen in OCT compound (Tissue Tek, Elkhart, IN), and stored at −80°C, and (c) stored at −80°C without fixation.

For light microscopy, these fixed tissue blocks were cut into thin sections (3–4 μm) and stained with hematoxylin-eosin. Histological grade was evaluated according to the classification of Tsuda *et al.*[14)] Immunohistochemical stainings were perfomed by the streptavidin-biotin-complex method (sABC kit, Nichirei, Tokyo). Before staining, in the formalin-fixed tissue sections in particular, antigens were retrieved by microwave heating in 0.1 *M* citrate buffer (pH 6.0). Sections were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min

at room temperature and then washed in PBS (pH 7.2). Following incubation with normal mouse or rabbit serum (Nichirei, Tokyo) for 10 min, the sections were incubated with the primary antibodies for 6 h in a moist chamber at room temperature. The primary antibodies used were: 1 : 500 dilution of anti-hsp90 (Affinity Bioreagents, Neshanic Station, NJ), 1 : 300 dilution of anti-hsp70 (Affinity Bioreagents), 1 : 100 dilution of anti-PCNA (PC-10, DAKO, Glostrup, Denmark), 1 : 300 dilution of anti-Ub (Chemicon International, Temecula, CA), 1 : 1 dilution of anti-ER (Immunotech S. A., Marseille, France) and 1 : 60 dilution of anti-c-*erb*B2 gene product (Nichirei, Tokyo). After incubation, the sections were washed twice in PBS and treated with biotinylated goat anti-rabbit IgG or rabbit anti-mouse IgG and peroxidase-conjugated streptavidin for 10 min. They were then reacted with 0.02% diaminobenzidine tetrahydrochloride containing 0.005% hydrogen peroxide for 4 min, and counterstained with hematoxylin or methyl green.

For ultrastructural study, sections were stained by the horseradish peroxidase labeling method, fixed with osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were observed under a Hitachi 7000 electron microscope without counter staining.

In situ hybridization was performed as follows. The frozen specimens fixed in 4% paraformaldehyde were cut at 4 μm and mounted on silane-coated slides with air drying at room temperature. An oligonucleotide anti-sense probe for hsp90α (5′-TTC AGG TTT GTC TTC CGA CTC TTT CTC TTC TTT TTC TTT TTC TTC TTC TTT GTC TTC CTT-3′) was prepared based on the nucleotide sequence (1432–1491) reported by Hickei *et al.*[15] and an anti-sense probe for hsp90β (5′-CTT GGG CTT TTC TTC ATC ATC TTT ATC TTC CTC TTC TTT CTC ACC TTT CTC-3′) was based on the nucleotide sequence (789–839) reported by Rebbe *et al.*[16] These probes were labeled with digoxigenin dUTP (Boehringer Mannheim, Bedford, MA) by the 3′-terminal tailing method. In addition, a complementary sequence for each probe was prepared, similarly labeled, and used as a sense probe. Hybridization was performed at 37°C for 18 h. The probes were washed twice at room temperature with 50% formamide/2×SSC for 15 min, then with 2×SSC for 15 min, and finally with 0.2 ×SSC for 15 min. The digoxigenin-labeled oligonucleotide probe was detected using the DIG nucleic acid detection kit (Boehringer Mannheim GmbH, Best, Nr, Germany). The sections were washed with buffer 1 (0.15 *M* NaCl, 0.1 *M* Tris-HCl, pH 7.5) for 30 min, then incubated with buffer 2 (1.0% (w/v) blocking reagents in buffer 1) for 1 h at room temperature. After washing again with buffer 1, the sections were incubated with a 1/2000 dilution of polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase in buffer 1 containing 0.2% Tween 20 for 30 min at room temperature. The sections were washed twice with buffer 1 for 15 min at room temperature and equilibrated with buffer 3 (0.1 *M* NaCl, 0.05 *M* $MgCl_2$, 0.1 *M* Tris-HCl, pH 9.5) for 2 min. The sections were incubated with chromogenic solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in a dark box for 3–5 h. The reaction was stopped with TE buffer (10 m*M* Tris-HCl, 1m*M* EDTA, pH 8.0), and the sections were mounted in aqueous mounting medium (Daido Sangyo, Tokyo).

For semiquantification by means of the reverse-transcriptase polymerase-chain reaction (RT-PCR), total RNA was extracted from unfixed frozen specimens 3 mm^3 in size, and cDNA was synthesized using a first strand cDNA synthesis kit (Boehringer Mannheim, Bedford, MA). Using this cDNA as the template DNA, the RT-PCR method was performed with 72 cancerous tissues and 10 non-cancerous tissues. For hsp90α, two oligonucleotides (5′-ACC CAG ACC CAA GAC CAA CCG -3′ and 5′-ATT TGA AAT GAG CTC TCT CAG-3′) were prepared as primers based on nucleotides 13–33 and 133–153, respectively.[15] For hsp90β, two oligonucleotides (5′-GTG CAC CAT GGA GAG GAG-3′ and 5′-ATT AGA GAT CAA CTC CCG AAG-3′) were prepared as primers based on nucleotides 105–122 and 210–230, respectively.[16] For hsc73, two oligonucleotides (5′-CAG AGT GCA GGA GGC ATG CCA GGA GGA ATG-3′ and 5′-AGA GGG AGG AGC TCC ACC ACC AGG AAA-3′) were prepared based on nucleotides 4522–4551 and 4561–4587, respectively.[17] For β-actin, an endogenous control, two primers (5′-TAC ATG GCT GGG GTG TTG AA-3′ and 5′-AAG AGA GGC ATC CTC ACC CT-3′) were used.[18] PCR was performed using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: template DNA 50–100 ng, dNTP 200 μ*M*, primer 2 p*M*, Taq DNA polymerase 2 U (Takara Shuzo, Tokyo); denaturation, 95°C, 1 min; annealing, 55°C, 1 min; primer extension, 72°C, 2 min; 30 cycles. Electrophoresis of the PCR products on a 2% agarose gel revealed an hsp90α band of about 141 bp, an hsp90β band of about 126 bp, an hsc73 band of about 68 bp, and a β-actin band of about 218 bp. Each band was analyzed using NIH image analysis software version 1.56, and the ratios of hsp90α, hsp90β, and hsc73 to β-actin were calculated and semiquantitatively analyzed.[18, 19] Thirty cycles of PCR were used because exponential amplification was maintained for all PCR products through 30 cycles in our preliminary experiment. As a negative control, specimens without addition of avian myeloblastosis reverse transcriptase at the time of cDNA synthesis were used. These results were analyzed statistically using the χ^2 test, Student's *t* test, the Wilcoxon signed-rank test and the Krüskal-Wallis test.

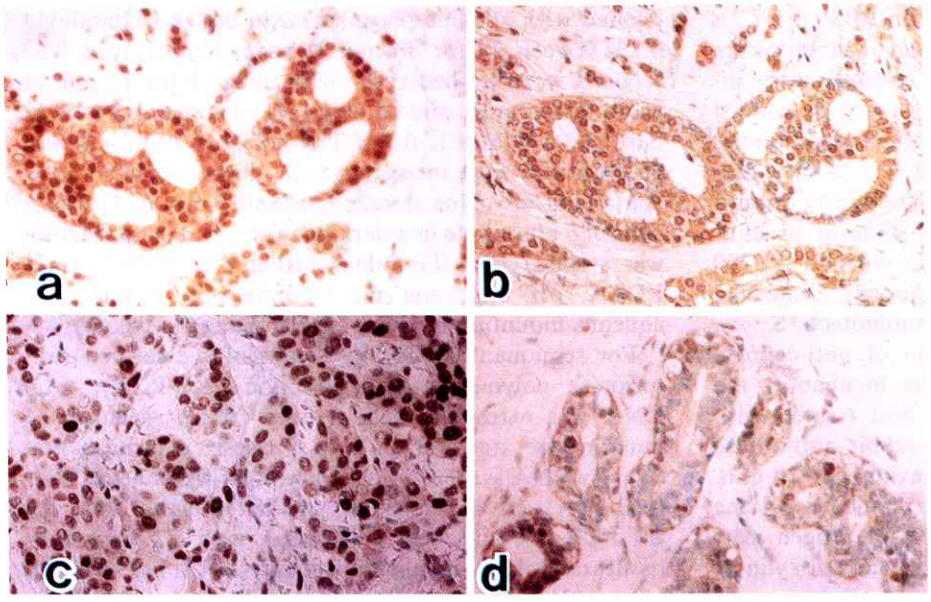

Fig. 1. Immunohistochemical staining in human breast cancerous and non-cancerous tissues. (a) Hsp70 is localized in the nuclei and cytoplasm of the cancer cells, ×300. (b) Ub is localized in the nuclei and cytoplasm of the cancer cells, ×300. (c) PCNA is localized in human breast cancer tissue, ×400. (d) PCNA is localized in non-cancerous breast tissue, ×400.

Table I. Intracellular Localization of Ubiquitin and Hsp70 in Human Invasive Ductal Carcinoma

Histological grade	Ubiquitin (dose)			Hsp70 (case)			PCNA LI
	Nucleus (+)	Cytoplasm only	—	Nucleus (+)	Cytoplasm only	—	Mean±SD
Pap	26	11	5	27	13	2	61.83±16.72
Sol	10	5	0	9	4	2	55.03±17.72
Sci	29	20	0	35	17	1	58.91±20.47

A close correlation of nuclear localization is observed between ubiquitin and Hsp70 (χ^2 test, $P<0.0000005$). The t test shows a correlation between nuclear localization of Hsp 70 and PCNA LI (t test, $P<0.01$).

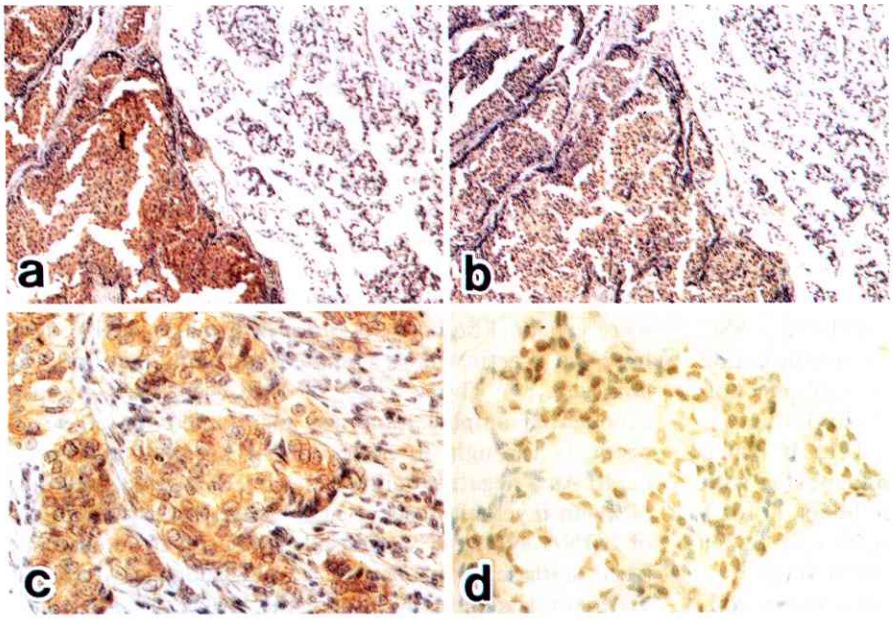

Fig. 2. Immunohistochemical staining in human breast cancer tissues. (a) Hsp90 is localized in cancerous tissue, ×100. (b) c-erbB2 protein is localized in cancerous tissue, ×100. (c) Hsp90 is diffusely localized in the nuclei of breast cancer cells, ×600. (d) ER is localized in the nuclei of cancer cells and reveals a weak positive staining in the cytoplasm, ×400.

RESULTS

Breast cancer specimens were classified as pap, sol, or sci on the basis of morphological criteria.

Immunohistochemically, hsp70 and Ub were localized in the cytoplasm of cancer cells, where they showed similar distributions (Fig. 1). However, a close correlation between the localization of hsp70 and Ub in the nucleus was observed, as shown in Table I (χ^2 test, $P<0.00001$). The mean PCNA LI for the cancerous tissue was 59.2, compared with 27.5 for the non-cancerous tissue. The relationship between hsp70 expression in the nucleus and a high PCNA LI was significant (t test, $t=2.99$, $P<0.01$, Table I).

Hsp90 and c-erbB2 protein were localized in the cytoplasm of cancerous cells (Fig. 2). However, some hsp90-positive specimens of the invasive ductal carcinomas did not show c-erbB2 protein staining. Hsp90 was found in 89.5% of the specimens, while only 34.0% were c-erbB2 protein-positive (Table II). Hsp90 was irregularly localized in healthy lactiferous ducts, but was localized throughout the nuclei and cytoplasm of the cancer cells (Fig. 2). Ultrastructurally, hsp90 was localized in the nucleus and in the cytoplasm on the luminal side of the cell (Fig. 3). ER was localized in the nuclei of the cancer cells and the cytoplasm was weakly ER-positive (Fig. 2). Ultrastructurally, ER was localized in the nucleus and the cytoplasm, including the endoplasmic reticulum, of cancer cells (data not shown). An inverse correlation between ER and c-erbB2 protein distribution in the tissue was observed in the breast cancer specimens (χ^2 test, $P<0.01$).

Table II. Localization of Hsp90, c-erbB2 and Estrogen Receptor (ER) in Human Invasive Ductal Carcinoma (%)

Histological classification	Hsp90	c-erbB2	ER
Pap	89.4	38.3	63.8
Sol	86.7	14.2	63.1
Sci	93.9	44.9	57.1

χ^2 test shows an inverse correlation between estrogen receptor and c-erbB2 protein in human breast cancer (χ^2 test, $P<0.01$).
No correlation is observed between Hsp90 and c-erbB2 protein or estrogen receptor.

Hsp90 has two isoforms with slight differences in primary structure[20] (hsp90α and hsp90β). ISH revealed marked expression of hsp90α mRNA and hsp90β mRNA in cancer cells and in fibroblasts (Figs. 4 and 5). The expression levels of hsp90α, hsp90β, and hsc73 mRNA were evaluated by RT-PCR using β-actin as an endogenous control (Fig. 6). The expression levels of hsp90α mRNA and hsp90β mRNA relative to the level of β-actin mRNA as determined by RT-PCR, were analyzed using the NIH image analysis software. The relationship between them and PCNA LIs was also examined. Hsp90α mRNA expression was much higher in the cancerous tissue than in the non-cancerous tissue ($P<0.0001$, 2-tailed t test, Table III). Hsp90α mRNA expression was significantly correlated with the PCNA LI (Wilcoxon signed-rank test, $P<0.001$, Table III). However, hsp90β mRNA expression was not correlated with the PCNA

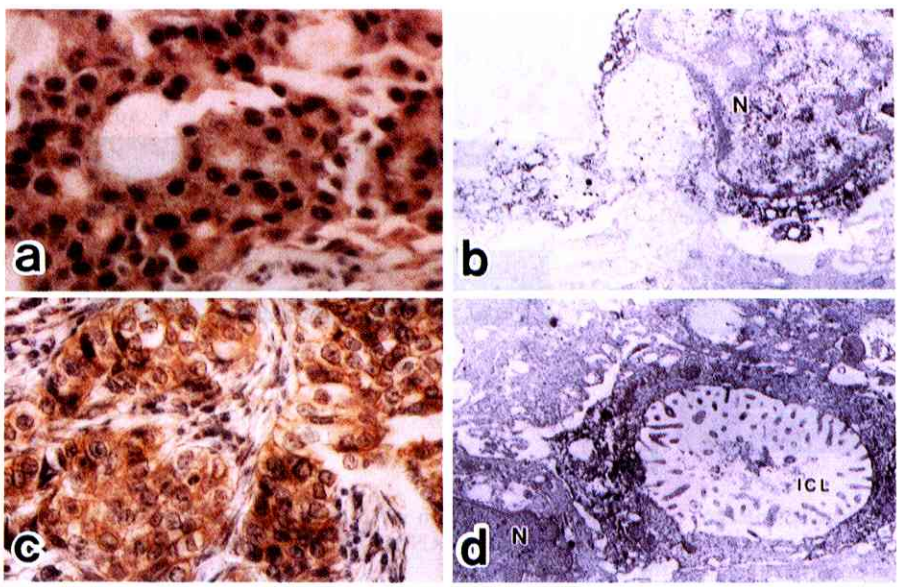

Fig. 3. Hsp90 localization in human breast cancer tissues. (a) Immunohistochemically, hsp90 is localized in the nuclei and cytoplasm of cancer cells, ×600. (b) Ultrastructurally, hsp90 is localized in the nucleus of a cancer cell, ×6000. (c) Immunohistochemically, hsp90 is localized in the cytoplasm of cancer cells, ×600. (d) Ultrastructurally, hsp90 is localized in the cytoplasm of a cancer cell, ×6000.

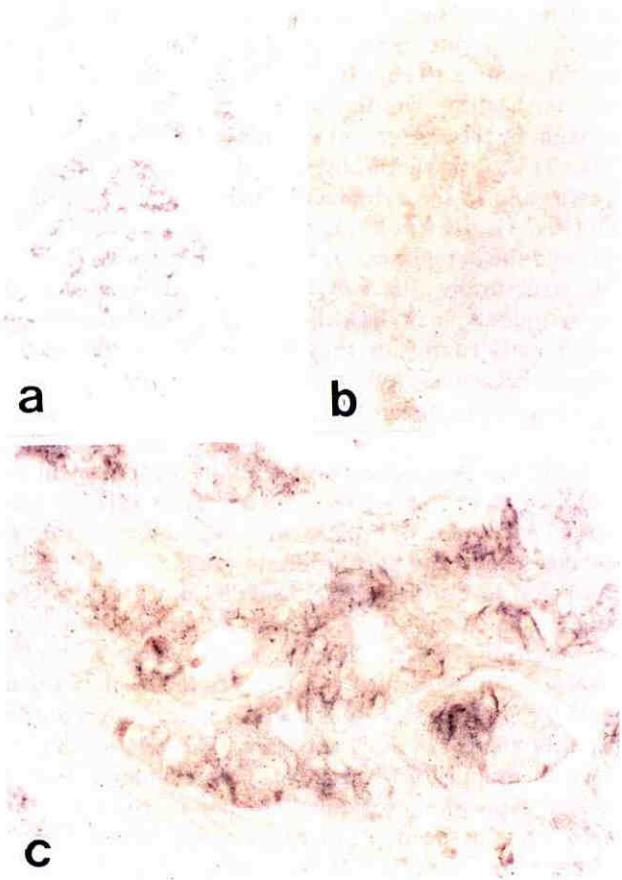

Fig. 4. Hsp90α mRNA shows marked expression in carcinoma cells and is also expressed in fibroblasts. (a) *In situ* hybridization for hsp90α with anti-sense probe, $\times$100. (b) *In situ* hybridization for hsp90α with sense probe, $\times$100. (c) *In situ* hybridization for hsp90α with anti-sense probe, $\times$600.

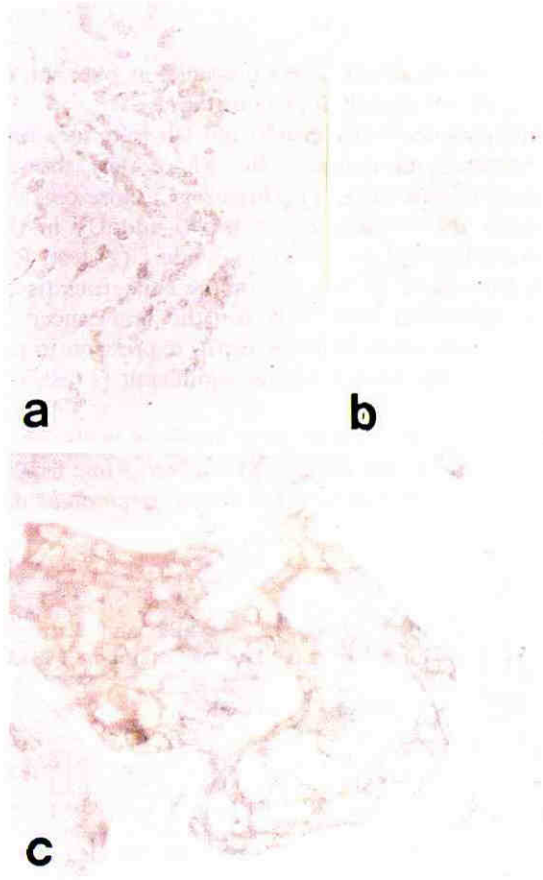

Fig. 5. Expression of hsp90β mRNA in human breast cancer tissues. (a) *In situ* hybridization for hsp90β with anti-sense probe, $\times$100. (b) *In situ* hybridization for hsp90β with sense probe, $\times$100. (c) *In situ* hybridization for hsp90β with anti-sense probe, $\times$600.

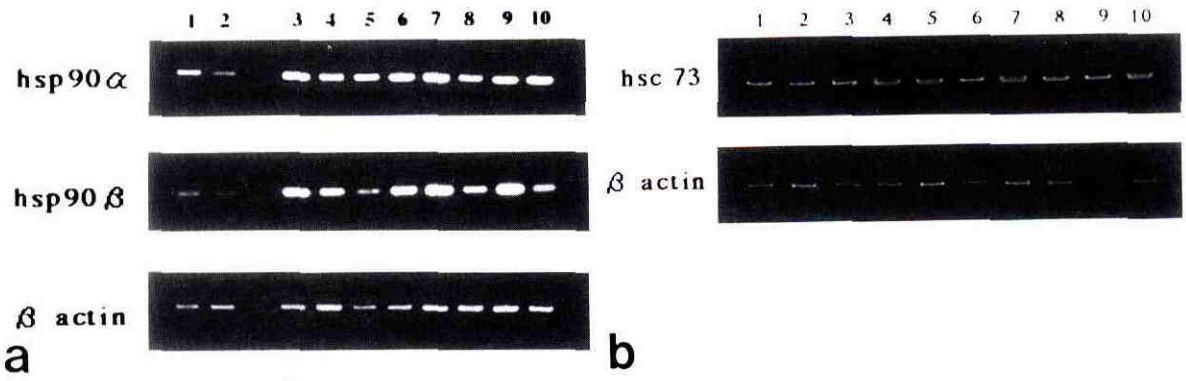

Fig. 6. RT-PCR analysis of hsp90α mRNA, hsp90β mRNA, hsc73 mRNA and β-actin mRNA in non-cancerous tissues (lanes 1 and 2) and in cancerous tissues (lanes 3–10) is shown. (a) Expression of hsp90α mRNA and hsp90β mRNA evaluated by the RT-PCR method. (b) Expression of hsc73 mRNA evaluated by the RT-PCR method.

Table III. Expression of Hsp90α, Hsp90β and Hsc73 mRNAs in Relation to PCNA LI in Human Breast Cancer by Semiquantitative RT-PCR (mean$\pm$SD)

	Hsp90α/β-actin	Hsp90β/β-actin	Hsp73/β-actin	PCNA LI
Cancer	1.95$\pm$0.77	1.35$\pm$1.22	2.14$\pm$1.42	61.9$\pm$16.7
Non-cancer	0.56$\pm$0.14	0.32$\pm$0.20	1.21$\pm$1.00	18.5$\pm$6.36

Hsp90α mRNA expression is significantly higher in cancerous tissue than in non-cancerous tissue (t test, P (2-tailed) <0.00001).
High PCNA LI score is also very significant (t test, P (2-tailed) <0.0001).
Hsp90α mRNA expression is correlated with high PCNA LI score (Wilcoxon signed-rank test, P-value <0.001).
Hsp90β mRNA expression is not correlated with high PCNA LI score.
Hsc73 mRNA expression is much more marked in cancerous tissue than in non-cancerous tissue, but the difference is not statistically significant. Hsc73 mRNA expression is also correlated with high PCNA LI score (Wilcoxon signed-rank test, $P<0.0005$).

Table IV. Expression of Hsp90β mRNA in Relation to Histologic Grade in Human Breast Cancer by Semiquantitative RT-PCR (mean$\pm$SD)

Histologic grade	Hsp90β/β-actin (mRNA, RT-PCR)
1	0.49$\pm$0.07
2	1.34$\pm$1.05
3	2.49$\pm$1.13

In cancers of high histologic grade, i.e., poorly differentiated, hsp90β mRNA expression is significantly high, as shown by the Krüskal-Wallis test ($P<0.05$).
No correlation is observed between histologic grade and expression of hsp90α mRNA.

LI. In breast cancer specimens showing a higher histological grade, hsp90β mRNA expression was significantly high (Krüskal-Wallis test, $P<0.05$, Table IV). Hsc73 mRNA expression was also significantly correlated with PCNA LI (Wilcoxon signed-rank test, $P<0.0005$, Table III) and appeared to be greater in the cancerous tissue than in the non-cancerous tissue, although the difference was not significant (2-tailed t test).

DISCUSSION

Recent studies have indicated that hsps are involved in cell cycle regulation, control of DNA damage, and the metabolism of gene products such as *p53*, and play important roles in carcinogenesis. In particular, hsp90,[2] hsp70,[3] and Ub[4, 5, 21] are thought to be closely linked to the cell cycle.

Hsp90 is constantly present at a low level in normal tissues and is necessary for the early S phase of the cell cycle.[22] Our immunohistochemical examination showed that irregular hsp90 localization was present in a small proportion of healthy lactiferous ducts. However, hsp90 was frequently localized in invasive ductal carcinomas, and showed a diffuse localization in the cytoplasm and the nuclei of cancerous cells. Hsp90 expression was observed ultrastructurally in the nucleus and cytoplasm of cancerous cells. Hsp90 exists in two isoforms, hsp90α and hsp90β, showing slight differences in their primary structures, and encoded by different genes, on chromosomes 14q32[23] and 6p12,[24] respectively. The two isoforms primarily form homo-dimers, although hsp90β is also present as monomers.[20] We studied the distribution of hsp90 isoform mRNAs by ISH. Both hsp90α mRNA and hsp90β mRNA were expressed in cancer cells. Image analysis of RT-PCR products showed significant expression of hsp90α mRNA in the cancerous tissue, and the PCNA LI was correlated with hsp90α mRNA, but not with hsp90β mRNA, in proliferating cells. Recently, hsp90α has been reported to contribute to cell proliferation by controlling the cell cycle in chicken cell culture[2] and yeast *in vitro*.[25] Other studies have shown excessive hsp90α expression in human pancreatic carcinoma cells[26] and leukemic cells.[27] Our results suggest that the detection of hsp90α indicates a poor prognosis in human breast cancer.[28, 29] Hsp90β mRNA expression was significantly raised in breast cancer cells showing poor histological differentiation. Hsp90 also forms complexes with actin[12] and tubulin,[13] which constitute the cytoskeleton, suggesting that hsp90 may play a role in structural conformation and cellular atypia. In addition, the associations between hsp90 and Raf/Mil, have been reported to be involved in cell differentiation,[30] an increase in hsp90 mRNA induced by mitogens,[31] and the inhibition of apoptosis and cell differentiation by hsp90β.[32] These findings also suggest the inhibition of cancer cell differentiation by hsp90β. Thus, hsp90α and hsp90β seem to have some different roles in cell differentiation.

On the other hand, Ub and hsp70 showed similar immunohistochemical localization patterns. The anti-hsp70

antibody used in this study reacts with hsp70A, hsp70B, hsp72, and hsc73. Only hsc73 was localized in the nucleus. In specimens showing hsp70 expression in the nuclei, the PCNA LI was significantly raised. Our results using RT-PCR showed that hsc73 mRNA levels were raised in the cancerous tissue, and that the PCNA LI was also significantly high. Hsc73 mRNA showed almost the same levels of expression in non-cancerous and cancerous tissues, but endogenous β-actin mRNA, as the control, was decreased in cancerous tissues compared with non-cancerous tissues. Thus, hsc73 mRNA expression is relatively increased in the cancerous tissues compared with non-cancerous tissues. By contrast, Ub is related to a response in the early G_1 stage.[33] These results indicate an important role for hsp70 and Ub expression in breast cancer proliferation. Detection of these parameters by immunohistochemical methods may be useful for predicting the prognosis of breast cancer.

It is also important to measure hsp90,[34] hsp70,[35] and Ub before selecting a treatment method. Human breast cancer cells rarely tolerate high temperature, but are resistant to various stresses such as hypoxia, low nutrition, and anti-cancer drugs. Assuming that these heat shock proteins are involved in the proliferation and differentiation of breast cancer cells, it is possible that their synthesis is inhibited by flavonoid compounds. Hsp inhibition may result in the acquisition of tolerance and the inhibition of multidrug resistant gene product expression associated with hsps, which would be applicable to the treatment of cancer.

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