

## Autoantibody to Heat Shock Protein Hsp40 in Sera of Lung Cancer Patients

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**Heat shock protein Hsp40 is a stress protein with chaperone activity and has a cooperative function with Hsp70 in mammalian cells. We examined the possible expression of Hsp40 in lung tumor tissues using immunoblotting and immunohistochemistry, and established an enzyme-linked immunosorbent assay (ELISA) method to detect IgG antibody to Hsp40 in the serum using purified human Hsp40. Sera were obtained from 130 normal subjects and 50 patients with lung cancer. Lung tumor tissues and cells specifically overexpressed Hsp40, and no such expression was detected in normal lung tissues. Compared with normal sera, significantly higher levels of autoantibody to Hsp40 were present in patients with lung cancer. The present study is the first to demonstrate overexpression of Hsp40 in human tumor tissue and the associated presence of autoantibody to Hsp40 in the serum. These results suggest that overexpression of Hsp40 in tumor cells may be recognized as a self-antigen.**

**Key words:** Stress protein — Heat shock protein — Autoantibody — Lung cancer — ELISA

A number of heat shock proteins (Hsps) are present in mammalian cells and exert a variety of important biological effects in response to environmental stimuli.<sup>1,2)</sup> Some of these proteins, such as Hsp60, Hsp70 and Hsp90, have been shown to act as molecular chaperones that are involved in folding of nascent polypeptide chains and translocation of precursor proteins across the membranes of the cytoplasmic organelles.<sup>1,3,4)</sup> Hsps with chaperone activity are not only expressed constitutively in cells, but also markedly induced by various stresses such as heat, ethanol, heavy metal ions, ultraviolet and anticancer drugs.<sup>2)</sup> Several recent studies have investigated the biological function of Hsps in various human diseases including cancer, infectious and autoimmune diseases.<sup>5)</sup>

Hsp40 family proteins have been identified as eukaryotic homologues of bacterial DnaJ protein,<sup>6)</sup> and human Hsp40, identified by Ohtsuka *et al.*,<sup>7,8)</sup> has a molecular chaperone activity.<sup>9)</sup> Moreover, Hsp40 is associated with Hsp70 (DnaK protein in *Escherichia coli*) in the cytoplasm under normal growth conditions.<sup>9)</sup> Hsp40 co-localizes with Hsp70 in the nuclei and nucleoli in heat-shocked cells,<sup>9)</sup> where it interacts and cooperates with Hsp70 as chaperones under stress conditions (Hsp70/Hsp40 chaperone system). However, its clinical significance, including expression and function, is quite unknown. In the present

study, we examined the expression of Hsp40 in lung tumor tissues, and the presence of IgG antibody to human Hsp40 in sera of lung cancer patients.

### MATERIALS AND METHODS

**Tissue and serum samples** Tissue samples were obtained from previously untreated patients with lung cancer. These included serum samples, lung tumor samples and sections from apparently normal lung parenchyma. We also obtained serum samples from normal volunteers in our hospital with informed consent, and the volunteers had normal organ functions and no apparent diseases. The histopathologic type of lung cancer was determined according to the criteria of the World Health Organization. Only patients with no apparent autoimmune or infectious diseases were included in the present study. The specimens and sera were immediately frozen at  $-80^{\circ}\text{C}$  and stored until processing for immunoblotting and enzyme-linked immunosorbent assay (ELISA), respectively.

We also examined NCI-H460 cells, a human large cell type lung cancer cell line (American Type Culture Collection, Rockville, MD), and used the cells as a positive control for immunoblotting. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4% L-glutamine, and 80 mg/liter kanamycin sulfate in a humidified incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

**Immunoblotting** For preparation of whole cell protein, cultured cells were washed three times with phosphate-buffered saline (PBS) and then collected into pH 7.4 TBS

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(137 mM NaCl, 25 mM Tris)-1 mM EDTA. The cell pellet obtained after centrifugation was resuspended in TBS containing protease inhibitors (20  $\mu\text{g/ml}$  aprotinin, 20  $\mu\text{g/ml}$  leupeptin, 40  $\mu\text{M}$  pepstatin, and 2 mM phenylmethylsulfonyl fluoride (PMSF)), and sheared with a needle. For tissue protein, approximately 500 mg of each tissue was suspended in TBS as described above, and sonicated. The cell and tissue suspensions were lysed in 2 $\times$  sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) sample buffer (125 mM Tris-HCl, 4 mM EDTA, 30% sucrose, 20% glycerol, 6% SDS, 10% 2-mercaptoethanol), and the proteins were denatured by boiling for 10 min. The concentration of proteins in each sample was measured according to the method of Bradford (Bio-Rad Protein Assay Kit; Bio-Rad Lab., Hercules, CA). Each 100  $\mu\text{g}$  of whole cell and tissue protein was separated on 7.5% SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking with 5% milk in TBS containing 0.1% Tween-20 (TBST), the membranes were incubated with rabbit polyclonal anti-human-Hsp40 antibody<sup>10</sup> at room temperature for 60 min followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 45 min. After washing with TBST, reactions were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Co., Bucks, UK).

**Immunohistochemistry** The tissue samples were fixed in buffered formalin and embedded in paraffin. Paraffin-embedded sections were cut at 5  $\mu\text{m}$  and placed on silanized glass slides. In the next step, the sections were deparaffinized by incubation for 10 min in xylene, ethanol and water. After blocking endogenous peroxidase activity with 1% hydrogen peroxide in methanol, the sections were treated with normal goat serum for 20 min to block non-specific binding. Primary rabbit polyclonal anti-human-Hsp40 antibody<sup>10</sup> was applied at a dilution of 1:300 and incubated in a moist chamber for 12 h at 4°C. After washing in PBS, biotinylated goat anti-rabbit IgG (Nichirei Co., Tokyo) was applied and the sections were incubated for 30 min at room temperature. After washing in PBS, the sections were developed in Tris-buffered saline containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide. The sections were finally washed in PBS and counterstained with hematoxylin.

**Purification of human Hsp40** The plasmid pQE9/Hsp40,<sup>11</sup> that encodes 6 $\times$  His-tagged Hsp40, was transferred into M15 *E. coli* cells and grown at 37°C. After 4-h incubation with 1 mM isopropyl-1-thio- $\beta$ -D-galactoside (IPTG), the cells were lysed with a sonicator in buffer A (50 mM Na-phosphate, 300 mM NaCl, pH 8.0) containing 10 mM MgCl<sub>2</sub>, 20  $\mu\text{g/ml}$  DNase I, 1  $\mu\text{g/ml}$  leupeptin, 10 mM pepstatin, 1 mM PMSF and 1% Triton X-100. The cleared lysate was loaded onto a Ni<sup>2+</sup>-NTA column (QIAGEN, Chatsworth, CA) previously equilibrated with buffer A. The column was washed with buffer A then with

buffer B (50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6.0), and the proteins were eluted with a linear gradient of 0–500 mM imidazole in buffer B. Peak fractions of His-Hsp40 were collected and dialyzed against a coating buffer. The purity of the His-Hsp40 protein was more than 90% as determined with SDS-PAGE followed by Coomassie blue staining.

**ELISA of anti-Hsp40 antibody** We used the ELISAmate Kit (Kirkegaard & Perry Lab., Gaithersburg, MD) to detect anti-Hsp40 IgG antibody in human sera. For this purpose, a microtiter plate was coated with the above purified human Hsp40 dissolved in coating buffer, at a final concentration of 10  $\mu\text{g/ml}$ . The plates were incubated for 1 h, washed 4 times with a washing solution and then blocked using bovine serum albumin (BSA) diluent/blocking solution for 15 min at room temperature. After the next washing step, 100  $\mu\text{l}$  of the control (the negative control was BSA diluent/blocking solution alone and the positive control was rabbit anti-serum against human Hsp40 which was diluted at 1:200 in BSA diluent/blocking solution) and test sera (1:200 dilution in BSA diluent/blocking solution) were added to each well and the plates were incubated for 1 h at room temperature. After washing, we added alkaline phosphatase-conjugated goat antibodies to human IgG(H+L) or rabbit IgG(H+L) (Kirkegaard & Perry Lab.) diluted at 1:200 in BSA diluent/blocking solution. After 1-h incubation at room temperature, the plates were washed and the enzymatic reaction was carried out using a substrate solution. The absorbance of test sera was measured at 405 nm by an MR600 microplate reader (Dynatech Co., Alexandria, VA)

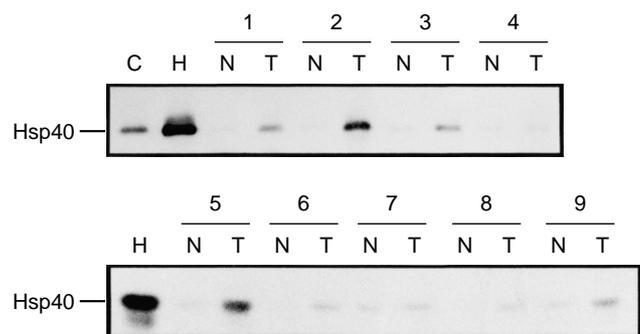


Fig. 1. Immunoblot of Hsp40 in paired normal lung and tumor tissues samples from patients with lung cancer. Each 100  $\mu\text{g}$  of whole cell and tissue proteins was separated on a 7.5% SDS-PAGE gel. Protein from heat-shocked NCI-H460 cells was prepared after incubation at 45°C for 30 min followed by 6-h recovery at 37°C. Hsp40 was highly induced in the cells by heat-shock, and constitutively overexpressed in at least six tumor tissues compared with the paired normal tissues. Patients 1, 2, 5, 7, 9, squamous cell carcinoma; patients 3, 4, 6, 8, adenocarcinoma. C, NCI-H460 cells; H, heat-shocked NCI-H460 cells; N, normal lung tissue; T, tumor tissue.

when positive control serum showed the same absorbance value in each experiment.

**Statistical analysis** Data were expressed as mean±SD (median, [range]). Differences in absorbance between groups were tested for statistical significance by Mann-Whitney's *U* test. A two-tailed  $P < 0.05$  denoted the presence of a statistically significant difference.

## RESULTS

**Hsp40 expression in lung tumor tissues** NCI-H460 cells, used as a positive control, constitutively expressed Hsp40, which was highly induced by heat shock at 45°C for 30 min, as previously reported in HeLa cells.<sup>10</sup> Immunoblotting showed that out of nine paired samples of normal lung and tumor tissues analyzed here, at least six tumors apparently overexpressed Hsp40 compared with normal tissues (Fig. 1). Overexpression of Hsp40 in lung

cancer tissues was also confirmed by using immunohistochemical methods (Fig. 2). Tumor cells in several Hsp40-overexpressing tumor tissues, including the above six tumors, were strongly stained with anti-Hsp40 antibody. These results indicate that tumor cells of human lung cancer constitutively overexpress Hsp40.

**Anti-Hsp40 antibody in sera** Using ELISA, we detected anti-Hsp40 IgG antibody in sera of 130 normal subjects (control) and 50 patients with four major histologic types of lung cancer (Fig. 3). The control absorbance was  $171.0 \pm 38.4$  (162.5, [103.0–289.5]). Based on this finding, the cut-off absorbance was set as  $247.8$ , representing the mean+2SD. The absorbance of samples from all patients was  $298.1 \pm 131.2$  (250.8, [165.5–958.5]), which was significantly higher than the control ( $P < 0.0001$ ). The absorbance was more than the cut-off level in 50% of patients, particularly in patients with adenocarcinoma (10 out of 15 patients). Specifically, the absorbance was  $353.9 \pm 192.8$

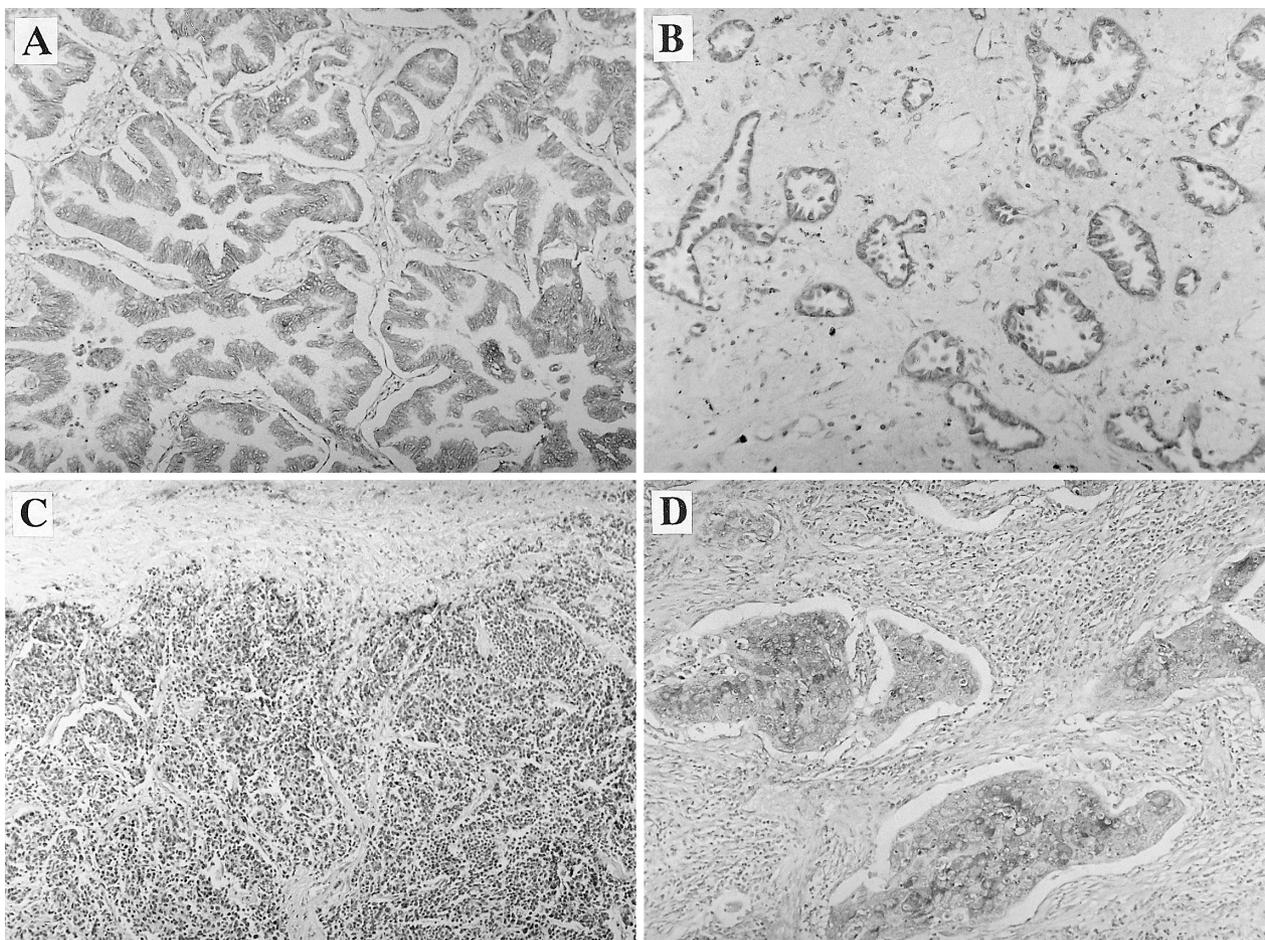


Fig. 2. Immunohistochemical staining for Hsp40 in lung tumor tissues. A, adenocarcinoma (original magnification,  $\times 100$ ); B, adenocarcinoma ( $\times 200$ ); C, small-cell carcinoma ( $\times 100$ ); D, squamous cell carcinoma ( $\times 100$ ).

(313, [165.5–958.5]) in adenocarcinomas,  $264.9 \pm 82.7$  (238, [167.5–483.5]) in squamous cell carcinomas,  $280.1 \pm 100.2$  (256, [186.5–582.0]) in small-cell carcinomas, and  $284.2 \pm 66.8$  (242, [231.5–372.0]) in large cell carcinomas. Each of these values was significantly higher than the control ( $P < 0.0001$ ), but no differences were seen among the different histologic types. In the preliminary analysis for clinical implications of anti-Hsp40 antibody, the absorbances in advanced-stage non-small-cell lung cancers were significantly higher than those in early-stage ones (data not shown). These results indicate that circulating autoantibody to overexpressed-Hsp40 in tumor cells is present in patients with lung cancers.

## DISCUSSION

The present study for the first time demonstrated that lung tumor tissues constitutively overexpresses Hsp40 compared with normal lung tissues. To date, a few Hsps such as Hsp60, Hsp70 and Hsp90 have been reported to be specifically overexpressed in breast, colon, lung, pancreatic, ovarian and brain tumor tissues.<sup>12–18</sup> Our preliminary data using immunohistochemical staining confirmed a specific and simultaneous overexpression of Hsp70 and Hsp40 in lung cancers. The constitutive overexpression of these Hsps with chaperone activity in tumor cells may serve to deal with abnormal proteins in these cells, since most oncoproteins or tumor suppressor proteins in tumor cells undergo certain changes during carcinogenesis. In fact, mutant p53 tumor suppressor protein is physically associated with Hsp70 as well as Hsp40.<sup>9</sup> In addition, in comparison with normal quiescent cells, rapidly proliferating tumor cells may need much larger amounts of molecular chaperones to support their protein metabolism. At present, however, independent expression mechanisms or functions of Hsp40 in tumor cells remain to be determined. Hsp itself may be also involved in carcinogenesis or malignant transformation of tumor cells, since a high expression of Hsp70<sup>12</sup> or Hsp90<sup>13</sup> in breast cancer and Hsp60 in ovarian cancer<sup>17</sup> correlates with poor prognosis or is associated with resistance against certain anticancer drugs *in vitro*.<sup>19</sup>

In the present study, we could detect IgG antibody to Hsp40 in sera of lung cancer patients, suggesting that the immune system perhaps recognizes overexpressed-Hsp40 in tumor cells and produces the autoantibody. However, it is not clear whether Hsp40, together with Hsp70, elicits or enhances tumor immunity. Hsp70 and Hsp90 with chaperone activity elicit tumor-specific immunity resulting in rejection of the tumor in experimental animal models.<sup>20</sup> They are potentially involved in tumor-specific antigen presentation linked to major histocompatibility complex (MHC) to immune cells.<sup>21</sup> A number of peptides binding to these Hsps (Hsp-peptide complexes) are recognized by

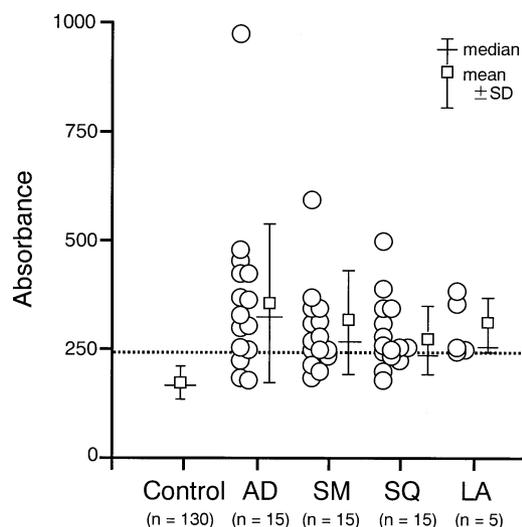


Fig. 3. Anti-Hsp40 IgG antibody in sera of patients with four major histologic types of lung cancer was detected by ELISA. The absorbance was more than the cut-off value (dash line) in 50% of patients. The absorbance value in each histologic type was significantly higher than the control ( $P < 0.0001$ , Mann-Whitney's *U* test), while no significant differences were observed among different histologic types. Control, normal human sera; AD, adenocarcinoma; SM, small-cell carcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma.

immune cells, and sequentially, specific cytotoxic T cells against tumor cells are elicited.<sup>21</sup> Concomitantly, the overexpressed Hsps in tumor cells may be recognized as self-antigens, resulting in detectable autoantibody to the Hsps in the serum. In fact, Conroy *et al.*<sup>22</sup> detected IgG antibody to Hsp90 in sera of breast cancer patients.

Speculatively, Hsp40/DnaJ as well as other Hsps may be associated with pathogenesis of non-malignant diseases. Human Hsp40 shows a high homology with bacterial and yeast DnaJ heat shock protein family.<sup>8</sup> Antibodies to Hsp60, Hsp70 and Hsp90 are present in sera of patients with autoimmune and inflammatory bowel diseases,<sup>23</sup> and pulmonary sarcoidosis.<sup>24</sup> "Common antigens," homologous peptide antigens of human Hsps shared with pathogen-derived Hsps that are the major antigens during infections,<sup>25</sup> are thought to be involved in their pathogenesis.<sup>5</sup> Briefly, the immune response directed against pathogen-derived Hsps in infected cells cross-react with human Hsps "common antigens," resulting in the production of autoantibodies and cytotoxic T cell against host cells.<sup>5</sup> The presence of such an association of Hsp40 should be further investigated in future *in vitro* and *in vivo* studies.

In conclusion, we established an ELISA method to detect antibody to Hsp40 in sera, and for the first time demonstrated the presence of autoantibody to Hsp40 in the sera of lung cancer patients. This ELISA method should

be useful to elucidate the roles of autoantibody to Hsp40 in various diseases.

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