It has also been reported that HSP110 is co-expressed with HSP70 and HSP90 during stress, and that it promotes HSP90 activity and may function as a nucleotide exchange factor for cytosolic HSP70 [36]. We elucidated whether HSP110 expression can facilitate cancer invasion through the activation of HSP70 and HSP90.

Hosaka *et al.* [37] reported that HSP110 suppression induces apoptosis in cancer cell lines but not in fibroblasts. Dorard *et al.* [30] identified a loss-of-function mutation of HSP110 (HSP110ΔE9) in colorectal cancer with microsatellite instability. HSP110ΔE9 overexpression enhanced cancer cell sensitivity to anticancer agents. Here, low nuclear HSP110 expression group had better prognosis compared with the high expression group, and HSP110 suppression was shown to increase cell sensitivity to 5-FU and cisplatin in human gastric cancer cell lines, which is consistent with the previous reports. Novel treatment strategies, combining an HSP110 inhibitor and an anticancer agent, may be effective for the treatment of gastric cancer patients with acquired anticancer drug resistance.

Previously, HSP110 was identified as a cancer antigen in various human carcinomas [31, 32]. We report here that the high expression of the nuclear HSP110 was observed in gastric cancer patients. Wang *et al.* [29] developed a vaccine composed of a recombinant protein coupled with large heat shock protein. Our results suggest that chemosensitivity may decrease due to heat stress-induced HSP110 expression. Therefore, various vaccines, which may utilize HSPs (*i.e.,* covalent coupling, isolation of HSPs with antigens attached, recombinant vaccines made by heat-denaturation of full-length antigens and HSP110) may be useful in anticancer treatments.

HSP110-specific siRNAs were used to suppress the expression of HSP110 in gastric cancer cell lines, which presents a limitation of this study. Therefore, total HSP110 expression was suppressed, and not only the specific nuclear HSP110 expression. Total HP110 expression was strongly suppressed in MKN7 and MKN45 by the HSP110-specific siRNA, but this is probably the consequence of the suppression of both nuclear and cytoplasmic HSP110 expression.

In conclusion, the high expression of nuclear HSP110 was shown to be associated with cancer progression, poor prognosis, and recurrence after adjuvant

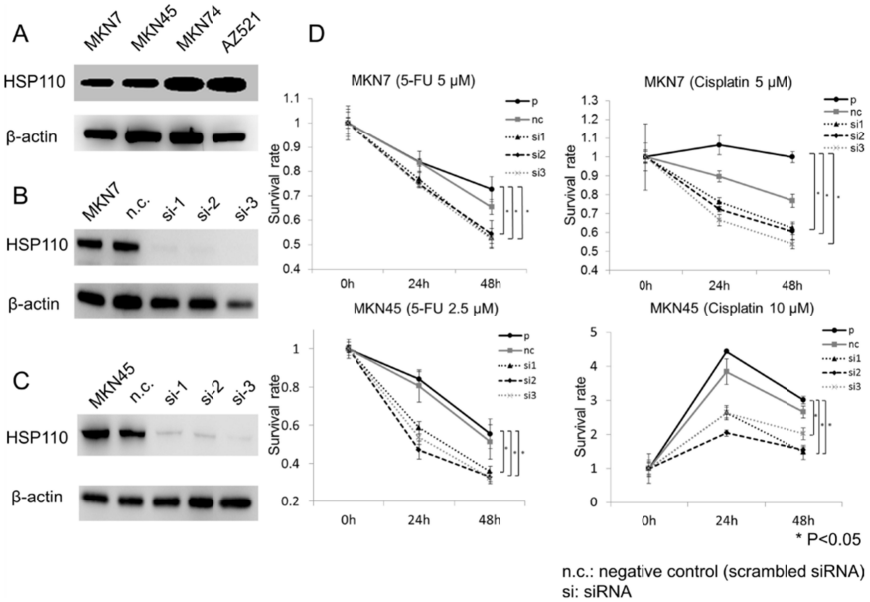

Figure 4: Functional analysis of human gastric cancer cell lines treated with HSP110 siRNA. (**A**) The expression of HSP110 in human gastric cancer cell lines was assessed by western blot. β-actin was used as the loading control. (**B**) HSP110 expression was suppressed using HSP110 siRNA (MKN7); (**C**) HSP110 suppression using HSP110 siRNA (MKN45). (**D**) The effects of HSP110 suppression on chemosensitivity of MKN7 and MKN45 cells. Both MKN7 and MKN45 cells showed a significantly increased sensitivity to 5-fluorouracil in HSP110 siRNA-treated groups, compared with the parent and control cells ($P < 0.05$). n.c.: negative control (scrambled siRNA), si: siRNA.

chemotherapy in gastric cancer patients. Furthermore, HSP110 suppression increased the sensitivity to 5-FU and cisplatin in the human gastric cancer cell lines. Our results suggest that nuclear HSP110 expression in gastric cancer may be a new prognostic and drug sensitivity marker, and HSP110 may serve as a new molecular therapeutic target for the treatment of refractory gastric cancer.

METHODS

Patients and samples

Primary gastric cancer tissues were obtained from gastric cancer patients (n = 210; 147 men and 63 women) who underwent radical gastrectomy at the Department of General Surgical Science, Gunma University Hospital, Japan, between January 1999 and May 2006. The stage of gastric cancer was described according to the classification of gastric carcinoma of the Japanese Gastric Cancer Association's 3rd English edition [38]. Forty-eight patients received 5-FU-based adjuvant chemotherapy between January 2003 and May 2006. The correlation between HSP110 expression and clinicopathological factors and prognosis was evaluated in these patients. Written informed consents were obtained from all patients according to institutional guidelines.

Tissue microarray analysis and immunohistochemical staining

Tumor samples were fixed in formalin, embedded in paraffin, and stored in the archives of the Clinical Department of Pathology, Gunma University Hospital, Japan. For 210 gastric cancer patients, one paraffin block containing representative non-necrotic tumor areas was selected, and gastric cancer tissue cores (2.0 mm diameter per tumor) were sampled from the representative areas and transferred into the paraffin block using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD, USA). Cores were arranged into quad tissue array blocks, with each containing 50–55 tumor cores. Tissue microarray blocks were cut into 3.5-μm thick sections, and were used for the subsequent immunohistochemical staining. Additionally, 4-μm sections were cut from the paraffin blocks of 10 gastric cancer samples, selected among 210 gastric cancer patients for validation.

All sections were incubated at 60°C for 60 min and deparaffinized in xylene. Afterward, these sections were rehydrated and incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature, in order to block endogenous peroxidase activity. Following the rehydration through a graded series of ethanol treatments, the sections were heated in boiling water and soaked in Immunosaver (Nishin EM, Tokyo, Japan) at 98°C for 90 min. Non-specific binding sites were blocked by incubating the sections with Protein Block Serum-Free (DAKO, Carpinteria, CA, USA) for 30 min. A rabbit monoclonal anti-HSP110 antibody (GeneTex, CA, USA) was applied at 1:100 dilution, for 24 h at 4°C. The primary antibody was visualized using the Histofine Simple Stain MAX-PO (MULTI) (Nichirei, Tokyo, Japan) according to the instruction manual. A chromogen, 3,3-diaminobenzidine tetrahydrochloride, was applied as a 0.02% solution containing 0.005% hydrogen peroxide in 50 mM ammonium acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained with Mayer's hematoxylin and mounted. The evaluation of immunohistochemical staining was performed by two independent researchers who were blinded to the patients' data. We focused on nuclear HSP110 expression, and the intensity of nuclear HSP110 staining was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. Gastric cancer patients were assigned to the nuclear HSP110 low expression group (0, 1+) or high expression group (2+, 3+), according to staining score (Figure 1C). Additionally, the tissues adjacent to the cancerous tissues in the tissue microarray samples were considered non-cancerous tissue. We evaluated the expression of HSP110 in the non-cancerous tissue of these 10 samples, for validation. The non-cancerous tissue was defined as the normal gastric mucosa tissue or stromal cells.

Cell culture

The human gastric cancer cell lines MKN7, MKN45, MKN74, and AZ521 were used in this study. These cell lines were obtained from RIKEN BRC through the National Bio-Resource Project of MEXT, Tokyo, Japan. The cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA).

siRNA transfection

HSP110-specific siRNA was purchased from Bonac Corporation (Fukuoka, Japan). MKN7 and MKN45 cells were plated at a density of 1.0×10^6 cells per well in 100 μl of Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA). Twenty nM of HSP110-specific siRNA 1, 2, 3 and scrambled siRNA (negative control) were added to the cells, and cells were transfected with siRNAs using an electroporator (CUY-21 EDIT II; BEX, Tokyo, Japan), according to the manufacturer's instructions. Poring pulses were applied at 125 V (pulse length, 10.0 ms; 1 pulse; interval, 40.0 ms), and transfer pulses were applied at 10 V (pulse length, 50.0 ms; 5 pulses; interval, 50.0 ms). After 72 h of incubation, further experiments were performed.

Protein extraction and western blot analysis

Western blotting was performed to confirm the expression of HSP110 and β-actin in gastric cancer cell lines. Transfected cells were incubated for 72 h, and total proteins were extracted from MKN7, MKN45, MKN74, and AZ521 cells using PRO-PREP Protein Extraction Solution Kit (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea). The proteins were separated on 4–12% Bis-Tris Mini Gels (Life Technologies Corporation, Carlsbad, CA, USA), and transferred to membranes using an iBlot Dry Blotting System (Life Technologies Corporation, Carlsbad, CA, USA). The membranes were incubated overnight at 4°C with rabbit monoclonal anti-HSP110 antibody (1:1000; GeneTex, CA, USA) and anti-β-actin antibody (1:1000; Sigma-Aldrich, St Louis, MO, USA). Following this, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and the target proteins were detected with the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) using Image Quant LAS4000 (GE Healthcare Life Sciences, UK).

Chemosensitivity assay

Water-soluble tetrazolium-8 (Cell Counting Kit-8; Dojindo Laboratories, Japan) was used in order to evaluate the sensitivity to cisplatin and 5-FU. After 72 h of incubation following the transfection, MKN7 and MKN45 cells were seeded (1×10^4 cells/well) into 96-well plates in 100 µl of RPMI 1640 medium containing 20% FBS before drug exposure. After 24 h of pre-incubation, 10 µl of Cell Counting Kit-8 reagent were added, and the cells were additionally incubated for 2 h at 37°C. The absorbance of each well was detected at 450 nm using an xMark Microplate Absorbance Spectrophotometer (Bio Rad, Hercules, CA, USA). Afterward, the cells were treated with various concentrations of cisplatin and 5-FU for 48 h. Viability was determined using colorimetry by measuring absorbance every 24 h.

Statistical analysis

Data for continuous variables were expressed as mean ± standard error of the mean. Significance was determined using Student's *t*-test and analysis of variance. The statistical analysis of the immunohistochemical staining results was performed using the chi-squared test. Survival curves were generated according to the Kaplan-Meier method and analyzed using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses using a Cox proportional hazards model. Results were considered statistically significant when P value was < 0.05. All statistical analyses were performed using JMP software, version 12 (SAS Institute Inc., Cary, NC, USA).

ACKNOWLEDGMENTS AND FUNDING

The authors thank Ms. Yukie Saito, Ms. Tomoko Yano, Ms. Tomoko Ubukata, Ms. Yuka Matsui, Ms. Ayaka Ishida, and Ayaka Ishikubo for their excellent assistance.

CONFLICTS OF INTEREST

None.

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