Basic Study

Nuclear heat shock protein 110 expression is associated with poor prognosis and hyperthermo-chemotherapy resistance in gastric cancer patients with peritoneal metastasis

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Author contributions: Kimura A collected the data and wrote the initial draft; Ogata K and Kuwano H supervised the experiments; all authors read and approved the final manuscript.

Institutional animal care and use committee statement: All animal studies were approved by the Gunma University Institutional Animal Care and Use Committee.

Conflict-of-interest statement: None of the authors have conflict of interests to disclose.

Data sharing statement: No additional data are available.

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Manuscript source: Unsolicited manuscript

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Received: June 29, 2017
Peer-review started: June 30, 2017
First decision: July 27, 2017
Revised: September 8, 2017
Accepted: September 19, 2017
Article in press: September 19, 2017
Published online: November 14, 2017

Institutional review board statement: This study was reviewed and approved by the institutional review board and ethics committee of Gunma University Hospital.
Abstract

AIM
To investigate the significance of heat shock protein 110 (HSP110) in gastric cancer (GC) patients with peritoneal metastasis undergoing hyperthermo-chemotherapy.

METHODS
Primary GC patients (n = 14) with peritoneal metastasis underwent intraperitoneal hyperthermo-chemotherapy using a Thermotron RF-8 heating device two weeks after surgery. We analyzed HSP110 expression in surgically resected tumors using immunohistochemistry. Additionally, the effect of HSP110 suppression on hyperthermo-chemosensitivity was assessed in vitro in the MKN45 GC cell line using the HSP inhibitor KNK437.

RESULTS
HSP110 immunohistochemical staining in 14 GC patients showed that five (35.7%) samples belonged to the low expression group, and nine (64.3%) samples belonged to the high expression group. Progression-free survival was significantly shorter in the HSP110 high-expression group than in the low-expression group (P = 0.0313). However, no significant relationships were identified between HSP110 expression and the clinicopathological characteristics of patients. Furthermore, high HSP110 expression was not an independent prognostic factor in GC patients with peritoneal metastasis (P = 0.0625). HSP110 expression in MKN45 cells was suppressed by KNK437 at the hyperthermic temperature of 43℃ in vitro. Comparison of MKN45 cell proliferation in the presence and absence of KNK437 at 43℃, revealed that proliferation was significantly decreased when HSP110 was inhibited by KNK437. Additionally, HSP110 suppression via HSP inhibitor treatment increased cellular sensitivity to hyperthermo-chemotherapy in vitro.

CONCLUSION
The expression of nuclear HSP110 in GC patients might be a new marker of chemosensitivity and a therapeutic target for patients who are tolerant to existing hyperthermo-chemotherapies.

Key words: Peritoneal metastasis; Hyperthermia; Hyperthermo-chemotherapy; Heat shock protein 110; Gastric cancer; Drug resistance

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Core tip: Gastric cancer remains one of the most common cancers worldwide. Peritoneal dissemination is the most common reason behind gastric cancer (GC) recurrence, and the median survival duration for patients with metastatic and recurrent GC is only 13-16 mo. In our department, we have used intraperitoneal hyperthermo-chemotherapy in patients with advanced rectal cancer and GC with peritoneal metastasis. In this study, we evaluated the significance of heat shock protein 110 (HSP110) expression in GC patients with peritoneal metastasis and assessed the effects of HSP110 suppression on hyperthermo-chemotherapy sensitivity in vitro.

INTRODUCTION
Despite progress in early diagnosis and improvements in treatment, gastric cancer (GC) is a central cause of cancer-related deaths worldwide. Furthermore, a particularly high GC mortality rate has been reported in Asia[1]. Peritoneal dissemination is the most common reason behind GC recurrence, and the first line of treatment for this disease is chemotherapy. New chemotherapies for metastatic and recurrent GC are being developed. However, the median survival duration for patients with metastatic and recurrent GC is only 13-16 mo[2-7].

Intraperitoneal hyperthermo-chemotherapy is an effective alternative treatment to standard chemotherapy in GC patients with peritoneal dissemination[6-12]. Indeed, hyperthermic intraperitoneal perfusion chemotherapy (HIPEC) with cisplatin combined with an intravenous chemotherapy regimen with paclitaxel, 5-fluorouracil, and leucovorin has improved survival rate and decreased the postoperative recurrence of locally advanced GC[12]. Furthermore, it has been suggested that hyperthermia and 5-fluorouracil act synergistically to promote apoptosis and enhance thermotolerance in the SGC-7901 GC cell line[9]. In our department, we have used intraperitoneal hyperthermo-chemotherapy in patients with advanced rectal cancer and GC with peritoneal metastasis[13-16]. A preliminary non-random study in a small number of patients revealed that patients in the postoperative intraperitoneal hyperthermo-chemotherapy (PIHC) group had a higher survival rate and better prognosis than did the patients in the control group[15].

Heat shock proteins (HSPs) have been characterized as molecular chaperones that prevent the formation of misfolded protein structures[17-19]. HSPs are induced by exposure to the stress condition, including fever,
irradiation and chemicals. HSPs in cancer maintain several oncoproteins homeostasis and promote cancer cell survival by inhibiting apoptosis induction\textsuperscript{[17,20]}. It was reported that overexpression of HSPs might be correlated with poor prognosis in several types of human carcinomas\textsuperscript{[21-26]}. Additionally, it was reported that high levels of various HSP family members are associated with increased chemoresistance in several malignancies\textsuperscript{[27-29]}. Previously, we found that high expression of nuclear HSP110 is associated with cancer progression and poor prognosis in GC patients and that HSP110 suppression increases chemosensitivity in human GC cell lines\textsuperscript{[17]}. However, the clinicopathological significance of HSP110 expression and localization and their relationship with hyperthermo-chemotherapy sensitivity in GC patients are still unclear. Therefore, the purposes of current study were to determine the significance of HSP110 expression in GC patients with peritoneal metastasis and to evaluate the impact of HSP110 inhibition on hyperthermo-chemotherapy sensitivity \textit{in vitro}.

**MATERIALS AND METHODS**

**Patients and samples**
The Institutional Review Board of Gunma University Hospital approved this study, and written informed consent was obtained from all patients. From April 2000 to December 2011, 14 GC patients with peritoneal dissemination underwent distal or total gastrectomy for cytoreduction at the Department of General Surgical Science, Gunma University. All patients were diagnosed with peritoneal metastasis (P1) or positive peritoneal lavage cytology (CY1) during surgery. Gastric cancer staging was performed according to the Classification of Gastric Carcinoma (third edition) of the Japanese Gastric Cancer Association\textsuperscript{[30]}. All patients underwent PIHC.

**PIHC**
Treatment regimens for PIHC were administered as previously described\textsuperscript{[15]}. Patients underwent distal or total gastrectomy with lymph node dissection, and a 19-Fr silicon drain was inserted in the left subphrenic lesion. PIHC was administered to patients diagnosed with P1 or CY1 during surgery. Two weeks after surgery, hyperthermia was induced using 8-MHz radiofrequency capacitive heating equipment (Thermotron RF-8, Yamamoto Vinyter, Osaka, Japan). Physiologic saline (1 L) containing 80 mg/m\textsuperscript{2} cisplatin was warmed to 37 °C in a dry incubator and introduced as quickly as possible into the peritoneal cavity via a catheter. After PIHC, the catheter was clamped for six hours. A minimum temperature of higher than 40 °C was achieved in the abdominal cavity and maintained for 60 min. The treatment was repeated every two weeks for a maximum of four cycles. After completing all PIHC courses, patients took S-1 (Taiho Pharmaceutical Company, Tokyo, Japan) for one year from the date of surgery.

**Immunohistochemical staining**
Resected surgical specimens were fixed in formalin, embedded in paraffin, cut into 4-μm thick sections, and mounted on glass slides. Immunohistochemical staining was performed as previously reported\textsuperscript{[17,31]}. All sections were deparaffinized in xylene. Sections were dehydrated in alcohol and soaked with 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to inhibit endogenous peroxidase. The sections were soaked in boiling water, and immersed in Immunosaver (Nishin EM, Tokyo, Japan) at 98 °C for 90 min. Non-specific binding sites were blocked by incubating the sections with Serum-Free Protein Block (DAKO, Carpinteria, CA, United States) for 30 min at room temperature. The sections were incubated with a 1:100 dilution of a rabbit monoclonal antibody against HSP110 (GeneTex, CA, United States) for 24 h at 4 °C. The signal from the primary antibody was visualized using the Histofine Simple Stain MAX-PO (MULTI) (Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. The chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) was applied as a 0.02% solution containing 0.005% hydrogen peroxide in 50 mmol/L ammonium acetate-citrate acid buffer (pH 6.0). All sections were counterstained with Mayer’s hematoxylin solution.

The immunohistochemically stained samples were analyzed by two researchers blinded to the patient information. Staining for HSP110 was assessed using the immunoreactive score (IRS) to evaluate the proportion of cells expressing HSP110 and their relative staining intensity as previously described\textsuperscript{[31,32]}. The intensity of nuclear HSP110 staining was graded as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining (Figure 1A). The percentage of nuclear HSP110-expressing GC cells was calculated based on at least 1000 cancer cells in total from five representative areas. The percentage of nuclear HSP110 staining was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining (Figure 1A). The percentage of nuclear HSP110-expressing GC cells was calculated based on at least 1000 cancer cells in total from five representative areas. The percentage of nuclear HSP110 staining was scored as follows: 0, no staining; 1+, 1%-10%; 2+, 11%-50%; and 3+, 51%-100% (Figure 1A). The IRS was calculated by multiplying the intensity and expression scores to arrive at values of 0, 1+, 2+, 3+, 4+, 6+, or 9+. IRS values of 0, 1+, 2+ and 3+ represent low HSP110 expression, while IRS values of 4+, 6+, and 9+ represent high HSP110 expression.

**Cell culture**
The human GC cell line, MKN45, was purchased from RIKEN BRC through the National Bio-Resource Project of MEXT, Tokyo, Japan. MKN45 cells were maintained in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and supplemented with 100
Nitrocellulose membranes using a wet transfer protocol. The membranes were incubated overnight at 4°C with rabbit monoclonal antibody against HSP110 (1:1000; GeneTex) and β-actin (1:1000; Sigma, St Louis, MO, United States). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and each protein was evaluated using the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) using Image Quant LAS4000 (GE Healthcare Life Sciences, United Kingdom).

**Cell proliferation assay**

Cell proliferation was measured with the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). MKN45 cells were seeded (3000 cells/well) into 96-well plates in 100 μL medium with KNK437 (50 or 100 nmol/L). To generate heat shock, cells were incubated at 43°C for 3 h before KNK437 (50 or 100 nmol/L) was added. Cell proliferation was assessed at 0, 12, 24, and 36 h. To assess cell proliferation, 10 μL of Cell Counting Kit-8 reagent was added to each well.
and incubated at 37 °C for 2 h. Next, the absorbance of each well was detected at 450 nm using an xMark Microplate Absorbance Spectrophotometer (Bio Rad, Hercules, CA, United States).

Chemosensitivity assay
Cell Counting Kit-8 was used to evaluate the sensitivity of MKN45 cells to hyperthermo-chemotherapy with cisplatin. MKN45 cells were plated (approximately 10000 cells per well) into 96-well plates in 100 μL of medium with KNK437 (50 or 100 nmol/L) before cisplatin exposure. The cells were subjected to heat shock at 43 °C for 3 h; then, 10 μL of Cell Counting Kit-8 reagent was added, and the cells were incubated for 2 h at 37 °C. The absorbance of each well was evaluated at 450 nm using an xMark Microplate Absorbance Spectrophotometer. Then, the cells were treated with various concentrations of cisplatin (0, 0.1, 1 or 10 μmol/L) for 48 h. As control, the sensitivity of the cells to chemotherapy with cisplatin in the absence of heat treatment was evaluated in the same manner.

Statistical analysis
Statistically significant differences were analyzed with Student’s t-test for continuous variables and the chi-square test for categorical variables. The data for continuous variables are expressed as the mean ± SE of the mean. Survival curves were calculated according to the Kaplan-Meier method and analyzed with the log-rank test. Univariate and multivariate analysis by the Cox proportional hazards model was used to identify prognostic factors. P < 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with JMP software (version 12; SAS Institute Inc., Cary, NC, United States).

RESULTS

The clinical significance of nuclear HSP110 expression in GC patients with peritoneal metastasis
We evaluated the expression of nuclear HSP110 in 14 GC patients by immunohistochemical staining. Five (35.7%) samples belonged to the low-expression group, and nine (64.3%) samples belonged to the high-expression group. The relationships between nuclear HSP110 expression with the clinicopathological features of 14 GC patients are shown in Table 1. There were no significant relationships between nuclear HSP110 expression and clinicopathological features of the GC patients. However, the three-year progression-free survival rate was significantly lower in the high HSP110 expression group than that in the low-expression group (P = 0.0313; Figure 1B). The results of univariate analyses of clinicopathological factors affecting progression-free survival rates after surgery are shown in Table 2. The relative risk for all factors including HSP110 expression was greater than 1. However, none of the results were statistically significant. Univariate regression analysis revealed that high HSP110 expression was not an independent
MKN45 cells were grown at 37 °C, there were no significant differences in cisplatin sensitivity among cells treated with KNK437 and parental and control cells (Figure 3B). Furthermore, the therapeutic sensitivity of MKN45 cells treated with cisplatin and KNK437 under hyperthermic conditions was higher than that observed with the other therapeutic combinations (Figure 3C).

**DISCUSSION**

In this study, we found that high nuclear HSP110 expression in GC patients with peritoneal metastasis undergoing hyperthermo-chemotherapy was associated with poor prognosis and poor progression-free survival. Our studies with MKN45 cells showed that the KNK437-mediated inhibition of HSP110 increased the hyperthermo-chemosensitivity of GC cells in vitro. However, there were no significant relationships between nuclear HSP110 expression and the clinicopathological features of the GC patients. Previously, we reported that nuclear HSP110 expression was associated with venous invasion in 210 GC patients[3]. However, we were unable to identify a significant association between HSP110 expression and venous invasion due to the small number of patients included in this study. It is possible that high nuclear HSP110 expression was also associated with venous invasion in the cases in the present study, which may have influenced prognosis. Additionally, our univariate regression analysis showed that high HSP110 expression was not an independent prognostic factor in GC. These results may also be attributed to the small number of patients in the current study.

In this study, we used the HSP inhibitor KNK437 to suppress HSP110 expression in MKN45 GC cells. However, the mechanism by which KNK437 inhibits HSPs is not fully understood. In COLO 320DM (human colon carcinoma) cells, KNK437 was shown to inhibit the acquisition of thermotolerance and the induction of various HSPs including HSP105, HSP70, and HSP40 in a dose-dependent manner[33]. Another study reported that thermotolerance is suppressed by KNK437 through the inhibition of heat-induced accumulation of HSP27 and HSP72 and the induction of p53-independent apoptosis[34]. Moreover, it has been reported that in SCC VII cells, the inhibition of thermotolerance by KNK437 can improve the efficacy of clinical fractionated hyperthermia[35]. Previous reports have linked certain protein expression with hyperthermo-chemosensitivity. Upregulation of miR-218 has been observed in GC patients after cytoreductive surgery and intraperitoneal hyperthermic chemotherapy, and this was shown to increase chemosensitivity to cisplatin[36]. Consistent with this report, our results show that the alteration of various protein levels can affect hyperthermo-chemosensitivity in MKN45 cells in vitro. Specifically, this study showed that hyperthermo-chemosensitivity to the intraperitoneal infusion of

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**Table 1 Relationship between clinicopathological characteristics of gastric cancer patients and nuclear heat shock protein 110 expression (%)**

| Factors                        | HSP110 expression in gastric cancer patients (n = 14) | P value |
|--------------------------------|------------------------------------------------------|---------|
|                                | Low (n = 5)                                          | High (n = 9) |
| Age (mean ± SE)                | 55.2 ± 3.8                                          | 59.3 ± 2.8 | 0.4025 |
| Sex                            |                                                      |          |
| Male                           | 2 (44.4)                                             | 7 (77.8) | 0.1575 |
| Female                         | 3 (60.0)                                             | 2 (22.2) |
| Histology                      |                                                      |          |
| Well, Moderate                 | 1 (33.3)                                             | 2 (66.7) | 0.9227 |
| Muc, Poor, Signet              | 4 (36.4)                                             | 7 (63.6) |
| Depth                          |                                                      |          |
| sn, mp, ss                     | 0 (0.0)                                              | 0 (0.0)  |
| se, si                         | 5 (55.6)                                             | 9 (63.4) |
| Lymph node metastasis          |                                                      |          |
| Absent                         | 0 (0.0)                                              | 2 (100.0)| 0.2549 |
| Present                        | 5 (41.7)                                             | 7 (58.3) |
| Lympatic invasion              |                                                      |          |
| Absent                         | 1 (33.3)                                             | 3 (66.7) | 0.4797 |
| Present                        | 4 (40.0)                                             | 6 (60.0) |
| Venous invasion                |                                                      |          |
| Absent                         | 4 (40.0)                                             | 6 (60.0) | 0.5967 |
| Present                        | 1 (25.0)                                             | 3 (75.0) |
| Peritoneal lavage cytology     |                                                      |          |
| Negative                       | 1 (25.0)                                             | 3 (75.0) | 0.5967 |
| Positive                       | 4 (40.0)                                             | 6 (60.0) |
| Peritoneal metastasis          |                                                      |          |
| Absent                         | 2 (50.0)                                             | 2 (50.0) | 0.4805 |
| Present                        | 3 (30.0)                                             | 7 (70.0) |

Well: Well-differentiated; Moderate: Moderately differentiated; Muc: Mucinous; Poor: Poorly differentiated; Signet: Signet ring cells; sn: Submucosa; mp: Muscularis propria; ss: Subserosa; se: Serosa exposed; si: Serosa infiltrating.

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The effect of HSP110 suppression on the proliferation of MKN45 GC cells

Compared to the untreated cells, the proliferation of MKN45 cells at 43 °C was significantly reduced when HSP110 was inhibited by KNK437 treatment. However, the same analysis performed at 37 °C revealed no significant difference in the proliferation of MKN45 cells grown in the presence or absence of KNK437 (Figure 3A).

Effect of HSP110 suppression on hyperthermo-chemosensitivity in GC cell lines

At 43 °C, the cisplatin sensitivity of MKN45 cells was significantly higher in the KNK437-mediated HSP110 inhibition group than that in the parental or control cell groups. However, when the MKN45 cells were grown at 37 °C, there were no significant differences in cisplatin sensitivity among cells treated with KNK437 and parental and control cells (Figure 3B). Furthermore, the therapeutic sensitivity of MKN45 cells treated with cisplatin and KNK437 under hyperthermic conditions was higher than that observed with the other therapeutic combinations (Figure 3C).
Figure 3  Functional analysis of the MKN45 human gastric cancer cell line treated with KNK437 under hyperthermic conditions. A: Proliferation of MKN45 cells with and without KNK437-mediated HSP110 suppression. The results for normal (37 °C) (right panel) and hyperthermic (43 °C) (left panel) conditions are shown. Proliferation of MKN45 cells in the KNK437-mediated HSP110 suppression group (under hyperthermic condition) was significantly lower than that of the parental and control groups (*P < 0.05); B: Cisplatin sensitivity in MKN45 cells in the presence or absence of KNK437-mediated HSP110 suppression. The results for normal (37 °C) (right panel) and hyperthermic (43 °C) (left panel) conditions are shown. Cisplatin sensitivity of MKN45 cells under the hyperthermic condition of 43 °C was significantly increased by KNK437-mediated HSP110 suppression (*P < 0.05); C: Treatment of MKN45 cells with various therapeutic combinations. Therapeutic sensitivity of MKN45 cells treated with cisplatin and KNK437 under hyperthermic conditions was greater than that of cells with other therapeutic combinations (*P < 0.05). HSP110: Heat shock protein 110; HT: Heat treatment.
cisplatin was enhanced by HSP110 suppression. Combination therapy with hyperthermo-chemotherapy and HSP110 inhibitors might be a new treatment strategy for GC patients with peritoneal metastasis.

This study has several limitations. First, the sample size was very small because hyperthermochemotherapy is not a common therapy for GC patients with peritoneal metastasis and because only a few institutions perform this therapy. Second, the KNK437 HSP inhibitor used in this study is not specific to HSP110 alone. Hence, further analysis are needed with the specific suppression of HSP110. Third, we assessed only resistance to cisplatin in this study. However, resistance to S-1 might also affect the progression-free survival of GC patients. Finally, we administered cisplatin via intraperitoneal infusion. Recently, the effectiveness of the hydrophobic drug paclitaxel has been demonstrated via intraperitoneal chemotherapy. In the future, we need to validate the results of combination therapy with an HSP110-specific inhibitor and hyperthermo-chemotherapy with paclitaxel.

In conclusion, nuclear HSP110 expression is associated with poor prognosis in GC patients with peritoneal metastasis who are treated via intraperitoneal hyperthermo-chemotherapy. Therefore, the IRS values related to HSP110 expression might be used as effective biomarkers for the prognosis of GC patients with peritoneal metastasis. Furthermore, HSP110 suppression in the MKN45 GC cell line increased their hyperthermo-chemosensitivity against cisplatin. Taken together, our results show that nuclear HSP110 expression in GC patients with peritoneal metastasis might be a clinically useful biomarker of prognosis and a therapeutic target for patients who are tolerant to existing chemotherapies or hyperthermia.

| Table 2  | Univariate analyses of clinicopathological features affecting progression-free survival rates in patients after surgery |
|---------|---------------------------------------------------------------|
| Clinicopathological variables | Univariate analysis | RR  | 95%CI | P value |
| Age (< 65 yr/ ≥ 65 yr) | 2.17 | 0.46-8.33 | 0.2988 |
| Sex (male/female) | 1.62 | 0.48-6.22 | 0.4412 |
| Histology (differentiated/undifferentiated) | 2.18 | 0.46-8.33 | 0.2988 |
| Lymph node metastasis (absent/present) | 1.25 | 0.19-5.02 | 0.7832 |
| Lymphatic invasion (absent/present) | 1.29 | 0.33-4.29 | 0.6934 |
| Venous invasion (absent/present) | 1.18 | 0.26-4.13 | 0.8057 |
| Peritoneal lavage cytology (negative/positive) | 1.32 | 0.38-6.10 | 0.6766 |
| Peritoneal metastasis (absent/present) | 2.28 | 0.58-15.08 | 0.2568 |
| HSP110 expression (low/high) | 3.40 | 0.94-16.01 | 0.0625 |

HSP110: Heat shock protein 110.

ACKNOWLEDGMENTS
The authors thank Ms. Yukie Saito, Ms. Tomoko Yano, Ms. Tomoko Ubukata, Ms. Yuka Matsui, and Atsuko Sato for their excellent assistance.

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