

Heat shock-induced HIKESHI protects cell viability via nuclear translocation of heat shock protein 70

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Abstract. Heat shock proteins (HSPs), particularly HSP70, help restore normal cellular function following damage caused by stressors. HSP expression in tumor tissues indicates cancer progression, and while the development of HSP inhibitors is progressing, these substances are not widely used to treat cancer. HIKESHI (C11orf73) does not control the intracellular movement of HSP70 at normal temperatures; however, it does regulate the function and movements of HSP70 during heat shock. In this study, we examined the intracellular movement of HSP70 during heat shock to investigate the significance of HIKESHI expression in gastric cancer (GC) and determine if HIKESHI inhibition has cytotoxic effects. We examined HIKESHI using GC cell lines and immunostaining in 207 GC tissue samples. HIKESHI expression in GC tissues was associated with the progression of lymphatic invasion. Suppressing HIKESHI using siRNA did not affect cell viability at normal temperatures. However, suppressing HIKESHI during heat shock inhibited HSP70 nuclear transport and suppressed cell viability. Our results suggest that HIKESHI is a marker of cancer progression and that the combination of HIKESHI inhibition and hyperthermia is a therapeutic tool for refractory GC.

Introduction

Gastric cancer (GC) is a central cause of cancer-related deaths worldwide (1). Recurrent GC with peritoneal dissemination is associated with poor prognosis and symptoms such as intestinal obstruction and abdominal bloating (2). Chemotherapy for peritoneal dissemination is sub-optimal due to insufficient drug delivery (3). In addition, overall survival time may be prolonged by treatments that combine chemotherapy and hyperthermia (4). Unfortunately, hyperthermia is poorly tolerated by most patients. Cellular stressors cause protein dysfunction and non-specific aggregation, and disrupt protein folding (5-7). Heat shock proteins (HSPs), which are induced by heat shock, are especially important for maintaining the normal cellular function against stresses such as heat shock, oxidative stress, and UV irradiation (8). Mammalian HSPs are classified into several protein families depending on their molecular weight: HSP27, HSP40, HSP60, HSP70, HSP90, and large HSPs (9,10). HSPs are over-expressed in almost all types of cancers and are indicators of tumor cell proliferation, differentiation, metastasis, and resistance to treatment (11,12). The elevated expression of HSP70 in clinical breast and endometrial cancer samples is associated with cancer progression and poor prognosis (13,14). Fang *et al* and He *et al* reported that HSP70 and HSP90 are associated with increased resistance to chemotherapy and may therefore be potential therapeutic targets in refractory malignancies (15,16). However, HSP70 inhibitors have high toxicity and do not exert tumor-specific effects (17,18).

The term 'HIKESHI' means to put out fire in Japanese. Kose and Imamoto in the Japanese institution first named c11orf73 as HIKESHI because the protein transports HSP70 into the nucleus to protect cellular homeostasis against heat shock (19). Therefore, HIKESHI is identified as both-full name and gene symbol.

HSP70 rapidly moves from the cytoplasm into the cell nucleus during heat shock stress (20-22). This movement is controlled by importin and exportin at 37°C, but is highly

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impaired at 43°C (23). The protein HIKESHI can transport HSP70 to the nucleus at high temperatures, when importin fails. During heat shock stress, facilitating this transport helps decrease cell damage caused by the loss of HSP70 within the cell nucleus (23). Consequently, suppression of HIKESHI may improve cellular resistance to hyperthermia treatment for cancer. Thus, tumor-specific, localized hyperthermia, with the addition of HIKESHI suppression, may be a promising treatment for recurrent GC.

The significance of HIKESHI expression in GC is unknown, and the relationship between HIKESHI and hyperthermia has not yet been investigated with GC cells. This study examined HIKESHI expression in tissue samples obtained from 207 patients with GC and assessed the role of HIKESHI in resistance to heat shock treatment in GC cell lines.

Materials and methods

Patients and samples. We obtained primary GC tissue samples from 207 patients diagnosed with GC (145 males and 62 females) who underwent radical gastrectomy at the Department of General Surgical Science, Gunma University, between 1999 and 2006. The gastric cancers were staged according to the Japanese Classification of Gastric Carcinoma: 3rd English edition, as developed by the Japanese Gastric Cancer Association (24). The University Institutional Review Board oversaw all aspects of this research, and we obtained written informed consent from all patients according to the institutional guidelines.

Tissue microarray production 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. For each of the 207 GC samples, one paraffin block containing representative non-necrotic tumor tissue was selected. We sampled GC tissue cores (2.0-mm diameter per tumor) from representative areas and transferred them onto paraffin blocks using a tissue-arraying instrument (Beecher Instruments, Silver Spring, MD, USA). Cores were arranged into quad tissue array blocks, each containing 50-55 tumor cores. We obtained 3.5- μ m sections from the microarray blocks and used them for immunohistochemical staining. All sections were incubated at 60°C for 60 min and deparaffinized in xylene, and then rehydrated and incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to block endogenous peroxidase activity.

After rehydration with 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 50 min. Non-specific binding sites were blocked by incubating with Protein block serum-free (Dako, Carpinteria, CA, USA) for 40 min. We applied a rabbit polyclonal anti-C11orf73 antibody (Abcam plc, Cambridge, UK) at a dilution of 1:100 for 24 h at 4°C. The primary antibody was visualized using the Histofine Simple Stain MAX-PO (Multi) kit (Nichirei, Tokyo, Japan) according to procedures described in the instruction manual. Chromogen 3,3'-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution containing 0.005% H₂O₂ in 50 mM ammonium

Table I. Relationship between clinicopathological factors and the cytoplasmic expression of HIKESHI in 207 gastric cancer tissue samples.

Factors	HIKESHI expression (n=207)		P-value
	Low (n=76)	High (n=131)	
Age (mean \pm standard error)	65.7 \pm 1.3	64.1 \pm 0.9	0.2612
Sex, n (%)			0.5776
Male	55 (37.9)	90 (62.1)	
Female	21 (33.9)	41 (66.1)	
Histology, n (%)			0.179
Well, Moderate	27 (31.4)	59 (68.6)	
Poor, Signet	49 (40.5)	72 (59.5)	
Depth of invasion, n (%)			0.7006
m, sm, mp	21 (38.9)	33 (61.1)	
ss, se, si	55 (36.0)	98 (64.0)	
LN metastasis, n (%)			0.4607
Absent	27 (40.3)	40 (59.7)	
Present	49 (35.0)	91 (65.0)	
Lymphatic invasion, n (%)			0.049 ^a
Absent	11 (57.9)	8 (42.1)	
Present	65 (34.6)	123 (65.4)	
Venous invasion, n (%)			0.2659
Absent	58 (38.7)	92 (61.3)	
Present	17 (30.4)	39 (69.4)	
Recurrence, n (%)			0.9432
Absent	41 (36.9)	70 (63.1)	
Present	35 (36.5)	61 (63.5)	
Stage, n (%)			0.8738
I, II	38 (37.3)	64 (62.7)	
III, IV	38 (36.2)	67 (63.8)	

acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained with Mayer's hematoxylin prior to mounting. We defined the intensity of HIKESHI staining in cytoplasm as follows: 0 = no staining, 1 = weak staining, and 2 = strong staining. We divided 76 of the 207 GC specimens into a cytoplasm HIKESHI low expression group (0) and 131 into a high expression group (1 and 2) according to this scoring procedure (Fig. 1).

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

Expression of HIKESHI by heat shock treatment. The MKN7 and MKN45 cells were seeded (1.0 \times 10⁶ cells/dish) into a 10-cm dish in 10 ml of medium. After 24 h pre-incubation

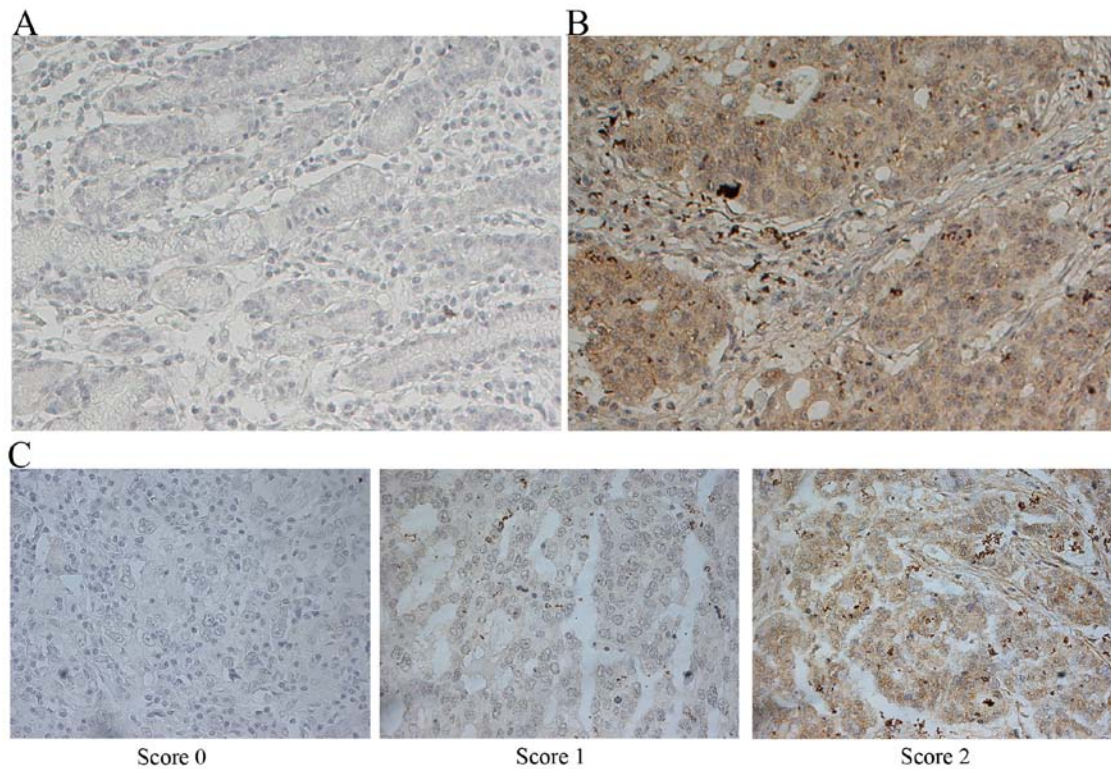


Figure 1. Immunohistochemical staining of HIKESHI in primary gastric cancer samples. (A) Non-cancerous tissue (original magnification, x100). (B) Cancerous tissue (original magnification, x100). (C) Tissue microarray samples (original magnification, x200). The intensity of cytoplasm HIKESHI staining was scored as follows: score 0 = no staining; score 1 = weak staining; score 2 = strong staining.

at 37°C, the cells were incubated for 1, 2, and 3 h at 43°C as heat shock treatment. Immediately after the treatment, total protein was extracted using the PRO-PREP Protein Extraction Solution kit (iNtRON Biotechnology, Sungnam, Korea).

siRNA transfection. We purchased HIKESHI-specific siRNA from Bonac Corp. (Fukuoka, Japan). MKN7 and MKN45 cells were used at a density of 1.0×10^6 cells per well in 100 μ l of Opti-MEM I reduced serum medium (Invitrogen), and mixed 20 nM of HIKESHI-specific siRNA-1, -2 and control siRNA into the cells. The siRNA was transfected via 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 msec; 1 pulse; interval, 40.0 msec) and transfer pulses were applied at 10 V (pulse length, 50.0 msec; 5 pulses; interval, 50.0 msec). After 72 h of incubation, the following experiments were performed.

Western blot analysis. Western blotting confirmed the expression of HIKESHI and β -actin proteins in the GC cell lines. Transfected cells were incubated for 48 h. Total protein was extracted from MKN7, MKN74, MKN45, and KATOIII. These proteins were separated and transferred onto membranes using 10% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight at 4°C with the rabbit polyclonal anti-C11orf73 antibody (1:1,000; Abcam) and β -actin (1:1,000; Sigma, St. Louis, MO, USA), and then treated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The proteins were detected

using the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) with Image Quant LAS4000.

Sensitivity assay of heat shock treatment. We used a water-soluble tetrazolium-8 assay [Cell Counting Kit-8 (CCK-8), Dojindo Laboratories, Kumamoto, Japan] to evaluate the relationships among cell viability, HIKESHI suppression, and heat shock treatment. MKN7 and MKN45 cells were seeded (3000 cells/well) into 96-well plates in 100 μ l medium after a 72-h incubation period following transfection. The cells were pre-incubated for 24 h, and then incubated for 3 h at 43°C on day 0 and day 1. We added 10 μ l of the CCK-8 reagent and incubated the cells for an additional 2 h at 37°C. The xMark Microplate Absorbance Spectrophotometer (Bio-Rad) was used to detect the absorbance of each well at 450 nm.

Fluorescent immunohistochemistry. After siRNA transfection, MKN7 cells were seeded (5000 cells/chamber) into Chamber Slide II (AGC Techno Glass Co., Ltd., Shizuoka, Japan) in 500 μ l medium for 72 h. These cells were fixed with 100% methanol at -20°C for 15 min, and then incubated with the rabbit C11orf73 antibody (1:100; Abcam) and mouse HSP70 antibody (1:100; Abcam) overnight at 4°C. To detect antibodies against C11orf73 and HSP70, fluorophore-labeled antibodies with anti-mouse fluorescein isothiocyanate and anti-rabbit Cy3 specificities (Molecular Probes) were used for 2 h at room temperature and at a dilution of 1:1,000. All sections were then counterstained with 4',6-diamidino-2-phenylindole and examined under an All-in-One BZ-X710 Fluorescence Microscope (Keyence Corp.).

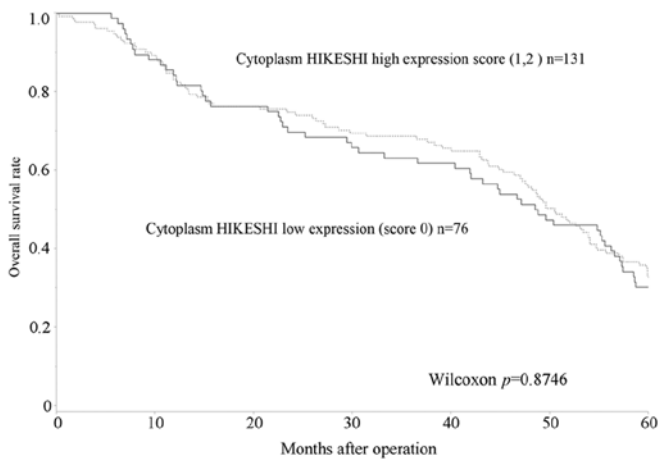


Figure 2. Overall survival of gastric cancer patients according to the cytoplasm HIKESHI expression. There was no significant difference in the overall survival of patients with GC and cytoplasm HIKESHI low expression and those with cytoplasm HIKESHI high expression ($P=0.8746$).

Statistical analysis. Data for the continuous variables were expressed as the mean \pm SEM. Significance was determined using Student's *t*-tests and ANOVA. Statistical analysis of the immunohistochemical staining data was performed using the chi-square test. Survival curves were generated according to the Kaplan-Meier method and analyzed with the generalized Wilcoxon test. Results were considered statistically significant when the relevant *P*-value was <0.05 . All statistical analyses were performed with JMP software, version 12.2.0 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical significance of HIKESHI expression in GC patients. We evaluated HIKESHI expression in 207 samples of GC tissue microarrays with immunohistochemical analysis.

HIKESHI expression in GC tissues was higher than that observed in non-cancerous tissues. In cancerous tissues, the HIKESHI immunoreactivity was positive, mainly in the cytoplasm (Fig. 1A and B). Cytoplasmic HIKESHI expression scores were as follows: 0, 76 (36.7%) samples; 1, 89 (43.0%) samples; 2, 42 (20.3%) samples (Fig. 1C). Seventy-six (36.7%) samples fell within the cytoplasm HIKESHI low expression group and 131 (63.3%) samples were within the cytoplasm HIKESHI high expression group. The relationship between cytoplasm HIKESHI expression and clinical and pathological factors exhibited by the 207 patients with GC is shown in Table I. The high expression of cytoplasmic HIKESHI showed a tendency to be associated with lymphatic invasion ($P=0.049$). There were no obvious differences between the cytoplasm HIKESHI low expression group and the high expression group, according to the overall survival rate ($P=0.8746$, Fig. 2).

Regulation of HIKESHI expression in GC cell lines. HIKESHI was expressed in human GC cell lines (MKN7, MKN74, MKN45) on western blotting (Fig. 3A). MKN7 with high baseline HIKESHI expression had more resistance to heat shock treatment than MKN45 with low HIKESHI (data not shown). We used both MKN7 and MKN45 to analyze the effects of HIKESHI suppression on *in vitro* GC cell lines. The expression of HIKESHI increased during heat shock treatment (Fig. 3B). HIKESHI expression was suppressed in MKN7 and MKN45 cells treated with the suppressor HIKESHI siRNA (Fig. 3C).

Effect of heat shock treatment and HIKESHI suppression on cell viability in GC cell lines. HIKESHI suppression was not associated with viability at 37°C (Fig. 4A). Therefore, we examined the relationships among cell viability, HIKESHI suppression, and heat shock treatment in GC cell lines. MKN7 and MKN45 cells treated with HIKESHI siRNAs were incubated at 43°C for 3 h, with heat shock every 24 h. The

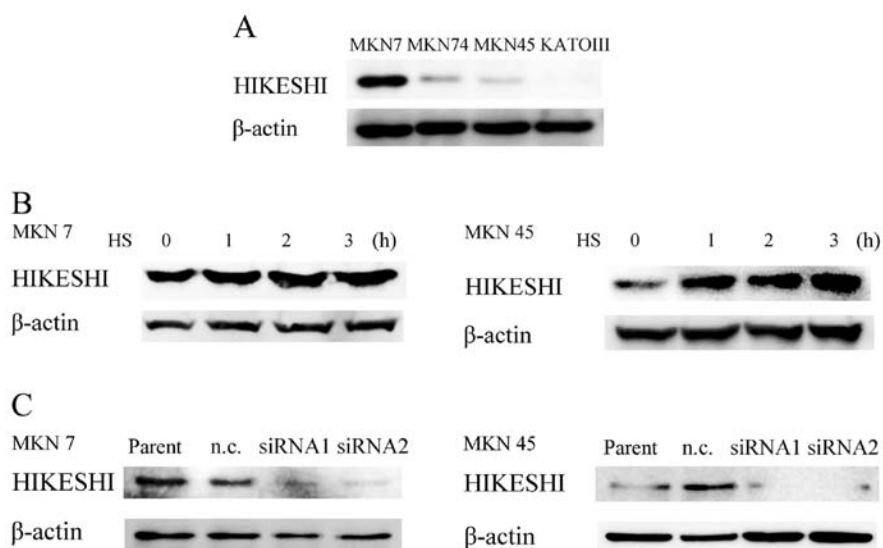


Figure 3. Functional analysis of human gastric cancer cell lines treated with heat shock treatment and HIKESHI siRNA. (A) The expression of HIKESHI in human gastric cancer cell lines was assessed by western blotting. β -actin was used as the loading control. (B) In MKN7 and MKN45 cells, the expression level of HIKESHI increased depending on time in heat shock treatment. (C) HIKESHI expression was suppressed using HIKESHI siRNA (MKN7 and MKN45).

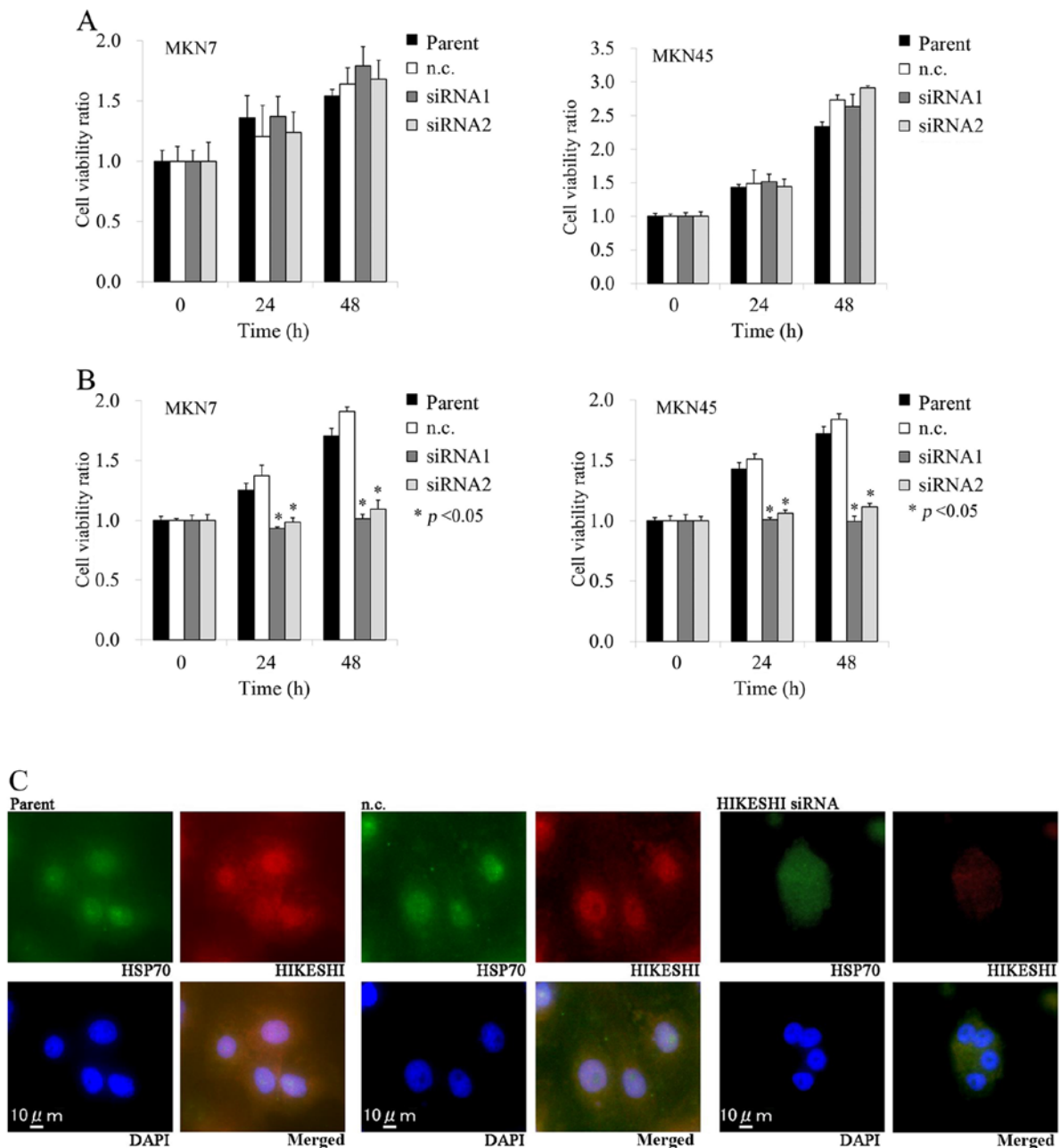


Figure 4. Functional analysis of human gastric cancer cell lines treated with HIKESHI siRNA only/and heat shock treatment. (A) The effects of only HIKESHI suppression of MKN7 and MKN45 cells. There was no difference in the proliferation ability among parent cells, negative control cells, siRNA1 cells, and siRNA2 cells. n.c., negative control (control siRNA). (B) The effects of HIKESHI suppression on heat shock treatment of MKN7 and MKN45 cells. Both MKN7 and MKN45 cells showed a significantly increased sensitivity to heat shock treatment in HIKESHI siRNA-treated groups, compared with the parent and negative control cells.

HIKESHI siRNA groups were significantly more sensitive to heat shock treatment than the parent cells and control cells ($P < 0.05$; Fig. 4B).

HIKESHI siRNA treatment suppressed the nuclear localization of HSP70 under heat shock. We evaluated the effects of HIKESHI siRNA on HSP70 movement during heat shock treatment using fluorescent immunostaining. In parent MKN7 cells and in the negative control group, HIKESHI and HSP70 were localized in the nucleus. However, in the HIKESHI siRNA group, nuclear localization of HSP70 was inhibited (Fig. 4C).

Discussion

In this study, we observed higher expression of HIKESHI in GC tissues than in non-cancerous tissues. This finding was associated with progressive invasion of lymphatic tissues. However, tissue samples obtained from patients with GC and high HIKESHI were not associated with poor prognosis. In GC cell lines, we induced HIKESHI expression with a 43°C heat shock treatment. Suppressing HIKESHI at 37°C did not affect cell proliferation; however, under heat shock stress, nuclear transport of HSP70 was suppressed, decreasing cell viability.

HIKESHI expression is related to the progression of lymphatic invasion in GC clinical samples. HIKESHI was important for nuclear transport of HSP70 and protected GC cell lines during heat shock stress. Some researchers reported that patients with GC and overexpression of HSP70, especially those with intestinal-type GC, exhibit poor prognosis (25,26). In primary GC, HIKESHI may activate HSP70, and can therefore serve as a marker for cancer progression. However, in our study, accumulation of HIKESHI in patients with GC, without hyperthermia, did not relate to other factors typically associated with progression, including tumor invasion, lymph node metastasis, and prognosis (despite oncogenic HSP70 protection by HIKESHI). Future studies should assess HIKESHI in GC samples treated with neoadjuvant therapy, including hyperthermia, in order to clarify the true significance of HIKESHI expression.

In this study, HIKESHI expression in GC cell lines was induced by a 43°C heat shock treatment. Kose *et al* reported that HIKESHI expression in HeLa cells was induced by heat shock treatment (19). Under non-heat shock conditions, importin transfer proteins containing the classical nuclear localization signal into the nucleus to maintain cellular homeostasis (27); however, these importins were inactivated under heat shock stress conditions. During heat shock, HIKESHI can carry several HSPs into the nucleus, preventing heat shock-induced cell death (28). Heat shock stress may activate HIKESHI to protect cancer cells during hyperthermia therapy. Presently, few studies elucidate the potential relationships between HIKESHI expression and cellular stressors including hyperthermia, chemotherapy, radiation therapy, and immunotherapy. It is necessary to develop methods to regulate HIKESHI expression and/or function to clarify the role of HIKESHI in the context of cellular stressors.

When HIKESHI is suppressed during 43°C heat shock treatment, HSP70 is unable to migrate to the nucleus and instead accumulates in the cytoplasm of GC cells, decreasing cell viability. HSPs, including HSP70, are overexpressed in cancer, including GC (29), and the therapeutic targeting of HSPs may be a promising strategy for future *in vitro* and *in vivo* analyses (30-32). However, system-wide targeting of HSPs may elicit undesirable side effects because HSPs protect both cancerous and non-cancerous cells from cellular stressors including heat shock (18). Therefore, highly specific tumor targeting is necessary. HIKESHI is a protein that transports HSP70 under heat shock conditions, indirectly assisting in cell recovery. At 37°C, suppressing HIKESHI does not affect HSP70 migration. This is because, at normal temperatures, inhibiting HIKESHI does not interfere with the protective effects of HSP70 in the presence of cellular stressors. The hyperthermia therapy of tumor tissue, with HIKESHI suppression, may produce antitumor effects by inhibiting the movement of HSP70 to the cell nucleus. Suppressing HIKESHI, along with hyperthermia treatment, might be a promising therapeutic strategy for patients with advanced GC or peritoneal dissemination of GC, as well as for patients unable to tolerate standard treatments that are highly cytotoxic. Patients unable to receive surgery and chemotherapy with double or triple anticancer drugs because of liver dysfunction, renal dysfunction, old age, or the presence of excessive comorbidities may also potentially benefit

from such an approach. In conclusion, high expression of HIKESHI was associated with the progression of lymphatic invasion. HIKESHI expression in clinical GC tissues may serve as a marker for cancer progression. Suppressing HIKESHI increased the susceptibility of human GC cell lines to heat shock stress. Thus, inhibiting HIKESHI, combined with targeted hyperthermia treatment, may emerge as a new therapeutic treatment for patients with refractory GC.

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