Short Communication

Inhibitory Effect of the HASPIN Inhibitor CHR-6494 on BxPC-3-Luc, A Luciferase-Expressing Pancreatic Cancer Cell Line

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Abstract
HASPIN acts in chromosome segregation via histone phosphorylation. Recently, HASPIN inhibitors have been shown to suppress growth of various cancer cells. Pancreatic cancer has no symptom in the early stages and may progress before detection. So, the 5-year survival rate is low. Here, we reported that administration of the HASPIN inhibitor, CHR-6494, to mice bearing pancreatic BxPC-3-Luc cancer cells significantly suppressed growth of BxPC-3-Luc cells. CHR-6494 might be a useful agent for treating pancreatic cancer.

Keywords: HASPIN Kinase, Histone H3, Pancreatic Cancer, Protein Kinase

All animal experiments were confirmed by the Guide for the Care and Use of Laboratory Animals and they were approved by the Institutional Committee of Laboratory Animal Experimentation and Research Ethics Committee of Nagasaki International University (Approval number 129).

The mouse histone H3 associated protein kinase *HASPIN* gene, encoding a nuclear-localized Ser/Thr kinase, was cloned from a cDNA library obtained by subtracting W/W^v mutant mouse testis mRNA (1). HASPIN phosphorylates Thr3 of histone H3 in mitotic cells. It is localized on centrosomes and spindles in mitosis, while regulates chromosome and spindle function during mitosis and meiosis (2). It is involved in the phosphorylation of Thr127 of TH2A, a germline-specific H2A variant, in elongating spermatids and early mitotic preimplantation mouse embryos (3). To further clarify its role, *HASPIN* gene-disrupted mice were generated. However, no obvious phenotype was detected in these mice. Light microscopy of the testes obtained from the *HASPIN* null mice showed a few seminiferous tubules, containing no germ cell (4). As part of the KINOME project, a HASPIN kinase inhibitor was isolated. Findings showed that this inhibitor suppresses growth of cancer cells *in vitro* and *in vivo* (5, 6). While HASPIN is essential for proliferation of the cultured cancer cells (5), no abnormality was observed in *HASPIN*-null mice. So function of HASPIN may be counterbalanced by its association with other molecules in normal cells. In addition, it may play important roles in cell division, during proliferation of male germ cells and cancer cells (4-6).

Pancreatic cancer is on the rise globally and it is an important cause of death (7). Pancreatic cancer is painless.

So it is generally detected late, in advanced stages. The main treatment is surgery. When invasion and metastases are likely, chemotherapy is also given (8). With few effective therapies, it is crucial to find more effective treatments and to explore the mechanisms involved in improving how to save patients with pancreatic cancer.

Recently, some small molecules have been found to inhibit HASPIN and growth of cancer cells derived from various tissues (5, 9-13). The natural product coumestrol inhibits HASPIN (14). Therefore, we examined whether CHR-6494 suppresses proliferation of pancreatic BxPC-3-Luc cancer cells.

First, a cell viability assay was used to examine whether CHR-6494 (Sigma-Aldrich, Japan) inhibits proliferation of pancreatic BxPC-3-Luc cancer cells (National Institutes of Biomedical Innovation, Health and Nutrition, Japan). Cell viability was assessed using the XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis[4-methoxy6-nitro] benzene sulfonic acid hydrate) assay (Sigma-Aldrich, Japan), according to the manufacturer's protocol (5). CHR-6494 was dissolved at 10 mM in DMSO and stored at -20°C. This solution was next sequentially diluted in DMSO and equal amounts were added to each well of 96-well plates. A density of 2×10^4 cells per well were seeded and incubated for 24 hours in order to the cell attachment. Then the medium was replaced with new medium containing different drug concentrations (0.01, 0.1, 0.5, 1, 5, 10, 50 and 100 μM), using eight wells for each CHR-6494 concentration, apart from the well with no CHR-6494, as a negative control. The XTT reagent was added 48 hours after drug administration and the absorbance was measured 2 hours later. The half-maximal inhibitory concentration (IC$_{50}$)

was determined using GraphPad Prism (GraphPad, USA). CHR-6494 inhibited cell growth dose-dependently and the IC_{50} was 849.0 nM (Fig.1).
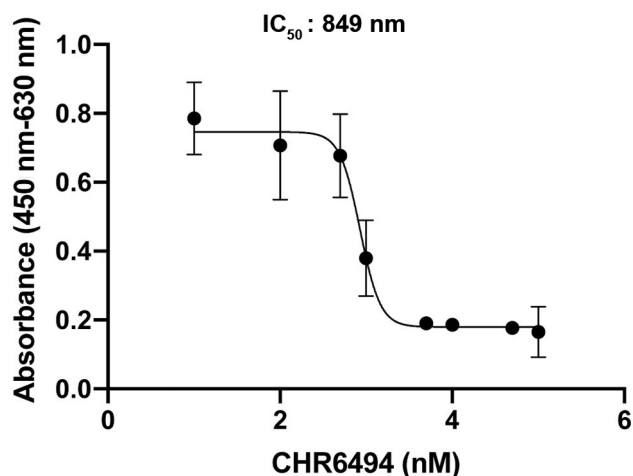

Fig.1: CHR-6494 inhibits proliferation of BxPC-3-Luc cells *in vitro*. Effects of CHR-6494 on the viability of BxPC-3-Luc cells were evaluated using the XTT assay.

Next, 200 µl of BxPC-3-Luc cell suspension, containing 2×10^6 cells, was added into the flanks of 12 nude mice (BALB/cAJcl- nu/nu, CLEA Japan, Japan). They were treated with CHR-6494 or vehicle control. Subsequently, potential effects of intraperitoneal administration of CHR-6494 on tumor size were examined. When the tumor long axis was reached to 130 mm, 2-3 weeks after administration of the BxPC-3-Luc cells, CHR-6494 administration was started. As negative controls, vehicle solution (10% DMSO) and co-solvent (20% 2-hydroxypropyl-b-cyclodextrin; Sigma-Aldrich, Japan) in saline were injected into the peritoneal cavities of six mice (three males and three females). For treatment, 5 µg/µl CHR-6494 in vehicle solution was injected into the peritoneal cavities of six other mice (three males and three females) at 50 mg/kg body weight by adjusting the injection volume. Considering the weight of mice (10-30 g), 100-300 µl solution was injected to each one. The injectable material was stored at -30˚C. Treatments were performed for five cycles consisting of five consecutive injection days and five successive non-treatment days for 4 weeks. The treatments were followed, as previously reported procedures (5). The volume of each tumor was determined by measuring long (L) and short (S) axes with calipers and using the formula L×S2/2. The tumor volume of tumor-bearing mice after 1–4 weeks of CHR-6494 administration was 1.5 ± 0.6, 2.5 ± 0.7, 3.5 ± 0.8 and 3.8 ± 0.9 mm³, respectively. The tumor size in untreated control mice after 1-4 weeks was 2.0 ± 0.5, 3.0 ± 0.6, 4.0 ± 0.8

and 6.0 ± 1.7 mm³, respectively (Fig.2). Means were compared between groups using Student's t test; significant differences were determined at the P<0.05 level. There was no significant difference in tumor size between the male and female groups. Administration of CHR-6494 for 4 weeks significantly inhibited tumor growth.
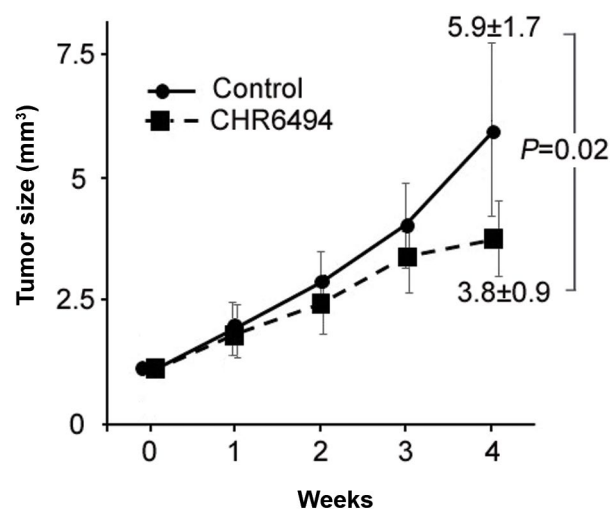

Fig.2: CHR-6494 prevents tumor development in xenograft mice treated with CHR-6494. Tumor size in treated (squares) and not treated (dots) mice with CHR-6494. The vertical axis indicates the average size of tumors in each group. Data are presented as the mean ± SD (P<0.05).

BxPC-3-Luc cells contain luciferase reporter gene. Luminescence of the xenograft tumors on nude mice was detected at the Central Institute for Experimental Animals (Kanagawa, Japan) using IVIS (IVIS Lumina XRMS Series III; Perkin Elmer, USA) with ᴅ-Lucifer in potassium salt (VivoGlo Luciferin, *in vivo* grade; Promega). The *in vivo* bioluminescence imaging showed no apparent signal of metastasis in the xenograft models (Fig.3).
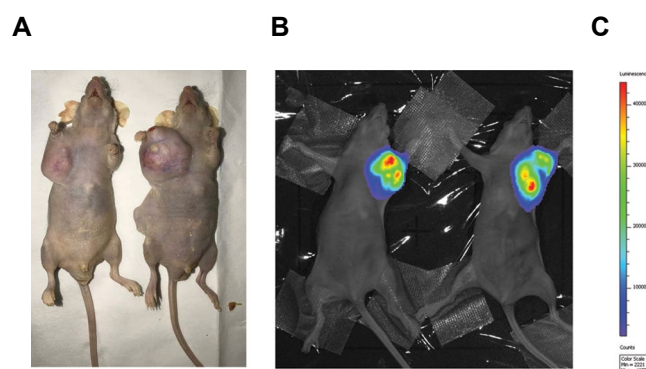

Fig.3: Images of BxPC-3-Luc cell tumors in nude mice. BxPC-3-Luc cell tumors in nude mice in bright images of **A.** Treated and B. Not treated mice with CHR-6494, and luminescence images of **C.** Untreated mice. No BxPC-3-Luc cell metastasis to other organs was observed in nude mice, three weeks after transplantation.

A few chemicals inhibit the kinase activity of HASPIN and suppress cancer cell growth. The HASPIN inhibitor, CHR6494, is more specific for HASPIN kinase activity and suppresses cancer cell growth (5, 9-14). We had established a HASPIN-disrupted mouse line, but contrary to our prediction no abnormality was observed in these mice. Deficiency in HASPIN-specific inhibition did not appear to cause severe abnormalities in normal cell differentiation and proliferation. Nevertheless, HASPIN plays a vital role in cell division via the phosphorylation of histone H3 in cultured cells. H3T3 is phosphorylated by VRK1, dephosphorylated by PP1A. It is also phosphorylated by HASPIN in a cell cycle-dependent manner (15). In HASPIN-null mice, H3T3 is phosphorylated by VRK1, but absence of HASPIN may affect the PP1A phosphatase activity and compensate for the phosphorylation state of H3T3. In dedifferentiated cancer cells, loss of HASPIN kinase activity may be not complemented (5, 6). Since treatment of pancreatic cancer is difficult, effect of CHR-6494 on pancreatic cancer BxPC-3-Luc cells were investigated. We found that CHR-6494 significantly inhibited growth of BxPC-3-Luc cells. In an RNA interference-mediated knockdown experiment, supressing expression of HASPIN, development of pancreatic cancer was suppressed (16); therefore, the HASPIN inhibitor CHR6494 is thought to have no side-effect in humans. These results suggested that, as a potentially useful treatment against pancreatic cancer.

Acknowledgments

Authors' Contributions

H.T.; Contributed to the study conception and design, experimental work, data and statistical analyses, data interpretation, and writing the manuscript. H.N.-F.; Contributed to the experimental work and data analyses. M.W., K.T., H.M., Y.A.; Contributed to the materials. All authors read and approved the final manuscript.

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