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Reduced expression of the *H19* long non-coding RNA inhibits pancreatic cancer metastasis

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Abstract

H19 is an oncofetal RNA expressed in the developing embryo as well as in bladder, breast, gastric, pancreatic, hepatocellular, and prostate cancers. Recent studies have shown that *H19* enhances cancer invasion and metastasis; however, its roles in cancer remain controversial. In the current study, *H19* exhibited the second largest increase (82.4-fold) and represented the only non-protein coding gene among 11 genes identified that were elevated over 10-fold in lung-metastasis-derived pancreatic cancer cells compared with their parental cells using a mouse metastatic model. Subsequently, we further clarified the roles of *H19* in pancreatic cancer growth and metastasis using in vitro and in vivo techniques. In situ hybridization showed that *H19* was detected in 23 of 139 invasive ductal carcinomas (17%), and that *H19* expression positively correlated with higher histological grades (P < 0.0001). Overexpression of *H19* in PANC-1 pancreatic cancer cells induced higher motilities, whereas *H19* inhibition using shRNA and siRNA showed opposite results; however, cell growth rates were not impacted. Intravenous injection of *H19* shRNA vector-transfected PANC-1 cells yielded marked inhibition of metastasis in the liver and lungs of immunodeficient mice. These findings suggest that *H19* has important roles in pancreatic cancer metastasis, and that inhibition of *H19* represents a novel candidate for pancreatic cancer therapy.

The number of cases of pancreatic cancer has been increasing every year such that it now represents the fourth leading cause of cancer death in the United States [1].

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Surgical resection, chemotherapy, and radiotherapy have been employed as treatment options for pancreatic cancer. However, the most recent 5-year survival rate for patients with pancreatic cancer is only 8% [1]. Pancreatic cancers readily metastasize to the other organs at the early stage of the cancer and the recurrence rate after surgery is high. Therefore, novel treatment strategies are required to improve the remission and cure rates of this devastating disorder.

According to the central dogma theory of biology, RNA constitutes the intermediary between DNA and protein. However, recent genome-wide transcriptome studies have identified that almost all of the human genome is transcribed and produces a large number of non-coding RNAs (ncRNAs) including both small ncRNAs and long ncRNAs (lncRNAs) [2–4]. Small ncRNAs have been defined as a ribonucleotide smaller than 200-mers including small interfering RNAs (siRNAs), microRNAs, piwi-interacting RNAs, and small nuclear RNAs [5]. Conversely, lncRNAs comprise mRNA-like transcripts longer than 200 ribonucleotides lacking significant open reading frames [2, 3, 6]. The expression and roles of small ncRNAs have been studied intensively in cancer and have demonstrated that small ncRNAs are involved in carcinogenesis. Recently,

accumulating evidence suggests that lncRNAs have crucial roles in carcinogenesis, and in the growth and metastasis of various types of cancer; however, the detailed mechanisms remain unclear [2, 3, 5, 6].

We previously established a novel pancreatic cancer cell line (PANC-lung1 cells) from lung metastatic lesions using super immunodeficient NOD/Shi-scid, IL-2R γ null (NOG) mice [7, 8]. PANC-lung1 cells exhibited a higher metastatic ability than the parental PANC-1 cells both in vitro and in vivo. To clarify the mechanisms of pancreatic cancer metastasis, we compared the RNA expression levels of the two cell lines using DNA microarray analysis and identified that *H19*, a lncRNA, represented the highest-expressing ncRNA in metastasis-derived PANC-lung1 cells.

H19 is known as an imprinted RNA that is transcribed exclusively from the maternal allele and also as an oncofetal RNA that is expressed in the developing embryo, as well as in some types of tumors [9, 10]. Recent evidence has shown that H19 enhances invasion and metastasis in bladder cancer [11], glioma [12], breast cancer [13], gastric cancer [14], and pancreatic cancer [15], whereas it suppresses the aggressiveness of hepatocellular carcinoma [16] and prostate cancer [17].

In the current study, we report that inhibition of H19 decreased the metastasis of pancreatic cancer in vivo, indicating that H19 represents a novel candidate for targeted pancreatic cancer therapy.

Materials and methods

Materials

The following were purchased for this study: anti-human leukocyte antigen (HLA) class I-A, B, and C antibody (clone EMR8-5) from Hokudo Co., Ltd (Tokyo, Japan); pancreas tissue microarrays (PA1001, PA1921, and PA2081a) from Biomax, Inc. (Rockville, MD); Histofine mouse stain kit from Nichirei (Tokyo, Japan); TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays for H19 (Hs00262142 g1) and 18S rRNA (Hs03928990_g1vv), and siRNAs targeting human H19 (siH19-A, n272453; siH19-B, n272446) and negative control siRNA (Silencer select Negative control 2), as well as pcDNA3.1(-) vector from Life Technologies Corporation (Carlsbad, CA); FuGene HD transfection reagent from Roche Diagnostics (Mannheim, Germany); TransITsiQUEST Transfection Reagent from Mirus Bio LLC (Madison, WI); Low Input Quick Amp Labeling Kit, Agilent human 44k oligonucleotide microarray, and Agilent Gene Expression washing buffer from Agilent Technologies (Santa Clara, CA); Qiagen RNeasy Mini kit from Qiagen (Hilden, Germany); QuantiGene ViewRNA ISH Tissue 2Plex Assay Kit, human *H19* lncRNA probe set (catalog number VA1-13208-01), human *ubiquitin-C* mRNA probe set (catalog number VA6-10503-01), and *Escherichia coli* K12 dapB mRNA probe set (catalog number VF1-10272-06) from Affymetrix, Inc. (Santa Clara, CA); FastPure RNA kit and pBAsi-hU6 Neo DNA vector from TaKaRa Bio (Shiga, Japan); WST-8 cell counting kit, gemcitabine hydrochloride, and 5-fluorouracil (5-FU) from Wako Pure Chemical Industries (Osaka, Japan); cell culture insert (8 µm pore size) from BD Biosciences (Franklin Lakes, NJ); paclitaxel from Taiho Phamaceutical Co. (Tokyo, Japan), and Diff-Quick staining kit from Sysmex Corp. (Kobe, Japan). All other reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Cell culture

Human pancreatic ductal carcinoma (PDAC) cell lines PK-1, AsPC-1, PK-45P, PK-45H, PK-9, PK-8, PANC-1, and MIAPaCa-2 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), and human immortalized pancreatic ductal epithelial cell lines HPDE4 and HPDE6 were obtained from Dr. Ming-Sound Tsao (Ontario Cancer Institute, Toronto, ON, Canada). These cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37 °C under a humidified 5% CO₂ atmosphere. The Capan-1 human PDAC cell line was purchased from American Type Culture Collection (Manassas, VA) and the cells were cultured in RPMI 1640 medium with 15% FBS.

Cell lines from PANC-1 cell-derived liver and lung metastases

Cell lines from liver and lung metastases derived from PANC-1 cells were established as previously reported [7]. Briefly, NOD/Shi-scid, IL-2ã^{null} (NOG; Central Institute for Experimental Animals, Kanagawa, Japan) mice were administered a single intrasplenic injection of 1×10^5 PANC-1 cells. Five to 8 weeks later, the involved liver and lungs of the mice were removed and fragments of the metastatic foci were dispersed in a culture medium (PANCliver1, PANC-liver2, PANC-lung1, and PANC-lung2). As negative control cells, the tumor fragments from subcutaneous injection of PANC-1 cells were dispersed in a medium containing antibiotics and two cell lines were established (PANC-skin A and PANC-skin B cells). Metastatic PANC-1 cells from the liver or lungs and subcutaneously injected PANC-1 cells were confirmed as being of human origin using PCR with human- or mouse-specific mitochondrial gene primers as previously described [7]. All animal experiments were carried out according to the institutional animal care guidelines of the Nippon Medical School Animal Ethical Committee (No. 26-109).

DNA microarray analysis

PANC-1 and PANC-lung1 cells were plated at a density of 2.5×10^5 cells in a 60 mm dish. After 3 days, total RNA was isolated using the FastPure RNA kit. For DNA microarray analysis, 50 ng RNA from these cells was labeled using the Low Input Quick Amp Labeling Kit. Labeled RNA was further purified using the Qiagen RNeasy Mini kit. Labeled cRNA was hybridized to the Agilent human 44k oligonucleotide microarray and washed using Agilent Gene Expression washing buffer. Microarrays were scanned in an Agilent DNA Microarray Scanner and expression data were obtained using Agilent Feature Extraction software. Data were analyzed using Gene Spring GX version 11 (Agilent Technologies).

Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed with the StepOnePlus PCR system (Life Technologies) using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays for *H19* and 18S rRNA. qRT-PCR was performed according to the manufacturer's protocol. qRT-PCR results were expressed as the ratio of target to 18S rRNA, the latter serving as an internal standard. Gene expression levels were measured in triplicate using qRT-PCR.

Branched DNA in situ hybridization

Branched DNA in situ hybridization (bDNA ISH) was performed according to the protocol of the QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit using normal, inflammatory, and neoplastic pancreas tissue microarrays [18]. Briefly, the pancreas microarray tissue sections purchased from Biomax Inc. were re-fixed in 10% formaldehyde/phosphate-buffered saline (PBS) to equalize tissue conditions, deparaffinized, boiled in Pretreatment Solution (1:100) for 10 min at 90-100 °C, and digested with protease (1:100) for 10 min at 40 °C. Sections were hybridized with either the H19 probe set, ubiquitin-C probe set (positive control), or E. coli K12 dapB probe set (negative control) diluted 1:40 for 2 h at 40 °C. The H19 probe binds to human H19 but does not react with mouse H19. After washing, the sections were hybridized with PreAmplifier Mix for 25 min at 40 °C, washed, hybridized with Amplifier Mix for 15 min at 40 °C, washed, and finally hybridized with Label Probe conjugated to alkaline phosphatase (1:1000) for 15 min at 40 °C. After washing, the sections were incubated with either the fast red substrate and counterstained with hematoxylin.

Generation of stable H19-overexpressing or H19shRNA-expressing PANC-1 cells

The *H19* full-length cDNA (2.3 kb) cloned into pcDNA3.1 (–) vector (a generous gift from Dr. Eric Adriaenssens, Science and Technology, Lille 1 University, France) and empty vector were transfected into PANC-1 cells using the FuGENE HD transfection reagent, according to the manufacturer's instructions. Briefly, 2×10^5 cells/mL were transfected with 5 µg DNA using FuGene HD, and the cells were passaged and cultured with 1 mg/mL Geneticin. Cell lysates were collected and *H19* RNA levels were measured by qRT-PCR.

To construct expression vectors for human *H19* short hairpin RNA (shRNA), a DNA fragment flanked by *Bam* HI and *Hind* III sites containing the sense target sequence for *H19* (5'-TCA TCA GCC CAA CAT CAA A-3'), the hairpin loop sequence (5'-TAG TGC TCC TGG TTG-3'), and the antisense target sequence were synthesized and inserted into the pBAsi-hU6 Neo DNA vector. Similarly, the scrambled sequence (5'-TCT TAA TCG CGT ATA AGG C-3') was used to construct the sham vector that served as a negative control. Transfections of the *H19* shRNA (shH19) and scrambled shRNA (Sc) vectors were performed as described above.

Transfection of siRNA targeting H19

We used two types of siRNA targeting different sites in the *H19* RNA: siH19-A (n272453) and siH19-B (n272446). The sense siRNA sequences of siH19-A and siH19-B were 5'-UCA UCA GCC CAA CAU CAA ATT-3' and 5'-CCC UCU AGC UUG GAA AUG-ATT-3', respectively. The cells were plated at a density of 2×10^5 cells in 35 mm dishes and transfected 24 h later with 5 nM of either siRNAs targeting *H19* or Silencer negative control siRNA (siNeg) using TransITsiQUEST according to the manufacturer's protocol. To confirm the effective transfection of siRNA into cells, total RNA was prepared at 48 h after the transfection and *H19* RNA levels were determined by qRT-PCR.

Cell proliferation assays

Cells were cultured in RPMI 1640 medium with 10% FBS at a density of 5×10^3 in 96-well plates followed by incubation for 24, 48, 72, or 96 h. The cells were then incubated with WST-8 cell counting reagent for 4 h. Optical density was measured using a plate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm.

Single-cell movement assay

To assess cell migration, a single-cell movement assay was performed [7]. Cells (5000 per well) were seeded onto fourwell glass-bottom dishes. Images of cell movement were captured every 5 min and monitored for 24 h by a Digital Eclipse TE 2000-E motorized inverted microscope (Nikon Insteck Co., Ltd). The total distance covered by individual cells (60 cells for each group) within 24 h was determined using Metamorph software 7.6 (Universal Imaging Corp. Ltd, Buckinghamshire, UK) according to the manufacturer's protocol.

Boyden chamber assay

A cell migration assay was also carried out using the Boyden chamber technique as previously reported [7, 19, 20]. Cell culture inserts (8 μ m pore size and 6 mm in diameter) were used according to manufacturer's instructions. For PANC-1 and PANC-lung1 cells, at 12 h after placing 5×10^4 cells/500 μ L onto the upper component of the inserts, the numbers of cells that had migrated through the membrane to the lower surface of the filter were counted in nine high-power fields (× 200).

Intravenous implantation of PANC-1 cells in NOG mice

PANC-1 parental cells, Sc cells, or shH19 cells (1×10^6) cells in 100 µL PBS) were injected into the tail vein of 8-week-old male NOG mice (N = 3 for each cell line). Eleven weeks after injection, the animals were killed and the internal organs were excised and weighed.

To analyze the metastases histologically, the organs including the liver, lungs, heart, spleen, kidneys, adrenal glands, pancreas, stomach, intestine, and colon were fixed in 4% paraformaldehyde, embedded in paraffin wax, and cut into tissue sections at 4 μ m thickness. The sections were stained with hematoxylin and eosin, and the serial sections were immunostained using a Histofine mouse stain kit with a mouse monoclonal antibody against HLA class I-A, B, and C. For analysis of the immunostaining results, we captured five images in each specimen at × 40 magnification and then measured the areas of positive cells using Image J software (NIH, Bethesda, MD).

H19 expression levels following the addition of anticancer drugs

Cells were plated in 24-well plates with growth medium. Each anti-cancer drug ($100 \mu M$ gemcitabine, 5-FU, or abraxane) was administered the next day and then the cells were further cultured for 24 h. The cells were collected and qRT-PCR was performed as described above.

Statistical analysis

All quantitative data are presented as the means \pm SEM. The Student's *t*-test or Dunnett's method for continuous variables, Mann–Whitney's *U*-test or Steel's method for nonparametric variables, and Fisher's exact probability test for categorical variables were used to examine significant differences. Spearman's correlation coefficient by rank test was used to compare *H19* expression and ductal adenocarcinoma differentiation. Differences were considered significant when *P* < 0.05. Computations were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA) and Statcel 3 software (OMS Publishing, Inc., Saitama, Japan).

Table 1 Highly expressed molecules in lung metastasis-derived PANC-1 cells

	Gene name	Symbol	Type of RNA	Function	Fold change
1	Cancer/testis antigen 1	CTAGIA	mRNA	Tumor cell antigen	84.4
2	H19, imprinted maternally expressed transcript (non-protein coding)	H19	Long non-coding RNA	Tumor suppressor or inducer	82.4
3	Cancer/testis antigen 2	CTAG2	mRNA	Tumor cell antigen	62.1
4	Receptor (G protein-coupled) activity modifying protein 1	RAMP1	mRNA	Calcitonin transport	36
5	Protocadherin 1	PCDH1	mRNA	Cell aggregation	25.4
6	Paired box 6	PAX6	mRNA	Neural and eye development	18.4
7	Eva-1 homolog A, regulator of programmed cell death	EVAIA	mRNA	Autophagy, apoptosis	17.3
8	Secreted protein acidic and cysteine rich	SPARC	mRNA	Calcification, metastasis	15.7
9	Collagen type VI alpha 1	COL6A1	mRNA	Cell shape, microfibril	13.5
10	Oxysterol binding protein like 6	OSBPL6	mRNA	Lipid metabolism	12.3
11	Tetratricopeptide repeat domain 39C	TTC39C	mRNA	Anaphase	11.5

Bold values emphasize the H19 expression levels

Results

DNA microarray analysis of metastatic adenocarcinoma

To investigate the underlying mechanisms of metastasis of pancreatic adenocarcinoma, we performed a DNA microarray analysis and compared the gene expression patterns between human parental PANC-1 and lung metastasisderived PANC-lung1 cells. We identified 11 genes that were over 10-fold elevated in PANC-lung1 cells as compared with PANC-1 cells (Table 1). Among these, *H19* exhibited the second largest increase (82.4-fold) and represented the only non-protein coding gene. We further confirmed the *H19* increase in PANC-lung1 cells using qRT-PCR analysis in triplicate. We subsequently focused

Table 2 H19 expression in human pancreatic cancer tissues

Grandular differentiation	Tumor grade	N <i>H1</i> 9		19	P-value
			Positive	Negative	
Ductal adenocarcinoma		139	23	116	
Well-differentiated	Grade 1	29	1	28 -	
Moderately-differentiated	Grade 2	77	8	69	< 0.0001
Poorly-differentiated	Grade 3	33	14	19 - 1	1

Fig. 1 *H19* expression in human pancreatic tissues. *H19* was strongly expressed in poorly differentiated adenocarcinoma **a**and **b** (arrows) but was not expressed in well differentiated adenocarcinoma forming tubular structures **c**. In normal pancreatic tissues, *H19* was weakly expressed in the intercalated and intralobular ducts **d** (arrows). Branched DNA in situ hybridization on *H19* in the following studies, because the roles of lncRNA in cancer metastasis have not been fully elucidated.

H19 expression in human pancreatic cancer tissues

First, we examined the localization of *H19* in human pancreatic cancer tissues using a highly sensitive ISH technique. Dotted *H19* signals were detected focally or diffusely in 23 of 139 invasive ductal carcinomas (17%, Table 2). In human PDAC tissues, *H19* was strongly expressed in tumor cells forming alveolar or small ductal structures (Fig. 1a,b, arrows), whereas it was not expressed in the majority of well-differentiated adenocarcinoma cells (Fig. 1c). *H19* expression was positively correlated with a higher histological grade of ductal adenocarcinomas (P < 0.0001, Table 2). In normal pancreatic tissues, sparse dotted positivity of *H19* was detected in the intercalated/intralobular ducts (arrows, Fig. 1d), but not in the interlobular ducts, acini, and islets.

H19 expression in pancreatic cell lines

Next, we examined the H19 expression levels in nine PDAC cell lines and in two cell lines from normal pancreatic ductal epithelial cells. Of the nine PDAC cell lines, five showed higher expression levels of H19 than observed in the two normal pancreatic ductal epithelial cell lines (Fig. 2a). We then examined the expression levels of H19 in several types of cell lines derived from PANC-1 cells. The cell lines established from subcutaneous tumors of PANC-1 cells (PANC-skin A and -skin B) showed similar H19



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Fig. 2 *H19* expression levels in human pancreatic cancer cells. Of nine pancreatic cancer cell lines tested, five showed higher expression levels of *H19* than the two normal pancreatic ductal epithelial cells **a**. *H19* expression levels were higher in cell lines derived from liver and lung metastases than their parental cells and subcutaneous tumor-

derived cells **b**. PANC-skin A and B, subcutaneously implanted PANC-1 cell-derived cell lines; PANC-lung1 and 2, lung metastasis of PANC-1 cell-derived cell lines; PANC-liver1 and 2, liver metastasis of PANC-1 cell-derived cell lines





Fig. 3 Stable transfection of an H19 expression vector into PANC-1 human pancreatic cancer cells. a H19 expression in H19 vector-transfected cells (H19) compared with wild-type (WT) and empty

vector transfected cells (EV). **b** Cell proliferation rates. **c**, **d** Single cell movement and Boyden chamber assays of cell motility. **P < 0.01



Fig. 4 Cell proliferation and migration of *H19* shRNA-transfected PANC-1 cells. **a** *H19* expression in *H19* shRNA-transfected (shH19), wild-type (WT), and scramble vector (Sc) transfected cells. **b** Cell

expression levels as the parental PANC-1 cells. Conversely, *H19* expression levels were higher in cell lines established from liver and lung metastatic foci that developed consequent to splenic injection of PANC-1 cells (PANC-liver1, -liver2, -lung1, and -lung2) as compared with the levels in parental PANC-1 cells (Fig. 2b). Furthermore, lung metastasis-derived PANC-1 cells showed higher *H19* expression levels than liver-derived PANC-1 cells in each mouse (PANC-liver1 vs. PANC-lung1 and PANC-liver2 vs. PANC-lung2, respectively).

Cell behaviors of H19-overexpressing PANC-1 cells

To examine the effects of H19 on the growth and migration of pancreatic cancer cells, we overexpressed H19 in PANC-1 cells using an H19 expression vector (H19 cells) and also prepared empty vector-transfected PANC-1 cells as a control (EV cells). H19 expression levels in H19 cells were approximately 10-fold higher than those in wild-type (WT) and emty vector (EV) cells as determined by qRT-PCR analysis (Fig. 3a).

Using these cells, we examined the effects of enhanced expression of *H19* on pancreatic cancer cell behaviors. Cell growth rates did not significantly differ between WT, EV, and H19 cells (Fig. 3b). Next, we confirmed the effect of *H19* on cell motility. H19 cells moved greater distances in comparison with EV cells (P < 0.01) (Fig. 3c). In addition, in Boyden chamber assays, a significantly larger number of H19 cells migrated through the pores of the membrane compared with EV cells (P < 0.01, Fig. 3d).



proliferation rates. c, d Single-cell movement and Boyden chamber assays showing cell motility. *P < 0.05, **P < 0.01

Effects of H19 silencing in PANC-1 cells on cell behaviors

Next, we suppressed *H19* expression in PANC-1 cells using a shRNA that targeted the *H19* transcripts. *H19* expression was lower in *H19* shRNA-transfected cells (shH19 cells) than in WT and scrambled sequence transfected shRNA (Sc) cells (Fig. 4a).

The growth rates of shH19 cells were not altered compared with those of Sc cells (Fig. 4b). In the single-cell movement assay, shH19 cells exhibited decreased motility in comparison with Sc cells (P < 0.05, Fig. 4c). Significantly fewer migrated shH19 cells than Sc cells were observed using the Boyden chamber assay (P < 0.01, Fig. 4d).

Effects of transient suppression of H19 in PANClung1 cells

We next sought to determine the effects of siRNAs targeting H19 RNA on the cell behaviors of PANC-lung1 cells. Two siRNAs targeting different sites of the H19transcript (siH19-A and siH19-B) were used to silence H19 in PANC-lung1 cells, which exhibited the highest level of H19 expression in our study. An siRNA that does not bind to any human RNA was also transfected into PANC-lung1 cells as a negative control (siNeg). H19 RNA levels in siH19-A and siH19-B cells were decreased to approximately 10% of those in WT or siNeg cells (Fig. 5a).



Fig. 5 Transient inhibition of H19 in PANC-lung1, lung-metastasisderived pancreatic cancer cells. **a** Two types of siRNA targeting H19(siH19-A and siH19-B) were used to inhibit H19 expression. **b** Cell

H19 silencing did not impact the cell growth rates (Fig. 5b). In contrast, the single cell movements of siH19-A and siH19-B cells were inhibited compared with those of WT and siNeg cells (siH19-A vs. siNeg, P < 0.01, Fig. 5c). The Boyden chamber assay showed that the numbers siH19-A and siH19-B cells that had migrated through the membrane were significantly less than those of siNeg cells (P < 0.01, Fig. 5d).

Metastatic ability of H19-silenced PANC-1 cells in immunodeficient mice

To assess the metastatic potential of H19-silencing in pancreatic cancer cells, we performed tail vein injections of PANC-1 cells, Sc cells, and shH19 cells into NOG mice. At 11 weeks post-injection, macroscopically visible tumors were observed in the liver, lungs, kidneys, and adrenal glands in each group of mice. WT and Sc cells formed larger numbers of macroscopically-visible fused tumors in the liver and lung, as compared with shH19 cells (Fig. 6a,c). The liver- and lungto-body weight ratios were lower in shH19 cells than WT and Sc cells (Fig. 6b,d), with the difference of liver-to-body weight ratio being statistically significant (P < 0.05). Histologically, human HLA class I positive-pancreatic cancer cells were focally localized in the liver and lungs of mice injected with shH19 cells, whereas WT and Sc cells proliferated diffusely in the liver and lung fields (Fig. 6b,d). The metastatic areas in the liver were statistically lower following shH19 cell administration (*P* < 0.01, Fig. 6b).



proliferation rate following siRNA *H19* inhibition compared to control cells. **c**, **d** Single-cell movement and Boyden chamber assays showing cell motility in siH19-A and siH19-B-transfected cells. **P < 0.01

Discussion

Accumulated gene mutations in pancreatic cancer cells have been reported to correlate with distant metastasis using rapid autopsy [21]. However, detailed mechanisms of pancreatic cancer metastasis including metastasis-related proteins or RNAs have not been well clarified. In the present study, a microarray analysis was applied to investigate the different gene expression levels between parental and metastatic pancreatic cancer cells. Notably, the 3 molecules showing the greatest expression increase in metastasisderived pancreatic cancer cells showed enhancement of over 60-fold compared with parental cell expression. Cancer/testis antigen 1 and 2 represented the tumor cell antigens with the highest protein-coding gene expression in metastatic cancer cells and lncRNA H19 was the most increased RNA. The human H19 gene is located on chromosome 11p15.5 and produces a 2.3 kb RNA. H19 was originally discovered in a screen of a murine fetal liver cDNA library for clones containing RNA sequences that decrease after birth [22]. H19 is expressed in various tissues of the developing embryo but is repressed in all tissues except skeletal and cardiac muscle shortly after birth in both mice and humans [22-24]. H19 has also been reported to be reactivated during regeneration and carcinogenesis in adult tissues [6, 10]. H19 was expressed in bladder cancer [11], glioma [12], breast cancer [13], gastric cancer [14], pancreatic cancer [15], hepatocellular carcinoma [16], and prostate cancer [17].



Fig. 6 Tail vein injection of H19 shRNA-transfected PANC-1 cells into NOG mice. Number of tumors in the liver **a** and lungs **c** at 11 weeks post-injection of wild-type (WT), scrambled vector (Sc)-, or shH19 transfected cells. Ratios of the liver- and lung-to-body weight

In the present study we used bDNA ISH to assess H19 expression, which yields highly sensitive and specific signals through amplification by branched DNA technology in formalin-fixed, paraffin-embedded tissues [25]. Here, H19 was found to be expressed in the intercalated/intralobular ducts but not in the interlobular ducts in normal pancreatic tissues. Some researchers have indicated that the intercalated ducts are involved in pancreatic regeneration from toxic injury [26]. These findings suggest that H19 contributes to the normal and regenerative process of smallsized ductal cells during pancreatic inflammation. Conversely, Micha et al. [27]. reported that H19 was imprinted in the normal pancreas and in chronic pancreatitis and that loss of imprinting was observed in one of four pancreatic cancer tissues but was not associated with increased transcript levels. Ariel et al. [24]. reported that H19 was expressed in the acini and islets but not in the ducts in the pancreas of the human fetus; in addition, they observed no expression in four PDACs using ISH. In contrast, Scaiewicz et al. [28]. described that 65% of PDACs exhibited positive



b, **d**. Representative images and positive areas of liver and lungs (**b**, **d**, arrowheads) following immunohistochemistry using human HLA class I

W/T

Sc

shH19

Wild

shH19

SC

signals for *H19* expression. In our study, using highly specific and sensitive bDNA ISH, *H19* was expressed in 17% of PDACs and its expression was correlated with poorly-differentiated histological types, suggesting that *H19* is associated with the aggressiveness of PDAC. Moreover, some proteins such as CD133 and ezrin are expressed in the intercalated ducts rather than interlobular large ducts and acini, as well as being overexpressed in PDAC [29, 30]. Accordingly, some researchers have considered that intercalated ducts may constitute the site of origin of PDAC [30].

Pancreatic cancer is known to be a highly heterogeneous tumor type [31]. Recently, Moffitt et al. [32]. divided pancreatic cancer into two groups (basal-like and classical) by using a gene expression clustering technique. Notably, in a study incorporating The Cancer Genome Atlas (TCGA), *H19* was highly expressed in a cluster including the largely basal-like subtype, which has worse outcome compared with another cluster that included the largely classical subtype [33]. Further research is therefore needed to clarify

the relationship between H19 expression and pancreatic cancer heterogeneity.

H19 expression in metastatic cells was increased as compared with their parental pancreatic cancer cells. Furthermore, the H19 levels of lung metastasis-derived cells were higher than those of liver metastasis-derived cells. However, the expression levels of H19 were not changed between parental cells and pancreatic cancer cells established from skin-growing tumors after subcutaneous injection. These results suggest that H19 might contribute to the metastatic process in pancreatic cancer.

Accordingly, we next examined the function of H19 in pancreatic cancer. H19 has been reported to induce cell proliferation in breast [34] and gastric cancer cells [35]. H19 also enhanced the migration and invasion of bladder cancer cells [11] and glioma cells [12]. In contrast, some researchers have reported that H19 suppressed the migration and invasion of hepatocellular carcinoma [16] and prostate cancer cells [17]. These findings suggest that the function of H19 varies with the histopathological type of cancer. In the present study, the analysis of gain- and loss-of-function experiments in pancreatic cancer cells demonstrated that H19 did not influence cell proliferation but increased the cell migration ability, which is consistent with a recent report [15]. Furthermore, we found that suppression of H19 expression was associated with a drastic decrease in the metastatic potential of PANC-1 cells in an experimental metastasis model using NOG mice. These in vitro and in vivo results indicate that H19 has key roles in cell migration and metastasis formation in pancreatic cancer but does not directly impact cancer cell proliferation. Notably, in approximately 70% of patients with pancreatic cancer, metastasis is reported as a main cause of death owing to multiple organ failure as determined using autopsy cases [36]. Thus, the inhibition of pancreatic cancer cell motility via siRNA-mediated H19 suppression might indicate the potential effectiveness of exogenous administration of nucleic acid medicine as a novel therapy for pancreatic cancer. However, in this study we did not obtain data regarding a correlation between H19 expression and prognosis. From a RNA-Seq dataset of TCGA database, 4 (2.2%) of 178 sequenced cases exhibited upregulated expression of H19 with a median duration of disease-free survival of 5.81 months, compared with 17.05 months in cases without H19 upregulation.

With respect to pancreatic cancer treatment and H19 expression, we detected significant increases of H19 level in 5-FU- or abraxane-treated PANC-1 cells compared with non-treated cells (Supplementary Figure 1). This is consistent with the results from a study using the breast carcinoma cell line MCF-7, wherein a subline (MCF-7/AdrVp cells) exhibiting the property of a multidrug resistance overexpressed H19 relative to the parental MCF-7 cell line

[37]. A similar phenomenon was also noted in NCI-H1688, a lung carcinoma cell line with a multidrug resistance phenotype [37]. The increased expression of H19 in anticancer agent-treated PANC-1 cells therefore suggests that H19 may be associated with drug resistance in pancreatic carcinoma cells.

Our results from both in vitro and in vivo experiments suggest that H19 expression correlates with poorly differentiated cell types and promotes pancreatic cancer metastasis. The restricted expression in normal tissues vs. the elevated expression in some high-grade cancer and metastatic cancer cells suggests that H19 might represent a novel therapeutic target for the treatment of pancreatic cancer.

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Compliance with Ethical Standards

Conflict of interest :The authors declare that they have no conflict of interest.

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