High levels of human epididymis protein 4 mRNA and protein expression are associated with chemoresistance and a poor prognosis in pancreatic cancer

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Abstract. Pancreatic cancer is associated with an exceedingly poor prognosis, warranting the development of novel therapeutic strategies and discovery of prognostic predictors. Given that chemoresistance-related molecules are reportedly associated with the poor prognosis of pancreatic cancer, the present study aimed to identify molecules that could be efficacious therapeutic targets for pancreatic cancer. First, 10 patient-derived xenografts (PDXs) were established from patients with pancreatic cancer. Subsequently, after treating tumor tissue generated from the PDXs with standard drugs, next-generation sequencing (NGS) was performed using these tissues. The results of NGS analysis and immunohistochemical analysis on 80 pancreatic cancer tissues revealed that human epididymis protein 4 (HE4) expression in the anticancer drug-treated PDX group was higher than that in the untreated PDXs. In addition, chemoresistance ability was observed in tumor cell lines overexpressing HE4. Furthermore, Kaplan-Meier analysis of tumor tissues from 80 patients with pancreatic cancer was performed and it was found that patients with a high HE4 expression level had a poor survival rate compared with those who had a low HE4 expression level. Multivariate analysis also indicated the high expression level of HE4 was an independent poor prognostic biomarker. Thus, it was concluded that high gene and protein expression levels of HE4 mediate chemoresistance and are independent prognostic factors for pancreatic cancer.

Introduction

Pancreatic cancer is known to be one of the most severe malignant tumors in humans and is the fourth-leading cause of cancer-related mortality in the USA (1). Due to the rapidly aging population, much of which is due to aging baby boomers, it is predicted that pancreatic cancer will be the second-leading cause of cancer-related mortality among the elderly, particularly those >65 years of age, by 2030 (2). This global trend has continued, leading to >200,000 deaths due to pancreatic cancer each year (3). Due to its local advancement or multiple metastasis, approximately 80% of patients with pancreatic cancer are diagnosed at an advanced stage where surgical resection of the tumor cannot be performed (4), leading to a 5-year overall survival (OS) rate of 6% (5). Moreover, the median survival time is reportedly <2 years for surgically treated patients (6).

Pancreatic cancer has not exhibited a significant improvement in OS over the past 30 years and has maintained similar mortality and incidence rates. The mortality rate has remained

high as several patients are diagnosed at an advanced and unresectable stage, where current treatment procedures have limited therapeutic efficacy (7). Resistance to chemotherapy is another reason for the high mortality rate (3,8). Gemcitabine (GEM) is a typical chemotherapeutic drug used in the treatment of advanced-stage pancreatic cancer based on randomized trials (9-11), indicating an improvement in the 1-year survival rate when using GEM compared to when using 5-fluorouracil (5-FU) (12). However, systemic chemotherapy for patients with pancreatic cancer has limited effects on the OS due not only to low response rates, but also to chemoresistant abilities generated by unknown mechanisms underlying the mode of action of GEM. On the other hand, nab-paclitaxel co-administered with GEM, another standard chemotherapeutic regimen, has been shown to improve OS and progression-free survival (PFS) compared to GEM monotherapy, based on a randomized trial for advanced pancreatic cancer (10). Currently, patients with advanced disease have an abysmal prognosis; therefore, effective screening methods and early diagnosis, along with the development of novel therapeutics, are essential for a more comprehensive management of the disease. Thus, the present study aimed to identify novel molecules related to chemoresistance in pancreatic cancer using patient-derived xenografts (PDXs). The present study identified a molecule, human epididymis protein 4 (HE4), that was associated with a poor prognosis and chemoresistance in pancreatic cancer.

In 1991, the *HE4* gene was cloned from human epididymis by Kirchhoff *et al* for the first time (13). As HE4 was identified in the epithelium of the distal epididymis, it was considered to act as a protease inhibitor during the process of sperm maturation (13,14). From structural analysis, HE4 is a member of the whey-acidic-protein (WAP) family consisting of 2 WAP-type 4-disulfide core (WFDC) domains (15,16). HE4 has a molecular weight of approximately 25 kDa and is a secretory glycosylated protein encoded by the WAP 4-disulfide core domain 2 (WFDC2) gene (17). The genomic location of *HE4* is chromosome 20, which often undergoes amplification and, as a result, has a high gene expression level in a number of types of cancer (15). Recently, HE4 has attracted attention as a biomarker in various types of neoplasia, such as ovarian (18,19), endometrial (20,21), lung (22-24), breast (25), gastric (26) and colorectal (27) cancers; its clinicopathological significance, cellular function and mechanisms of action, however, remain unknown. While some normal tissues and various malignant tissues exhibit HE4 expression, low levels have been observed in the epithelium of some normal tissues (28,29).

Recent studies have demonstrated that HE4 is expressed in several pancreatic carcinomas (30-33) and that the normal pancreas does not generally express HE4 (17,30). Although HE4 seems to be associated with pancreatic cancer, the association between HE4 and resistance to anticancer drugs or prognosis remains unclear. The present study aimed to reveal the mechanisms underling chemoresistance or the poor prognosis of pancreatic cancer, and to determine the roles of HE4 expression in these mechanism *in vitro* and *ex vivo*.

Materials and methods

Establishment of pancreatic cancer PDXs. All experiments involving laboratory animals were performed in accordance with the care and use guidelines and approved by the Ethics Committee of the Kanagawa Cancer Center Research Institute (approval no. 176).

The establishment of the PDX model has been previously described by the authors (34). A total of 1,204 NSG mice were obtained from the Jackson Laboratory. Briefly, fresh tumor tissues excised from 36 patients with pancreatic cancer; 18 males and 18 females, aged 48-69 years, with stage I-II disease [for human research, the study protocol was approved by the Ethics Committee of the Showa University School of Medicine (Tokyo, Japan); approval no. 2611; consent was not obtained from the individual patients; however, the patients were notified of the details of the study using an opt-out form and were given the right to refuse study participation. This is a method widely used in Japan] were minced into small sections. They were then subcutaneously implanted into the dorsal upper part of the backs of 40 NSG mice (12 weeks old; female, weighing 20-30 g), as previously described (35,36). For the induction of anesthesia, all mice were exposed to 4% isoflurane at a 400 ml/min airflow in a chamber. Anesthesia was then maintained with 1-2% isoflurane at a 200 ml/min airflow. The mice were euthanized by cervical dislocation prior to the excision of the tumor tissues. The first 140 PDXs established through implantation were labeled as generation 1 (G1). When the tumor volume reached 1,000 mm^3 in this group, the tumor was once excised and re-implanted into other mice following the same procedure and in total, 160 mice were used to re-implant the tumor tissues in each passage. This passage process was repeated and PDXs up to G7 were successfully generated. A total of 1,140 mice were used to establish each generation of PDXs.

Characteristics of pancreatic cancer PDXs. Immunohisto-chemistry (IHC) and gene analysis were conducted to confirm whether PDXs retained the histological and genetic characteristics of the original patient tissues even following repeated passaging. For the methodology in this section, the same procedures we used as described in a previous study by the authors (34).

Designation of chemoresistance-related molecules in the PDXs from pancreatic cancer. Anticancer drugs (GEM as monotherapy or GEM and nab-paclitaxel as combination therapy) or saline as an untreated control were intraperitoneally injected into the PDXs as described in a previous study by the authors (34). In brief, chemotherapeutic drugs or saline were administered to the PDX models by an intra-abdominal injection on days 1, 4 and 7; no injection was performed on day 10. The tumor volume was measured each week for both the chemotherapy (treatment) and saline (non-treatment) groups. Each group included 32 PDXs.

Tumor tissues from PDXs of both the treatment and non-treatment groups were implanted when the tumor volume surpassed 1,500 mm^3 in the non-treatment group. Tissue DNA and RNA were extracted and subjected to NGS by Illumina HiSeq 4000 system.

Transcriptome analysis. For the methodology for transcriptome analysis, the same methods and procedures were used as previously described (34). Paired-end reads were mapped to all

or any human RefSeq transcripts (hg38 coordinates) and mouse RefSeq transcripts (mm 10 coordinates) by bowtie 1.1.2 (37), with one mismatch at most. When the mapped reads belonged to both species or more than one gene was found, they were removed. The primary 100 bp of every read for samples with 150-bp read length were applied to mapping to avoid bias from the difference in read length. Since reads mapped to noncoding transcripts were discarded, the remaining reads were suitable for estimating the overview of organic phenomenon of human cancer cells and mouse stromal cells, consistent with strategies that have been previously described (34,38).

Bioinformatics analysis. The TCGA database of the National Cancer Institute has published a huge number of RNA sequencing data from pancreatic adenocarcinoma patients. The database is available to all researchers. These data were retrieved and used for Kaplan-Meier analysis based on HE4 expression.

Cells and cell culture. Various cell lines were prepared, including 293 (permanent primary cell line), SK-N-AS (human neuroblastoma), KATOIII (human gastric cancer), AsPC-1 (human pancreatic ductal cell carcinoma), PANC-1 (human pancreatic ductal cell carcinoma), SUIT-2 (human pancreatic ductal cell carcinoma), MIA PaCa-2 (human pancreatic ductal cell carcinoma), IMR-32 (human neuroblastoma), HCT116 (human colon cancer) and HeLa (human cervical epithelioid carcinoma); in addition, 3 non-cancerous cell lines were prepared, namely human umbilical vein endothelial cells (HUVECs), human renal glomerular endothelial cells (HRGEC) and H6c7 (human pancreatic duct epithelial cell line). The SK-N-AS, IMR-32 and HCT116 cell lines were obtained from Chiba Cancer Center Research Institute. The other tumor cell lines were obtained from the Department of Cancer Immunotherapy at the Kanagawa Cancer Center. The HUVEC and HRGEC cell lines were obtained from the Department of Anatomy at Showa University School of Medicine. The H6c7 cell line was purchased from Kerafast. The 293, KATOIII, AsPC-1, MIA PaCa-2, HCT116 and HeLa cells were grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.), and the PANC-1, SUIT-2, SK-N-AS and IMR-32 cells were grown in Roswell Park Memorial Institute medium (Thermo Fisher Scientific, Inc.). The HUVECs were cultured in endothelial cell growth medium-2 (EGM-2; PromoCell), while the HRGECs were cultured in endothelial cell medium (ScienCell Research Laboratories, Inc.). Each medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cytiva), 100 μg/ml streptomycin and 100 U/ml penicillin. The H6c7 cells were cultured in keratinocyte-SFM supplemented with human recombinant epidermal growth factor (EGF), bovine pituitary extract (Invitrogen; Thermo Fisher Scientific, Inc.) and 1X antibiotic-antimycotic (Invitrogen; Thermo Fisher Scientific, Inc.). Each cell line was cultured at 37°C in 5% CO_2.

Reverse transcription-quantitative PCR (RT-qPCR). HE4 expression levels were determined in each cell line. After collecting cultured cells, total RNA was prepared with an RNeasy Mini kit (Qiagen GmbH) and subsequently reverse transcribed with Superscript IV VILO Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. The cDNA was then amplified using the StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with TaqMan Fast Advanced Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) to quantify the mRNA expression of HE4 and ACTB. The optimal thermal cycling conditions for the master mix were as follows: 40 cycles of a two-step PCR (95°C for 1 sec and 60°C for 20 sec) after the initial enzyme activation (50°C for 2 min and 95°C for 2 min). The quantification cycle (Cq) values were determined using StepOne Software v2.0 yielding amplification plots. The $\Delta\Delta$Cq method was then used to calculate relative gene expression, as previously described (39). The specific TaqMan probes were as follows: HE4, Hs00197437_m1; ACTB, Hs01060665_g1 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cell viability assay of tumor cell lines treated with anticancer drugs. Cell viability assays of various malignant tumor cell lines (SK-N-AS, KATOIII, AsPC-1, PANC-1, SUIT-2, MIA PaCa-2, IMR-32, HCT116 and HeLa) treated with GEM were performed as previously described (34). After examining cell viability, endogenous HE4 gene expression levels were measured in these cell lines by RT-qPCR. First, each cell line was inoculated at 1×10^4 cells/well in a 96-well plate and cultured for 24 h GEM was then added at varying concentrations (0-10,000 nM). Following a 72-h culture, the cell viability under each condition was measured using MTT Reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The relative cell number was then determined by calculating the rate of change, where the cell number of untreated cells (0 nm GFM) was set as 100%. For transient overexpression experiments, an expression plasmid vector for HE4 (pCMV6-AC-HE4-GFP tag plasmid, NM_006418) was obtained from OriGene Technologies, Inc. and an empty vector (pCMV6-AC-GFP) was adopted for control transfection. The 293 cells were seeded at 3×10^5 cells/well in 6-well plates for transfection. Following a 24-h culture, transfection was performed using 6 μl FuGENE 6 (Promega Corporation) with 2 μg the expression plasmid for GFP-tagged HE4 or with the empty plasmid, based on the manufacturer's protocol. At 24 h following transfection, the cells were suspended and seeded in a 96-well plate with or without GEM. Following a 48-h culture, cell viability was measured as described above.

Immunohistological analysis of pancreatic cancer tissues. The present study comprised a successive cohort of 80 patients with histopathologically-confirmed pancreatic cancer who had undergone surgical excision at the Showa University Hospital), from January 1, 2008 to December 31, 2017. The patient cohort of the present study is identical to that of a previous study by the authors (34). The study protocol was approved by the Ethics Committee of the Showa University School of Medicine (approval no. 2611), and all study procedures abided by the principles of the Declaration of Helsinki. Consent was not obtained from the individual patients. However, the patients were notified of the details of the study using an opt-out form and were given the right to refuse study participation. This is a method widely used in Japan. Patients had no history of chemotherapy prior to surgical excision. For further stratification to analyze the clinicopathological factors, invasive factors

were assessed according to the classification of pancreatic cancer by the Japan Pancreas Society.

By using a Leica Bond system, HE4 protein expression in patient tissues was evaluated immunohistologically. Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and pretreated with heat-mediated antigen retrieval solution with sodium citrate buffer for 20 min. The sections were then incubated with a primary antibody against HE4 (cat. no. ab200828; Abcam) for 15 min at 25±1˚C. Bond™ Primary Antibody Diluent (cat. no. AR9352; Leica Biosystems, Bannockburn, IL) was used to dilute the antibody (1:1,000 dilution). HE4 detection was carried out using a horseradish peroxidase-conjugated compact polymer system (HRP-polymer secondary antibody; goat anti-rabbit IgG H&L (HRP polymer); product code ab214880; pre-diluted; Abcam). And DAB was applied as the chromogen and incubated for 5 min at 25±1˚C. Counterstaining was performed with hematoxylin for 5 min at 25±1˚C, and the sections were mounted for microscopic observation.

The immunostained tissue sections were assessed by two pathologists who had no clinical information of the patients. The degree of positiveness was evaluated by the staining intensity and the percentage of positively stained cells. The criteria for the staining intensity grade were as follows: 0, negative (uncolored); 1, weak (light yellow); 2, moderate (yellowish brown); and 3, strong (brown). Based on the relative number of stained tumor cells, staining percentages were graded as follows: 0 (<19% positive tumor cells), 1 (20-49% positive tumor cells), 2 (50-69% positive tumor cells) and 3 (≥70% positive tumor cells). HE4 expression was classified referring to the staining index (scored as 0, 1, 2, 3, 4, 6, or 9). The staining index scores of <3 (low HE4 expression) and >4 (high HE4 expression) were regarded as the optimal cut-off values.

Statistical analysis. All statistical analyses were carried out using JMP Pro 14.0 software (SAS Institute Inc.). *HE4* gene expression in 9 cell lines was statistically analyzed using one-way analysis of variance (ANOVA) and subsequently by the Tukey-Kramer post-hoc test. The difference in cell viability between the control 293 cells, which had been transfected with an empty vector, and the HE4-expressing 293 cells, which had been transfected with the HE4 expression vector, at each concentration of GEM, was statistically analyzed by two-way ANOVA with the Bonferroni post hoc test for multiple comparisons. In addition, associations between the IHC status of HE4 expression and various clinical and pathological parameters were assessed using the Student's t-test or Fisher's exact test. In this study, OS was defined as the interval time from the first surgery to either patients' decease or last observation. Kaplan-Meier analysis and log-rank tests were adopted to estimate the difference in OS due to high or low HE4 expression level. Using the Cox proportional hazards regression model, univariate and multivariate analyses were performed. All statistical tests were two-tailed, and a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of a PDX model from pancreatic cancer. A total of 10 PDX lines of pancreatic cancer were established.

By histopathological observation, it was found that the PDXs had identical histological characteristics to the patient specimens, even following repeated PDX passaging procedures. In addition, it was found that the expression of HLA class I molecules in PDX specimens was maintained even after passaging (Fig. 1). Thus, the PDXs established in the present study retained the pathological characteristics of the original patient tissues. Furthermore, gene mutation characteristics were investigated by DNA/RNA extraction and high-throughput sequencing. In addition to the pathological features, the tumor cells of PDXs retained the genetic characteristics of the original pancreatic tissues from the patients (data not shown).

Designation of a chemoresistance-related molecule. The antitumor effects of GEM monotherapy or GEM and nab-paclitaxel combination therapy were examined using the PDX model to identify a molecule related to chemoresistance, and tumor growth was analyzed following antitumor treatment. The curve of the tumor volume of a typical PDX is shown in Fig. 2.

The mRNA expression level in the tumor tissues was then assessed by NGS analysis. The ratio of the control to treated PDXs was calculated by referring to the data for normalized expression (NE) values. In total, 6 PDX lines were examined for GEM monotherapy, as well as 10 PDX lines for GEM and nab-paclitaxel combination therapy; genes with NE values >10 and NE ratios (treated group to control group) >2 were selected (Fig. 3A and B). *HE4* was identified as having a robust expression in the treatment group. The ratio of the NE values for *HE4* expression (Fig. 3C and D) between the chemotherapy-treated and untreated groups tended to be >1.0 for some PDXs treated with GEM or GEM and nab-paclitaxel. The results revealed that the mRNA expression level of *HE4* increased when PDXs were treated with anticancer drugs.

Analysis of pancreatic cancer prognosis using the TCGA database. The association between *HE4* expression and OS in 166 pancreatic cancer samples from the TCGA database was analyzed, and the association between the mRNA expression level of *HE4* and patient survival was also assessed (Fig. 4). As a result, it was found that patients with a higher *HE4* expression level exhibited lower survival rates than those with a low *HE4* expression level (P=0.049).

In vitro confirmation of HE4 function. To investigate the specific role of HE4 in chemoresistance, the gene expression levels of endogenous *HE4* were examined in various tumor cell lines (SK-N-AS, KATOIII, AsPC-1, PANC-1, SUIT-2, MIA PaCa-2, IMR-32, HCT116 and HeLa) and 2 non-cancerous cell lines (HUVECs and HRGECs) by RT-qPCR. It was found that the endogenous *HE4* levels differed between the cell lines (Fig. 5A). As the HeLa cells exhibited the lowest *HE4* expression levels, they were used as the controls. In addition, the 2 non-cancerous cell lines, HUVECs and HRGECs, also exhibited a significantly low expression of *HE4*, similar to that in the HeLa cells, and were also considered as controls. As a result, *HE4* expression was significantly higher in the SK-N-AS, KATOIII and ASPC-1 cell lines than in the others (Table SI). Moreover, in the non-cancerous pancreatic cell line, H6c7, the *HE4* expression level was relatively high compared to that in the HeLa cell line (Fig. S1). Subsequently, cell viability
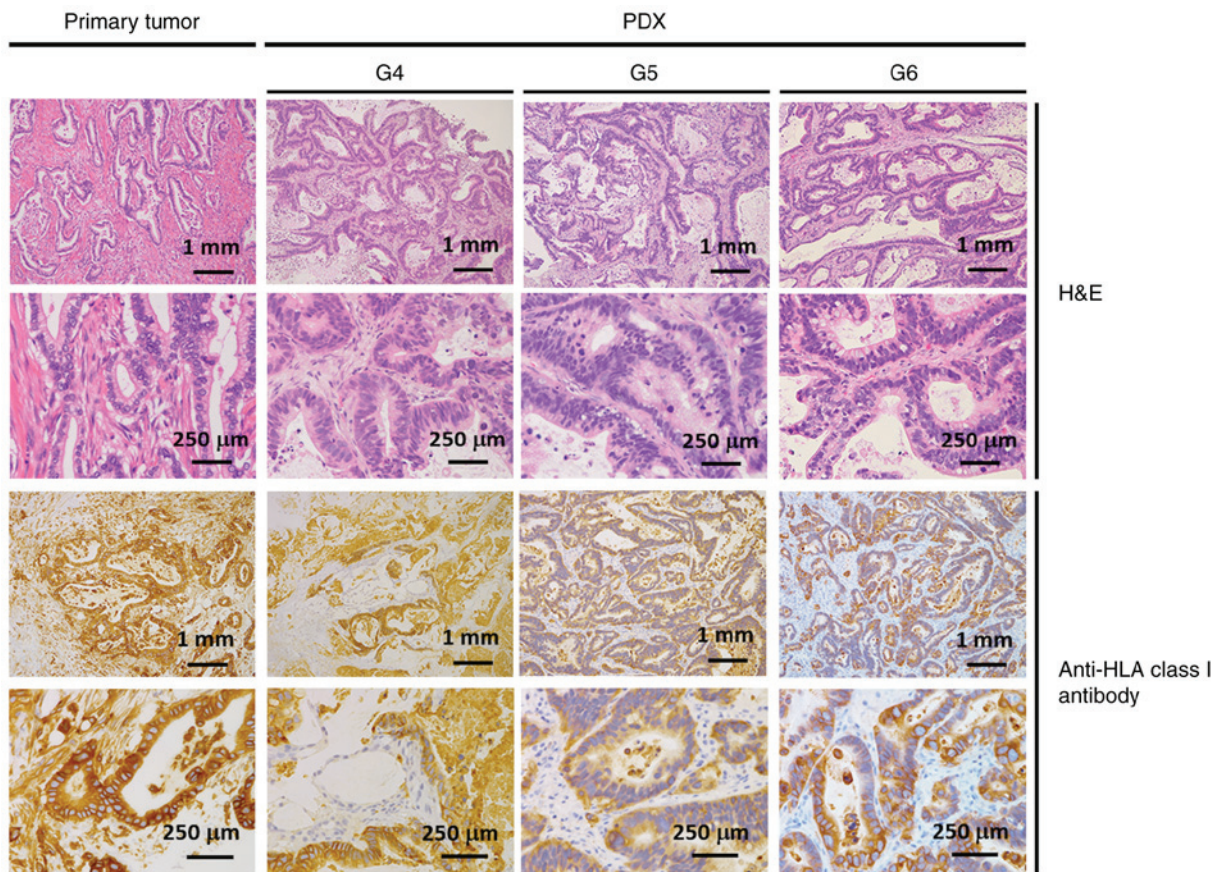
Figure 1. Morphological characteristics of PDXs and verification of anticancer effects in PDX tumors. Pathological features found in the xenograft tumors. H&E and immunohistochemical staining with antibodies against HLA class I for both the primary tumor and generated PDXs are shown. Poor differentiation of tubular adenocarcinoma was discovered in the original tumor by pathological diagnosis. HLA^{+} cancer cells, which were derived from patients, remained during the passaging processes. The morphology of the xenograft was retained. PDX, patient-derived xenograft.
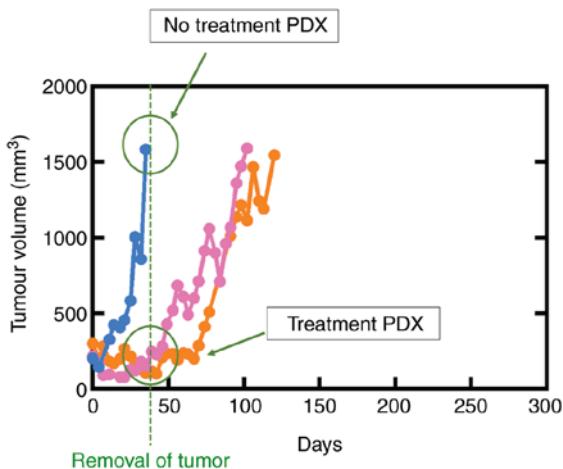

Figure 2. Tumor growth curves of typical PDXs. Until the volume of tumor exceeded 1,500 mm^3, PDXs belonging to the chemotherapy group were treated with GEM or GEM + nab-paclitaxel. PDX, patient-derived xenograft; GEM, gemcitabine.

assays were performed on these cell lines treated with various concentrations of GEM (Fig. 5B). A statistically significant difference in cell numbers was observed among the 9 cell lines at the GEM concentrations of 100, 1,000 and 10,000 nM. It was found that the 3 cell lines with a relatively high *HE4*

expression (SK-N-AS, KATOIII and ASPC-1) exhibited higher cell survival rates than the 4 cell lines with a low *HE4* expression (HeLa, HCT116, IMR-32 and MIA PaCa-2) at GEM concentrations of 1,000 and 10,000 nM (Table SII). As regards the pancreatic cancer cell lines, the AsPC-1 cells, which exhibited a significantly high expression of *HE4*, had higher viable cell numbers than did the SUIT-2 and MIA PaCa-2 cells at GEM concentrations of 1,000 and 10,000 nM (Fig. 5B). These results suggest that HE4 expression is partially associated with resistance to GEM.

The 293 cells were prepared for the induction of exogeneous HE4 expression, and an MTT cell viability assay for the 293 cells treated with GEM was conducted (Fig. 6A). With the increased GEM concentration, the number of tumor cells decreased in the control group; in the HE4-expressing cells, however, a partial decrease in tumor cell growth was observed following GEM treatment. At each GEM concentration, apart from those of 0.001 and 100 nM, the viability of the HE4-expressing cells was significantly higher than that of the controls (Fig. 6B and C), suggesting that HE4 partially contributes to chemoresistance *in vitro*.

Immunohistological and prognostic analyses in patients with pancreatic cancer. To corroborate the discoveries on HE4 expression in the current PDX model with that in clinical specimens, IHC was performed and HE4-positive tumor cells
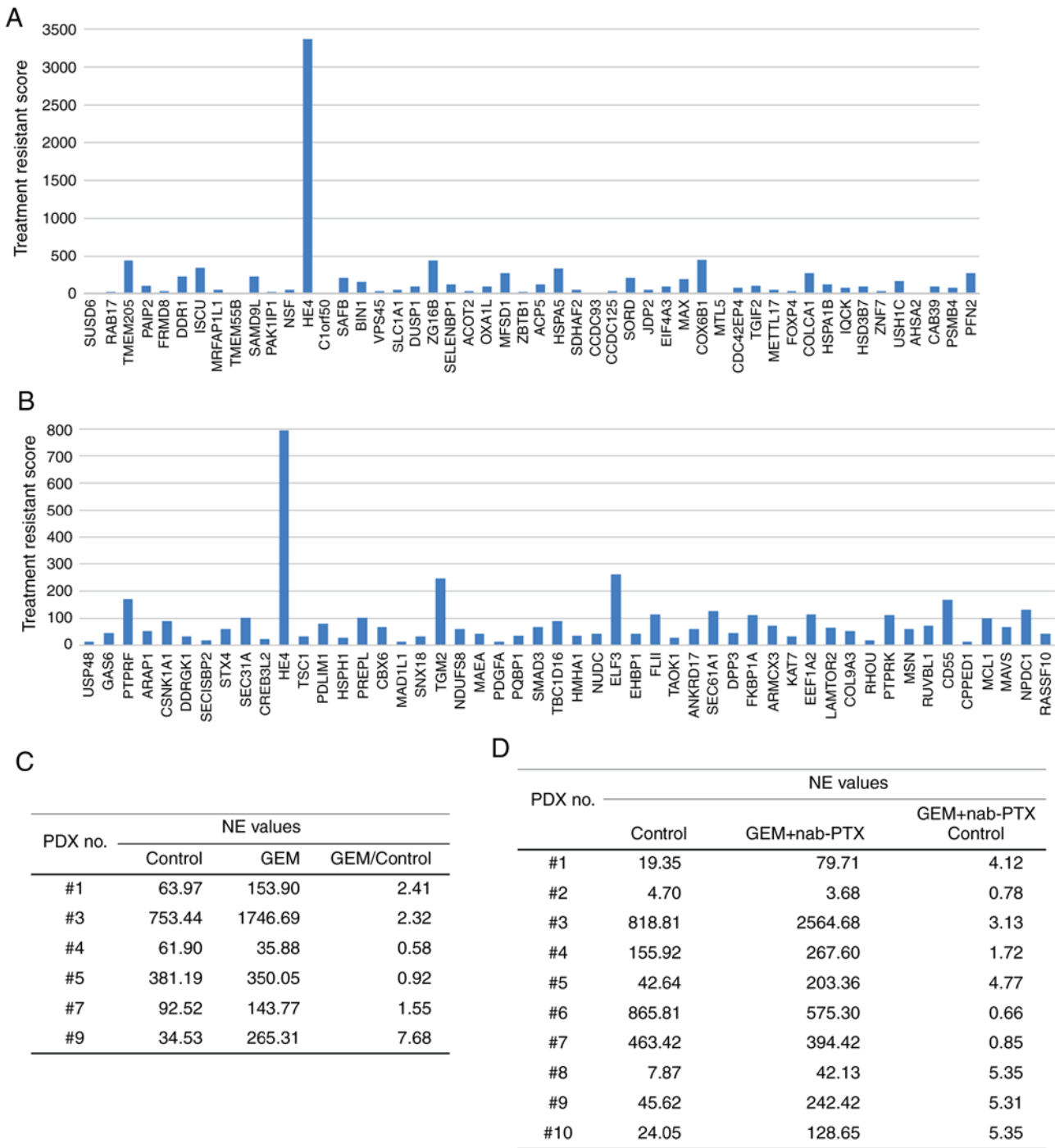
Figure 3. Identification of chemotherapy resistance molecules. (A and B) Results of NGS analysis of (A) GEM and (B) GEM + nab-PTX administration. The genes are shown on the horizontal axis, and the treatment resistance score is shown on the vertical axis. Treatment resistance score was calculated by the ratio of NE value (treated group/control group) multiplied by the difference in NE value (treated group-control group). In both cases (A and B), HE4 was upregulated in the treatment group. (C and D) The NE value of *HE4* mRNA. The NE value ratio of *HE4* expression between the treatment and control groups was often >1.0 in PDXs treated with GEM or GEM plus nab-PTX. HE4, human epididymis protein 4; PDX, patient-derived xenograft; GEM, gemcitabine; nab-PTX, nab-paclitaxel.

were found (Fig. 7A). The samples were then divided into 2 groups as follows: High HE4 expression [55.0% (44/80)] group and low HE4 expression [45.0% (36/80)] group, as described in Fig. 7B. No obvious associations were found between the 2 groups in each category, including age (P=0.46), sex (P=0.26), tumor location (head or body/tail, P=0.059), histological type (adenocarcinoma or others, P=0.62), TNM stage (IIA or IIB, P=0.999), lymphatic invasion (P=0.999),

venous invasion (P=0.50) and the presence of adjuvant chemotherapy (P=0.82; Table I). The associations between the expression level of HE4 and survival rate were examined by Kaplan-Meier analysis and log-rank tests (Fig. 7C). From the results presented in Fig. 7C, the higher HE4 expression was, the poorer the survival rate (P=0.028) in the current cohort of 80 patients with pancreatic cancer. In total, 3 prognostic parameters were identified from the univariate analysis of OS:

Table I. Association between HE4 expression and clinico-pathological features in 80 cases of pancreatic cancer.

| Characreristics | HE4 expression | | P-value |
	Low n=36	High n=44	
Age, years (mean ± SD)	72.00±9.38	71.75±12.49	0.46[a]
Sex			0.26[b]
Male	15	25	
Female	21	19	
Tumor location			0.059[b]
Head	19	33	
Body/tail	17	11	
Histological type			0.62[b]
Adenocarcinoma	35	41	
Others	1	3	
TNM (UICC 7th)			0.999[b]
IIA	9	11	
IIB	27	33	
Lymphatic invasion			0.999[b]
ly0, ly1	14	17	
ly2, ly3	22	27	
Venous invasion			0.50[b]
v0, v1	3	6	
v2, v3	33	38	
Adjuvant chemotherapy			0.82[b]
Absent	23	26	
Present	13	18	

[a]Data analyzed using the Student's t-test. [b]Data analyzed using Fisher's exact test.

Sex (P=0.033), adjuvant chemotherapy (P=0.037) and HE4 expression (P=0.031), while the multivariate analysis revealed that sex (P=0.045), adjuvant chemotherapy (P=0.020) and HE4 expression (P=0.029) were independent factors of a poor prognosis (Table II). The data for age in Table I and sex in Table II are similar to those of a previous study by the authors (34) as the same patient cohort was used in both studies.

Discussion

The distinctive features of pancreatic cancer, known as one of the lethal malignancies, are a delay in making the diagnosis, the metastatic progression of cancer in the early stages and chemoresistance. Thus, it is imperative to discover novel and effective prognostic markers as well as targets for anticancer therapy. In a previous study by the authors, it was found that HE4 expression increased in chemotherapy-treated PDXs compared with that in untreated PDXs, suggesting that chemotherapy treatment may have induced the expression of HE4 or that the increased expression of HE4 may be involved in the mechanisms of chemoresistance. The present study also investigated HE4 gene expression levels and cell viability in various
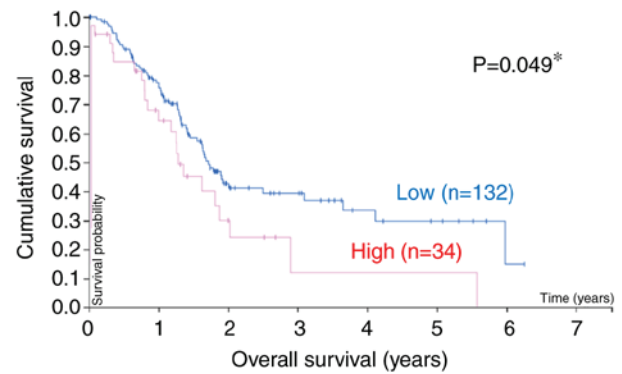

Figure 4. Kaplan-Meier plots demonstrating the correlation between HE4 mRNA expression and patient survival in TCGA database for pancreatic cancer (n=166). Red line, high expression (n=34); blue line, low expression (n=132). HE4, human epididymis protein 4. The asterisk (*) indicates a statistically significant difference (P<0.05).

tumor cell lines, including pancreatic cancer cells. From the results, the cell lines with a relatively higher expression of HE4 tended to have a higher cell viability than did those with a lower HE4 expression when the GEM concentration was increased. Of note, the viability of HE4-overexpressing 293 cells largely increased in response to GEM treatment compared to that in non-treated cells. Furthermore, it was demonstrated that a high HE4 expression was an independent prognostic factor, which is in association with the survival rate of pancreatic cancer. The current discoveries for pancreatic cancer were supported by an analysis of TCGA data that highlighted the use of HE4 expression as a prognostic factor.

The present study generated numerous PDX models; herein, pancreatic cancer PDXs were established in order to understand the mechanisms of chemoresistance and develop a novel biomarker. Several types of tumor cell lines and mouse tumor models have been applied in a number of experiments to determine the effects and potential toxicities of anticancer agents in cancer patients (35,40). The results of this research, however, do not always represent the data from human clinical trials (41). Furthermore, mouse studies are not always translatable to human cases (42,43). As a result, substantial mouse models that are applicable to the pathology in human clinical trials are required.

In recent years, drug screening using the latest animal models, including PDXs that are produced by transplanting tiny pieces of human tumors into mice, have been drawing attention; with these techniques, the environment inside the human body has been mimicked in host sites (44). As the noble PDX methods have been established by grafting freshly resected samples directly into immunodeficient mice, relevant and effective in vivo models for human tumors have been developed (45). Thus, PDX models that retained the molecular signatures and morphology of the resected original tumors were established for the speedy screening of latent therapy (46,47). In the present study, PDXs that were generated from pancreatic cancer were established and it was proven that they preserved the pathological features of human tumor tissues. It was confirmed that the PDXs maintained the specific histological features of their donor tumors, which were stable throughout the repeated passaging. The amount
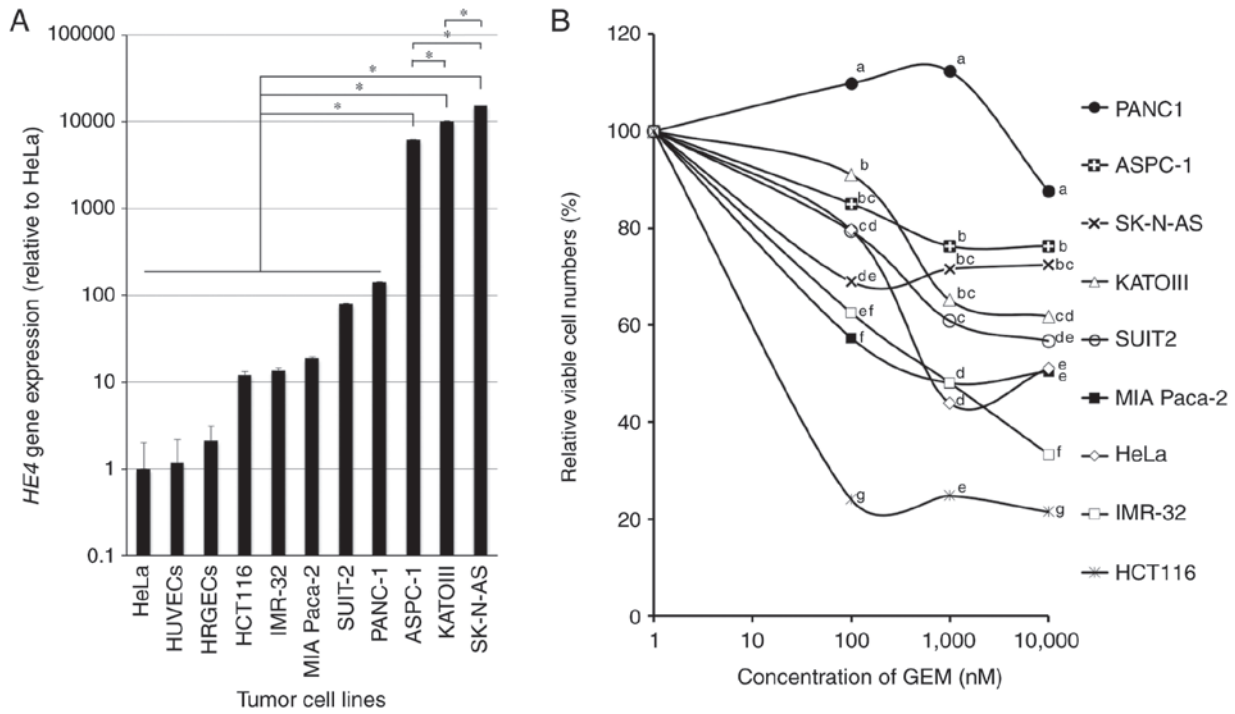
Figure 5. Expression of HE4 and cell viability assays of cell lines. (A) The levels of *HE4* gene expression in 9 tumor cell lines (SK-N-AS, KATOIII, ASPC-1, PANC-1, SUIT-2, MIA-Paca2, IMR-32, HCT116 and HeLa) and 2 non-cancerous cell lines (HUVECs and HRGECs) measured by RT-qPCR. Endogenous HE4 expression levels differed among cell lines. *P<0.05. (B) Cell viability assays of tumor cell lines in response to GEM treatment. When the concentration of GEM was increased, the relative viable cell numbers tended to be higher in the cell lines in which expression of endogenous HE4 was relatively higher. Cell lines with different lowercase letters (a-g) indicate statistically significant differences at each GEM concentration. [a]P<0.05 vs. b-g; [b]P<0.05 vs. c-g; [c]P<0.05 vs. d-g; [d]P<0.05 vs. e-g; [e]P<0.05 vs. f and g; and [f]P<0.05 vs. g. HE4, human epididymis protein 4; GEM, gemcitabine; HUVECs, human umbilical vein endothelial cells; HRGECs, human renal glomerular endothelial cells.
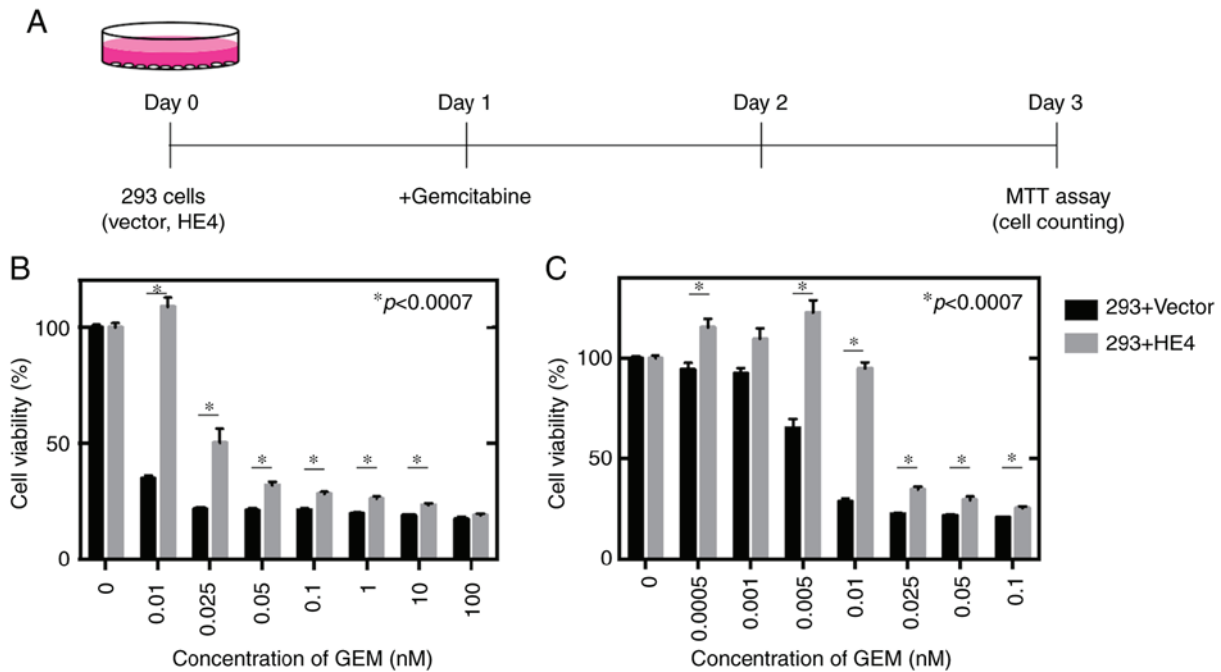

Figure 6. Viability assay of 293 cells. (A) Expression of HE4 and control vectors in 293 cells followed by treatment with GEM after 24 h (day 1). MTT assay was performed 48 h following treatment with GEM at various concentrations (day 3). (B and C) Histograms representing the rates of change of each measured OD value of control vector and HE4-expressing 293 cells. *P<0.0007, control vector vs. HE4-expressing cells at each GEM concentration, as determined by two-way ANOVA for multiple comparisons with the Bonferroni post hoc test. HE4, human epididymis protein 4; GEM, gemcitabine.

of sample tissue collected from pancreatic cancer is limited due to the condition of the disease; PDX models are, therefore, considered ideal animal models to investigate the pathology of pancreatic cancer.
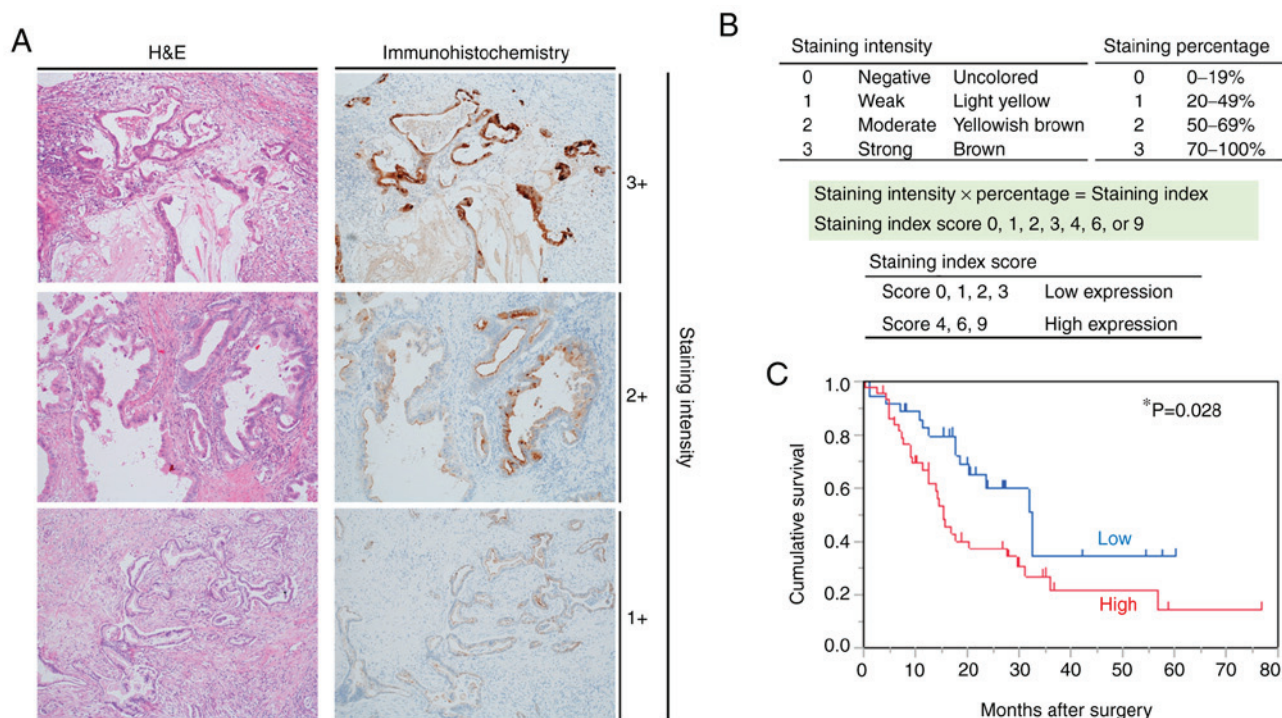
Figure 7. Association between HE4 expression and patient prognosis. (A) IHC analysis of HE4 in patient tissues (magnification, x100). Images are of the same sample tissue sections and representing the intensity of stain. Images in the left panel show H&E staining, and those in the right indicate IHC detection of HE4. (B) The criteria for evaluation of HE4 expression levels. The intensity of stain and percentage criteria are designated. (C) Kaplan-Meier survival analysis in pancreatic cancer patients (n=80). Overall survival is shown in accordance with the protein expression level of HE4. The red line represents the group of patients with high HE4 expression (n=44), and the blue line indicates those with low HE4 expression (n=36). HE4, human epididymis protein 4.

PDX experiments revealed that the mRNA expression of *HE4* increased in the chemotherapy-treated group compared with that in the untreated group, indicating that the elevation of mRNA expression of *HE4* elicited the chemoresistance. Nevertheless, variations in mRNA expression do not necessarily reflect an alteration in protein expression, as it may be affected by a variety of post-transcriptional regulations (48-50); therefore, mRNA levels do not necessarily reflect protein abundance. The proteomics of rectal and colon cancers formerly qualified in the TCGA database were analyzed, and it was revealed that the difference in protein expression could not be estimated from the quantity of mRNA transcripts between these cancers (51). To confirm the concordance between mRNA and protein levels of HE4, we examined whether there is a relationship between HE4 protein and clinicopathological factors by evaluating HE4 expression in 80 pancreatic cancer patient tissues. As a result, it was regarded that the increased expression level of HE4 as a prognostic factor that caused poor survival in patients with pancreatic cancer.

A better understanding of the association between HE4 expression and a poor prognosis is provided by reviews of the literature in this field; the cellular and molecular mechanisms may be proven by the role of HE4 in the progression of ovarian cancer (52,53). Recent studies have inferred that HE4 may play a pivotal role in the occurrence and development of tumors. They provide sufficient evidence that HE4, when overexpressed in cancer cells localized in the ovary or endometrium, is capable of improving cell proliferation, adhesion and invasion (54-56). There are a few reports on the functions of HE4 in pancreatic cancer. Lu *et al* demonstrated that extracellular

recombinant HE4 protein purified from human cells was able to enhance the proliferation of pancreatic cancer cell lines (33). They also observed that extracellular HE4 increased DNA synthesis and modulated the expression of cell cycle regulators, such as p21 and PCNA. Therefore, it is possible that HE4 plays an important role in the progression of pancreatic cancer and poor prognosis.

The association between HE4 and resistance to anticancer drugs has been elucidated in studies on ovarian cancer. For instance, Lee *et al* (57) found that HE4 enhanced drug resistance against cisplatin and paclitaxel. Moreover, Moore *et al* (58) demonstrated that HE4, when overexpressed in SKOV-3 clones, was not so sensitive to cisplatin and paclitaxel compared to controls *in vitro*. These studies revealed that the localization of HE4 expression is related to the active state of growth factors, such as EFG and VEGF, which induce nuclear translocation. They also indicated that insulin is associated with HE4 localization; insulin does not stimulate nuclear translocation but nucleolar translocation. From these findings, it was inferred that EGF, VEGF, insulin and their receptors are responsible for ovarian tumor progression and chemoresistance (59-61). An OVCAD study assessed the association between platinum response and HE4 concentration in plasma and ascites and found that HE4 levels were significantly higher in the subgroup of platinum-non-responder patients than in that of platinum-sensitive patients (62). In addition, Ribeiro *et al* (63) reported that HE4 conferred resistance to the anticancer drugs, cisplatin and paclitaxel; HE4 overexpression promoted chemoresistance to cisplatin and paclitaxel, which was partially reversed by the downregulation of HE4. It has been indicated

Table II. Univariate and multivariate analyses of prognostic factor for overall survival in 80 pancreatic cancer patients.

Clinicopathological factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value[a]	HR	95% CI	P-value[a]
Age, years (≤71 vs. >71)	1.1	0.60-2.09	0.76	-	-	-
Sex (Male vs. female)	0.52	0.28-0.95	0.033[b]	0.54	0.29-0.97	0.045[b]
TNM stage UICC 7th (IIA vs. IIB)	0.55	0.25-1.12	0.12	-	-	-
Tumor location (Body/tail vs. head)	1.68	0.90-3.31	0.12	-	-	-
Histological type (Adeno vs. others)	0.6	0.10-1.20	0.48	-	-	-
Lymphatic invasion (ly0, ly1 vs. ly2, ly3)	1.4	0.77-2.62	0.28	-	-	-
Venous invasion (v0, v1 vs. v2, v3)	1.13	0.51-3.00	0.78	-	-	-
Adjuvant chemotherapy (Absent vs. present)	0.49	0.25-0.93	0.037[b]	0.45	0.22-0.86	0.020[b]
HE4 (Low vs. high)	1.98	1.08-3.80	0.031[b]	2.01	1.09-3.86	0.029[b]

95% CI, 95% confidence interval. [a]Cox proportional hazard model. [b]Statistically significant difference.

that multiple factors can facilitate HE4-mediated chemoresistance, related to the deregulation of MAPK signaling, which induces apoptosis, as well as alterations in tubulin levels or stability (63). Wang *et al* (64) demonstrated that HE4 can attenuate apoptosis induced by carboplatin by reducing the mitochondrial Bax/Bcl-2 ratio; HE4 markedly increased Bcl-2 expression, while inhibiting Bax expression (64). Moreover, Angioli *et al* (65) proposed that evaluating the serum values of HE4 concentration may aid in the prediction of the response to chemotherapy in epithelial ovarian cancer. The present study demonstrated that HE4 contributed to GEM chemoresistance based on cell viability assays of several tumor cell lines. To the best of our knowledge, there are no previous reports available on the role of HE4 in GEM resistance. Based on the PDX experiment, it is possible that HE4 is also involved in resistance to nab-paclitaxel. As nab-paclitaxel is a nanoparticle albumin-bound form of paclitaxel, the findings of the present study may be similar to those of previous studies that evaluated paclitaxel resistance in ovarian cancer patients. Although further investigations are required to determine whether these results are applicable to pancreatic cancer, the data support a role for HE4 in multidrug resistance.

Previous studies have demonstrated that HE4 is upregulated in pancreatic cancer cell lines and tissues (30,31). Moreover, Huang *et al* (32) demonstrated that HE4 mRNA and protein expression increased in pancreatic adenocarcinoma tissues and that the level of serum HE4 was elevated in patients with pancreatic adenocarcinoma. However, to the best of our knowledge, no studies to date have reported the involvement of HE4 in the poor prognosis and GEM resistance in pancreatic cancer. The present study seems to be the first to demonstrate that HE4 is associated with a poor prognosis and GEM resistance in pancreatic cancer, thereby highlighting that HE4 may be a candidate biomarker to predict prognosis and chemoresistance.

Multivariate analysis indicated that high expression levels of HE4 are a substantive prognostic factor that forecast a poorer outcome. In addition to HE4 expression, the lack of adjuvant chemotherapy and being male were statistically significant poor prognostic factors. An effect of sex on pancreatic cancer

was demonstrated in the present study, although there is little established evidence. With respect to the direct prognosis of pancreatic cancer, several studies have demonstrated significant differences in the sub-analysis of larger studies. Although the studies had patient heterogeneity and included not only stage IV (66-68), but all stages (69-72), the male sex exhibited a poor prognosis with a significantly reduced OS. Another study demonstrated that post-operative male patients exhibited a reduction in disease-specific survival (6). Liu *et al* (73) reported that it is more likely to accidentally identify distant metastases in male patients with resectable pancreatic cancer. The cause of the poor prognosis for males has not yet been clarified. It is conceivable that females have fewer comorbidities than males do at the same ages. Moreover, considering that pancreatic cancer is often unresectable, there is a possibility that sex differences may exist in the effectiveness of chemotherapy. Hohla *et al* (74) reported that despite a lack of statistical significance, OS and PFS tended to be better for females than for males, and they demonstrated that women had better response rates with significant differences in the FOLFIRINOX therapy group. Women also tended to have a better prognosis after GEM chemotherapy (75). Thus, compared with females, males reportedly have a poorer prognosis in pancreatic cancer, corroborating the findings of the present study. Although serum HE4 sensitivity exhibits a sex effect, the association with prognosis is unknown and is a topic for future research.

The present study had certain limitations. First, the present study could not examine the association between HE4 expression and chemotherapy in cancer patients. In the present study, all patients did not receive chemotherapy prior to surgery, since neoadjuvant chemotherapy has not yet been permitted in Japan. Nevertheless, it was considered advantageous to include some patients who had received neoadjuvant chemotherapy prior to surgery. Second, the present study was not able to conduct additional PDX experiments to confirm the involvement of HE4 in chemoresistance; nonetheless, endogenous *HE4* gene expression was measured in several cell lines by RT-qPCR. Third, the absence of western blot analysis data is another limitation. Fourth, although cell viability assays of

various cell lines were conducted, including HUVECs and HRGECs, in response to GEM, inherent mutations in these cell lines may be involved in resistance to GEM. To overcome this limitation, it is necessary to further examine the association between gene mutations in each cell line and GEM resistance. In *in vitro* assays using cell lines, *HE4* gene expression in the PANC-1 cell line was statistically significantly lower than that in the SK-N-AS, KATOIII and ASPC-1 cell lines (Table SI). However, following treatment with GEM, regardless of the concentration, the viable cell number of the PANC-1 cell line was significantly higher than that of other cell lines, including ASPC-1, KATOIII and SK-N-AS. Due to these inconsistent results regarding HE4 expression and GEM resistance, PANC-1 cells may possess a different mechanism of GEM resistance. Moreover, HE4 was highly expressed in the normal pancreatic duct cell line (H6c7). Hence, although the present study demonstrated that HE4 expression may be applied as a predictive marker of a poor prognosis, the results for H6c7 cells suggested that HE4 expression was not cancer-specific, and thus, cannot serve as a diagnostic marker for pancreatic cancer. The regulatory mechanisms associated with a high or low HE4 expression in this normal pancreatic cell line, and in pancreatic cancer cell lines remain unclear, and will need to be clarified in future investigations.

Taken together, the findings of the present study demonstrated that HE4 induced chemoresistance. As HE4 expression was associated with a poor prognosis and is a promising novel prognostic marker for pancreatic cancer, HE4 as a potential therapeutic target for pancreatic cancer is hereby proposed. However, further studies evaluating the clinical applicability of HE4 in the prognosis and treatment of pancreatic cancer are essential.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ROh was involved in the investigative aspects of the study, and in the methodology, validation, visualization and writing of the original draft. EY was involved in data curation, formal analysis, methodology, project administration and in providing resources. SI and DK were involved in formal analysis. YK, KH, AH, TI, YH and HA provided resources and analyzed the general data and patient indications, as well as in the study methodology. MS, MW, RO and KA were involved in the investigative aspects of the study, as well as in the study methodology. JT and KY provided resources and were involved in study methodology and project administration. TS was involved in the study methodology. TA and MM provided resources and were involved in study methodology and project administration. TN, NO and MT were involved in the investigative aspects of the study, as well as in the study methodology. SK and TT were involved in study supervision, methodology, project administration and reviewed and revised the manuscript. SW was involved in the study conceptualization, methodology, project administration, supervision and validation, as well as in the writing, reviewing and editing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For human research, the study protocol was approved by the Ethics Committee of the Showa University School of Medicine (Tokyo, Japan; approval no. 2611), and all study procedures adhered to the principles of the Declaration of Helsinki. Consent was not obtained from the individual patients. However, the patients were notified of the details of the study using an opt-out form and were given the right to refuse study participation. This is a method widely used in Japan. For animal research, all experiments involving laboratory animals were performed in accordance with the care and use guidelines of the Kanagawa Cancer Center Research Institute. All protocols were approved by the ethics committee of the Kanagawa Cancer Center Research Institute (approval no. 176).

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

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