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Transcriptional regulation of *ETV5* by mitogen-activated protein kinase via ETS-1 in human pancreatic cancer cells

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Pancreatic cancer is characterized by constitutive activation of mitogen-activated protein kinase / extracellular signal-regulated kinase 1/2 (ERK1/2) driven by gain-of-function mutations of *KRAS*. Our previous transcriptome sequencing of ERK1/2-attenuated cultured pancreatic cancer cells unveiled numerous downstream genes activated by ERK1/2 including *ETV5*. In this study, we explored the mechanism of transcriptional regulation of *ETV5* by ERK1/2 in human pancreatic cancer cells. Detailed reporter assays uncovered a core promoter region spanning between −350 and −985 from the transcription start site of *ETV5* as a strong responsive element to ERK1/2 activity. Moreover, ETS proto-oncogene 1, transcription factor (ETS-1) was found to bind to one of consensus binding sites in the core region and to promote ERK1/2-mediated upregulation of *ETV5*. Investigation of functional significances of ETS variant transcription factor 5 (ETV5) expression in the pancreatic cancer cells revealed that ETV5 was associated with resistance to gemcitabine; while no significance in proliferation, migration, and invasion. ETV5 expression in pancreatic ductal adenocarcinoma tissues resected from patients undergoing neoadjuvant chemotherapy was associated with *KRAS* mutations, which was consistent with *ETV5* as a downstream upregulated molecule of RAS-ERK1/2 pathway. This study elucidated the mechanism of ERK1/2-mediated transcriptional regulation of *ETV5* in human cancer cells, which could contribute to understand pancreatic cancer pathobiology.

Pancreatic cancer is currently the third leading cause of cancer deaths in the United States[1]. Furthermore, its incidence is on the rise and is anticipated to become the second leading cause of cancer deaths in the United States by 2030[2]. Despite multidisciplinary treatment, the 5-year survival rate for pancreatic cancer patients is very poor, less than 10%[3]. To improve this dismal prognosis, it is necessary to develop novel diagnostic and/or therapeutic procedures. Elucidating the molecular pathobiology of pancreatic cancer may guide such development.

The molecular hallmark of pancreatic cancer is constitutive activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) pathway principally caused by *KRAS* mutations. The activated ERK1/2 phosphorylates various transcription factors in the nucleus, altering the expression of downstream genes and triggering various cellular responses, which is thought to play an important role in malignant phenotypes of pancreatic cancer[4]. Inhibition of downstream molecules of ERK1/2 can attenuate some of such malignant phenotypes[5].

By comparing transcriptome profiles of human pancreatic cancer cells treated with and without mitogen activated protein kinase kinase (MAP2K/MEK) inhibitor, we identified genes whose expression is modulated by ERK1/2 pathway activity[6,7]. In this study, we focused on *ETV5*, whose gene expression was markedly downregulated by inhibition of ERK1/2 pathway[7]. *ETV5* encodes ETS variant transcription factor 5 (ETV5), a member of the E26 transformation-specific (ETS) family of transcription factors, also known as ETS-related molecules (ERM)[8]. ETV5 has been reported to be a downstream factor of the ERK1/2 pathway[9–11], and the activation of ETV5 in cancer cells is known to be associated with various malignant phenotypes, particularly

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proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, and acquisition of drug resistance[12]. Furthermore, elevated protein expression of ETV5 in colon cancer tissues[13] and elevated RNA expression of *ETV5* in ovarian cancer tissues[14] have been shown to be associated with poor prognosis. Functions of *ETV5* in the pancreas have been reported to be involved in regeneration of pancreatic ductal cells from pancreatitis[15,16] and insulin secretion in islet cells[17,18]. However, it is unclear how *ETV5* expression is regulated in cancer cells. In this study, we aimed to uncover the mechanism of ERK1/2-associated transcriptional regulation of *ETV5* in pancreatic cancer cells as well as significances of ETV5 on proliferation, migration, invasion, and drug resistance of human pancreatic cancer cells and clinicopathological impacts of ETV5 in patients with surgically resected pancreatic ductal adenocarcinoma (PDAC). The results of this study demonstrate that the transcriptional expression of *ETV5* in human pancreatic cancer cells is significantly regulated by activated ERK1/2. Furthermore, the exact promoter region of *ETV5* that responds to ERK1/2 activity was determined, revealing that ETS-1 likely binds to the *ETV5* promoter and mediates active ERK1/2 signaling.

Results

Positive correlation between ETV5 expression and ERK1/2 pathway activity

We previously performed a transcriptome analysis to determine what genes were altered in expression when U0126, MEK inhibitor, was administered to cells of human pancreatic cancer cell lines (NCBI Gene Expression Omnibus Accession: GSE268817)[7]. Five genes including *ETV5*, *ETV4*, *COL13A1*, *HIST1H4L/H4C13*, and *WDR62* were found to be particularly downregulated by the inhibition of ERK1/2 pathway, meaning that these genes were preferentially upregulated by the active ERK1/2 (Table 1, Supplemental Table S1). The expression of *ETV5* was most modulated, suggesting that *ETV5* is strongly associated with the ERK1/2 activity and may play a role in human pancreatic cancer. Therefore, we focused on *ETV5* in this study. We validated the results of transcriptome analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and protein expression of ETV5 by immunoblots of pancreatic cancer cell lines, namely, AsPC-1, MIAPaCa-2, and PCI-35, incubated with U0126. These cell lines harbor *KRAS* mutations as follows: a homozygous G12D mutation in AsPC-1, a homozygous G12C mutation in MIAPaCa-2, and a heterozygous G12D mutation in PCI-35[19]. The results indicated that the expression of ETV5 was downregulated along with the inhibition of ERK1/2 (Fig. 1).

Identification of a promoter element of *ETV5* responding to ERK1/2 activity

The above results suggested that ETV5 might be a downstream molecule of the ERK1/2 pathway, and that ERK1/2 was supposed to upregulate ETV5 expression. Then, we performed a promoter assay to elucidate the mechanism of transcriptional regulation of *ETV5* associated with ERK1/2. As a candidate promoter region, the 2483 bp genomic upstream region spanning between −2458 and +25 from the transcription start site of *ETV5* (chr3:186109065–186111547; GRCh38/hg38) was cloned into a reporter vector (Fig. 2A). Reporter assays were performed in the human pancreatic cancer cell lines, which indicated that the candidate promoter region indeed showed a strong promoter activity (Fig. 2B). Although the protein expression was variable, the promoter activity was fairly strong in these pancreatic cancer cells. However, because the reporter activity was relatively low in PCI-35 compared to AsPC-1 and MIAPaCa-2, subsequent experiments were performed by using AsPC-1 and MIAPaCa-2.

To determine an exact site responsible for the promoter activity in the candidate promoter region, reporter vectors containing various truncation sequences were constructed (Fig. 2C). The constructed reporter vectors were transfected into AsPC-1 and MIAPaCa-2, and their reporter activities were measured. Compared to the full promoter genomic fragment, truncated fragments spanning between −985 and +25 from the transcription start site (#2 in Fig. 2C) revealed the maximum promoter activity that exceeded the full promoter activity. Since the region between −350 and −44 was inferred to contain a TATA box, an adjacent upstream region between −985 and −350 were considered to be responsible for the promoter activity positively regulated by ERK1/2, which we designated core promoter region. In addition, the upregulation of promoter activity in truncated fragments compared to the full fragment (Fig. 2C) suggested that the region upstream from −985 might have an inhibiting promoter activity.

To test whether the ERK1/2 pathway would be indeed involved in the promoter activity of *ETV5*, we examined alterations in the activity by modulation of ERK1/2 activity. It was revealed that the promoter activity was downregulated when ERK1/2 activity was inhibited (Fig. 2D).

Identification of a responsive transcription factor for ERK1/2-mediated *ETV5* regulation

Next, we moved on to find a responsible transcription factor binding site that mediates ERK1/2 activity in the core promoter region. By searching of possible transcription factor binding sites, we found 8 candidate binding sites for ETS transcription factor ELK1 (ELK-1) or ETS-1, representative targeted transcription factors

Gene symbol	U0126 vs. DMSO (fold change)
ETV5	-6.42
ETV4	-5.05
COL13A1	-3.49
HIST1H4L/H4C13	-3.21
WDR62	-3.05

Table 1. The genes whose expression was markedly downregulated by ERK1/2 pathway Inhibition.

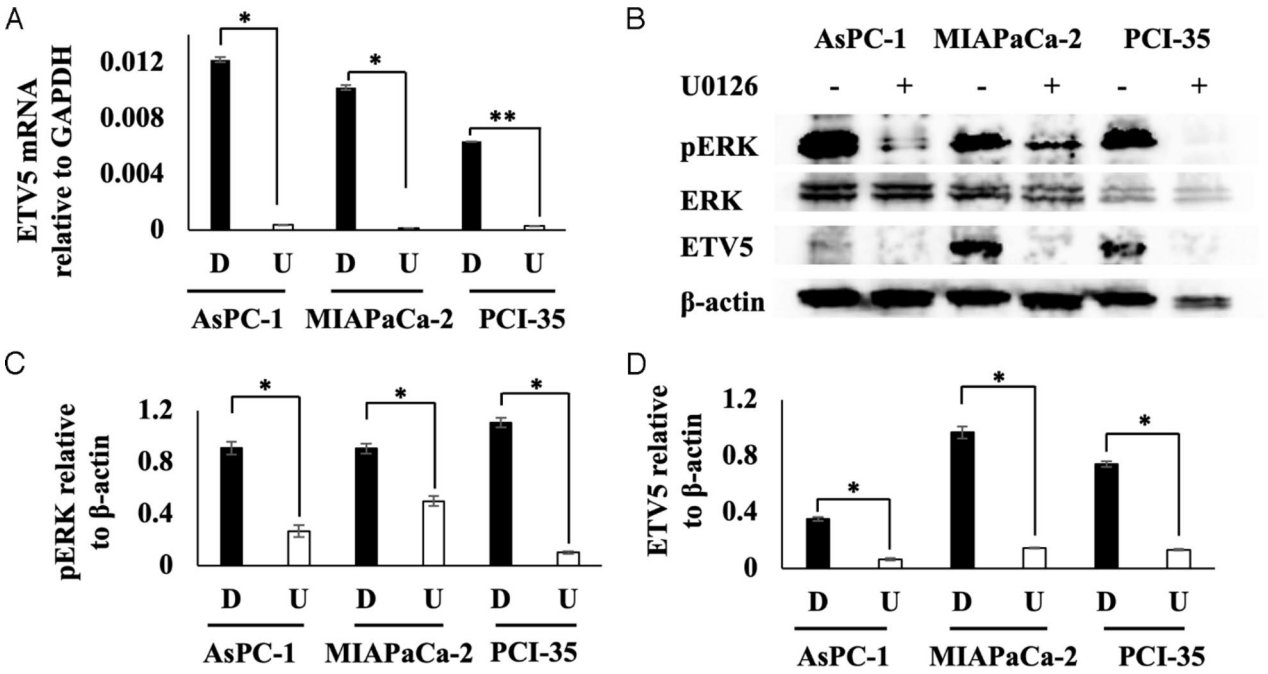


Fig. 1. Alterations of *ETV5* expression by ERK1/2 inhibition. (**A**) qRT-PCR of human pancreatic cancer cells treated with U0126 (U) or dimethyl sulfoxide (D). (**B**) A representative immunoblot of human pancreatic cancer cells treated with (+) or without (-) U0126 probed for the expression of phosphorylated ERK (pERK), ERK, ETV5, and β-actin. Original blots are shown in Supplemental Figure S1. (**C** and **D**) Densitometry analyses of the expression of pERK (**C**) and ETV5 (**D**) relative to that of β-actin in immunoblots of pancreatic cancer cells treated with U0126 (U) or dimethyl sulfoxide (**D**). Asterisks indicate that *, $p < 0.05$; and **, $p < 0.01$. Graphs show means and standard errors of duplicate experiments.

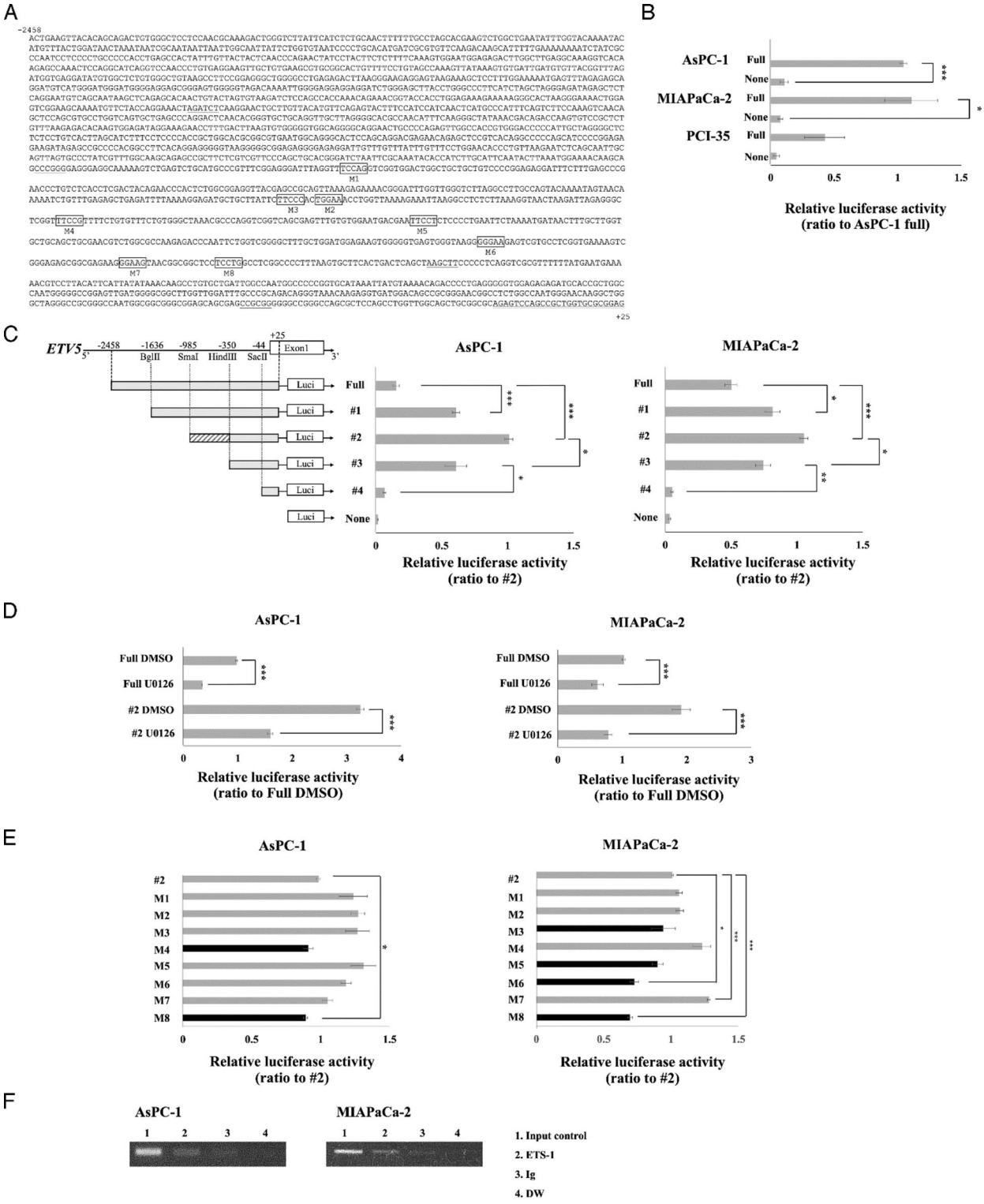
of ERK1/2, namely, 2 sites for both ELK-1 and ETS-1 (M1, M2), 5 sites for ELK-1 (M3-M7), and 1 site for ETS-1 (M8) in the core region (Table 2; Fig. 2A). Then we tested promoter activities of the core region containing mutations in each of M1-M8 binding site in AsPC-1 and MIAPaCa2, which revealed that M8 was consistently responsible for the core promoter activity (Fig. 2E). M8 was a consensus binding site for ETS-1, therefore, this result indicated that ETS-1 that binds to the region was likely to mediate ERK1/2 activity to promote ETV5 expression. We performed chromatin immunoprecipitation (ChIP) assay, which revealed that ETS-1 binds to the aforementioned site (Fig. 2F). These results indicated that ETV5 is promoted by ERK1/2 via ETS-1 through the promoter region spanning between − 985 and − 350 from the transcription start site of *ETV5*.

Effects of attenuated ETV5 expression on phenotypes of human pancreatic cancer cells

Next, we moved on to investigate pathobiological significances of ETV5 in pancreatic cancer. We performed knockdown of ETV5 via short interference RNAs (siRNAs) and examined its effects on proliferation, migration, invasion, and resistance to gemcitabine of the pancreatic cancer cells. The siRNA-mediated downregulation of ETV5 was confirmed by qRT-PCR and immunoblotting without affecting pERK expression (Fig. 3A-D). Subsequent assays showed that the downregulation of ETV5 did not significantly influence on proliferation, migration, and invasion; however, it significantly decreased the resistance to gemcitabine of the pancreatic cancer cells (Fig. 3E-H, Supplemental Figures S2-S3).

To validate functional changes in downstream factors during *ETV5* knockdown, we checked changes in expression of snail family transcriptional repressor 1 (SNAI1) and vascular endothelial growth factor (VEGF), presumed downstream molecules of ETV5 associated with EMT and angiogenesis, respectively[10,20]. We supposed that these molecules would be downregulated by knockdown of *ETV5*; however, no obvious changes in VEGF nor downregulation of SNAI1 were observed upon the knockdown of *ETV5*, but rather SNAI1 expression was upregulated in AsPC-1, without affecting expression of phosphorylated ERK and ETS variant transcription factor 4 (ETV4) (Supplemental Figures S4, S5).

In qRT-PCR using samples after the scratch assay, mRNA expression of *ETV5* was confirmed to be decreased (Supplemental Figure S6), indicating that *ETV5* was knocked down even 48 h after the siRNA treatment. However, we found that the downregulation of ETV5 revealed no consistent significant effects on cell proliferation, migration, and invasion of the tested pancreatic cancer cells. These results suggest that upon knockdown of ETV5, no effect on these phenotypes of human pancreatic cancer cells can be observed, supposed to be due to some compensatory mechanisms at work.



Effects of ETV5 expression on clinicopathological features of patients with surgically resected pancreatic ductal adenocarcinoma with neoadjuvant chemotherapy

To know clinicopathological significances of ETV5 in patients with PDAC, we performed immunohistochemical evaluation of the expression of ETV5 in 112 resected PDAC specimens at Tohoku University Hospital. These patients specifically underwent neoadjuvant chemotherapy with gemcitabine and S-1 combination. There were 87 cases with high ETV5 expression and 25 cases with low ETV5 expression (Table 3; Fig. 4). Among these 87 cases, 37 cases were previously examined for *KRAS* mutations in PDAC tissues[21] and found that ETV5 expression was associated with *KRAS* mutations ($p = 0.0420$). The *KRAS* mutation is assumed to upregulate ERK1/2 activity; therefore, this result further endorsed the tight association between ERK1/2 and ETV5 expression. On the other hand, no other specific clinicopathological features including prognosis were found to be associated with ETV5 expression in PDAC tissues in this 87 patients' cohort (Table 3; Fig. 4B). Moreover, we extracted

◀**Fig. 2**. Identification of *ETV5* promoter element responding to ERK1/2 activity. (**A**) Human genomic sequence retrieved from GRCh38/hg38 between − 2458 and + 25 from the transcription start site of *ETV5*. Underlines indicate restriction enzyme sites. The double underline indicates exon 1 of *ETV5*. Boxes indicate putative binding sites of ETS transcription factors in the most probable responsive region to ERK1/2 (the hatched box in #2 of **C**). (**B**) Reporter assays using PGV-P2 reporter vectors replaced SV40 promoter region with the human genomic sequences between − 2458 and + 25 from the transcription start site of *ETV5* (Full) and no genomic sequences (None) in human pancreatic cancer cells, AsPC-1, MIAPaCa-2, and PCI-35, showed strong promoter activities of Full. (**C**) Reporter activities of various truncated promoter sequences using AsPC-1 and MIAPaCa-2, The significance level was corrected by Bonferroni method to 0.0125. $^{*}p < 0.0125$, $^{**}p < 0.001$, $^{***}p < 0.001$. (**D**) Reporter activities of Full and #2 in AsPC-1 and MIAPaCa-2 treated with U0126. (**E**) Site directed mutagenesis of consensus binding sites of ETS transcription factors in #2 promoter sequence (M1-M8; Table 2) on reporter activities in AsPC-1 and MIAPaCa-2. Black bars indicate less activities compared to the non-mutated #2 promoter sequence. The significance level was corrected by Bonferroni method to 0.00625. $^{*}p < 0.00625$, $^{**}p < 0.001$, $^{***}p < 0.001$. (**F**) Chromatin immunoprecipitation assay using anti-ETS1 antibody for candidate ETS1 binding site (M8). PCR products shown were input control (lane 1), immunoprecipitants with anti-ETS1 antibody (lane 2), immunoprecipitants with nonspecific immunoglobulin (lane 3), and negative control (lane 4). Unless otherwise noted, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All data are means and standard errors of du-plicates.

	Primer	Candidate transcription factor binding sites	Transcription factors
M1	CCGTTTCGGAGGGATTTAGGTTT**TT**AGGTCGGTGGAC	aggtTTCCAggtcg/ ggttTCCAGg	ELK-1/ ETS-1
M2	GAGATGCTGCTTATTCTTCCCACT**AA**AAACCTGGTTAAAAGAAATTAAGG	cccacTGGAAacct/ aCTGGAaacc	ELK-1/ ETS-1
M3	TGAGATTTTAAAAGGAGATGCTGCTTATTCTT**TT**CACTGGAAACCTGGTTA	attcTTCCCactgg	ELK-1
M4	AAGATTAGAGGGCTCGGTTT**TT**GTTTTCTGTGTTTCTGTGGG	cggtTTCCGttttc	ELK-1
M5	AGCGAGTTTGTGTGGAATGACGAATT**TT**TCTCCCCTGAATTC	cgaaTTCCTctccc	ELK-1
M6	GGTGAGTGGGTAAGGG**TT**AAGAGTCGTGCCTCGG	taaggGGGAAgagt	ELK-1
M7	GAGAGCGGCGAGAAG**AA**AAGTAACGGCGGCTC	gagaagGGAAGtaacg/ gagaaGGGAAgtaa	ELK-1
M8	AAGTAACGGCGGCTCCT**TT**TGGCCTCGGCC	ctccTCCTGg	ETS-1

Table 2. List of primers used for site-directed mutagenesis for candidate binding sites of ETS transcription factors. Underlined bold letters indicate mutations.

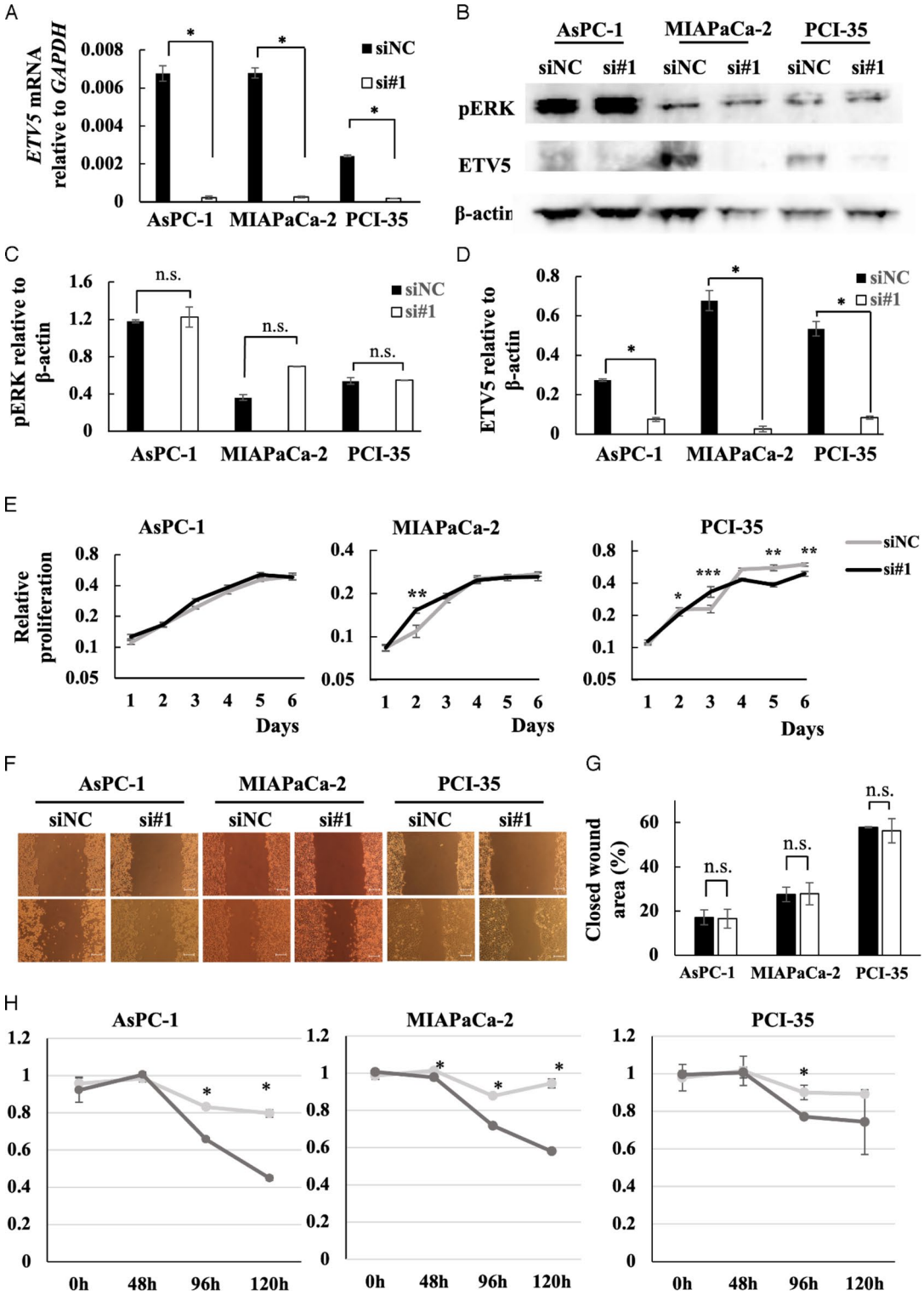
the Cancer Genome Atlas (TCGA) data to examine the relationship between the transcriptional expression of *ETV5* in pancreatic cancer tissues and patients' survivals and PDAC subtypes of classical and basal-like[22] which resulted in finding of no significant associations (Supplemental Figures S7, S8). These results suggest that ETV5 expression may not be particularly significant in clinicopathological phenotypes of patients with resected PDAC.

Discussion

In this study, we identified *ETV5* as a downstream molecule strongly associated with ERK1/2 activity in human pancreatic cancer cells. By transcriptome sequencing of ERK1/2-attenuated cultured pancreatic cancer cells, we found that *ETV5* expression was markedly modulated by active ERK1/2. Then, we performed detailed promoter assays and determined the exact promoter region of *ETV5* responding to ERK1/2 activity. Moreover, we demonstrated that ETS-1 was likely to be associated with the ETV5 promoter to mediate the active ERK1/2 signal. We also revealed that ETV5 expression was associated with resistance to gemcitabine in human pancreatic cancer cells and *KRAS* mutations in PDAC tissues of patients undergoing neoadjuvant chemotherapy. On the other hand, we did not find any significances of ETV5 expression in cell proliferation, migration, and invasion of the pancreatic cancer cells or clinicopathological features including survivals in the PDAC patients.

Pancreatic cancer is characterized by the constitutive activation of ERK1/2 pathway principally driven by the frequent gain-of-function mutation of *KRAS* and synergistic epigenetic loss of expression of DUSP6[23]. The active ERK1/2 induces expression of numerous downstream genes that are supposed to be involved in malignant phenotypes of pancreatic cancer[4–6]. We identified that *ETV5* is one of such downstream genes significantly associated with the ERK1/2 activity in pancreatic cancer cells.

ETV5 (also known as ERM) is a member of ETS transcription factors[12], and it has been shown to play pivotal roles in cell biology and cancer pathogenesis by numerous studies: ETV5 promotes G1-S phase transition by directly repressing the transcriptional activity of p21/CDKN1A[24,25] and the transcription of cell cycle genes involved in G1-S and G2-M progression by promoting the expression of forkhead box M1[26]. ETV5 promotes EMT in papillary thyroid cancer cells by regulating transcriptions of *TWIST1* directly and *SNAI1* indirectly[10]. Overexpression of ETV5 in endometrial carcinomas is associated with increased expression of fibronectin, α5 integrin, β1 integrin, and N-cadherin; while it is associated with decreased expression of cyclin D1 and β-catenin, which promotes invasion[27]. ETV5 promotes angiogenesis by activating *VEGFA* expression through direct binding to its promoter and inducing secretion of C-C motif chemokine ligand 2 via signal transducer and activator of transcription 3[20]. Moreover, ETV5 is shown to be associated with chemotherapy resistance: ETV5 mediates resistance to paclitaxel in ovarian cancer cells[28] and inhibitors of B-Raf proto-oncogene, serine/



threonine kinase (BRAF) in melanoma cells[29]. ETV5 can be a biomarker of sensitivity to cobimetinib and selumetinib, MEK inhibitors, in genotype-driven molecular targeted cancer therapy settings[30,31]. Hence, *ETV5* is a noteworthy gene as an oncogenic transcription factor involved in cancer development, progression, and resistance to chemotherapy.

ETV5 is known to be associated with MAPK/ERK1/2 by several interacting ways. ETV5 expression is upregulated via active MAPK in *BRAF*-mutated tumor cells[32]. *ALK*-mutated neuroblastoma cells show high ETV5 levels downstream of RAS/MAPK axis[11]. MEK/ERK activation is demonstrated to be required for *Etv5* mRNA induction in response to nerve growth factor in dorsal root ganglion cells[33]. Brain-derived nerve growth factor promotes ETV5 mRNA expression via activation of ERK1/2 in dorsal root ganglion cells[34]. The active form of MAP2K1 up-regulates *Etv5* gene expression in mouse germline stem cells[35]. Fibroblast growth factor receptor type 3 signaling induces a MAPK/ERK-mediated increase in ETV5 in bladder cancer cells[36]. ETV5

◀**Fig. 3**. Effects of *ETV5* knockdown by small interfering RNAs on human pancreatic cancer cell lines. (**A**) Alterations in *ETV5* expression at the mRNA level measured by qRT-PCR in pancreatic cancer cells treated with si#1, small interfering RNAs for *ETV5*, and siNC, small interfering RNAs for a negative control. (**B**) A representative immunoblot showing the expression of pERK, ETV5 and β-actin in pancreatic cancer cells with knockdown of *ETV5* (si#1) or a negative control (siNC). Original blots are shown in Supplemental Figure S2. (**C** and **D**) Densitometry analyses of immunoblots probed for the expression of pERK, ETV5 and β-actin in pancreatic cancer cells with knockdown of *ETV5* (si#1) or a negative control (siNC). (**E**) MTT assays for proliferations of pancreatic cancer cells with knockdown of *ETV5* (si#1) or a negative control (siNC). (**F**) Scratch wound healing assays for migration of pancreatic cancer cells with knockdown of *ETV5* (si#1) or a negative control (siNC). (**G**) The graph shows the rate of closing of the wound area in each pancreatic cancer cell line. (**H**) Chemosensitivity assays for gemcitabine in pancreatic cancer cells. The chemosensitivity was assessed as relative amount of siRNA (either si#1 for *ETV5* (black lines) or siNC for negative control (gray lines)) transfected viable cells of gemcitabine-treated compared to amount of viable cells of the control without siRNA transfection and gemcitabine treatment in average of those of 5-wells at each time point. Abbreviations: n.s., not significant. Asterisks indicate *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Scale bars in Fig. 3D indicate 100 μm. Graphs show means and standard errors obtained from duplicate experiments unless otherwise specified.

protein is stabilized by ERK-dependent inhibition of COP1 E3 ubiquitin ligase (COP1) mediated proteasomal destruction[37]. Therefore, ETV5 is demonstrated to be modulated by MAPK/ERK1/2 activation in transcriptional as well as post-translational protein levels in various kinds of cells; however, the detailed mechanism of transcriptional modulation has not been well understood. In this study, we demonstrated that *ETV5* expression was closely associated with ERK1/2 pathway activity via transcriptional control. The maximum promoter activity positively responding to ERK1/2 activity was observed in the region spanning between −350 and −985 from the transcription start site of *ETV5*. This region contained several consensus binding sites for ETS transcription factors, and one of the sites proved to be responsible for the transcriptional activity. ChIP assay revealed binding of ETS-1 on this region. ETS-1 is a member of the ETS transcription factor family, which is known to be a substrate of ERK1/2 and to play an important role in cancer pathobiology[38,39]. ETS-1 is known to be involved in malignant phenotypes of cancer cells by regulating various downstream factors[40], and in addition, our results suggest that ETS-1 possibly regulates the transcriptional activity of ETV5. Although no drugs targeting ETV5 have been developed to date, inhibiting ETV5 interacting proteins such as co-activators and suppressors, as well as post-translational modifications of ETV5, could be a new strategy to inhibit ETV5 function[12]. On the other hand, we found in our experiments using the human pancreatic cancer cell lines that translational protein levels of ETV5 were not in accord with transcriptional levels. This could be due to post-translational modifications as known to be the COP1-mediated proteasomal destruction[18]. Nevertheless, our results suggest that inhibition of ETS-1 may reduce the transcriptional activity of ETV5, suggesting that ETS-1 may be targeted as a therapeutic strategy for ETV5. Elucidation of the molecular mechanism of *ETV5* expression in cancer cells may pave the way for understanding cancer pathobiology and development of novel diagnostic and therapeutic procedures.

As stated above, ETV5 has been reported to be associated with oncologic phenotypes including proliferation, migration, invasion, angiogenesis, EMT, chemotherapy resistance, and prognosis[11,13,14,20,24,26,27,36,37,41–44]. In our in vitro experiments using human pancreatic cancer cell lines, the knockdown of ETV5 showed decreased resistance to gemcitabine despite showing no significant inhibitions of proliferation, migration, and invasion. Furthermore, immunohistochemical staining for ETV5 using resected pancreatic cancer specimens after neoadjuvant chemotherapy using gemcitabine and S-1 showed differences in the intensity of ETV5 expression in pancreatic cancer tissues, but no differences in clinicopathological characteristics including prognosis depending on ETV5 expression. However, the prognosis of patients with PDAC with low ETV5 expression seemed slightly better than that with high ETV5 expression especially until 4 years post-surgery. This temporary better prognosis could be attributed to the decreased resistance to gemcitabine, which warrants further examination in a large scale. The result that *KRAS* mutations were more frequent in cases with high ETV5 expression is reasonable, since ETV5 is a downstream factor of ERK1/2 as we proved. The results indicating little significance of ETV5 in proliferation, migration, and invasion of pancreatic cancer cells, although somewhat contradictory to that in other cancers, could be due to compensations by other molecules for downregulation of ETV5. ETV5 belongs to the polyomavirus enhancer activator 3 (PEA3) subfamily of ETS transcription factors. The PEA3 subgroup includes ETS variant transcription factor 1 (ETV1/ER81), ETV4/PEA3, and ETV5/ERM, and these molecules share a highly similar structure consisting of PEA3-type ETS transcriptional factor N-terminal domain and ETS domain[45]. The N-terminus domain harbors conserved ERK1/2 phosphorylation sites[46]. Especially, ETV4 and ETV5 are known to share specific functions in development and signal transductions including hippocampal dendrite development[47], retrograde signaling and axonal growth of dorsal root ganglion sensory neurons[33], and transcriptional regulation of cyclooxygenase-2 in granulosa and cumulus cells[48]. In cancer tissues, ETV1, ETV4, and ETV5 are highly expressed and their biological functions are synergistic[49]. ETV4 and ETV5 play a compensatory role each other and necessary for efficient clonogenic expansion in small cell lung cancer[50]. Multiple ETS factors have been shown to regulate the transcription of downstream factors in thyroid cancer[51]. Therefore, it is possible that some compensatory mechanisms involving other ETS factors could work in the functional expression of downstream factors of ETV5 in human pancreatic cancer cells. Indeed, we found that ETV4 expression was preserved in cells with ETV5 knockdown, which could play the compensatory role. Extensive additional experiments may be required to explore associated/redundant molecular pathways more than that performed in this study to test this possibility.

	ETV5 expression		
	Low ($n=25$)	High ($n=87$)	*P* value
Age (mean ± SD)	66.0 ± 8.6	67.5 ± 7.9	0.5245
Gender (male), n (%)	18 (72.0)	50 (57.5)	0.2473
Pretreatment CA19-9 (mean ± SD)	418.8 ± 659.2	569.1 ± 1174.5	0.3384
Pretreatment T classification (UICC), n (%)			0.1519
T1	13 (52.0)	33 (37.9)	
T2	5 (20.0)	36 (41.4)	
T3	1 (4.0)	1 (1.2)	
T4	6 (24.0)	17 (19.5)	
Pretreatment N classification (UICC), n (%)			0.1499
N0	20 (80.0)	55 (63.2)	
N1, 2	5 (20.0)	32 (36.8)	
Pretreatment M classification (UICC), n (%)			1.0000
M0	24 (96.0)	84 (96.5)	
M1	1 (4.0)	3 (3.5)	
Pretreatment TNM stage (UICC), n (%)			0.7798
IA	10 (40.0)	29 (33.3)	
IB	5 (20.0)	19 (21.8)	
IIA	1 (4.0)	1 (1.2)	
IIB	3 (12.0)	18 (20.7)	
III	5 (20.0)	17 (19.5)	
IV	1 (4.0)	3 (3.5)	
NCCN resectability, n (%)			0.4956
R	12 (48.0)	41 (47.1)	
BR	12 (48.0)	35 (40.2)	
UR	1 (4.0)	11 (12.7)	
Residual tumor, n (%)			
R0	20 (80.0)	74 (85.1)	0.5453
R1	5 (20.0)	13 (14.9)	
CAP grade, n (%)			0.1103
1	4 (16.0)	5 (5.7)	
2	9 (36.0)	24 (27.6)	
3	12 (48.0)	58 (66.7)	
Histopathological grade, n (%)			0.5403
Well differentiated adenocarcinoma	7 (28.0)	19 (21.8)	
Moderately differentiated adenocarcinoma	12 (48.0)	46 (52.9)	
Poorly differentiated adenocarcinoma	6 (24.0)	16 (18.4)	
Others (undifferentiated/adenosquamous)	0 (0.0)	6 (6.9)	
Next generation sequencing, n	8	29	
Mutation of *KRAS*, n (%)	6 (75.0)	29 (100.0)	0.0420
Mutation of *TP53*, n (%)	6 (75.0)	21 (72.4)	1.0000
Mutation of *CDKN2A*, n (%)	0 (0.0)	6 (20.7)	0.3053
Mutation of *SMAD4*, n (%)	2 (25.0)	7 (24.3)	1.0000

Table 3. Correlation of clinicopathological characteristics and gene mutations with ETV5 expression in PDAC. Abbreviations are BR, borderline resectable cancer; CA19-9, carbohydrate antigen 19 – 9; CAP, College of American Pathologists; NCCN, National Comprehensive Cancer Network; R, resectable cancer; SD, standard deviation; UICC, Union for International Cancer Control; and UR, unresectable cancer.

There are number of limitations in this study. The number of samples used in the experiments was small and the experimental results need to be validated in larger number of samples. Knockdown experiments using single siRNA is not enough to avoid off-target effects. The ChIP assay was performed only for the M8 site in this study, we may need to examine associations of ETS-1 with other candidate binding sites. To confirm the ETS-1-driven ETV5 promotion, it may be ideal to perform a screening using the clustered regularly interspaced short palindromic repeats system to disrupt ETS-1 in the cells. Furthermore, it would be ideal to conduct further experiments using other cancer cell lines to verify whether the regulation of ETV5 by the ERK1/2 pathway and ETS-1 shown in the present experiments is specific to pancreatic cancer cells or is a universal mechanism in other cancer cells. The experimental results of this study did not reveal any association between ETV5 and

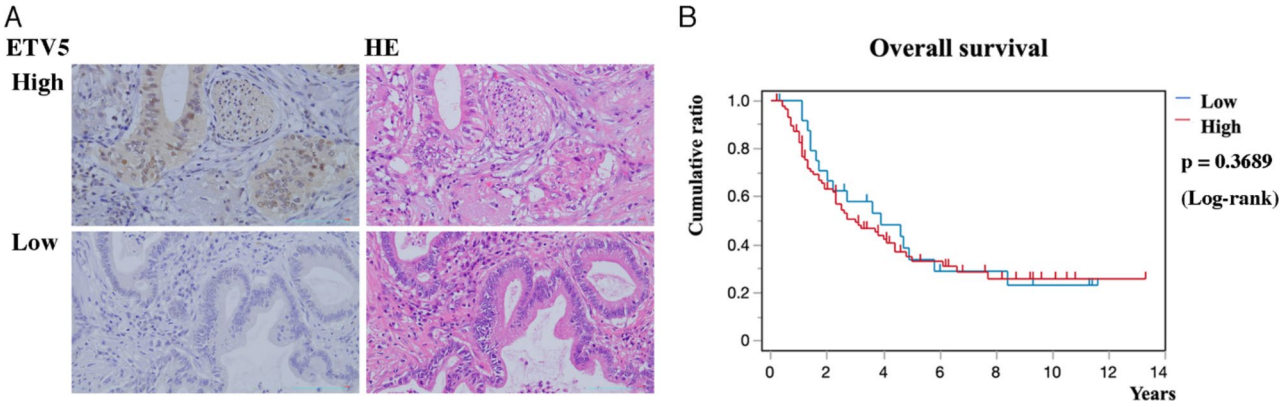


Fig. 4. ETV5 expression evaluated by immunohistochemistry in surgically resected pancreatic ductal adenocarcinoma (PDAC) undergoing neoadjuvant chemotherapy. (**A**) Representative images of ETV5 immunohistochemistry. Left upper panel shows high expression of ETV5 in PDAC cells. Left lower panel shows low expression of ETV5 in PDAC cells. Right panels show corresponding images of hematoxylin and eosin staining (20 × objective lens; Scale bars indicate 100 μm). (**B**) Kaplan-Meier curves for comparing overall survivals of patients with PDAC surgically resected after neoadjuvant chemotherapy with gemcitabine and S-1 depending on ETV5 expression.

oncologic phenotypes of pancreatic cancer cell lines including proliferation, migration, and invasion. Further studies examining the dynamics of downstream factors of ETV5 related to EMT or the cell cycle during ETV5 knockdown, and whether there are pathways or molecules that compensate for the reduced expression of ETV5 will be needed. In addition, because little has been reported on the function of ETV5 in human pancreatic cancer cells, whether or not ETV5 functions in conjunction with other transcription factors and regulators needs to be assessed. More extensive as well as in vivo studies may be required to clarify the importance of ETV5 in pancreatic cancer. Furthermore, we may need more patients to find an association between ETV5 expression in pancreatic cancer tissues and patients' survivals. Detailed classification of ETV5 expression in pancreatic cancer tissues, including expression intensity and expression rate, may provide more specific insights into the relationship between ETV5 expression and clinicopathological characteristics.

Besides the ERK1/2 pathway, which is closely associated with malignant phenotypes of pancreatic cancer, there are two other major MAPK pathways, also called stress-activated protein kinase pathways including the c-Jun N-terminal kinase (JNK) pathway and the p38 MAPK pathway. The JNK and p38 MAPK pathways regulate the activity and expression of key inflammatory mediators, including cytokines and proteases, which are known to be involved in carcinogenesis and drug resistance[52]. Upregulation of JNK has been shown to be important in pancreatic cancer cells to promote the development of the malignant phenotype[53]. In addition, activation of p38 has been reported to be involved in drug resistance to gemcitabine and 5-fluorouracil, the main drugs against pancreatic cancer[54,55]. Thus, the MAPK pathway is supposed to be deeply involved in the acquisition of malignant phenotypes and therapeutic resistance in pancreatic cancer. Further elucidation of the MAPK pathway, focusing on the ERK, JNK and p38 pathways, may lead to the development of effective treatments for pancreatic cancer.

Materials and methods

Human pancreatic cancer cell lines

Human pancreatic cancer cell lines AsPC-1, MIAPaCa-2, and PCI-35 were used in this study. AsPC-1 (https://www.atcc.org/products/crl-1682) and MIAPaCa-2 (https://www.atcc.org/products/crm-crl-1420) were obtained from the American Type Culture Collection (Manassas, VA, USA), and PCI-35 (https://cancercelllines.org/cellline/?id=cellosaurus:CVCL_5191) was provided by Dr. Hiroshi Ishikura[56]. Unless otherwise stated, AsPC-1 and PCI-35 were cultured with RPMI-1640 with 10% fetal bovine serum, and MIAPaCa-2 was cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum.

Inhibition of ERK1/2 pathway

Cells of the human pancreatic cancer cell lines were seeded at 5×10^5 cells per dish in 10-cm culture dishes and cultured under 37 °C in 5% CO2. After 24 h, the medium was replaced with medium containing U0126 (Sigma-Aldrich, St. Louis, MO, USA), a MEK/MAP2K inhibitor, dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 μmol/L, or with medium containing only the same amount of DMSO without U0126 as a control. After incubation for 24 h, RNA isolation and protein extraction were performed.

Whole transcriptome sequencing

This study used data from RNA sequencing previously performed in our laboratory[7]. The procedure was as follows. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The isolated RNA was constructed into a fragment library using TruSeq Stranded Total RNA LT Sample Prep Kit Gold (Illumina, San Diego, CA, USA). Constructed libraries were subjected to

total transcriptome enrichment using a NovaSeq 6000 S4 Reagent Kit. The prepared transcriptome libraries were sequenced on an Illumina NovaSeq 6000 platform using the paired-end sequencing method. All procedures were performed according to the manufacturer's instructions. Reads were aligned to GRCh38 using HISAT2. Around one hundred million reads were mapped per sample. Known genes and transcripts were assembled with StringTie based on the reference genome model. During data preprocessing, low-quality transcripts were filtered out. Afterward, log2 transformation of fragments per kilobase of exon per million mapped reads (FPKM) + 1 and quantile normalization were performed. Genes showing two-fold or more changes in expression by ERK1/2 attenuation were interpreted as significant.

qRT-PCR

Complementary DNA (cDNA) was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Subsequently, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of *ETV5* was performed using TaqMan Gene Expression Assay, TaqMan Fast Advanced Mix, and Applied Biosystems 7500 Real-time PCR system (Applied Biosystems). Expression levels were calculated by the ΔΔCt method using *GAPDH* levels as an internal control. All procedures were performed according to the manufacturer's instructions.

Immunoblotting

Cells were lysed in RIPA buffer (Sigma-Aldrich) with Complete Mini, proteinase inhibitor, and PhosSTOP, phosphatase inhibitor (Roche Diagnostics, Rotkreuz, Switzerland). The cell lysates containing 20 μg of protein was added to 6×SDS sample buffer and then boiled at 95 °C for 5 min. The boiled samples were centrifuged briefly and supernatants were used for electrophoresis in a 5-12.5% or 10–20% gradient polyacrylamide gel (DRC, Tokyo, Japan). The gel was blotted to Clear Blot Membrane-p (ATTO, Tokyo, Japan). The blot was blocked using PBS with 0.1% Tween-20 containing 2% bovine serum albumin or 2% ECL Prime Blocking Reagent (GE Healthcare, Chicago, IL, USA). Primary antibodies used were monoclonal anti-activated MAP kinase (diphosphorylated ERK1 and 2) (clone MAPK-YT; 1:1000; Sigma-Aldrich), monoclonal anti-p44/42 MAPK (Erk1/2) (clone 137F5; 1:2000; Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-ETV5 (clone E5G9V; 1:1000: Cell Signaling Technology), monoclonal anti-ETV4 (clone 16; 1:200: Santa Cruz Biotechnology Inc., Dallas, TX, USA), monoclonal anti-SNAI1 (clone G-7; 1:200: Santa Cruz Biotechnology Inc.), monoclonal anti-beta actin (clone AC-15; 1:1000; Sig-ma-Aldrich), and monoclonal anti-VEGF (clone JH121; 1:100; Thermo Fisher Scientific, Waltham, MA, USA). Secondary antibodies used were a horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (1:30,000; GE Healthcare) or an HRP-conjugated anti-rabbit immunoglobulin (1:10,000; Cell Signaling Technology). These antibodies, except the anti-ETV5, anti-SNAI1 and anti-ETV4 antibodies, were diluted in Can Get Signal (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. The anti-ETV5 antibody was diluted in PBS with 0.1% Tween-20 containing 5% bovine serum albumin (Sigma-Aldrich). The anti-SNAI1 and anti-ETV4 antibodies were diluted in PBS with 0.1% Tween-20 containing 2% ECL Prime Blocking Reagent (GE Healthcare). The primary antibody reactions were performed overnight. Signals were visualized by reaction with ECL Prime Detection Reagent (GE Healthcare) and digitally processed using LAS 4000 mini-CCD camera system (Fujifilm, Tokyo, Japan). The protein levels of pERK, ERK and ETV5 were quantified by densitometry using ImageJ software[57]. The band densities were normalized with β-actin.

Promoter assay

A candidate promoter region of 2458 base pairs (bp) in adjacent upstream of exon 1 of *ETV5* (UCSC Genome Browser GRCh38/hg38; https://genome-asia.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu, accessed on 18 May 2022) was amplified using CloneAmp HiFi PCR Premix (TaKaRa Bio Inc., Shiga, Japan) with paired primers of 5′-CGAGATCTGCGATCTACTGAAGTTACACAGCAGAC-3′ and 5′-AGTACCGGAATGCCACTCCGCGCACCAGCGGCTGG-3′, which contained part of vector sequences for the in-fusion cloning, and human genomic DNA as a template under the following condition: initial denaturation for 2 min at 94 ∘C, 40 cycles of reactions comprising 10 s at 98 ∘C, 10 s at 55 ∘C, and 150 s at 72 ∘C. The amplified products were pretreated with Cloning Enhancer and cloned into the reporter vector PGV-P2 (TOYO B-NET, Tokyo, Japan) using In-Fusion HD Cloning Plus CE (TaKaRa Bio Inc.) according to manufacturers' instructions. The original PGV-P2 vector was digested with HindIII (Roche Diagnostics) and XhoI (TaKaRa Bio Inc.), followed by blunting digested ends and self-ligation to create None vectors that lacks the SV40 promoter region for constant expression of the luciferase gene. Truncated promoter vectors were created by digestions with various restriction enzymes including BglII (Roche Diagnostics), Hind III, NheI (New England Biolabs), SacII (New England Biolabs), and SmaI (New England Biolabs), Nucleotide sequences were confirmed using Bigdye terminator (Thermo Fisher Scientific) and 3500xL Genetic Analyzer (Applied Biosystems) according to manufactures' instructions.

Cells of the human pancreatic cancer cell lines were seeded at 1×10^5 cells per well in 6-well plates, and 24 h after seeding, 0.5 μg of either of the constructed reporter vectors, 0.05 μg of phRL-TK vector (Promega, Madison, WI, USA) were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent according to manufacturer's instructions. 24 h after the transfection, cells were washed with PBS and lysed in Lysis buffer (Promega). A dual luciferase assay was performed using Dual Luciferase Assay Kit (Promega) and Centro LB960 (Berthold, Bad Wildbad, Germany) according to manufacturers' instructions.

To examine alterations of promoter activities when the ERK1/2 pathway was inhibited, co-transfectants of both any of the constructed reporter vectors and phRL-TK vector were incubated with media containing U0126 dissolved in DMSO to a final concentration of 10 μmol/L, or with medium containing only the same amount of DMSO without U0126 as a control. 24 h later, cells were collected and subjected to the dual luciferase assay.

The web-based transcription factor binding site search program, Match (http://www.gene-regulation.com/cgi-bin/pub/ programs/match/bin/match.cgi, accessed June 20, 2023), was used to find candidate transcription factor-binding sites. Reporter vectors with mutated sequences of candidate consensus binding sites were created by the site-directed mutagenesis using the QuikChange II Site-directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) (Table 2). The sequence of the constructed vectors was confirmed by Sanger sequencing using Bigdye terminator and 3500xL Genetic Analyzer (Applied Biosystems).

ChIP assay

Cells of AsPC-1 and MIAPaCs-2 were seeded 4×10^6 cells per dish in 10-cm culture dishes. 24 hours after the seeding, cells were fixed with 1% formaldehyde solution and collected. The collected cells were sonicated in a Biorupter (Cosmobio, Tokyo, Japan) and immunoprecipitated with monoclonal anti-ETS-1 antibody (clone D8O8A; Cell signaling Technology) or Normal Rabbit IgG, a nonspecific immunoglobulin (Cell signaling Technology) using ChIP-IT Express kit (Active Motif, Carlsbad, CA, USA). Paired primers of 5'-CTTGCTGGA TGGAGAAGTG-3' and 5'-GGAAGCTTAGCTGAGTCAGTG-3' to amplify a 148 bp region centered on the M8 site and Platinum PCR SuperMix High Fidelity (Invitrogen) were used for the PCR reaction.

Cell proliferation assay

Cells of the human pancreatic cancer cell lines were seeded 3×10^3 cells per well in 96-well plates or 3×10^5 cells per well in 6-well plates for sample collection of western blotting. 24 h after seeding, the cells were transfected with siRNA for *ETV5*, si#1, sense: 5′-CGAUUAUACUUUUGACGACAtt-3′, antisense: 5′-UGUCGUCAAAGUAUAAUCGgg-3′ (Thermo Fisher Scientific), or Silencer Select Negative Control (Thermo Fisher Scientific) at 100 nmol/L using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. 24 h after the transfection, qRT-PCR was performed with samples collected from 96 well plates and western blotting was performed with samples collected from 6 well plates to evaluate alterations of *ETV5* expression. Every 24 h up to 6 days, the medium was replaced with 100 µL of 0.05% 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyltetrazolium bromide (MTT, Sigma-Aldrich) and incubated for 1 h under 37 °C in 5% CO2. After the incubation, the MTT solution was removed, and the cells were suspended in 100% ethanol. Absorbance was measured at 590 nm using VersaMax Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA).

Scratch wound assay

The human pancreatic cancer cells were seeded 6×10^5 cells per well in 6-well plates and then transfected with si#1 in the same manner as described above when 90% confluent. 24 h after the transfection, the plates were straightened with sterile 10 µl pipette tips, washed slowly with PBS, and incubated in medium containing 1% fetal bovine serum. Images were taken using inverted microscope ECLIPSE TE200 (Nikon, Tokyo, Japan) 12 and 24 h after scratching. Only 12 h data were used for analysis in PCI-35 because the wound was completely filled 24 h after scratching. Alterations of wound area associated with cell migration were calculated using Image J software[57]. RNA isolation and qRT-PCR were performed on these samples after the assays (48 h after the transfection of si#1) as described above.

Invasion assay

Cell invasion of AsPC-1 was assessed using CytoSelect 24-Well Cell Invasion Assay, Basement Membrane (Cell Biolabs, San Diego, CA, USA). AsPC-1 was seeded in 10-cm dishes and transfected with si#1 in the same manner as described above. 24 h after the transfection, 5×10^5 transfected AsPC-1 were seeded in the inserts, and cells that passed through the membrane were evaluated after another 24 h. Other processes were performed according to the manufacturer's instructions.

Chemosensitivity assay

Cells of the pancreatic cancer cell lines, AsPC-1, MIAPaCa-2, and PCI-35, were seeded at 2.0×10^5 /well in 6 -well plate. The siRNA, either si#1 or siNC, was administered as described above. Cells were replated at three days later at 1.0×10^3 /well in 96-well plate. Gemcitabine was administered at either 0 (controls with DMSO only) or 10 µg/ml at final concentration at 24 h later. AlamarBlue™ assay (Thermo Fishers Inc.) was performed at 48, 96, and 120 h later. The chemosensitivity was assessed as relative amount of siRNA transfected viable cells of gemcitabine-treated compared to amount of viable cells of the control without siRNA transfection and gemcitabine treatment in average of those of 5-wells at each time point.

ETV5 immunohistochemical expression of pancreatic Cancer resection specimens after neoadjuvant chemotherapy

Formalin-fixed and paraffin embedded (FFPE) tissues obtained from 112 pancreatic cancer patients who underwent pancreatectomy at Tohoku University Hospital between January 2010 and March 2020 after neoadjuvant chemotherapy with gemcitabine and S-1 for routine histopathological diagnoses were used for immunohistochemical assays for ETV5 expression. Immunohistochemistry was done using polyclonal anti-ETV5 antibody (HPA063065, 1:100; Sigma-Aldrich) and Histofine Simple Stain MAX-PO kit (Nichirei Biosciences, Tokyo, Japan). Specificity and appropriateness of the anti-ETV5 antibody for immunohistochemistry was validated by the manufacturer and provided on the manufacturer's website (https://www.sigmaaldrich.com/US/en/product/sigma/hpa063065). A chromogen reaction was performed using 3,3'-diaminobenzidine. The specimens were classified as positive when obvious ETV5 expression was found in some of the pancreatic cancer tissues, and negative when no obvious ETV5 expression was observed. Immunohistochemistry specimens were evaluated by a Japanese Medical Specialty Board-certified pathologist (T.F.) blinded with clinical information.

Clinical information was collected retrospectively from the electronic medical record through April 30, 2023. Survival of the subject patients was defined as the date of surgery for pancreatic cancer up to the latest date when survival was confirmed in the electronic medical record. Among these samples, 37 samples were examined for *KRAS* mutations as described previously[21]. The relationship between *KRAS* mutations and the expression of ETV5 was evaluated.

TCGA open-access database analysis

RNA sequencing data from 260 pancreatic cancer patients with available clinical data were downloaded from TCGA portal (https://portal.gdc.cancer.gov, accessed 8 August 2023). To investigate the relationship between *ETV5* RNA expression and overall survival, 260 patients were divided into high ($n=130$) and low ($n=130$) expression groups, using the median value of *ETV5* transcripts per million as a threshold. According to Moffit classification of PDAC into basal-like and classical[22], the RNA expression levels of *ETV5* were compared in 62 cases with the basal-like subtype and 72 cases with the classical subtype available from TCGA portal (accessed 7 January 2025).

Statistical analysis

All experiments were conducted at least twice. For analysis of in vitro experiments, a Welch's t-test was performed for comparison between two groups. When comparisons between the two groups were needed more than once, we used the Bonferroni method and corrected for the level of significance. For the analysis of immunostaining for ETV5 in pancreatic cancer resection specimens after neoadjuvant chemotherapy, two groups divided by the pattern of ETV5 expression were compared by the Wilcoxon rank-sum test for continuous variables and by the Fisher's exact test for categorical variables. Kaplan-Meier curves were generated to compare overall survival rates between two groups according to differences in ETV5 expression using the log-rank test. Cases that were lost to follow-up at the hospital and whose survival was unknown were treated as censored. Unless otherwise noted, P values less than 0.05 in two-tailed were judged as significant. JMP Pro software version 17.0.0 (SAS Institute, Cary, NC, USA) was used for statistical analyses.

Data availability

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

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A.N.: Investigation, Formal analysis, Writing-Original draft preparation. M.K., Y.S., T.I., T.K., Y.O., Y.O., and Y.M.: Investigation. M.I., M.M., and K.N.: Resources. M.U.: Resources, Supervision. T.F.: Conceptualization, Methodology, Supervision, Writing - Review & Editing, Visualization, Funding acquisition. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
This study was approved by the Research Ethics Committee of the Tohoku University Graduate School of Medicine (2023-1-075; approved date 2023/4/26). All procedures involving human participants were conducted in accordance with the ethical standards of the Research Ethics Committee and Declaration of Helsinki (1964). Informed consent to use clinically obtained specimens for non-specified research purposes was obtained from all patients when the surgical materials were collected.

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