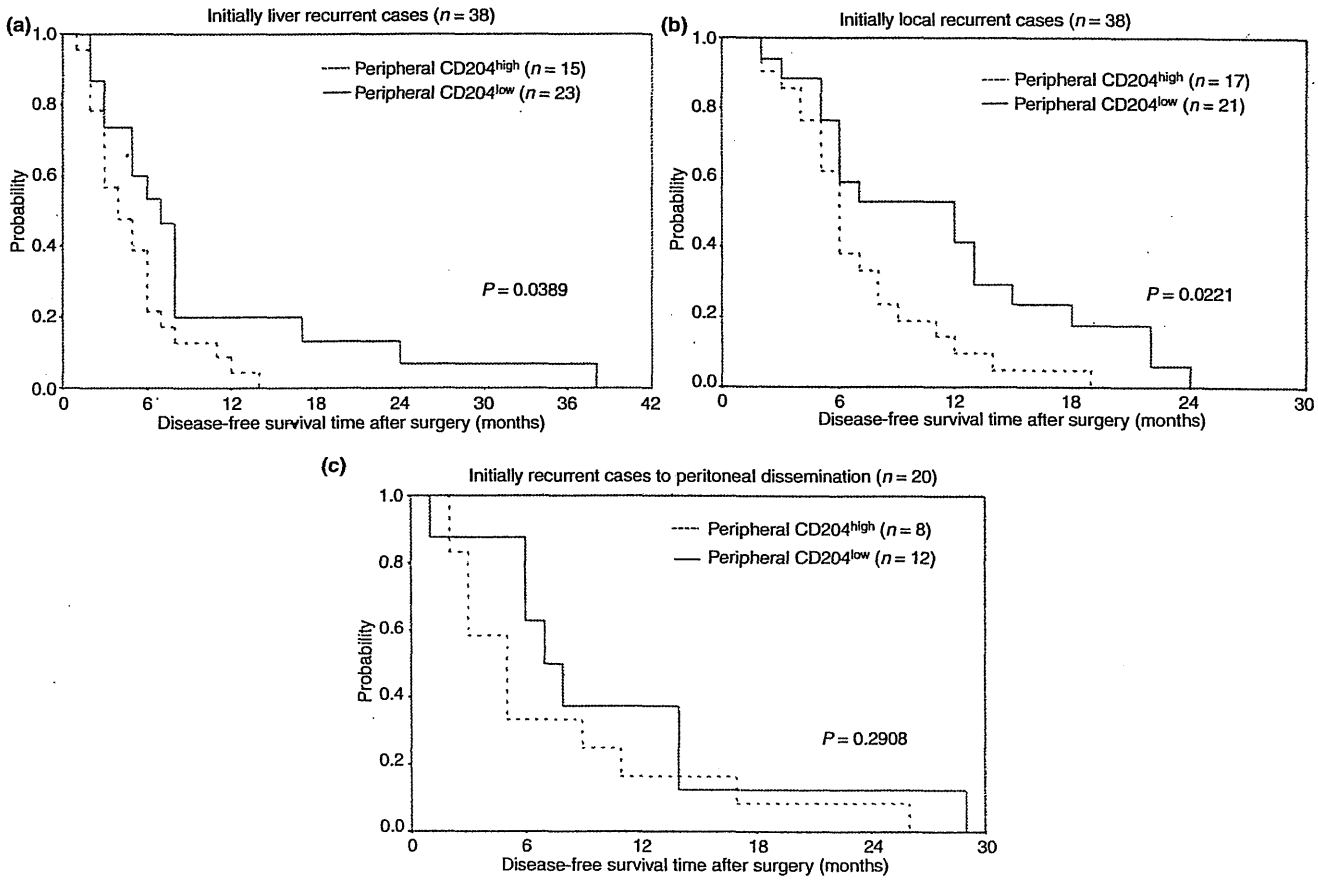

Fig. 4. Disease-free survival curves of invasive ductal carcinoma of the pancreas according to the area ratio of peripheral CD204-positive cells >3.39% and ≤3.39% in three groups that showed initial recurrence in the liver (a), local recurrence (b), and peritoneal dissemination (c). Peripheral CD204^{high} cases showed significantly shorter disease-free survival times in the groups with initial liver metastasis and initial local recurrence.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlation between immunopositive cell count and cell area ratio.

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MOLECULAR CANCER

RESEARCH Open Access

Targeting of MAPK-associated molecules identifies SON as a prime target to attenuate the proliferation and tumorigenicity of pancreatic cancer cells

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Abstract

Background: Pancreatic cancer is characterized by constitutive activation of mitogen-activated protein kinase (MAPK). Activation of MAPK is associated with the upregulation of genes implicated in the proliferation and survival of pancreatic cancer cells. We hypothesized that knockdown of these MAPK-associated molecules could produce notable anticancer phenotypes.

Methods: A RNA interference-mediated knockdown screening of 78 MAPK-associated molecules previously identified was performed to find molecules specifically associated with proliferation of pancreatic cancer cells *in vitro*. Expression of an identified molecule in pancreatic cancer tissues was examined by immunohistochemistry. *In vivo* tumorigenicity of cancer cells with stable knockdown of the molecule was assayed by using xenograft models. Flow cytometry and live cell imaging were employed to assess an association of the molecule with cell cycle.

Results: The knockdown screening revealed that knockdown of *SON*, the gene encoding SON, which is a large serine/arginine-rich protein involved in RNA processing, substantially suppressed pancreatic cancer cell proliferation and survival *in vitro* and tumorigenicity *in vivo*. SON expression was higher in ductal adenocarcinomas than in cells of normal ducts and precursor lesions in pancreatic cancer tissues. Knockdown of *SON* induced G2/M arrest and apoptosis in cultured cancer cells. The suppressive effect of *SON* knockdown on proliferation was less pronounced in cultured normal duct epithelial cells. SON formed nuclear speckles in the interphase of the cell cycle and dispersed in the cytoplasm during mitosis. Live cell imaging showed that SON diffusely dispersed in the early mitotic phase, accumulated in some foci in the cytoplasm in the late mitotic phase, and gradually reassembled into speckles after mitosis.

Conclusion: These results indicate that SON plays a critical role in the proliferation, survival, and tumorigenicity of pancreatic cancer cells, suggesting that SON is a novel therapeutic molecular target for pancreatic cancer.

Keywords: SON, MAPK, RNA interference, Speckle, Cell cycle

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Background

Pancreatic cancer is a leading cause of cancer-related deaths [1,2]. Despite advancements in diagnostic and therapeutic modalities, the 5-year survival rate of patients with pancreatic cancer is less than 10% [3]. This poor prognosis elicits an urgent need for the development of effective diagnostic and therapeutic measures to improve patient survival. Molecular medicine may be able to fulfill this need, as exemplified by imatinib in the treatment of chronic myeloid leukemia [4]. Pancreatic cancer is characterized by constitutive activation of mitogen-activated protein kinase (MAPK), due to gain-of-function mutations in *KRAS* or *BRAF* and loss-of-function of dual specificity phosphatase 6 (DUSP6) [5-7]. Active MAPK translocates to the nucleus, activates transcription factors, and induces the expression of a variety of genes [8]. In a previous study, we screened the genome for downstream targets of MAPK and identified 78 molecules specifically associated with MAPK activity in pancreatic cancer cells [9]. These MAPK-associated molecules include molecules implicated in DNA replication, RNA editing, spindle formation, mitosis, signal transduction, and membrane trafficking. These biological processes play critical roles in the survival, maintenance, and proliferation of pancreatic cancer cells. We hypothesized that molecular targeting of these MAPK-associated molecules could result in notable anticancer phenotypes, as we previously observed by targeting *AURKA* [9,10]. In this study, we performed a systematic knockdown screening of MAPK-associated molecules in pancreatic cancer cells.

Results

Knockdown screening of MAPK-modulated genes in pancreatic cancer cells

We performed knockdown screening using a pancreatic cancer cell line, MIA PaCa-2, and custom-designed short interfering RNAs (siRNAs) targeting all the 78 MAPK-modulated genes that were previously identified and isolated in the cell line (Additional file 1: Table S1) [9]. The cells were transiently transfected with each of the 78 siRNAs, and *in vitro* proliferation was subsequently examined for 5 consecutive days. This screening showed that proliferation of cancer cells was suppressed to variable degrees depending on the individual gene targeted (Figure 1). Knockdown of *AURKB, CENPA, EBNA1BP2, GOLT1A, KIF11, NEDD4L, SON, TPX2,* or *WDR5* suppressed proliferation by more than 50% compared with control. Among these targets, we focused on *SON* for further study because it showed the most substantial suppressive effect. This gene encodes a nuclear speckle protein, SON, which is involved in RNA processing.

Knockdown of *SON* attenuates proliferation in vitro, considerably in pancreatic cancer cells but less remarkably in normal phenotype cells

The *in vitro* suppressive effect of siRNA targeting *SON* on proliferation was reanalyzed in detail by using MIA PaCa2; PCI-35, a pancreatic cancer cell line with an aggressive phenotype; and HPDE, an immortalized normal human pancreatic duct epithelial cell line [7,11-13]. The suppressive effects of *SON* knockdown on cell proliferation appeared to be fatal in MIA PaCa-2, static in PCI-35, and insignificant in HPDE (Figure 2A). The effects of siRNA on SON expression were assayed by an immunoblotting method, which showed 77%, 10%, and 48% reduction of SON expression in MIA PaCa-2, PCI-35, and HPDE, respectively (Figure 2B). These results indicated that *SON* knockdown attenuated the *in vitro* proliferation of pancreatic cancer cells. The attenuation of proliferation depended on the efficiency of SON knockdown in pancreatic cancer cells, but was less remarkably affected in normal phenotype cells.

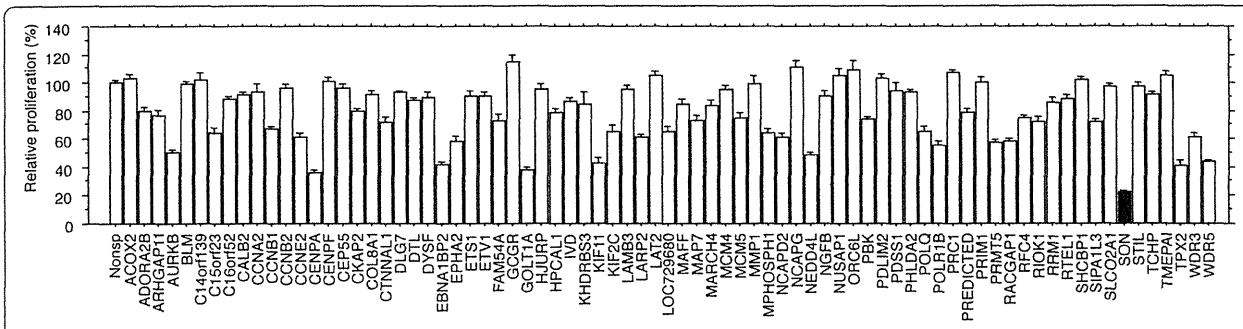

Figure 1 Knockdown screening of MAPK-associated genes in pancreatic cancer cells. Proliferation of MIA PaCa-2, a pancreatic cancer cell line, transfected with siRNA targeting various genes associated with MAPK (indicated on the horizontal axis), was determined by MTT assay on day 5 post-transfection. Plotted values are expressed relative to cells infected with control siRNA to a nonspecific sequence (Nonsp). Plots represent an average of 2 independent experiments; each experiment includes data from 8 independent transfection wells. Knockdown of *SON* (closed column) showed the most remarkable anti-proliferative phenotype.

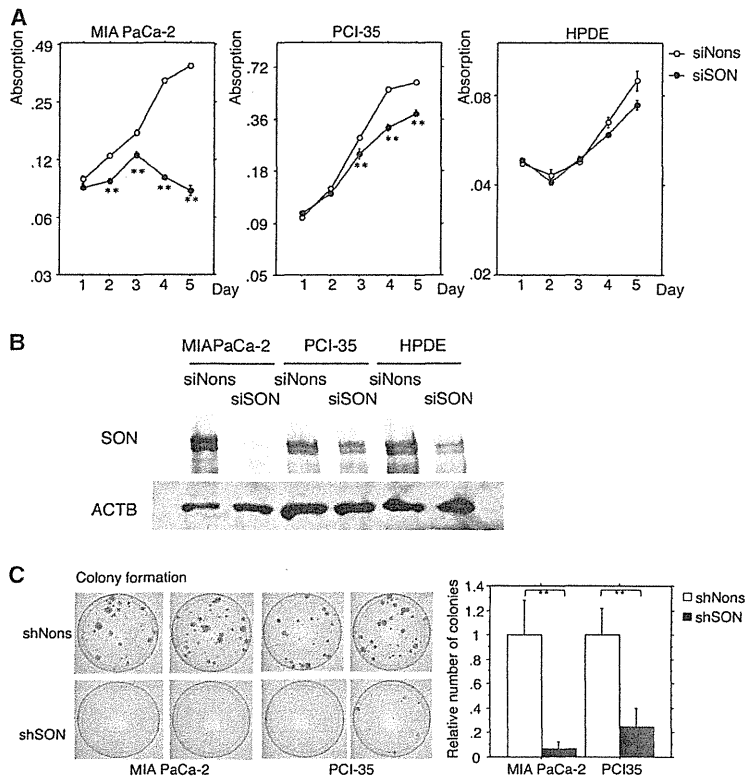

Figure 2 A. Proliferation of pancreatic cancer cells (MIA PaCa-2 and PCI-35) and normally phenotypic duct epithelial cells (HPDE) transfected with siRNA against *SON* (siSON) or a nonspecific sequence (siNons) and measured by MTT assay. The plots represent an average of 2 independent experiments; experiment includes data from 8 independent transfection wells. **B.** Expression of *SON* in cells transfected with siSON or siNons is shown in immunoblots probed with anti-SON antibody (SON) or anti-beta actin antibody (ACTB). **C.** Colony formation assay of pancreatic cancer cells transfected with vectors expressing shRNA targeting *SON* (shSON) or a non-specific sequence (shNons).

Stable knockdown of *SON* reduces the survival of pancreatic cancer cells in vitro

We next constructed a vector expressing short hairpin RNA (shRNA) identical to the *SON* siRNA when processed. We examined the effect of stable knockdown of *SON* on the survival of pancreatic cancer cells *in vitro* using a colony formation assay. We found that stable knockdown of *SON* strongly attenuated the survival of cancer cells, even in PCI-35 cells, in which transient transfection of siRNA targeting *SON* modestly suppressed proliferation (Figure 2C).

SON is overexpressed in pancreatic ductal adenocarcinomas

To establish the native expression of SON in pancreatic cancer, we examined 34 tissues with pancreatic ductal adenocarcinoma that were surgically resected. Immunohistochemistry showed that SON was strongly expressed in the nuclei of cancer cells in most ductal adenocarcinomas significantly more obviously than in the nuclei of non-neoplastic ducts or pancreatic intraepithelial neoplasia (PanIN), a precursor lesion of ductal adenocarcinoma ($p < 0.001$ by ANOVA) (Figure 3 and Table 1). This result indicates that SON is specifically overexpressed in pancreatic cancer.

Knockdown of *SON* retards the tumorigenicity of pancreatic cancer cells in vivo

We then performed a tumorigenicity assay using stably transfected pancreatic cancer cell clones carrying the shRNA vector targeting *SON*. Several stably transfected clones of MIA PaCa-2 and PCI-35 cells were obtained, and expression of *SON* was determined by real-time quantitative PCR. *SON* expression was lowest, reduced by 50%, in an MIA PaCa-2 clone (Figure 2D). We could not obtain any stably transfected PCI-35 clones in which *SON* expression was obviously reduced. This was probably because PCI-35, unlike MIA PaCa-2, could not survive modest knockdown of *SON*, which strongly suppresses the survival of cancer cells *in vitro*. The stably transfected clone of MIA PaCa-2 was inoculated into the subcutis of nude mice, and tumorigenicity was monitored. After 4 weeks, tumorigenicity was significantly retarded (Figure 4A).

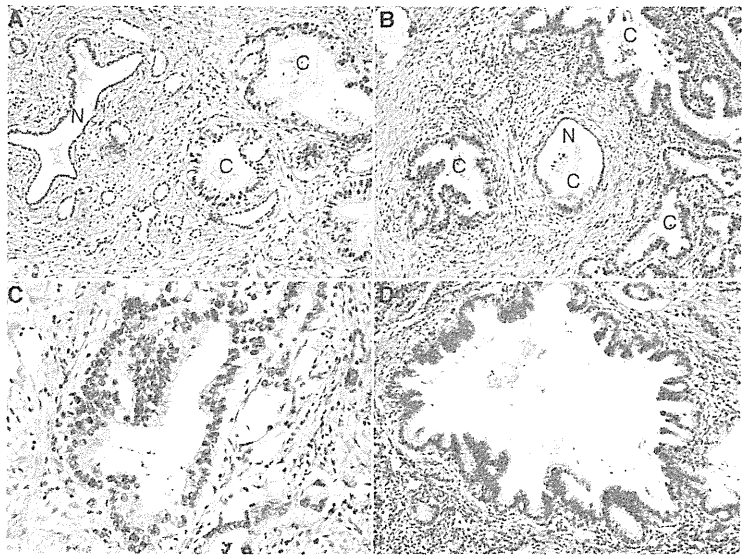

Figure 3 Immunohistochemical examination of SON expression in pancreatic cancer tissues. Diaminobenzidine and hematoxylin was used as a chromogen and a counter stain, respectively. **A** and **B**. Ductal adenocarcinomas (ducts labeled in C) strongly express SON in nuclei, more obviously than normal ductal cells (ducts labeled in N). The normal duct in panel B was partially (lower half) involved with carcinoma cells (original magnification, 100×). **C**. A high-powered view of ductal adenocarcinoma shows strong expression of SON in nuclei (original magnification, 200×). **D**. Pancreatic intraepithelial neoplasia, a precursor lesion of ductal adenocarcinoma, shows less obvious expression of SON (original magnification, 100×).

Knockdown of *SON* induces cell cycle arrest and apoptosis

To determine the mechanism by which *SON* knockdown suppresses the proliferation and survival of pancreatic cancer cells, the DNA content of siRNA-transfected MIA PaCa-2 and PCI-35 cells was measured by flow cytometry, and the cell cycle was assessed. Knockdown of *SON* increased the fraction of cells in G2/M and sub-G1, indicating that the cells were in G2/M arrest and apoptosis (Figure 4B).

SON shuttles between the nucleus and cytoplasm depending on the cell cycle

To investigate the dynamics of intracellular SON expression and its role in mitosis, a vector expressing SON, tagged with enhanced green fluorescence protein (EGFP) at the amino terminus (EGFP-SON), was constructed and transfected into 293 cells. The dynamics of intracellular SON expression were then analyzed. Expression of EGFP-SON was confirmed by immunoblotting by using specific antibodies against SON or EGFP (Figure 5A). Confocal laser scanning images showed that EGFP-SON was expressed as speckles in the nuclei of cells in the interphase and was dispersed in the cytoplasm of cells in the mitotic phase (Figure 5B). Time-lapse live imaging of cells expressing EGFP-SON showed that SON dispersed diffusely in the cytoplasm in metaphase and anaphase, accumulated in some foci in the cytoplasm during telophase and cytokinesis, and gradually reassembled in nuclear speckles after cytokinesis as foci in the cytoplasm faded (Figure 5C). From metaphase, the reassembly into nuclear speckles took approximately 2 hours. These results indicate that SON shuttles between the nucleus and the cytoplasm depending on the phase of the cell cycle, transitioning from nuclear speckles and through diffuse dispersion and subsequent temporal accumulation in the cytoplasm, to slow reassembly into nuclear speckles during mitosis and the early G1 phase.

Table 1 Expression of SON in ductal lesions evaluated by immunohistochemistry

Ductal lesion	Total number of lesions	Intensity score			*P* (ANOVA)
		1, weak	2, moderate	3, strong	
Ductal adenocarcinoma	34	0	3	31	< 0.001
PanIN	23	15	8	0	
Normal	29	24	5	0	

Discussion

In this study, among many genes associated with MAPK, we found that knockdown of *SON* remarkably suppressed the proliferation, survival, and tumor formation of pancreatic cancer cells. The suppressive effect was less pronounced in normally phenotypic ductal cells. In primary pancreatic cancer tissues, *SON* was overexpressed in

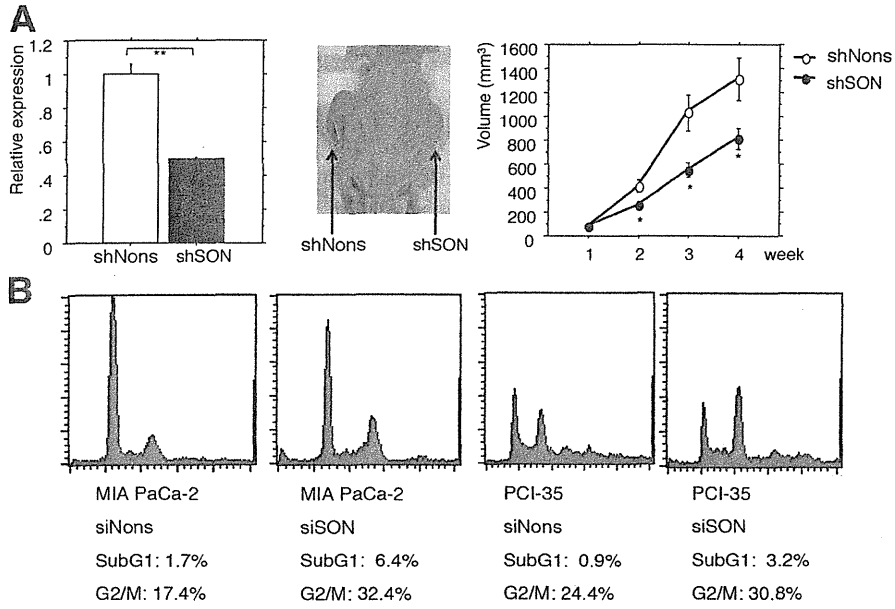

Figure 4 A. Left panel: Expression of SON in cloned cells (MIA PaCa-2) stably transfected with shSON or shNons. Middle panel: An example image of a nude mouse with a xenograft of MIA PaCa-2 clones stably transfected with shSON or shNons at 4 weeks after inoculation in the subcutis. Right panel: Average growth of tumors from xenografts of MIA PaCa-2 cells stably transfected with shSON or shNons in the subcutis of 4 nude mice. One (*) and 2 (**) asterisks indicate $p < 0.05$ and $p < 0.01$, respectively. Error bars denote 1 value of standard error. **B.** Cell cycle fractions of pancreatic cancer MIA PaCa-2 and PCI-35 cells transfected with short interfering RNA (siRNA) against *SON* (siSON) or a nonspecific sequence (siNons) as determined by flow cytometry.

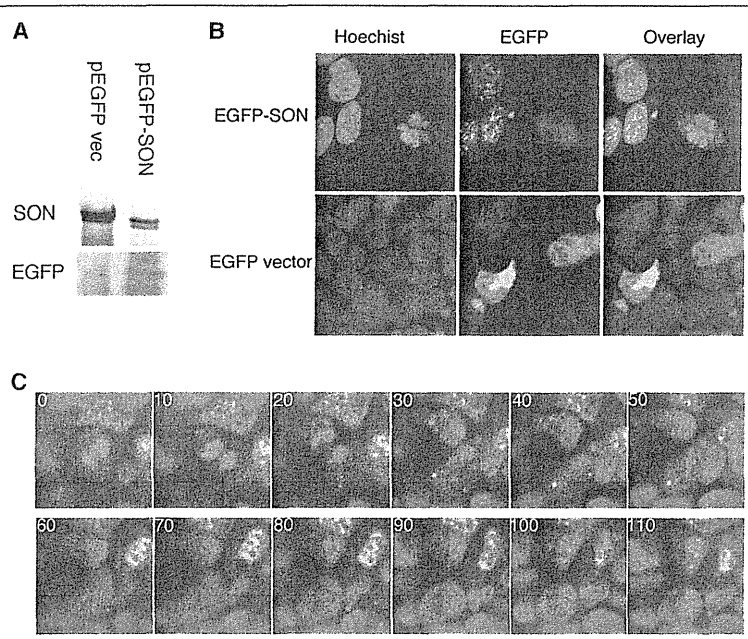

Figure 5 A. Immunoblot of total cell lysate from 293 cells transfected with pEGFP vector or pEGFP-SON probed with antibodies against EGFP or SON. B. Fluorescence images of 293 cells transfected with pEGFP-SON or pEGFP-vector. Note that EGFP-SON was speckled in the nuclei of interphase cells and diffusely dispersed in mitotic cells. Original magnification, 600×. **C.** Time-lapse images of 293 cells transfected with EGFP-SON. Sequential images taken at 10-minute intervals are shown (the number on each image indicates minutes from starting). EGFP-labeled SON dispersed in the cytoplasm during metaphase and anaphase (panels at 0 and 10 minutes), accumulated in small foci in the cytoplasm during telophase and cytokinesis (panels between 20 and 50 minutes), and then gradually reassembled in nuclear speckles (panels at 60 minutes and later). Original magnification, 400×.

ductal adenocarcinomas compared with normal duct cells and PanINs. Knockdown of *SON* induced G2/M arrest and apoptosis. SON shuttled between the nucleus and cytoplasm depending on the phase of cell cycle. These results indicate that SON plays a crucial role in the proliferation, survival, and tumorigenicity of pancreatic cancer cells, thus suggesting that this molecule could be a prime therapeutic molecular target for pancreatic cancer.

Our investigation showed that knockdown of MAPK-associated molecules suppressed the proliferation of pancreatic cancer cells *in vitro* to variable degrees. We found that knockdown of *AURKB, CENPA, EBNA1BP2, GOLT1A, KIF11, NEDD4L, SON, TPX2,* or *WDR5* strongly suppressed the proliferation. *AURKB* encodes aurora kinase B (AURKB), which is involved in chromosome segregation and cytokinesis during mitosis [14]. *CENPA* encodes centromere protein A (CENPA), which, by functioning as a replacement for histone H3 in centromeric nucleosomes, plays an essential role in kinetochore formation and functions in cellular mitosis [15]. *EBNA1BP2* encodes a ribonucleoprotein, Epstein-Barr virus nuclear antigen 1-binding protein 2 (EBNA1BP2), which serves as a scaffold for ribosome biogenesis [16]. *GOLT1A* encodes Golgi transport 1A (GOLT1A), which functions as a transporter on the Golgi membrane [17]. *KIF11* encodes a microtubule-dependent motor protein, kinesin family member 11 (KIF11), which plays a critical role in chromosome positioning during mitosis [18]. *NEDD4L* encodes neural precursor cell expressed, developmentally down-regulated 4-like, an E3 ubiquitin protein ligase (NEDD4L) that plays a role in polyubiquitination and proteasomal destruction of SMAD2/3 [19]. *TPX2* encodes a homologue of Tpx2 of *Xenopus* (TPX2), a binding partner of aurora kinase A (AURKA) that plays a role in microtubule spindle formation [20]. *WDR5* encodes WD repeat domain 5 (WDR5), which binds methylated histone H3 lysine 4 (H3K4) and is required for recruiting H3K4 methyltransferase [21]. Among these, AURKB, CENPA, KIF11, and TPX2 are involved in functions of the microtubule spindles and kinetochores, which are considered essential for cell mitosis. Because we screened by assaying the effects of knockdown of the MAPK-associated genes on *in vitro* proliferation of pancreatic cancer cells, molecules associated with the microtubules and kinetochores might be selectively represented in our screening. Interestingly, these microtubule kinetochore-associated molecules have already been studied as molecular targets in various cancers [22-25]. Nevertheless, of these MAPK-associated molecules, we found that knockdown of *SON* most remarkably suppressed proliferation, which led us to investigate *SON* in detail as a candidate molecular target.

SON encodes SON, a large protein harboring a serine or arginine-rich domain. It was first cloned as a gene encoding a protein with DNA-binding activity. However, subsequently, it turned out to be a nuclear speckle protein involved in RNA processing and required for proper and efficient splicing of pre-mRNAs [26-30]. In our study, knockdown of *SON* attenuated the proliferation, survival, and tumorigenicity of pancreatic cancer cells. These suppressive effects were attributable to cell cycle arrest at the G2/M phase and apoptosis induced by depletion of SON. The association between the depletion of SON and G2/M arrest has been reported to be associated with impairment of spindle pole separation, microtubule dynamics, and genome integrity due to inadequate RNA splicing of a specific set of cell cycle-related genes with weak splice sites, i.e., splice sites without the conserved sequence [30].

Pancreatic cancer cells were more susceptible to depletion of SON than normally phenotypic cells. This may be due to rapid progression through the cell cycle in cancer cells, which results in exaggerated dependence on SON to maintain efficient RNA processing of the cell cycle-related genes. This interpretation could be endorsed by the overexpression of SON we found in most ductal adenocarcinomas, compared with normal ductal cells or precursor lesions, which suggests that adenocarcinoma cells depend on *SON* more strongly than normal ductal cells and precursor lesions to maintain their phenotypes. These results suggest that depletion of *SON* may specifically lead to an anticancer phenotype. *SON* overexpression is purportedly due to the constitutive activation of MAPK in ductal adenocarcinoma; however, other possible causes, such as gene amplification or aberrations in protein turnover, cannot be ruled out and will be a subject of further study.

The dynamics of SON distribution during the cell cycle is not well known. We performed live-cell imaging of cells expressing EGFP-SON and observed that SON dispersed in the cytoplasm during early mitotic phase formed small foci in the cytoplasm in the late mitotic phase, and gradually redistributed as speckles in the nucleus as foci in the cytoplasm faded. The cytoplasmic small foci are supposed to be mitotic interchromatin granules that correspond to accumulations of nuclear speckle proteins in the cytoplasm in the late mitotic phase [31,32]. These dynamics seem similar to the dynamics of another speckle protein, SF2, and are consistent with the idea that SON plays a role in the appropriate organization of RNA splicing factors [29,33,34].

The knockdown of SON by RNA interference showed sufficient anti-cancer phenotypes experimentally. For the RNA interference, vector-mediated stable transduction appeared to be more effective than oligonucleotide-based

transient transduction as shown in Figure 2. Although the stable knockdown of *SON* by RNA interference could be an efficient molecular therapy for pancreatic cancer, the lack of a conventional method for tissue-specific, stable delivery of short, double-stranded RNA could limit the use of this approach in clinical therapeutics. Indeed, the use of RNA interference in clinical practice is generally not warranted. Recently, however, systemic delivery of siRNA combined with a special nanoparticle successfully knocked down a target gene in melanoma in a clinical trial [35]. The use of such a technique to attempt specific knockdown of *SON* in pancreatic cancer cells in a clinical model is worth trying and is an issue to be resolved in a future study. The results of this study also suggest that development of a molecule-oriented chemical substance against SON as therapy for pancreatic cancer is warranted.

Conclusion

This study indicates that SON is overexpressed and plays a critical role in the proliferation, survival, and tumorigenicity of pancreatic cancer cells, suggesting that SON is a novel therapeutic molecular target for pancreatic cancer.

Methods

Cell culture

Human pancreatic cancer cell lines, MIA PaCa-2 and PCI-35, and the human embryonic kidney cell line 293 were obtained and cultured as previously described [7,9]. The immortalized human pancreatic duct-epithelial cell line, HPDE, was kindly provided by Dr. MS Tsao (Princess Margaret Hospital and Ontario Cancer Institute, Toronto, ON) and cultured as previously described [12].

Transfection of siRNA and cell proliferation assay

siRNAs targeting each downstream MAPK-associated molecule were custom designed and manufactured (RNAi Co. Ltd., Tokyo, Japan) (Additional file 1: Table S1). Cells were seeded at 5×10^3 cells/well in 96-well plates with 100 μL of appropriate culture medium and incubated at 37°C with 5% CO_2 for 24 hours. Then, the medium was replaced with OPTI-MEM (Life Technologies, Carlsbad, CA), and the cells were transfected with siRNA at 10 nM with Oligofectamine (Life Technologies) according to the manufacturer's recommendations. After 4 hours of incubation, the transfection reagent was replaced with the appropriate culture medium. A colorimetric cell proliferation assay—3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay—was performed daily for 5 days as previously described [7].

Colony formation assay with shRNA vectors

pSUPER vector (Oligoengine, Seattle, WA) was used for the construction of vectors expressing shRNAs by cloning the oligonucleotides 5′-GATCCCCGCATCTA GACGTTCTATGATTCAAGAGATCATAGAACGTCT AGATGCTTTTTA-3′ and 5′-AGCTTAAAAAGCATC TAGACGTTCTATGATCTCTTGAATCATAGAACGTC TAGATGCGGG-3′ to target *SON* (shRNA-SON), and 5′-GATCCCCGTACCGCACGTCATTCGTATTCAAG AGATACGAATGACGTGCGGTACTTTTTA-3′ and 5′-AGCTTAAAAAGTACCGCACGTCATTCGTATCTCT TGAATACGAATGACGTGCGGTACGGG-3′ to serve as a control harboring a nonspecific sequence against the human genome (shRNA-Nons) according to the manufacturer's instructions. MIA PaCa-2 and PCI-35 cells were seeded at 1×10^5 cells/well in 6-well plates and incubated for 24 hours at 37°C with 5% CO_2. The shRNA-SON vector or shRNA-Nons vector were transfected into the cells with Lipofectamine™ reagent (Life Technologies) according to the manufacturer's recommendations. The cells were dissociated with trypsin 48 hours after transfection and reseeded in three 10-cm tissue-culture dishes, containing the appropriate culture medium supplemented with 10% FBS and G418 (Life Technologies) at 400 μg/mL for PCI-35 and 500 μg/mL for MIA PaCa-2. After 3 weeks, the cells were fixed with 10% formalin solution and stained with hematoxylin. The number of colonies was assessed with the COLONY program (Fujifilm Co. Ltd., Tokyo, Japan).

Immunohistochemistry

Thirty-four formalin-fixed, paraffin-embedded tissues of pancreatic ductal adenocarcinoma that were surgically resected during 2006 and 2007 at Tokyo Women's Medical University Hospital were studied. Indirect immunohistochemical staining was performed as previously described [36] by using a polyclonal anti-SON antibody (1:1200 dilution, Sigma, St. Louis, MO), a secondary antibody against rabbit immunoglobulin (Nichirei, Tokyo, Japan), and streptavidin solution (Nichirei). Use of the archival pathological tissues was approved by the ethics committee of Tokyo Women's Medical University. Immunohistochemical results were evaluated among ductal lesions classified into adenocarcinoma, PanIN, or normal duct by scoring intensities of staining into 1, weak; 2, moderate; and 3, strong by comparing with normal ductal cells that showed weak staining or acinar cells that showed moderate staining. The scores were statistically analyzed by ANOVA by using PASW Statistics software (IBM Japan, Tokyo, Japan).

Quantitative real-time polymerase chain reaction assay

The TaqMan Gene Expression Assay and a 7500 Real-time PCR system (Life Technologies) were used to analyze the transcriptional expression of *SON* by using the absolute quantitative assay according to the manufacturer's instructions. The expression of *SON* was

assessed relative to the endogenous expression of *GAPDH*.

In vivo *tumorigenicity assay*

Pancreatic cancer cells stably transfected with shRNA vectors were isolated by cloning the surviving cells from the colony formation assay. These clones, in 50% matrigel/culture medium without FBS, were inoculated into the subcutis of BALB/c nude mice (Clea Japan Inc., Tokyo, Japan). Tumorigenicity was monitored weekly, and the tumor volume was calculated using the following formula: $V = D \times d^2 \times 0.4$ (V, tumor volume; D, largest dimension; d, smallest dimension).

Flow cytometry

Flow cytometric analyses for cell cycle and apoptosis were performed as previously described [7].

Construction of the EGFP-SON vector

An expression vector containing the full coding sequence of *SON* cDNA (NM_138927) was constructed by assembling amplified products using KOD Plus DNA Polymerase and its specific buffer (TOYOBO, Osaka, Japan), appropriate paired primers, and pooled cDNA obtained from a fetal brain cDNA library (Stratagene/Agilent Technologies Inc., Santa Clara, CA) as follows. Paired primers used for amplification of cDNA fragments were C51, 5′-TTTAAGCTTATGGCGACCAACATCGAGCAG-3′ (melting temperature [Tm], 58°C) and C12, 5′-TAAGGGTGTTCTTGATCGCC-3′ (Tm, 52°C); C7, 5′-AGCCGCCGGAGAAGATCAAGG-3′ (Tm, 59°C) and C10, 5′-CAGGCTCTGAGGGCAAATTG-3′ (Tm, 53°C); and C5, 5′-TAAACTCAGTGAACCCAAACC-3′ (Tm, 50°C) and C52, 5′-TTTGGTACCTCAATACCTATTCAA GAAAAACATAC-3′ (Tm 48°C). Products amplified by PCR were sequentially cloned into the pFLAG-CMV-4 vector (Sigma, St. Louis, MO) at *Hin*dIII-*Eco*RI-*Kpn*I sites to obtain pFLAG-SON. The pEGFP-C2 vector (Clontech, Mountain View, CA) was modified by fill-in of its *Xho*I site to adjust the reading frame. The coding region of *SON* cDNA was prepared from pFLAG-SON by digestion with *Hin*dIII and *Kpn*I for the 3′ fragment and *Hin*dIII for the 5′ fragment. These fragments were sequentially cloned into the modified pEGFP-C2 vector at *Hin*dIII and *Kpn*I sites to obtain the pEGFP-SON vector. DNA sequences were confirmed by using BigDye® Terminator and a 3130x Genetic analyzer (Life Technologies).

Immunoblot

Denatured total cell lysate was separated in a 5–15% polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane by using an XV Pantera MP System (DRC Co., Ltd. Tokyo, Japan) according to the manufacturer's recommendations. The blotted membrane was probed with anti-SON antibody (Sigma), anti-beta actin antibody (Sigma), or anti-EGFP antibody (Clontech). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (GE Healthcare UK Ltd., Buckinghamshire, UK) were used for the secondary antibody reaction. Blocking conditions and concentrations of antibodies were determined according to the manufacturers' recommendations. Signals were visualized by reaction with ECL Detection Reagent (GE Healthcare UK Ltd.) and captured digitally by using an LAS 4000 Mini (Fujifilm Co. Ltd.) or by autoradiography. Intensities of bands were measured digitally using Image Gauge software (Fujifilm Co. Ltd.).

Laser scanning fluorescence imaging

The pEGFP-SON vector was transfected into 293 cells using Lipofectamine Plus (Life Technologies) according to the manufacturer's recommendations. The transfected cells were incubated with Eagle's Minimum Essential Medium (Sigma) supplemented with 10% FBS and 400 μg/mL G418. Stably transfected clones were obtained by cloning surviving cells using a cylinder cup. The isolated clones were seeded in a glass-bottom dish and incubated for 24 hours. The cells were incubated with a medium supplemented with 0.1 μg/mL Hoechst 33342 (Life Technologies) for 30 minutes. The medium was then replaced with fresh growth medium and examined under a confocal laser scanning microscope (LSM5, Carl-Zeiss Microimaging GmbH, Goettingen, Germany). Time-lapse images were obtained for 2 layers at 0- and 5-μm depth with 10-minute intervals over a total of 230 minutes.

Statistics

Student's t-test was applied to analyze statistical differences using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA). P values of <0.05 were considered statistically significant.

Additional file

Additional file 1: Table S1. Short interfering RNAs used in a systematic knockdown screening of MAPK-assoicated genes in pancreatic cancer.

Abbreviations

AURKA: Aurora kinase A; AURKB: Aurora kinase B; CENPA: Centromere protein A; DUSP6: Dual specificity phosphatase 6; EBNA1BP2: Epstein-Barr virus nuclear antigen 1-binding protein 2; EGFP: Enhanced green fluorescence protein; GOLT1A: Golgi transport 1A; H3K4: Histone H3 lysine 4; KIF11: Kinesin family member 11; MAPK: Mitogen-activated protein kinase; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NEDD4L: Neural precursor cell expressed, developmentally down-regulated 4-like, an E3 ubiquitin protein ligase; Nons: Non-specific sequence; PanIN: Pancreatic intraepithelial neoplasia; shRNA: Short hairpin RNA; siRNA: Short interfering RNA; TPX2: A homologue of Tpx2 of *Xenopus*; WDR5: WD repeat domain 5.

Competing interests
TF applied a patent on siRNAs used in this study. Other authors declare that they have no competing interests.

Authors' contribution
TF designed the study. TF and ET carried out *in vitro* and *in vivo* experiments and analyzed data. TF, YK, TH, MY, KShim, NS and KShir obtained, examined and analyzed surgical materials. TF wrote the manuscript. All authors had final approval of the submitted and published versions.

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Japan Pancreatic Cancer Registry; 30th Year Anniversary

Japan Pancreas Society

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Objectives: Since 1981, the Japan Pancreas Society has been hosting a nationwide pancreatic cancer registry. To commemorate its 30th anniversary, we review its history and latest achievement.

Methods: During 3 decades, more than 350 leading institutions in Japan contributed voluntarily to register and periodic follow-up. The registry was modified to protect privacy by encrypting and hash algorithm.

Results: From 1981 to 2007, 32,619 cumulative records were analyzed. The overall survival of invasive cancer was improved significantly. More patients with earlier stage or with intraductal and cystic neoplasms underwent resection. The strongest prognostic factor of Union for International Cancer Control (UICC) stage IIA and IIB tubular adenocarcinoma in the pancreatic head was histological grade, followed by tumor size, extent of lymph node dissection, and postoperative chemotherapy. The 5-year survival rate of Union for International Cancer Control stage 0 reached 85%. The improvement of survival of patients with invasive cancer in Japan can be attributed to the introduction of effective chemotherapies, regionalization, and the earlier diagnosis and treatment. Simple definition of "early pancreatic cancer" is needed.

Conclusions: At the 30th year anniversary, the Japan Pancreas Society nationwide pancreatic cancer registry is more shining than ever for current perspectives and for future diagnostic and treatment tactics.

Key Words: pancreatic cancer, nationwide registry, early diagnosis, surgical treatment, adjuvant therapy, classification

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The Japan Pancreas Society (JPS) has been conducting nationwide pancreatic cancer registry since 1981. This accomplished a magnificent and only-one database of not only pancreatic cancer but also other neoplastic disease including intraductal neoplasms, cystic neoplasms, neuroendocrine tumors (NETs), and others. Every record consists of more than 300 items regarding patients' background, diagnostic parameters, disease extension, treatment, and outcome. More than 350 leading institutions in Japan voluntarily contributed to its data collection and annual follow-ups. We have previously provided the progress and update[1,2] of our pancreatic cancer registry, and in this manuscript, we will review the history of pancreatic cancer registry in Japan and present its current accomplishment for the perspectives of diagnosis and treatment of pancreatic cancer.

HISTORY

After the establishment of JPS in 1969, the society grew rapidly, with clinicians and researchers exceeding 2000 in membership in 1981, when the nationwide pancreatic cancer registry was started. Before discussing the history of pancreatic cancer registry, we have to describe the history of pancreatic cancer classification in Japan and the world.

To make the registry successful, there has to be a rule for tumor classification. Otherwise, no scientific comparison is possible between the institutions, countries, and even with the historical controls. The TNM classification of cancer was developed in the late 1940s by Pierre Denoix at the Institute Gustave-Roussy.[3] The Union for International Cancer Control (UICC) first published TNM classification in 1953 and its first pocket book in 1968. The American Joint Committee on Cancer (AJCC) began publishing separate TNM classifications in the early 1980s, but AJCC and UICC classification was unified in 1987. As for pancreatic cancer, the TNM classification is currently in its seventh edition, which was not changed from the sixth edition revised in 2002.[4,5]

Partly owing to the difference of native language and partly owing to the difference of types of cancer-related death, the Japanese have developed their own tumor classifications. The first established Japanese tumor classification was for gastric cancer in 1963.[6] The JPS established the first version of rules for classification of pancreatic cancer in 1980. The rules had been periodically revised to the fourth edition, which resembles the UICC TNM classification in 1993. The first English version of the JPS classification was published based on this fourth edition in 1996.[7] The fourth JPS classification required grading description in every category, such as PV_0 (no infiltration to the portal venous system), PV_1 (suspicious infiltration), PV_2 (definite infiltration), and PV_3 (portal vein is stenotic by the invasion), which made the classification and registry complicated. In 2002, the JPS revised this grading simply to yes/no description in the JPS fifth version (English second version[8]) so that the classification can be as equal as the UICC/AJCC classifications. In the meantime, however, UICC had revised to its sixth version in 2002, which is the same with the current/seventh version. The JPS has published its seventh version in Japanese, and the third

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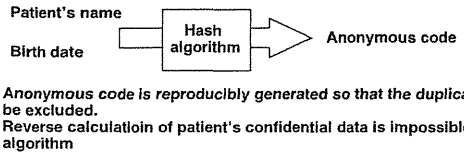

FIGURE 1. Anonymization by encrypting personal data using hash function. If a patient's name and birth date is perfectly the same, the anonymous code is the same. Same character in the anonymous code can be generated from multiple combination of name and birth date, making it impossible to recalculate the original name or birth date. The possibility of generating same anonymous code from different name is less than 1×10^{-20}. Each institution can identify individual patients easily.

English version will appear soon, but the concept of TNM is the same as its previous version in 2002 like UICC/AJCC.

From the beginning, the JPS conducted the pancreatic cancer registry, aiming at not only invasive cancer but also all neoplastic diseases including even benign adenomas, and the registry required the detailed description of the extent of the disease, so that the raw data were durable during several changes of the classification rules. For example, current JPS-T factor is as follows;

Tis: Noninvasive tumor (including mucinous cystic neoplasm, intraductal papillary mucinous neoplasm [IPMN], carcinoma in situ [CIS])
T1: Tumor limited to the pancreas, 2 cm or less in greatest dimension
T2: Tumor limited to the pancreas, more than 2 cm in greatest dimension
T3: Tumor that has extended into any of the following: bile duct, duodenum, peripancreatic tissue (anterior, and posterior [RP])
T4: Tumor that has extended into any of the following: adjacent large vessels (portal venous system, PV; and arteries [A]), extrapancreatic nerve plexus (PL), other organs (OO).

If bile duct, duodenum, A, RP, PV, arterial venous system, PL, and OO factors have been registered, the resulting T factor can be recalculated according to the change of rules. The invasive site was also recorded, such as superior mesenteric vein, portal vein, splenic vein, together with its arterial and plexus details. Similarly, the stations of lymph node metastasis and site of distant metastasis were reported according to the rules. In the change of 2002, PV_0 was converted to PV(−); PV_1, PV_2, and PV_3 were converted to PV(+); and all the data were recalculated according to the latest rule.

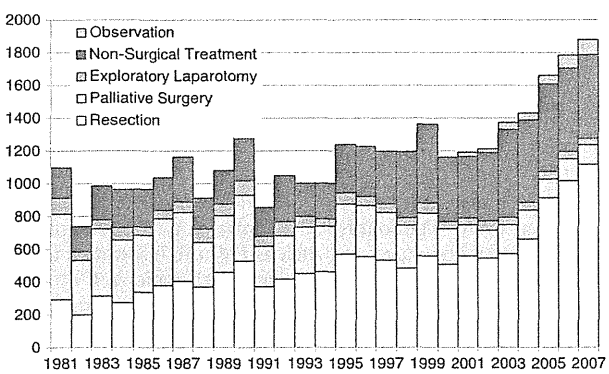

FIGURE 2. Trend of annual registry of all neoplasms. The number of patients treated and registered in each year. The number of patients who underwent pancreatectomy and nonsurgical treatment is increasing, whereas that with palliative surgery is decreasing.

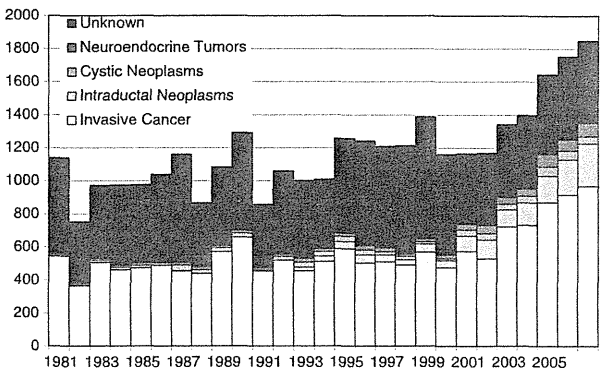

FIGURE 3. Trend of histological classification of all neoplasms. The number of patients with invasive cancer and INs is increasing, whereas that without histological confirmation is decreasing.

The pancreatic cancer registry was first conducted by Ryoichi Tsuchiya in Nagasaki University in 1981. The National Cancer Center jointly sponsored this registry because at that time, many other organizations and societies started their cancer registry. Because the registry required detailed recording on a data sheet and the rule should be widely spread, the manual of staging for the registration was published in 1986.[9] The annual report was published in Suizo in Japanese every year or every other year, and the retrospective review of surgical treatment was published in 1990.[10,11] Of the 7687 patients who were registered until 1990, 5826 cases (75.7%) underwent laparotomy, of whom 2311 (39.7%) underwent resection. At that time, the operative mortality rate was 4.5%. It should be noted that the rates for small carcinomas (>2 cm) were significantly higher than those for the tumors larger than 2 cm, and they insisted on early diagnosis. Then the registry was conducted by Yoichi Saito in Kobe University since 1989. Using the database, Satake et al[12] described the survival rate of patients with resected pancreatic cancer as much higher than that of patients with conservative treatment and emphasized the importance of early diagnosis of resectable pancreatic cancer, again. He offered the effectiveness of CA19-9 and elastase-1 as part of a screening program for early detection of cancer. Although the annual reporting in Suizo in Japanese continued,[13] the next English publication of pancreatic cancer registry appeared in

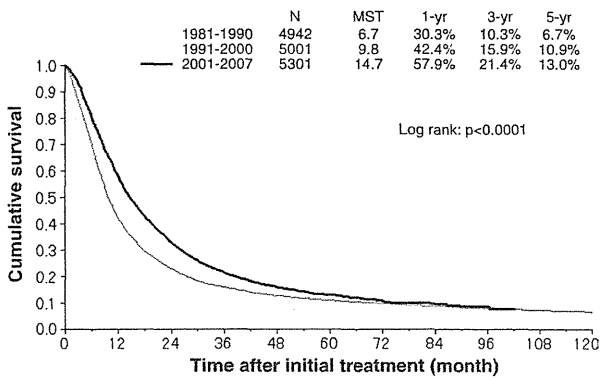

FIGURE 4. Survival of overall patients with invasive cancer. The overall survival significantly improved in the second and third decades.

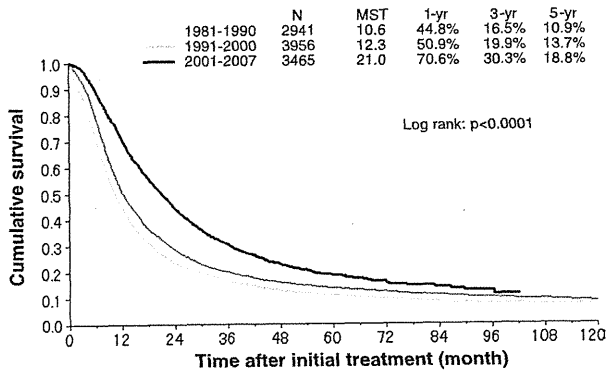

FIGURE 5. Survival of patients who underwent pancreatectomy for invasive cancer.

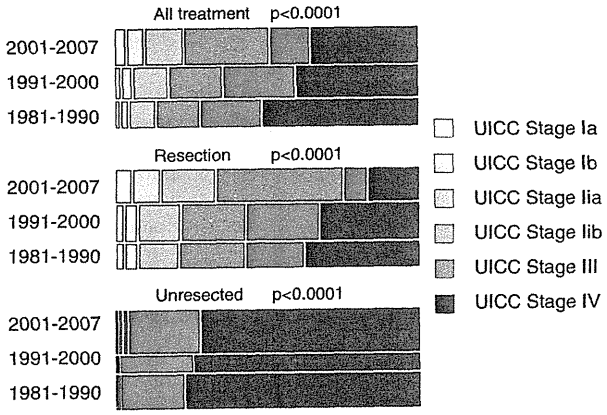

FIGURE 7. Union for International Cancer Control stage of patients in each treatment. In each decade, patients with earlier UICC stage disease underwent resection and nonsurgical treatment.

1998.[14] Using the data of 17,130 patients from 1981 through 1995, various aspects of diagnosis and treatment were reviewed. Ultrasonography and computed tomography have become increasingly important as the methods of detection. Tumor resection was performed in 36% of the patients, and the 5-year survival rate of the patients who underwent resection was 18.2%. They concluded that the rate of resection and results of surgical treatment had improved, which may be attributed to the increase in detection of resectable tumor and benefits of aggressive and extended surgery.

From 1998 to 2004, the registry was conducted by Seiki Matsuno in Tohoku University. Thanks to the development of computer, the data were integrated in a relational database in 1998, and the registration was first performed using electronic submission in 2003 after both UICC and JPS rule had been revised to their current form in 2002. Registry itself had a role in spreading the new rules of classification. The review was published periodically.[15,16] In 2004, "Pancreatic Cancer in Japan" was the special issue in *Pancreas*. The summarized data of 20 years of pancreatic cancer registry[1] and the clinicopathological characters of small pancreatic cancer[2] were included together with the achievements of Japanese pancreatologists. The cumulative number of records from 1981 to 2000 reached 23,302. In 2003, however, personal data protection law was enforced, and every kind of cancer registry faced the serious ethical problem of how to protect personal data and obtain a reliable data because the law requires the anonymization in clinical research if informed consent is not given. Actually, there is 2% to 5% of duplicated registry from multiple institutions in pancreatic cancer registry every year. There is an increasing possibility that different institutions or different specialties treat the same patient and make the registration separately. Thus, without knowing the personal name or birth date, correct exclusion of duplicated data is required. We have originated encrypting technique using a hash function to generate a code to distinguish the records (Fig. 1). Since 2005 and on, the registry has been conducted by Masao Tanaka in Kyushu University. After legal solution with approval of the ethical committee in Kyushu University, the data collection of 2005–2007 was achieved using the anonymous code. Pancreatic cancer registry report 2007[17] was published online with English subtitles because the data consisted of a huge number of tables and figures, summarizing not only each item but also the trend of outcome in every decade. Currently, the data of 2008-2010 are being collected.

The Japan Surgical Society and other collaborative surgical societies have established the National Clinical Database (NCD) to collect the data of all surgeries in Japan and has been working since January 1, 2011. The NCD is going to incorporate cancer registry of not only surgical cases but also nonsurgical cases. Pancreatic cancer registry is moving forward to collaborate with NCD, aiming at the registry of wider population and to grasp the reality of pancreatic cancer diagnosis and treatment. Several issues should be improved, saving the efforts of every clinician by hiring medical record administrators, automatic extraction of medical information from electronic medical records, and standardization of description. However, pancreatic cancer registry should be continued because only by this registry can we compare the outcome between institutions, nations, and historical controls and obtain the future perspectives.

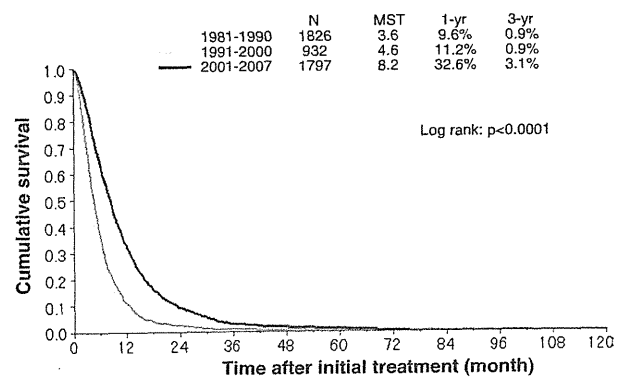

FIGURE 6. Survival of patients with unresectable invasive cancer.

THE VISION

The most important vision and perspective of pancreatic cancer registry is the correction of patients' background, treatment, and follow-up of outcome. The leading 350 institutions are contributing more than 1200 records each year, but the annual death from pancreatic cancer in Japan exceeds 25,000, yielding less than 10% of the whole nation. Most of the patients are still diagnosed too late and are missing the chance of treatment. Widening of the registry is a suspended problem. Annual follow-up is another important vision. So far, continuous follow-up gives the most reliable outcome, survival; and these 30 years of experience will make it possible to define if our strategy is improving the patients benefit.

ACCOMPLISHMENTS

Periodical reports from the conductors and others described the on-time review of the diagnostic and treatment status.[8–10,12,13,17,18] Many spinouts focusing on specific issue were published using this database. Dividing the invasive cancer by tumor size revealed that as the tumor grows larger, the pathological grade and the vascular, lymphatic, or perineural infiltration are worsened, suggesting that pancreatic cancer gains its aggressiveness during the tumor development.[19] Many Japanese surgeons tried to cure the patients with pancreatic cancer by extended retroperitoneal dissection and combined resection of large vessels. In 628 patients with UICC stage IIA and UICC stage IIB disease, the PV, RP, and PL infiltrations had a significant impact on the accomplishment of R0 resection in univariate and multivariate analyses. There was no advantage of PV resection for both PV(−) and PV(+) disease among patients with UICC stage IIA or IIB, suggesting no benefit of prophylactic PV resection.[20] Acinar cell carcinoma is a rare histological type, and no single institution has the power to collect a hundred case series. Using the database, of 115 patients with acinar cell carcinoma, 76.5% underwent resection; and the 5-year survival rate was 43.9%. It was concluded that preoperative diagnosis of acinar cell carcinoma is difficult, but once resected, favorable outcome may be expected.[21] In the UICC classification,[4] pancreatic NETs are classified according to the rules for pancreatic cancer. The JPS classification deals with pancreatic NET from its beginning and collected a large series of 177 patients with NETs. Of the 177 patients, 100 patients had nonfunctioning tumor. The survival after treatment correlated well with JPS stage.[22,23] In addition, the tumor extent of 122 patients with invasive cancer derived from IPMN and 31 patients with invasive cancer concomitant with IPMN were significantly smaller, less invasive, and less extensive than ordinary invasive cancer. The median survival of patients with the 2 conditions was significantly longer than for those with ordinary invasive cancer, suggesting that these 2 categories have more favorable biological behaviors or are diagnosed earlier than ordinary pancreatic cancer.[24]

SUMMARY OF THE LATEST DATA

The cumulative number of records with pancreatic neoplasms from 1981 to 2007 was 35,903. Duplicated 1711 records and the 1573 records without prognostic information were excluded. Resulting 32,619 records were analyzed. The database is maintained in FileMaker Pro software (FileMaker Inc, Santa Clara, Calif), and the data were statistically processed by JMP software (SAS Inc, Cary, NC). Because the whole registry data are excessive to describe in one paper, representative summary of latest outcome is presented.

TREND OF REGISTRY

Figure 2 shows the trend of registry of all patients according to the treatment. The total registration is increasing owing to the increase in the number of patients who undergo pancreatectomy and who receive nonsurgical treatment. Additionally, the number of patients who are observed without any treatment mainly owing to a lesion, for example, branch type IPMN, is simply followed up. Figure 3 shows the trend of histological distribution. The improvement of endoscopic ultrasound-guided fine needle aspiration made a great advance in histological confirmation of cancer and other neoplastic diseases. The number of patients without histological diagnosis is decreasing.

TREND OF SURVIVAL OUTCOME OF INVASIVE PANCREATIC CANCER

As Figure 4 shows, the overall survival of patients with invasive pancreatic cancer is improving decade by decade. The survival curve is divided to that of patients who underwent pancreatectomy (Fig. 5) and those who had unresectable disease (Fig. 6). There was a significant increase of survival rate in the patients who underwent resection. The UICC stage distribution is shown in Figure 7. The number of patients with earlier UICC stage is increasing, but as shown in Figure 8, the survival of patients with UICC stages IIA, IIB, III, and IV disease is improving. In patients with UICC stages IA and IB in which the pancreatic cancer is confined to the pancreas, the survival rates among these 3 decades are not statistically different.

PROGNOSTIC FACTORS

Collecting detailed clinicopathological factors enables us to identify prognostic factors based on a large number of patients. For example, Table 1 shows the multivariate analysis of prognostic factors of 995 patients who underwent pancreatectomy

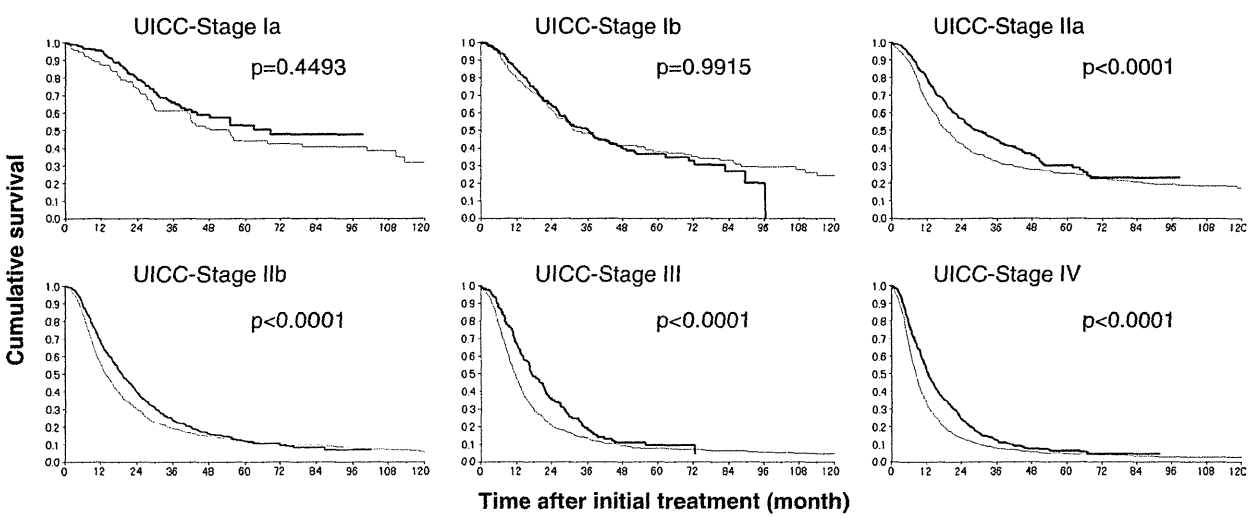

FIGURE 8. Survival of patients who underwent pancreatectomy by UICC stage. In UICC stages IA and IB, the outcome of surgery was not different statistically. In the advanced UICC stage, the survival was improved significantly.

TABLE 1. Multivariate Analysis of Prognostic Factors of Patients Who Underwent Pancreatectomy Within 2001–2007 for UICC Stage IIA and IIB Tubular Adenocarcinoma in the Pancreatic Head Using Cox Proportional Hazard Model (n = 995, censored 369)

Factor	Degree of Freedom	*P* (Prob > χ^2)	Hazard Ratio
Sex, M/F	1	0.0192	1.228:1
Histological Classification	2	<0.0001	
G1			1
G2			1.451
G3			2.301
Interstitial Abundance (Medullary/ Moderate/Scirrhous)	2	0.3112	
Interstitial Infiltration (INFα/β/γ)	2	0.1144	
Lymphatic Infiltration (0–3)	3	0.1570	
Venous Infiltration	3	0.0309	
v0			1
v1			1.048
v2			1.314
v3			1.479
Perineural Infiltration (1–3)	3	0.8102	
Tumor Size	3	0.0005	
TS1			1
TS2			1.265
TS3			1.899
TS4			2.898
Anterior Surface Invasion (No/Yes)	1	0.3156	
Bile Duct Invasion (No/Yes)	1	0.8046	
Duodenal Invasion (No/Yes)	1	0.6423	
Retroperitoneal Invasion (RP No/Yes)	1	0.5702	
Portal Vein Invasion (PV, No/Yes)	1	0.0819	
Arterial Invasion (No/Yes)	1	0.1805	
Plexus Invasion (PL, No/Yes)	1	0.1067	
Other Organ Invasion (No/Yes)	1	0.4408	
JPS-T (T1/T2/T3/T4)	3	0.3818	
JPS-N	2	0.0480	
N0			1.741
N1			1
N2			3.935
JPS Stage (I/II/III/IVa/IVb)	4	0.2232	
UICC-T (T1/T2/T3)	2	0.7594	
UICC-N (N0/N1)	1	0.0726	
Degree of Lymph Node Dissection	3	0.0086	
D1			1.490
D2			1.063
D3			1
Plexus Resection (No/Yes)	1	0.0933	
Portal Vein Resection (No/Yes)	1	0.1283	
Arterial Resection (No/Yes)	1	0.3536	
Preoperative Chemotherapy (No/Yes)	1	0.8566	
Postoperative Chemotherapy (No/Yes)	1	0.0146	
No			1.261
Yes			1
Preoperative Radiation (No/Yes)	1	0.9873	
Postoperative Radiation (No/Yes)	1	0.9362	

INF indicates interstitial infiltration.

from 2001 to 2007 for UICC stages IIA and IIB tubular adenocarcinoma in the pancreatic head using Cox proportional hazard model. Interestingly, the strongest factor was histological grade, followed by tumor size, the extent of lymph node dissection, postoperative chemotherapy, sex, venous infiltration, and JPS-N. Because UICC stages IIA and IIB are the most frequently encountered, these prognostic factors give us an insight not only about the biological aggressiveness of the tumor but also what we should do. In patients with UICC IIA and IIB diseases, the hazard ratio of male-to-female patients was 1.228. If the histology is G3, the hazard ratio is 2.3 times that of G1. Among various histological parameters of tubular adenocarcinoma, only venous infiltration had a statistically significant impact on survival at UICC stages IIA and IIB. If the tumor is larger than 6 cm, the hazard ratio is 2.898. It seems paradoxical that the hazard ratio of JPS-N0 is larger than that of JPS-N1, but JPS-N0 in the same UICC stage means that the tumor extent is more severe. The hazard ratio of JPS-N2 was highest at 3.935. Although, statistically, significance was not reached, the hazard ratio of UICC-N1 was 2.661 (data not shown). In what we did, the extent of lymph node dissection had a $P = 0.0086$. The hazard ratio of lymph node dissection (D)1 was significantly worse than D2 or D3. In the same cohort, the Kaplan-Meier method shows that the survival rate of patients who underwent D1 resection is significantly lower than that of patients with D2 and D3 resection (Fig. 9). In Japan, D2 resection is most frequently performed for UICC stage IIA and stage IIB disease. There was no statistically significant difference between the survival with D2 and D3 resection. Any of the combined resection of portal vein, artery, and extrapancreatic nerve plexus did not have significant positive or negative impact on survival at this stage. Postoperative adjuvant chemotherapy had lowered the hazard ratio significantly. However, the actual impact on survival seems to extend

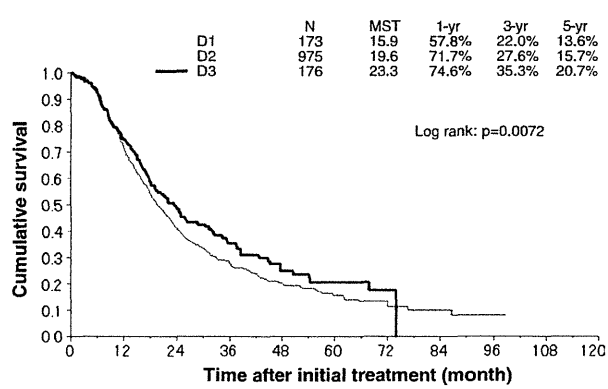

FIGURE 9. Survival of patients with UICC stages IIA and IIB tubular adenocarcinoma in the pancreatic head according to the extent of lymph node dissection. The 1374 records from 2001 to 2007 were analyzed. The survival rate between D1 and D2 was significantly different ($P = 0.0246$), whereas that between D2 and D3 was not statistically different ($P = 0.0887$).

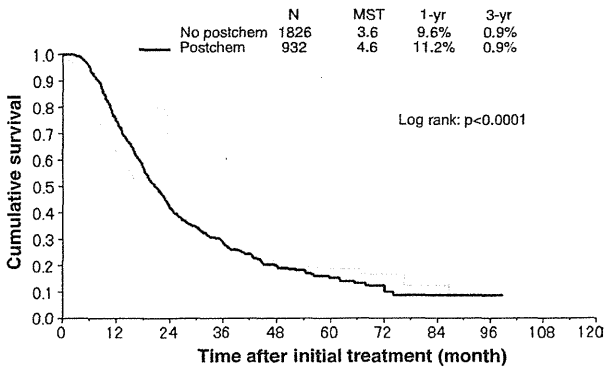

FIGURE 10. Survival of patients with UICC stages IIA and IIB tubular adenocarcinoma in the pancreatic head according to the postoperative chemotherapy. The patients without postoperative chemotherapy at the time of registration may receive chemotherapy after the recurrence was detected.

the disease- free survival for a short period of time (3 months in median) because the curves become close as shown in Figure 10. The numbers of patients with preoperative chemotherapy, with preoperative radiotherapy, and with postoperative radiotherapy were too small (<10% of the cohort) to draw any conclusion.

EARLY PANCREATIC CANCER

Because pancreatic cancer is one of the deadliest diseases, the effort for the earlier detection has been continued. In the JPS registry, the statistics of pancreatic cancer starts by definition from invasive stage, and there has been no simple definition of early pancreatic cancer.[25] With the accumulation of knowledge about molecular carcinogenesis and biological behaviors of premalignant disease such as PanINs,[26,27] IPMNs[28] and mucinous cystic neoplasms,[29] together with their relationships with chronic inflammation,[30] the definition of early pancreatic cancer cannot be made with the data of invasive cancer alone. There should be a seamless transition between intraepithelial premalignant change, microinvasion, and invasive cancer.

To define early pancreatic cancer, we have to think about the size of the tumor and the depth of invasion. Figure 11 shows

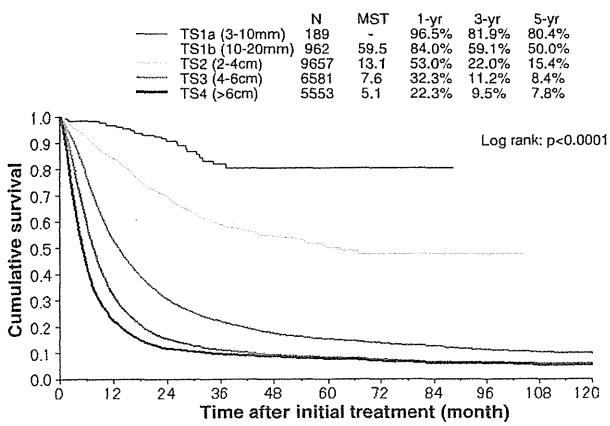

FIGURE 11. Survival of patients with invasive cancer according to tumor size. The actual tumor size is available from the records in 2000. The records that have contradiction between the actual size and TS rank were excluded from the analysis.

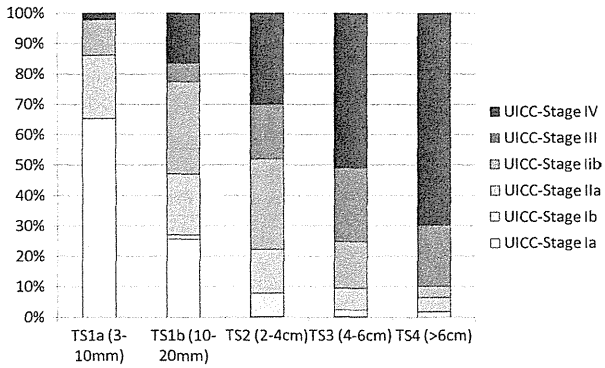

FIGURE 12. Union for International Cancer Control stage according to the size of invasive cancer. The frequency of advanced stage increased as the tumor grew.

the survival of patients with invasive cancer according to the size of tumor. When the tumor is 10 mm or less (TS1a), the survival rate was significantly higher than that of patients with tumor larger than 10 mm (TS1b and more). The 5-year survival rate of patients with TS1a invasive cancer is more than 80%. Furthermore, as the tumor grows, the rate of advanced UICC stage increases (Fig. 12). In patients with TS1a tumor, 65% of them had UICC stage IA disease, whereas only 25% of the patients with TS1b had UICC stage IA disease. You may notice that none of the patients with invasive cancer has UICC stage 0 disease, although the tumor is 10 mm or less. Thus, we should next take the depth of invasion into account to define early pancreatic cancer. Figure 13 shows the trend of UICC stage distributions of all patients including invasive cancer, intraductal neoplasms (INs), cystic neoplasms (CNs), and NETs (same patient cohort with Fig. 3). Increasing numbers of patients with UICC stage 0 (in situ), IA, and IB disease are registered. The overall survival rate of patients with INs, CNs, and invasive cancer is shown in Figure 14. Intraductal neoplasms includes IPMA, IPMC, PanIN1 to PanIN3, CIS with or without microinvasion, and their invasive counterparts. Cystic neoplasms include mucinous cystadenoma, mucinouscystadenocarcinoma, serous cystadenoma, and serous cystadenocarcinoma, with or without microinvasion, and their invasive counterparts. Invasive cancer includes papillary adenocarcinoma, tubular adenocarcinoma, adenosquamous carcinoma, anaplastic carcinoma, mucinous carcinoma, and undifferentiated carcinoma. The 5-year survival of patients with UICC stage 0

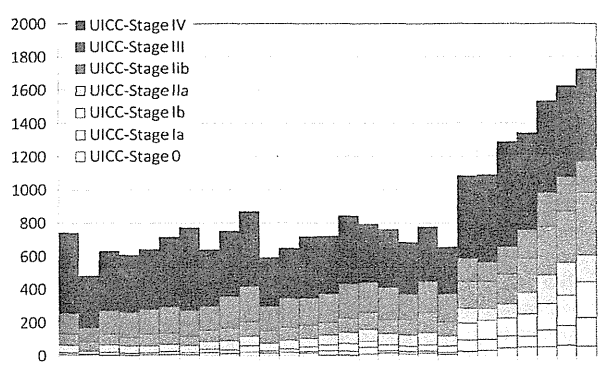

FIGURE 13. Trend of UICC stage of all neoplasms. Same patient cohort with Figure 3.

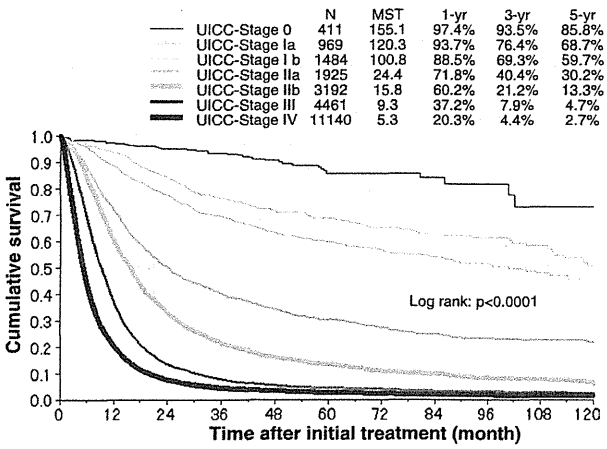

FIGURE 14. Survival of patients with INs, CNs, and invasive cancer according to UICC stage. Patients with NETs were excluded. Both adenomas and carcinomas are included.

is 85.8%, followed by UICC stage IA of 68.7% and UICC stage IB of 59.7%.

DISCUSSION

The JPS nationwide pancreatic cancer registry is an original and unique database that gives us the perspective of current diagnostic and treatment measure based on 30 years of experience and insight to the future. Without the continuous understanding and cooperation from the whole country, it was not possible to obtain a large amount of data that is durable for detailed analysis. We appreciate the effort of former conductors and every physician, collaborator, and patient who had this intractable disease.

The improvement of survival of patients with invasive cancer may be attributed to mainly 3 reasons. First, gemcitabine (GEM) and S-1 (an oral 5-fluorouracil derivative consist of tegafur: 5-chloro-2,4-dihydroxypyridine: potassium oxonate at a 1: 0.4: 1 molar ratio) were approved for pancreatic cancer in Japan in 2001 and 2006, respectively. According to the several clinical trials,[31–33] postoperative adjuvant chemotherapy had become a standard treatment. Gemcitabine is currently the most used regimen, but several randomized trials are ongoing to test postoperative S-1 regimen or GEM/S-1 (GS) combination for an adjuvant therapy. This may have contributed to the improvement of survival in each UICC stage, as shown in Figure 8. A large-scale randomized phase 3 study performed in Japan and Taiwan that compared GS versus S-1 versus GEM in unresectable advanced pancreatic cancer (GEST study: American Society for Clinical Oncology 2011 abstract numbers 4007 and 9070) revealed that GEM and S-1 are equivalently effective in the treatment of advanced unresectable pancreatic cancer in overall survival. The combined GS therapy showed significantly longer progression-free survival than each monotherapy. Crossover usage of GEM and S-1 may have also contributed to the longer survival because nearly half of the patients had received second-line therapy in all arms, and this resulted in the median overall survival with GEM (8.8 M), S-1 (9.7M), and GS (10.1M), respectively. New therapies, such as GEM/erlotinib[34] or FOLFIRINOX,[35] that showed superior outcome than GEM will be introduced in Japan in the future.

The second reason is that the treatments are mainly performed and could be improved in the high-volume centers. In diagnostic process, ultrasound-guided fine needle aspiration is playing a more important role in the differential diagnosis, and recent clinical trials require histological confirmation before enrolling the patients. Evidence-based JPS clinical guidelines for pancreatic cancer 2009[36] indicate that the frequency of complications after pancreaticoduodenectomy is lower, and management of complication after pancreas resection is superior in high-volume centers. Because postoperative adjuvant chemotherapy had become a standard treatment and the combination of surgery and chemotherapy enhanced the regionalization too, patients are moving to large centers more frequently these days, sometimes to enter in a clinical trial and sometimes to obtain a second opinion.

Third, the pancreatic neoplasms are getting diagnosed earlier than before as shown in Figures 7 and 13. Pancreatic cancer registry requested to submit the real size of the tumor from the records in 2000 and the collected large number of records with detailed clinicopathological parameters. As the tumor size grows, the frequency of higher grade of histology increases. Accordingly, the frequency of lymphatic, vascular, and perineural infiltrations increases, resulting in advanced UICC stage of the disease as shown in Figure 12. If the tumor is 10 mm or less, most of the case is UICC stage IA, with favorable survival. However, as long as we start the definition of pancreatic cancer from invasive ones, it seems impossible to define an early pancreatic cancer. On the other hand, the JPS classification of INs include "intraductal" neoplasms with "microinvasion" and "invasive cancer derived from IPMN." PanINs are also included in Ins, although PanIN1 and PanIN2 are not regarded as tumors by themselves. PanIN3 is regarded as CIS with or without microinvasion. Thus, we should carefully correct the data of size and depth together with clinical outcome to define an early pancreatic cancer regardless of the histological classification. As shown in Figure 14, there seems to be an "early pancreatic cancer" with favorable long-term survival.

CONCLUSION

The JPS pancreatic cancer registry has fulfilled the vision and mission of its founding. This nationwide pancreatic cancer registry has been an indispensable tool in evaluating the progress of diagnosis and management of pancreatic cancer over 30 years of experience. It also provides a great database for comparative studies with other national databases. As the registry continues to expand to include other types and early stages of pancreatic cancer, it will undoubtedly improve the management strategy of pancreatic cancer and provide a much improved outcome in the near future.

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