Identification of a novel alternative splicing transcript variant of the suppressor of fused: Relationship with lymph node metastasis in pancreatic ductal adenocarcinoma

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal diseases, and the median survival time is very short. Upregulation of hedgehog signaling pathway activity is a vital factor in pathogenesis of PDAC. However, as a negative regulator of hedgehog signaling, it is not very clear what role the suppressor of fused (SUFU) plays in PDAC tissue. In our study for the identification of alternative splicing transcripts of *SUFU* gene in human PDAC cells, a novel transcript variant of *SUFU* (SUFUvN) was discovered by 3' rapid amplification of cDNA ends (3'RACE) and cDNA clone. SUFUvN contained an additional new protein-coding exon compared with the transcript variant 1 of *SUFU* (SUFUv1, NM_016169) published in NCBI website. The sequence of the new protein-coding exon was the same as a fragment of intron between exon 10 and 11 of SUFUv1. Thus, an exon skipping occurred in transcription of SUFUv1. Compared with the expression vector of SUFUvN transfected PDAC cells, the corresponding protein expression encoded by SUFUvN (SUFU isoform N) was detected in PDAC tissue. Furthermore, it was observed that elevated SUFUvN transcription level was related with lymph node metastasis in PDAC tissues, while neither SUFUv1 nor transcript variant 2 of *SUFU* (SUFUv2, NM_001178133) did. Our data indicate that there exists a novel transcript variant of SUFU which can be transcribed and translated into corresponding protein and its transcription is related with metastasis of lymph nodes in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), also known as pancreatic cancer is one of the most fatal diseases, and the overall 5-year survival rate among patients with pancreatic cancer is <5%. In pancreatic cancer, 90% of tumors have activating mutations in the *KRAS2* oncogene (1). In addition, activation of hedgehog signaling pathway is vital in tumor development. Activity of hedgehog signaling pathway is gradually increased in the progress from pancreatic intraepithelial neoplasia (PanIN) to PDAC (2-4). Mutations of components lead to activation of hedgehog signaling pathway in some types of tumors (5-8). However, no mutations have been detected in components of this pathway in pancreatic cancer. SUFU is an essential negative regulator of mammalian hedgehog signaling pathway. Kasai *et al* (9) provided evidence that physical binding inhibition of GLI1 by SUFU is dismissed by tethering of SUFU in the cytoplasm of PDAC cells by overexpressed SCL/TAL1 interrupting locus (SIL) and KRAS mutation. However, Chen *et al* (10) provided convincing data indicating that mouse Sufu sequesters Gli2/3 protein in the cytoplasm and protects them from Spop-mediated protein degradation, providing a Gli protein pool for the production of Gli2/3 activators and repressors. Sufu is necessary for maximal activation of hedgehog pathway. Thus Sufu has an unexpected positive role in controlling mammalian hedgehog signaling. Thus, we proceeded to clarify the function of SUFU in human pancreatic cancer. There are two transcript variants of *SUFU* published in NCBI website: transcript variant 1 of *SUFU* (SUFUv1, NM_016169) and transcript variant 2 of SUFU (SUFUv2, NM_001178133). We started by making cDNA clones of alternative splicing transcript variants of *SUFU* gene in human pancreatic cancer cells.

Materials and methods

Cell culture and RNA extraction. Seven pancreatic cancer cell lines SW1990, PATU8988, AsPC-1, BxPC-3, CFPAC-1, Capan-2, PANC-1 were cultured in DMEM medium supplemented with 10% FBS and incubated in a 5% CO_2 humidified incubator at 37°C. Total RNA was extracted from cells using RNA iso Plus (Takara, Japan) according to the manufacturer's instructions. The yield of RNA was determined using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide.

3' rapid amplification of cDNA ends (3'RACE) and nested PCR. Two reference sequence transcript variants of *SUFU*

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Table I. Primers used in 3'RACE and RT-PCR of SUFUvN 5'cDNA.

Primers	Sequences
First cycle of nested PCR	
P1	5'-CGC AAA GAC AGC CTG GAA AGT GAC-3'
3'RACE outer primer	5'-TACCGTCGTTCCACTAGTGATTT-3'
Second cycle of nested PCR	
P2	5'-TCC TGC ATG GAC GGC ACT TTA C-3'
3'RACE inner primer	5'CGCGGATCCTCCACTAGTGATTTCAC TATAGG-3'
RT	
SUFU ORF reverse transcription primer	5'-CTAGTGTAGCGGACTGTCGAACA-3'
PCR	
P3	5'-CTCCAGGTTACCGCTATCG TC-3'
P4	5'-TTCGGTCAACAGAATCAGGTTTC-3'

gene are published on NCBI. They are transcript variant 1 of *SUFU* (SUFUv1, NM_016169) and transcript variant 2 of *SUFU* (SUFUv2, NM_001178133). 3'RACE was made to obtain the 3' cDNA of SUFU transcripts.

3'-Full RACE Core Set Ver.2.0 (product code, D314, Takara) kit contains: 3'RACE adaptor (a reverse transcription primer adaptor which can addict to mature mRNA poly A tail by its poly T sequences), M-MLV reverse transcriptase, 3'RACE outer primer (reverse primer of the first nested PCR), 3'RACE inner primer (reverse primer of the second nested PCR), etc. The steps are shown in Fig. 1A.

We performed reverse transcription. The reaction consisted of 1 μl total RNA (500 ng/μl), 1 μl 3'RACE adaptor, 0.25 μl M-MLV reverse transcriptase, 0.25 μl RNase inhibitor, 2 μl 5X M-MLV buffer, 1 μl dNTP mixture and 4.5 μl RNase-free dH_2O, in a total volume of 10 μl. Reaction was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) for 60 min at 42˚C, followed by heat inactivation of RT for 15 min at 70˚C.

First cycle of nested PCR. The forward primer of the first nested PCR was designed by us according to the kit instructions (Table I). We call this forward primer P1. It is situated in the 9th exon of SUFUv1. The first base pair of P1 is situated in the 1,218 bp of the whole RNA sequences. The reverse primer of the first nested PCR is in the kit called 3'RACE outer primer. The reaction consisted of 3 μl reverse transcription product, 7 μl 1X cDNA dilution buffer, 2 μl P1 (10 μmol/l), 2 μl 3'RACE outer primer, 5 μl 10X Ex Taq buffer (including Mg_2), 0.25 μl Ex Taq enzyme and 30.75 μl dH_2O. Reactions were performed in a GeneAmp PCR System 9700 for 3 min at 94˚C, followed by 25 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 5 min, and a last step of 72˚C for 10 min.

Second cycle of nested PCR. The forward primer of the second nested PCR was also designed by us according to the kit instruction (Table I). We call this forward primer P2. It is situated in the 10th exon of SUFUv1. The first base pair of P2 is situated in the 1,357 bp of the whole RNA sequences. The reverse primer of the second nested PCR is in the kit called 3'RACE inner primer. The reaction consisted of 1 μl the first nested PCR product, 8 μl dNTP mixture, 5 μl 10X Ex Taq buffer (including Mg_2), 0.5 μl Ex Taq enzyme, 2 μl P2 (10 μmol/l), 2 μl 3'RACE inner primer and 31.5 μl dH_2O. Reactions were performed in a GeneAmp PCR System 9700 for 3 min at 94˚C, followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 5 min, and a last step of 72˚C for 10 min.

The product of second nested PCR was ligated with pMD-18T vector, and infected DH5α. Then, we selected 10 positive clones, and made sequence detection.

RT-PCR to identify the 5' cDNA sequence of novel transcript variant of SUFU (SUFUvN). We performed reverse transcription by SUFU ORF reverse transcription primer (Table I), then, by PCR. The forward primer of PCR was designed by us called P3 (Table I). It is situated in the first exon of SUFUv1. Reverse primer of PCR was also designed by us called P4 (Table I). It contains partial 11th exon of SUFUv1 and partial new exon. Product of PCR was used for sequence detection. The steps are shown in Fig. 1B.

Construction of expression clones of SUFUvN and SUFUv1. The forward primer terminal is added with protection base pairs, *Bam*HI enzyme sites and Kozak sequences. The reverse primer terminal is added with protection base pairs, *Age*I enzyme sites. The forwad primer and reverse primer were designed to clone open reading frame (ORF) of *SUFU*. We performed reverse transcription by SUFU ORF reverse transcription primer (Table I), then by PCR. The sequence of forward primer is: 5'-**cg**ggatccGGGATGGC GGAGCTGCGGCCTA-3'. The sequence of reverse primer is: 5'-**gcgc**accggtCTAGTGTAG CGGACTGTCGAACA-3'. *Bam*HI and *Age*I double enzyme digestion was made in both PCR product and pcDNA3.1mychisA (+) expression vector. After ligation of PCR product with pcDNA3.1mychisA (+) expression vector and DH5α infection we selected positive clones and sequence detection was performed.

Cell transfection. SW1990 cells were transiently transfected with empty vector, SUFUv1 and SUFUvN expression vector, respectively, by use of Lipofectamine 2000 kit (Takara) according to the manufacturer's instructions. The cells were cultured for 24 h after transfection. Then the cells were harvested for further studies.

Western blotting. The empty vector, SUFUv1 and SUFUvN expression vector transfected SW1990 cells were collected. Cells were dissolved in cell lysis buffer. Protein of pancreatic cancer tissue was extracted by RIPA lysis buffer. The lysates were cooled with ice for 30 min, and then centrifuged at 13,000 x g for 30 min. Proteins (10 μg) in the collected supernatant were separated by SDS-PAGE on 12% gels and then transferred to PVDF membranes. The membrane, after a block with 5% skim milk, was incubated with primary antibodies to SUFU (rabbit anti-human monoclonal antibody, catalog no. NBP1-40515, Novus, USA) and GAPDH (ab97626,

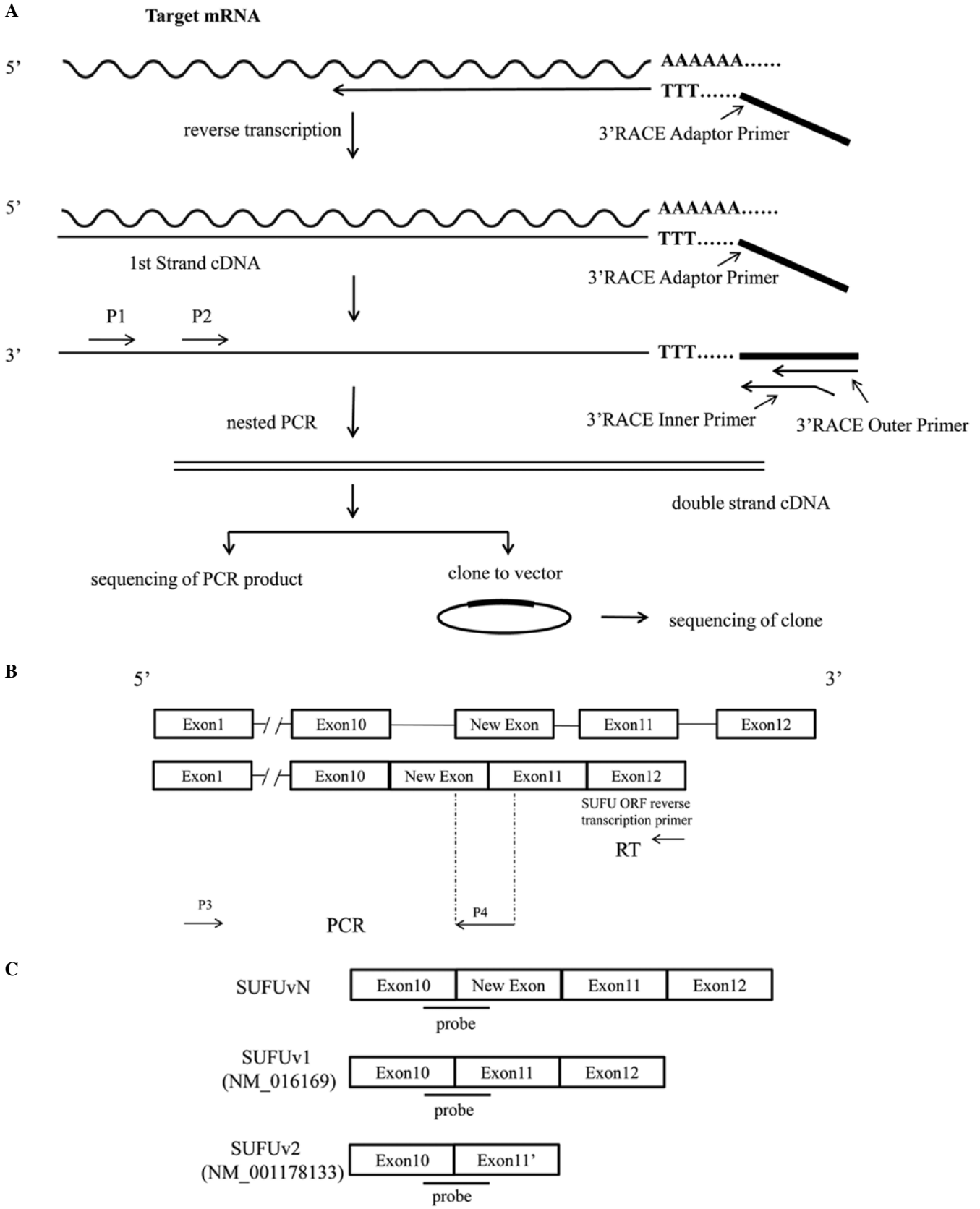

Figure 1. Schematic diagram. (A) Steps of 3'RACE (3' rapid amplification of cDNA ends). (B) Description of experiment for identifying 5' cDNA of SUFU novel variant. (C) MGB probe site detecting transcription level of different variants of SUFU.

Abcam, Cambridge, UK). This SUFU antibody is made by N terminal of SUFU antigen, so it can bind the N terminal of protein encoded by SUFUv1, SUFUv2 and SUFUvN (SUFU isoform 1, isoform 2 and isoform N). Primary antibody is 1:200 diluted to detect SUFU isoforms. The secondary antibody is HRP labeled goat anti-rabbit polyclonal IgG antibody.

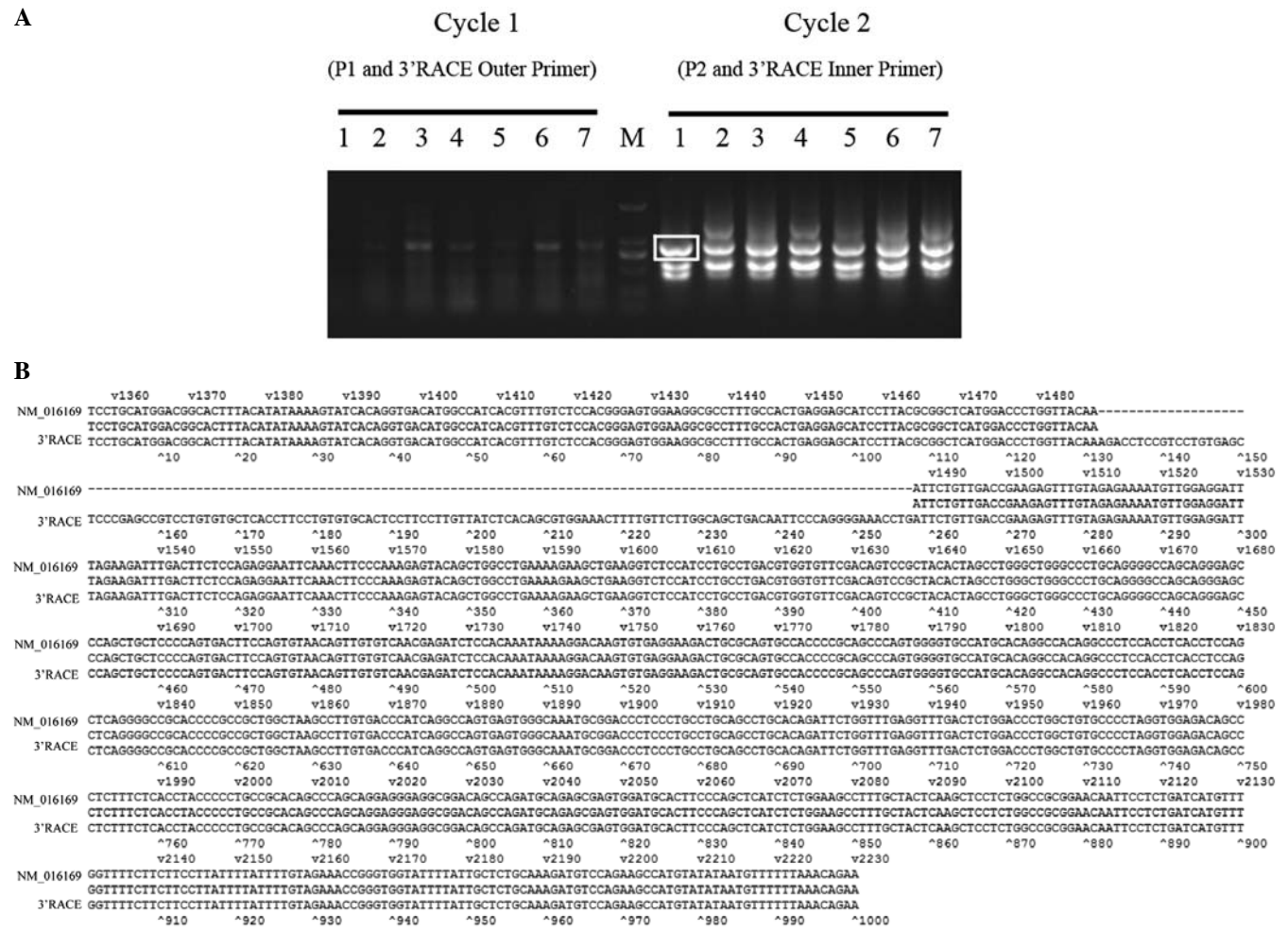

Figure 2. 3'RACE of *SUFU*. (A) The products of the first cycle and second cycle of 3'RACE nested PCR amplification for *SUFU* cDNA from pancreatic cancer cells. Lane M, DNA marker (2,000, 1,000, 750, 500, 250 and 100 bp). Lanes 1-7, SW1990, PATU8988, AsPC-1, BxPC-3, CFPAC-1, Capan-2, PANC-1. The indicated band ~1,000 bp in SW1990 was cloned and sequenced. (B) Sequence alignment of NM_016169 and 3'RACE clone. The mismatch sequence is the new exon.

GAPDH is used as inner control. ECL kit (product code, 34096, Thermo Fisher Scientific, USA) and the camera are used to capture the results.

Patients, tissue samples and total RNA extraction of tissue samples. Tumors were obtained from 40 adult patients diagnosed with pancreatic ductal adenocarcinoma who undergwent surgery at The Second Military Medical University affiliated Changhai Hospital between 2013 and 2014. This study was approved by the Ethics Committee of Changhai Hospital. The total RNA of the pancreatic cancer tissue was extracted by mirVana™ miRNA isolation kit (am1561, Takara). DNA contamination was removed by DNase in this kit. Total RNA was stored at -70˚C.

Real-time quantitative RT-PCR using TaqMan MGB probe method to detect transcription levels of different SUFU variants in PDAC tissues of 40 patients. The 18s control (Hs99999901_s1) MGB probe and primers kit were purchased from Invitrogen Co., USA. The primer pairs and the TaqMan MGB probe sequences of SUFU different variants were designed by Primer Express 3.0 software and were synthesized by Invitrogen Co. The sequences are shown in Table II, and the probes are shown in Fig. 1C.

Quantification was performed with a two-step reaction process: reverse transcription (RT) and PCR. Each RT reaction consisted of 1 μg RNA, 2 μl of PrimerScript buffer, 0.5 μl of oligodT, 2 μl of random 6 mers and 0.5 μl of PrimerScript RT Enzyme Mix I (Takara), in a total volume of 10 μl. Reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems) for 15 min at 37˚C, followed by heat inactivation of RT for 5 sec at 85˚C. Real-time PCR was performed using LightCycler® 480 II Real-time PCR Instrument (Roche, Swiss) with 20 μl PCR reaction mixture that included 10 μl of 2X TaqMan mix (Takara), 1 μl of 20X probe and primers mixture (Invitrogen), 2 μl of cDNA, and 7 μl of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec. Each sample was run in triplicate for analysis. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The expression levels of mRNAs were normalized to 18s rRNA and were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses. The relation of SUFU variant mRNA RQ values (-ΔΔCt) with clinical characteristics were assessed by Mann-Whitney U test. Statistical analysis was processed by

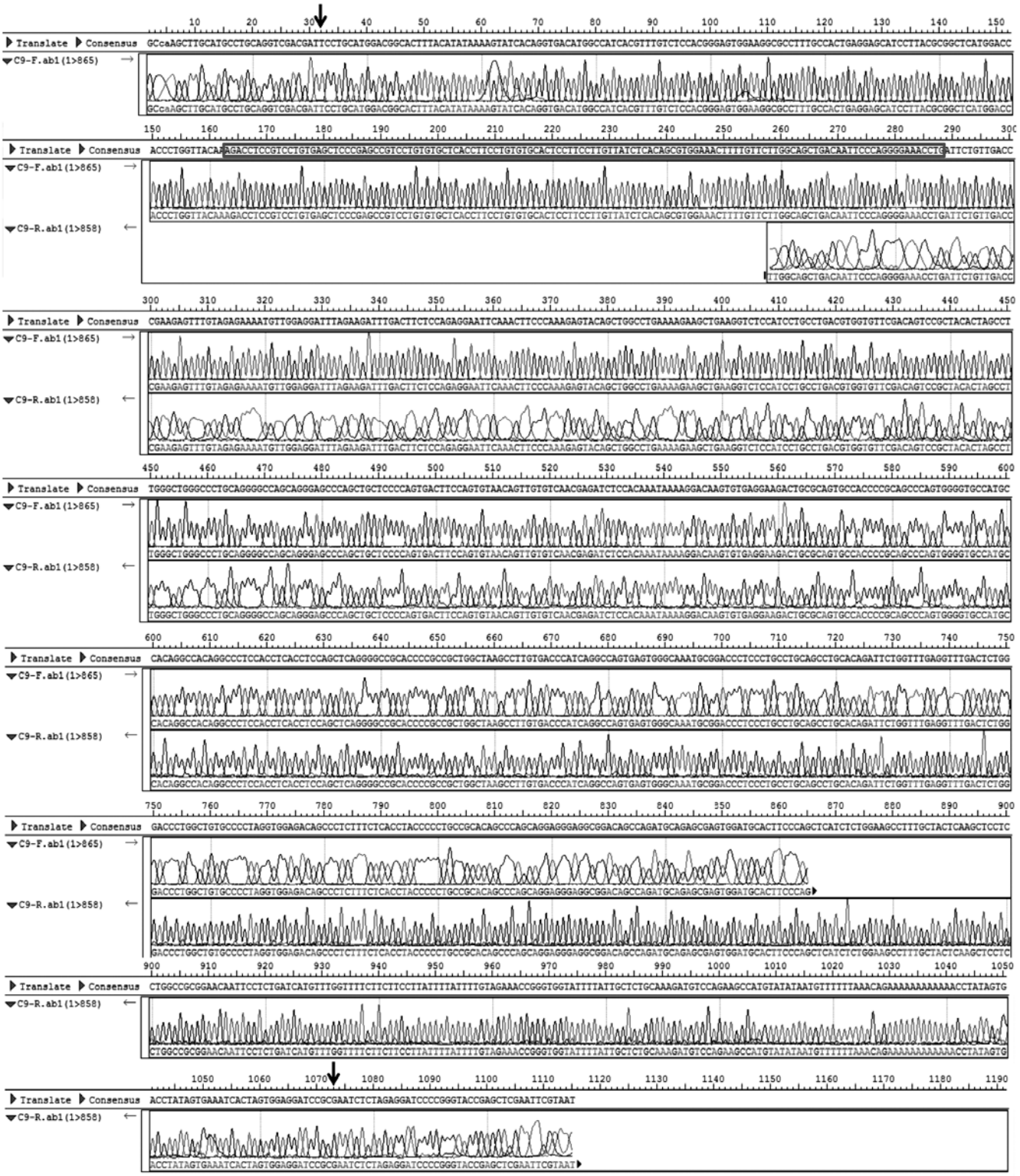

Figure 3. 3'RACE clone sequencing data. This is the direct sequence of clone 9 of the second cycle PCR products. The triangles indicate the insert sites of cloning vector, the frame indicates the new exon.

the SPSS 18.0 software package. P-values <0.05 were considered statistically significant.

Results

Identification of 3'- and 5' cDNA sequences of novel SUFU transcript variant (SUFUvN). Following the strategy described in Fig. 1A, the 3'RACE amplification for 3' cDNA of *SUFU* in pancreatic cancer cells was performed. The products of first cycle (using P1 and 3'RACE outer primer) and second cycle of nested PCR (using P2 and 3'RACE inner primer) are shown in Fig. 2A. A cDNA fragment of ~1,000 bp products from SW1990 cells was cloned into pMD18-T and sequenced. As shown in Figs. 2B and 3, a novel cDNA fragment sequence

Table II. Probes detecting different transcription variants of *SUFU*.

Transcript variants	Primer pairs and MGB probe
NM_016169	
Forward primer	CACTGAGGAGCATCCTTACGC
Reverse primer	AGCTGTACTCTTTGGGAAGTTTGAA
Probe	FAM-CCTGGTTACAAATTCTGTT-MGB
NM_001178133	
Forward primer	CCACTGAGGAGCATCCTTACG
Reverse primer	GCTGAAAATTGGAGATGCTGACT
Probe	FAM-CTGGTTACAACTCTGAACC-MGB
Sufu-vN	
Forward primer	AGGCGCCTTTGCCACTG
Reverse primer	ACACAGGAAGGTGAGCACACAG
Probe	FAM-TGGTTACAAAGACCTCCGT-MGB

was found at 3' termination of *SUFU* gene compared with cDNA of transcript variant 1 of *SUFU* (SUFUv1, NM_016169) and transcript variant 2 of *SUFU* (SUFUv2, NM_001178133) published in NCBI database. This fragment sequence contains a new protein-coding exon (126 bp) the same as a fragment of the intron sequence (102,625,811-102,625,936 of the human chromosome 10, hg38 version) between exon 10 and 11 of SUFUv1. We hypothesized that there exists a novel alternative splicing transcript variant of *SUFU* (SUFUvN) which contains an additional new exon compared with SUFUv1.

In order to identify the full length of SUFUvN cDNA squence, the 5' cDNA fragment of SUFUvN was amplified by RT-PCR following the strategy described in Fig. 1B, in which a specific primer 4 (P4) covering the new exon and exon 11 of SUFUv1 pairing with a specific primer 3 (P3) siting in exon 1 of SUFUv1 were used. As shown in Fig. 4A, a cDNA fragment of 1,400 bp products from SW1990 cells was obtained by RT-PCR and its sequence was the same as the exon 2-exon 10 of SUFUv1 cDNA. The combination of exon 10 and new exon was also been detectable in 1,400 bp product (data not shown). Thus, the new exon is combined with exon 1-10 in 5'-terminal and with exon 11-12 in 3' terminal.

Clone of full length of SUFUvN. RT-PCR product of SUFU transcript was double enzyme digested by *Bam*HI and *Age*I and was ligated with expression vector pcDNA3.1mychisA (+). After infection to DH5α, positive clones were selected and sequenced. From the sequencing and alignment result (data not shown), we identified two kinds of SUFU cDNA clones. They are SUFUv1 and full length of SUFUvN as described above. Figure of BLAT in UCSC web site show us SUFUvN, 5'cDNA of SUFUvN, 3'cDNA of SUFUvN, and other two SUFU variants (Fig. 4B).

Table III. Relation of SUFU variant transcription level in the pancreatic cancer tissues with clinical characteristics of pancreatic cancer patients.

		SUFUvN		SUFUv1		SUFUv2	
Characteristics	N	-ΔΔCt	P-value	-ΔΔCt	P-value	-ΔΔCt	P-value
N stage							
N0		1.39±0.91	<0.0001	2.13±1.04	0.08	2.38±1.49	0.05
N1		5.84±1.07		2.80±0.68		3.22±1.11	
T stage							
T0-2		2.85±2.44	0.23	2.44±1.20	0.95	2.38±1.47	0.29
T3-4		3.98±2.43		2.48±0.79		3.00±1.29	
Age (years)							
<65		3.56±2.56	0.86	2.40±1.05	0.64	2.97±1.37	0.16
≥65		3.77±2.25		2.66±0.37		2.31±1.31	
Gender							
Male		3.68±2.40	0.83	2.47±0.99	1.00	2.76±1.39	0.84
Female		3.47±2.67		2.47±0.81		2.89±1.38	
Tumor size (cm)							
<3		3.18±2.58	0.80	2.81±0.73	0.22	2.51±1.37	0.55
≥3		3.76±2.45		2.35±0.97		2.90±1.37	
Tumor differentiation							
Poor		3.30±2.55	0.98	2.11±0.99	0.33	2.67±1.51	0.76
Well		3.67±2.48		2.53±0.92		2.82±1.36	

Novel transcription variant of *SUFU* (SUFUvN), transcription variant 1 of *SUFU* (SUFUv1, NM_016169), transcription variant 2 of *SUFU* (SUFUv2, NM_001178133). Statistically significant, P<0.05.

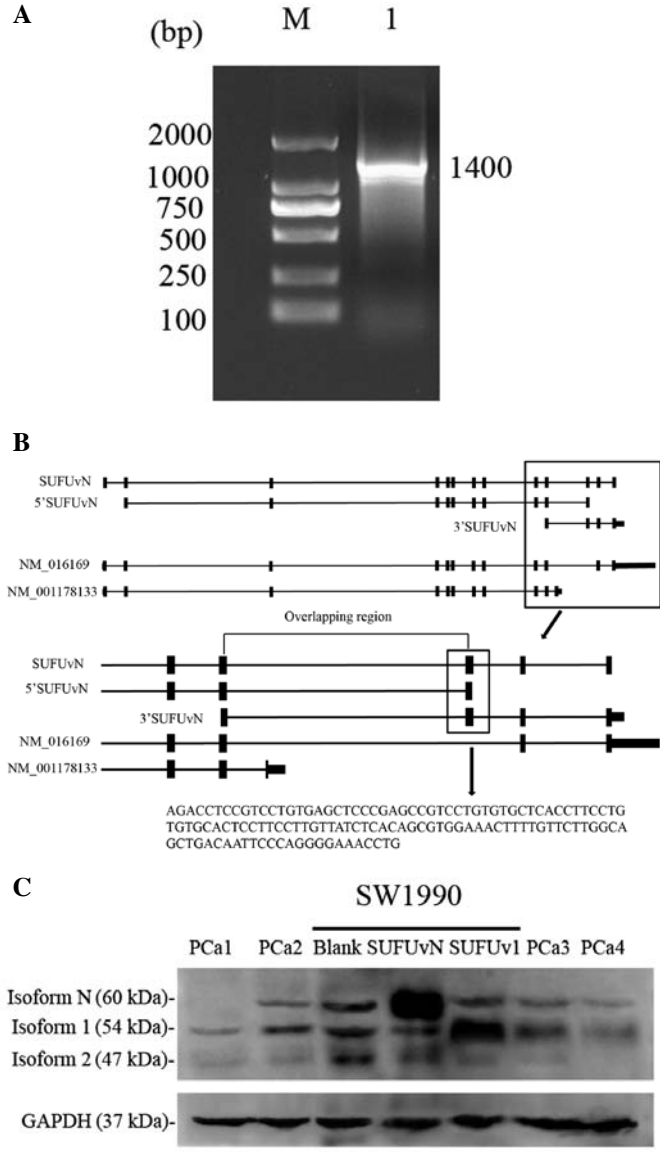

Figure 4. Identification of SUFUvN full length and protein expression. (A) PCR product of 5' cDNA fragment of SUFU novel variant. (B) BLAT figure of SUFUvN cDNA clone. The upper three lines are SUFUvN, 3' and 5' cDNA fragment of SUFUvN. The lower two lines are reference sequences of SUFU published in NCBI. The longer one of the reference sequences is NM_016169 (SUFUv1) while the shorter one is NM_001178133 (SUFUv2). (C) Protein expression encoded by SUFUvN was detected by western blotting. Two bands were identified to be protein encoded by SUFUv1 (isoform 1) and isoform N in PDAC tissues. Isoform 1 is ~54 kDa, while isoform N is ~60 kDa. The additional band ~47 kDa may be protein encoded by SUFUv2. GAPDH ~37 kDa is used as inner control. Lanes 1, 2, 6 and 7, human pancreatic cancer (PCa) tissues. Lane 3, empty vector transfected SW1990 serum. Lane 4, SUFUvN transfected SW1990 serum. Lane 5, SUFUv1 (NM_016169) transfected SW1990 serum. Primary antibody, anti-SUFU and anti-GAPDH.

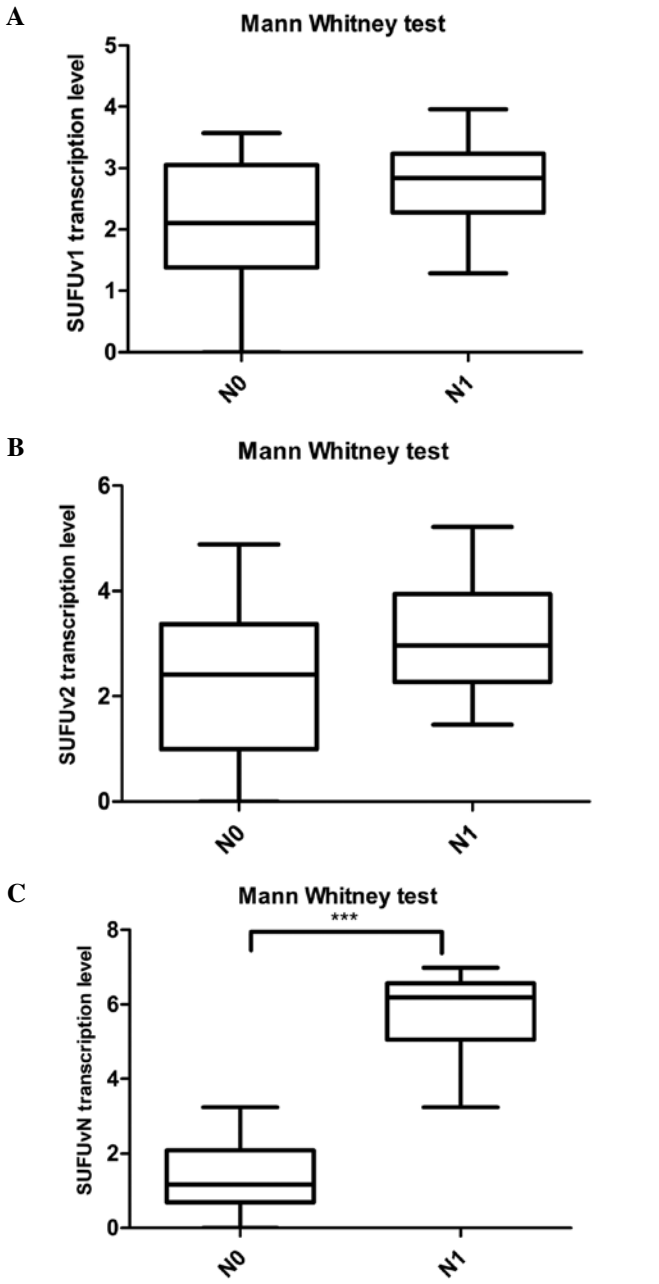

Figure 5. Relationship between transcription of SUFUvN and lymph node metastasis. Transcription levels of SUFUvN were significantly higher in N1 stage than in N0 stage. Transcription levels of SUFUv1 (NM_016169) in N1 and N0 stage show no difference, neither SUFUv2 (NM_001178133). ***P<0.01.

Identification of protein expression encoded by SUFUvN (isoform N). There were three bands present in the western blotting figure of PDAC tissue (Fig. 4C). Compared with lanes with SUFUv1 and SUFUvN expression vector transfected SW1990 serum, two bands were identified to be protein encoded by SUFUv1 (isoform 1) and isoform N in PDAC tissues. Isoform 1 is ~54 kDa, while isoform N is ~60 kDa. The additional band ~47 kDa may be protein encoded by SUFUv2 (isoform 2).

Detection and clinical relevance of SUFUvN in PDAC tissue. We designed TaqMan MGB probes to identify different transcript variants of SUFU. Each probe is described in the figure, so each probe can uniquely detect the corresponding SUFU variant (Fig. 1C). After detecting transcription levels of SUFU variants in PDAC tissues of 40 patients, we found that SUFUvN transcription levels are significantly higher in PDAC tissues of N1 stage patients than in N0 stage patients, while there is no relationship between other variants transcription levels and lymph node metastasis (Fig. 5). As shown in Table III, we found that the elevated transcription of SUFUvN was related with high N stage, while no correlation was found with other clinical features, including T stage, age of onset, gender, tumor size and tumor differentiation.

Discussion

Hedgehog (Hh) signaling pathway plays an important role in pathogenesis and development of pancreatic cancer, so investigating this pathway is very important. Mammalian Hh pathway is composed of Hh ligand, target cell receptor patched (Ptch), smoothened (Smo), and transcription factor family Gli. Gli family contains Gli1, Gli2 and Gli3. Gli2 and Gli3 are the targets of primary Hh signaling. When Hh ligand is absent, Gli2/3 undergo proteolysis to generate a transcriptional repressor form. They are processed into active form when Hh ligand is present. Gli1 cannot undergo the same kind of regulated proteolysis and is used exclusively as gene activator. Gli1 is not only a transcription activator but also a transcription target gene of Hh signaling pathway. Activation of Gli2 and/or Gli3 by Hh often leads to expression of Gli1, which then becomes important in a second wave of activation (11). The formation of either Gli repressor or activator are dependent on primary cilia structure. As a physically binding protein of Gli transcription factor, Sufu is recognized as a negative regulator of hedgehog pathway. However, Chen *et al* (10) provided convincing data indicating that mouse Sufu regulates Gli2/3 stability by antagonizing the Spop protein of the BTB domain containing protein family. Spop binds to Gli2 and Gli3 and promotes their ubiquitination and degradation in a proteasome-dependent manner. The overexpression of Sufu appears to block Spop-dependent Gli protein reduction. They speculated that Sufu sequesters Gli2/3 protein in the cytoplasm and protects them from Spop-mediated protein degradation, providing a Gli protein pool for the production of Gli2/3 activators and repressors. Sufu is necessary for maximal activation of Hh pathway. Thus Sufu is a positive regulator of Hh signaling pathway in this aspect. Clarification is required to establish whether Sufu is a negative or positive regulator of the Hh pathway. We found a novel alternative splicing transcript of *SUFU* named SUFUvN by 3'RACE of *SUFU* gene and cDNA clone in human pancreatic cancer SW1990 cells. In SUFUvN, a new exon is inserted, which is from intron between 10th and 11th exon of SUFUv1 (NM_016169). The inserted exon makes the carboxyl terminal of protein encoded from SUFUvN (isoform N) different from protein encoded from SUFUv1 (isoform 1) and may alter the function of the expressed protein. A further statistical analysis of clinical data revealed that transcription of SUFUvN is related to metastasis of lymph nodes. A study made by another group gives us some tips on SUFU and GLI interaction (12). The study found the ability of GLI binding of different SUFU isoforms will differ because of their different carboxyl terminal structure. To our knowledge, direct binding of Sufu to all three Gli transcription factors has been well documented (13-17). Sufu binds Gli: the amino and carboxyl terminal of Sufu bind the carboxyl and amino terminal of Gli, respectively. Thus we hypothesize that SUFU functions mentioned in our experiment are loaded by different variants of it according to their affinity to GLI. SUFUvN is possibly a positive regulator of Hh pathway because it has more affinitive to GLI2 and GLI3, while SUFUv1 is more likely to be a negative regulator of Hh pathway because of its affinity to GLI1. So, in pancreatic cancer tissue, endogenous SUFUvN binds with GLI2/3 to antagonize their degradation. High level of endogenous SUFUvN leads to more activation of Hh pathway, and accelerates tumor metastasis.

Research reports describing gene alternative splicing variants and different function of their proteins are limited. It is partially due to the complicated and limited methods in studying splicing variant. In our study, we designed TaqMan MGB probe and primers according to the unique exon of different variants. This can distinguish transcription of different variants. Although the corresponding protein isoforms can be recognized by western blotting because of the different molecular weights, the subcellular situation of different protein isoforms can not be distinguished by immunohistochemistry stain in tissue section unless unique antibodies are produced. Recognition of subcellular situation of different isoforms is very helpful for studing their functions. Additionally, if *in situ* hybridization of different variant mRNA is required, new technology and method should be applied, for example the LNA probe (18,19). In addition, the crystal and small-angle X-ray scattering structures of full-length human SUFU and its complex with the key SYGHL motif conserved in all GLIs have been reported by Cherry *et al* (20). It is demonstrated that GLI binding is associated with major conformational changes in SUFU. If alternative splicing transcription variants of *SUFU* including SUFUvN were to be added to their study in the future, we could improve the understanding of the affinity between SUFU variants and GLIs.

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