Identification of bile duct cancer specific fusion genes in patient tissues

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Directed by Professor Si Young Song

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먼저 이 논문이 완성되기까지 많은 관심과 격려로 늘 변함없이 따뜻하게 지도해주신 송시영 교수님께 진심으로 감사 드립니다. 또한 바쁘신 와중에도 귀중한 시간을 내주시어 논문에 대한 좋은 조언을 해주신 김상우 교수님과 전경희 교수님께 감사인사를 드립니다.

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감사합니다.

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Identification of bile duct cancer specific fusion genes in patient tissues

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(Directed by Professor Si Young Song)

Gene fusion occurs when a part of one gene fuses with or attaches to a part of another gene by genome rearrangement and the result in gene fusion may possess oncogenic properties; fused gene may be translated into a unique protein that may promotes cancer properties. Bile duct cancer are one of the most lethal cancer types with low 5-year survival rates, but lack of proper diagnostic or prognostic markers. Our aim was to investigate bile duct cancer specific fusion genes found in patient's specimens. We extracted total RNA from five bile duct cancer tissues and normal tissues from the same patients. We performed RNA sequencing and the result data was analyzed using ChimeraScan, Jaffa or Fusionchacher softwares to detect gene fusion. We selected one fusion gene that is PUM1-TRAF3. We analyzed the expression of a selected fusion gene in 55 bile duct cancer patients and cancer cell lines by conventional PCR and Droplet Digital PCR. We found PUM1-TRAF3 fusion gene in 28 patients out of 55 patients and it has 50.9% frequency.

In order to predict the function of PUM1-TRAF3 in vitro, PUM1-TRAF3 was transfected into SNU1196 to induce overexpression. Expression of PUM1-TRAF3 fusion gene leads to upregulation of EMT markers and cell proliferation, migration, invasion and clonogenicity. These findings indicate the presence of novel fusion genes as well as its possible application for the early diagnosis or prognosis of bile duct cancer.

Key words : bile duct cancer, RNA sequencing, fusion gene, PUM1-TRAF3

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I. INTRODUCTION

Cholangiocarcinoma (CCA) is a relatively rare but highly lethal malignancy arising from the biliary tract epithelium, and an increasing incidence of CCA has been reported worldwide in recent years.[1] The cure for CCA is attainable by curative radical surgical resection or liver transplantation, but the difficulty in early diagnosis often results in inoperable cases and hence a poor outcome of the patients with a 5-year survival of less than 5% and a median survival period of 6 months in advanced cases.[2] Liver transplantation is useful in the treatment of CCA. However, recurrence of the disease is common due to the unique biological characteristics involved, such as cholangiocyte differentiation and abundant stromal desmoplasia. Due to the lack of an early diagnosis, most patients are not eligible for surgical resection.[3,4] Therefore, novel treatment strategies against CCA are needed to improve survival, particularly in high-risk subgroups.

Although many frequently mutated genes have been identified in cholangiocarcinoma, such as TP53 (37-44%) and KRAS (17-54%),[5] none of these signature genes have become targets of therapy. Sequencing efforts are continuously conducted in order to generate in-depth information with regard to the somatic alterations in CCA.

Genomic rearrangements can have dramatic impact on gene function by amplification, deletion, or gene disruption and can create fusion gene proteins with new functions or locations. The characteristic genome rearrangements in CCA imply that recombination events such as gene fusions should be common. Moreover,

if the activity of a fusion gene represents a common oncogenic mechanism, the same fusion gene is likely to occur in many patients.

Recurrent fusion genes are important for understanding cancer mechanisms and developing useful clinical biomarkers and anticancer therapies. For instance, the BCR-ABL fusion gene in chronic myeloid leukemia is known to initiate oncogenesis through formation of a misregulated BCR-ABL fusion kinase. The BCR-ABL fusion gene is also a clinical biomarker of high diagnostic and prognostic utility in chronic myeloid leukemia. In addition, this fusion protein serves as a therapeutic target for the successful drug imatinib. [6] In solid human tumors, fusion genes such as the TMPRSS2-ERG fusion in prostate cancer, [7] FGFR-TACC in glioblastoma, [8] and DNAJB1-PRKACA in liver cancer [9] are important molecular signatures for understanding cancer mechanisms and stratifying patient groups. In CCA, one specific fusion(FIG-ROS1) is detected in two out of 23 patients (9%) in a series of Chinese Bile duct carcinomas(BTC) patients.[10] This frequency was not confirmed by another group of 45 BTC patients.[11]

Identifying additional molecular biomarkers in CCA genomes will potentially result in early diagnosis and new treatment strategies. In this study, we identify a bile duct cancer specific gene fusion PUM1-TRAF3 in a series of Korean CCA patients. Pumilio 1, also known as PUM1 is a homolog of the Drosophila Pumilio protein and belongs to the PUF family. The PUF family is comprised of evolutionarily conserved proteins that contain a C-terminal RNA-binding domain made up of eight highly conserved tandem repeats. Pumilio 1 is a typical PUF protein expressed in fetal tissues as well as adult stomach, kidney, intestine, muscle, brain and heart tissues. Pumilio 1 localizes to the cytoplasm and is believed to participate in cell fate, cell development, cell differentiation and maintenance of somatic stem cells.[12] TNF receptor–associated factors (TRAFs) are multifunctional intracellular proteins which participate in the recruitment and activation of a plethora of protein kinases involved in immune and death receptor signaling. The impact of TRAF3 deregulation extends to the pathogenesis of malignancy. Inactivating mutations and loss of heterozygosity in the TRAF3 locus have been reported in a significant proportion of myeloma patients, suggesting that TRAF3 may function as a tumor suppressor.[13,14]

II. MATERIALS AND METHODS

1. Sample Preparation

Patient sample and clinical information were obtained from consenting individuals who had undergone surgery for Bile duct cancer at the Severance Hospital, Yonsei University College of Medicine. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration, and the Ethical Committee and Institutional Review Board of Yonsei University College of Medicine approved the protocol of tissue acquisition from the patients' specimens. Tissue samples were divided and kept in RNalater (Ambion, TX, US) and stored at -80℃ until further use, or fixed with 4% paraformaldehyde and embedded in paraffin for histologic evaluation.

2. RNA Sequencing

Total RNA from human tissues were extracted using an RNeasy Mini Kit (Qiagen, Valentia, CA, US) and mRNA libraries were prepared from 20 to 50 ng, depending on the DV200 value, of the extracted RNA using the capturing chemistry of the TruSeq RNA Access Library Prep Kit (Illumina, San Diego, CA, US), according to the manufacturer's recommendations. The quality of the mRNA library was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US) and the concentration was measured on a Qubit Fluorometer using the Qubit RNA HS Assay kit (Life Technologies, Rockville, MD, US). We obtained RNA-seq data for five tumor-normal pairs of bile duct cancer. RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation kit (Illumina). The libraries were sequenced on the HiSeq 2500 platform (Illumina) as manufacture's recommendation. We generated on average, ~43 million paired-end (2 ×100bp) reads per sample.

3. Fusion identification and analysis

Three fusion detection tools were used to identify cancer somatic fusion: ChimeraScan (version 0.4.5), FusionCatcher (version 0.99.4d), JAFFFA (version 1.06). The hg19 build was used as the human reference genome. For ChimeraScan, we conducted with default parameter using each sample of paired tumor-normal.

For identification of somatic fusions, tumor fusion list detected in the individual tumor were removed normal fusions detected all normal samples. To focus on functional fusions with impact on cancer, both genes involving fusion within CDS (coding sequence) were remained. To remove false positive fusion, we remained somatic fusion genes with at least one spanning reads mapping to the fusion junction. For FusionCatcher, we used default parameters with paired tumor-normal samples to identify somatic fusions. In this tool, we applied no filtering process because FusionCatcher tool provides a few fusion events detected in sample, whose main goal is good real-time PCR validation rate. For JAFFA, we carried out with Direct mode due to 100bp read length as authors recommended. To obtain high confidence and reliable cancer somatic fusions, we extracted fusions with high and moderate categories, representing genes of fusion partner with breakpoints aligning to exon-exon boundaries, which may result in true fusion transcript. All cancer somatic fusion lists detected from three fusion finding software tools were removed normal fusions in present normal samples using Geuvadis RNA sequencing project and public RNA-seq data. For detection of normal fusions using Geuvadis project data, RNA-seq of 463 lymphoblastoid cell line samples from five populations of the 1000 Genomes project with 76bp read length, we used ChimeraScan with parameter r, 280. For collection of known normal fusions, we used normal fusions of BodyMap provided by ChimeraScan authors and those of Greger and colleagues (2014, PMID: 25133550) and removed the normal fusion list from tumor fusion list. Finally, we selected somatic fusions detected at least two software tools. To identify and predict functional fusion, for partner genes of fusion, we searched for kinase, oncogene, tumor suppressor gene and known bile duct cancer genes. To annotate fusions, we used COSMIC v77 fusion list and Cancer census gene list, Mitelman Database of chromosome Aberrations in cancer from ChimerDB2.0, FusionCancer (a database of fusion genes in human cancers) (PMID: 26215638) and PanCanFusionV2, which tumor fusions detected among multiple cancers from TCGA. Seven candidate fusions were chosen for further validation by conventional PCR amplification followed by Sanger sequencing.

4. Validation of the PUM1_TRAF3 fusion by RT-PCR

Total RNA from human tissues and cell pellets of harvested cultured cells were extracted using an RNeasy Mini Kit (Qiagen) and total RNA extraction from slides was performed using RNeasy FFPE Kit(Qiagen). Human normal pancreas cDNAs were purchased from Ambion (Life Technologies) and Takara. Total RNA was quantified using an ND-1000 Nanodrop spectrometer (NanoDrop Technologies, Wilmington, DE, US). Reverse transcription was performed using Superscript II, RNaseOUT, oligo(dT) primer, and dNTPs (all from Invitrogen, Carlsbad, CA, US). PCR, using Ex Taq HS DNA polymerase (TaKaRa, Shiga, Japan), was carried out for one cycle of denaturation at 95℃ for 1 minute 40 cycles of denaturation at 95℃ for 15 seconds, annealing at 58.4℃ for 30 seconds, and extension at 72℃ for 30 seconds; and a final extension at 72℃ for 7 minutes. PCR products were analyzed using agarose gel electrophoresis. Primers used were PT1 (forward) 5'- TGT ATG GCT GCC GTG TTA TC -3' and (reverse) 5'- ATG TCG TGC ACA CTC AGC AT -3', AD (forward) 5'- CCT TCT GAA ACC CCA GCT AA -3' and (reverse) 5'- AGC AAT CGT GCT GAC AAG AG-3' and GAPDH (forward) 5'- GTC TCC TCT GAC TTC AAC AGC G -3' and (reverse) 5'- ACC ACC CTG TTG CTG TAG CCA A -3'

5. Quantitative RT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed using a SYBR Green (Applied Biosystems, Foster City, CA, US) and PCR amplification consisted of an initiation step at 95℃ for 10 minutes, followed by 40 cycles at 95℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 15 seconds. qRT-PCR was performed using an ABI Prism 7300 Sequence Detection System (Applied Biosystems), and relative gene expression was calculated with Ct method (ΔCt_{GAPDH}-$\Delta Ct_{target\ gene}$=$\Delta\Delta Ct$; relative quantity=$2^{-\Delta\Delta Ct}$). All qRT-PCR was performed in triplicate with three independent total RNA samples. Primers were used as following; PT2 for (forward) 5'- GCA ATC ACG TGG TTC AGA AA-3' and (reverse) 5'- TGC TCT TCA TGC TGT CTG CT-3'), NIK for (forward) 5'- TCC ACT CAC GAA GGA TTC TGC' and (reverse) 5'- TGA GCA AGG ACT TTC CCA GG-3') IKKa for (forward) 5'- CAA ATG AGG AAC AGG GCA AT-3' and (reverse) 5'- TCA TGG

GGG AAA AAC ACA TT-3'), NFkB2 (forward) for 5' GAA CTC CTC CAT TGT GGA ACC-3' and (reverse) 5'- TCG GAA GCC TCT CTG CTT AG-3') and GAPDH for (forward) 5'- GTC TCC TCT GAC TTC AAC AGC G -3' and (reverse) 5'- ACC ACC CTG TTG CTG TAG CCA A -3'

6. Droplet Digital PCR

The PT2 (forward) 5'- GCA ATC ACG TGG TTC AGA AA-3' and (reverse) 5'- TGC TCT TCA TGC TGT CTG CT-3') primer was at final concentrations of 100nM, 10ul 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, Contra Costa, CA, US) and 1ug of template in a final volume of 20 µl. The 20-µl droplet digital PCR (ddPCR) reaction mixture was then loaded into the Bio-Rad DG8 disposable droplet generator cartridge. A volume of 70 µl of droplet generation oil was loaded into the oil well for each sample. The cartridge was placed into the droplet generator. The generated droplets were transferred to an ddPCR 96-well plate(Bio-Rad Laboratories). The plate was heat-sealed with a pierceable foil heat seal(Bio-Rad Laboratories) and then Thermal-cycling conditions were 95℃ × 5 minutes (1 cycle), 95℃ × 30 seconds (ramp rate 2.5℃/second), and 60℃ × 1 minute (ramp rate 2.5℃/second) (40 cycles) and 90℃ × 5 minutes (1 cycle). After PCR, the 96-well PCR plate was loaded on Bio-Rad's QX100 droplet reader. Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.3.2.0 that accompanied the QX100 droplet reader.

7. In Situ Proximity ligation Analysis

Sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by treatment of sections with methanol containing 0.3% hydrogen peroxide at room temperature for 20 minutes. Antigen retrieval was performed in citrate buffer (0.01 M, pH 6.0), followed by blocking in 10% normal donkey serum for 1 hour at room temperature to reduce nonspecific background staining. The tissue slides were incubated overnight at 4℃ with rabbit polyclonal antibody against PUM1(Santa Cruz Biotechnology, CA, US), and mouse monoclonal antibody against TRAF3(Santa Cruz Biotechnology) in antibody diluent(Gibco, Grand Island, NY,

US). After the washing, we used Duolink® In Situ Red Starter Kit (SIGMA, St. Louis, MO, US) to visualize PUM1 and TRAF3 fusion protein. The PLA probe solution as added to the slide and the two PLA probes were diluted in antibody diluent with 1:5 ratio and incubate for 1hour at 37℃. For ligation, ligation-ligase solution was added and the slides were washed and incubated for 30 minutes at 37℃. Then Amplification-polymerase solution was added to the washed slides and incubated for 100 minutes at 37℃. After the washing, the slides were air dried and slides were mounted with mounting solution containing DAPI (Vector, Burlingame, CA, US).

8. Cell culture

All bile duct cancer cell lines including SNU 245, SNU 308, SNU 478, SNU 869, SNU 1079 and SNU 1196 were purchased from KCLB(Korean cell line bank, Seoul, Korea) and were cultured in RPMI1640(Invitrogen Gibco, Grand Island, NY, US) and 10% fetal bovine serum(FBS; Hyclone, Logan, UT, US). HuCCT1 cell line was kindly donated by Dr. Ming Sound Tsao (University of Toronto) and was cultured in keratinocyte medium (Invitrogen). Cells were maintained at 37℃ in a humidified incubator containing 5% CO_2.

9. Gene cloning and expression

Fusion transcript sequence was polymerase chain reaction (PCR) amplified from the patient sample using PT3 (forward) 5'- CCA TGT TGT CGG AGT GAA AG-3' and (reverse) 5'- AGA CAG ACC GGT TCA AAT CC-3') primers. PCR amplification consisted of an initiation step at 95℃ for 3 minutes, followed by 37 cycles at 95℃ for 15 seconds, 58.4℃ for 15 seconds, and 72℃ for 4 minutes. The PCR product was eluted and amplified using primer PT4 (forward) 5'- ATT AAG CTT GCG GCC ATG AGC GTT GCA TGT GTC TT-3' and (reverse) 5'- GAT GAA TTC GCG GCC TTG GGA TCG GGC AGA TCC G-3') at 95℃ for 3 minutes, followed by 37 cycles at 95℃ for 15 seconds, 77.7℃ for 15 seconds, and 72℃ for 4 minutes. The PCR product was eluted and the PT was inserted into Not1 site of p3XFLAG-CMV™-14 Expression vector(SIGMA) using In-fusion cloning kit(Takara, Shiga, Japan) by mixing 50ng of PCR product, 50ng of linearized vector

and 2ul of recombinase enzyme at 50℃ to generate PT-FLAG. Bile duct cells were transiently transfected with the plasmid using Lipofectamine 2000(Invitrogen) and gene or protein levels were determined at 72 hours post transfection.

10. Protein extraction & Western blotting

Cells were homogenized in lysis buffer A (0.25 M sucrose, 20 mM Tris pH 7.6, 1.5 mM MgCl2, 10% glycerol, 1 mM EDTA and Complete Mini protein inhibitor cocktail (Roche Diagnostics) incubated on ice for one hr and centrifuged at 12,000 rpm for one hour at 4℃ for supernatant collection. Protein concentration of the resulting supernatant was determined using the BCA(Bicinchoninic acid solution) Protein Assay Kit(Thermo scientific, Rockford, US). 30ug of total Protein per lane were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore, Bedford, MA, US). The membrane was blocked in TBST containing 5% Skim milk. Primary antibodies were used as following; anti-FLAG(diluted 1:1000, SIGMA), anti-PUM1 (diluted 1:1000, Santa Cruz Biotechnology, CA, US), anti-TRAF3(diluted 1:1000, Santa Cruz Biotechnolgy), anti-N-Cadherin(diluted 1:1000, Abcam, Cambridge, MA, US), anti-NIK(diluted 1:1000, Santa Cruz Biotechnolgy), anti-NF-κB (diluted 1:1000, Santa Cruz Biotechnolgy), anti-Slug(diluted 1:1000, Cell Signaling, Danvers, USA) and anti-GAPDH(diluted 1:5000, Santa Cruz Biotechnology). For secondary antibody, goat anti-mouse IgG-HRP(diluted 1:5000, Santa Cruz Biotechnology) and goat anti-rabbit IgG HRP(diluted 1:5000, Santa Cruz Biotechnology) were used. Proteins were visualized by the Super Signal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, US)

11. Proliferation

Cells were detached and plated in triplicate at a density of 2×10^3cells/well in 96-well plates in 100 μl complete medium. Cells were cultured in DMEM (Gibco, Grand Island, NY, US) with 10% FBS and 1% antibiotics for proliferation assay and lactate assay. Twenty-four hrs after plating, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium), EZCytox, DaeilLab, Korea) was added and incubated for one hour. Absorbance at 450 nm was read with

a microplate reader

12. Cell migration and invasion assay

For the migration assay, cells were detached and suspended at 1.5×10^5cells/mL in 1%FBS and plated at a density of 3×10^4 cells/well in 24-well Transwell plates (Costar, Bethesda, MD). For the invasion assay, the upper chamber was pre-coated with Matrigel (BDbiosciences, Bedford, MA), and cells were seeded at 3×10^4 cells/well. The bottom chamber was filled with culture medium containing 20% FBS. After incubation at 37℃, cells were fixed with 5% glutaraldehyde for 30 minutes and stained with 0.1% crystal violet. Cells were completely removed from the upper surface of the membrane with a moist cotton swab. Migrated and invaded cells were counted and photographed under a BX51 microscope.

13. Colony formation assay

Colony-forming efficiency was determined using a double-layer soft agar method. A total of 5000 cells were plated in 0.35% agar over a layer of 0.5% agar containing DMEM and 10% FBS in 6-well plates. Cells were incubated for 4 weeks in a CO2 incubator, and colonies were observed under an inverted Olympus MVX10 microscope.

14. Statistical analysis

Fisher's Exact test was used for calculating statistical significance between the differences of with fusion versus without fusion. The level of statistical significance was set at $P < 0.05$. Kaplan–Meier survival curves (Kleinbaum and Klein, 2005) were computed via R package. The recurrence-free survival rate was compared among patients with fusion and without fusion to investigate whether fusion event is an indicator of prognosis.

III. RESULTS

1. Discovery of Candidate Fusions in Bile Duct cancers

To identify bile duct cancer specific gene mutation, we performed RNA-seq analysis with five pairs of fresh-frozen bile duct cancer and adjacent normal samples. The RNA-seq data was analyzed using three fusion detection tools, including ChimeraScan, FusionCatcher and JAFFA. As a result, a list of 52 candidates results were obtained. To determine somatic fusion in bile duct cancer, fusions found in any normal samples and previously known normal fusions were removed. To select bile duct cancer somatic fusion with reliable and high confidence, we analyzed according to tool with some strategies described in the method section. Moreover, the fusions detected at least two software tools were analyzed additionally. The data was compared with the previously reported gene fusions using ChimerDB2.0, FusionCancer, PanCanFusionV2, and COSMICv77 softwares and cancer-related genes were screened by Sanger Cancer gene census (Figure 1). As a result, we selected seven different fusions that were identified in more than two tools. Individual fusions were analyzed with tissue samples previously analyzed for RNA-seq analysis by conventional PCR. To determined site of fusion, Sanger sequencing was also performed. To validate the gene fusion, we constructed a primer to confirm the fusion of backward and forward genes, followed by conventional PCR, and confirmed by Sanger sequencing that the fusion gene was consistent with the RNA-seq data. Among seven fusions, we excluded five fusions whose expression was confirmed in cancer as well as normal tissues adjacent to the cancer. Though we tried to exclude the fusion genes found in normal tissues based on the previously reported data, Five genes were out and only two genes remained. The remaining two fusions ASH1L-DOCK7 and PUM1-TRAF3 were analyzed in cancer tissues. We excluded ASH1L-DOCK7 from future experiments because it is too long to study functions. To confirm that the gene fusion is cancer specific, we analyzed with two normal human pancreatic tissue cDNAs. We were verified using normal human pancreatic cDNA. Because normal bile duct cDNA could not be obtained. Both ASH1L-DOCK7 and PUM1-TRAF3 fusion was occurred in a single tumor sample that was detected in all three fusion finding tools. It is worth to mention that the two candidate fusions were

identified in a single patient's tissue (presented as #2150 patient) where the patient diagnosed with intrahepatic cholangiocarcinoma and tumor was recurred and metastasis in liver was also observed. It is likely that the fusion gene related to the outcome of the patient. We analyzed whether the fusion gene was repeatedly observed in patients with other bile duct cancer.

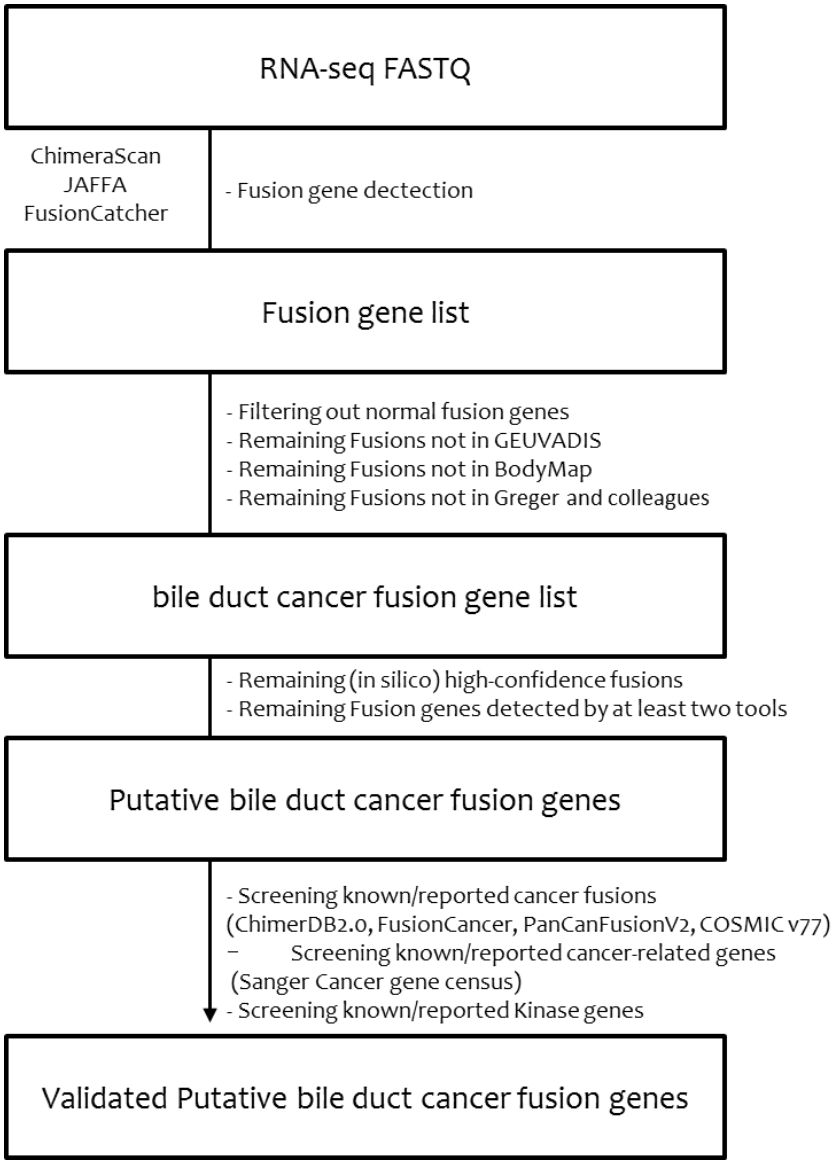

Figure 1. Workflow for fusion analysis

2. PUM1-TRAF3 is translated into an In-Frame fusion protein

PUM1-TRAF3 fusion gene occurs by chromosomal rearrangement of pumilio RNA binding family member 1 (PUM1) on chromosome 1p35.2 and TNF receptor associated factor (TRAF3) on chromosome 14q32.32 and Exon 18 of PUM1 and exon10 of TRAF3. As a result some of the puf domains present at the c-terminus of pum1 were deleted and the Zinc Finger domain at the n-terminus of traf3 was entirely deleted but MATH domain(MATH: Meprin And Traf-Homology) was remained (figure 2A). PUM1 is known for its roles in cell division, differentiation and development[12]. TRAF3 is known for tumor suppressor gene. Also TRAF3 inactivation is likely to promote cell survival via NF-κB pathway activation.[13,14]

In order to confirm the expression of PUM1_TRAF3 in a patient tissues(#2150) that conducted RNA sequencing, We performed conventional PCR and it was confirmed that it was expressed only in a cancer tissue (figure 2B). To confirm the fusion site, we performed gel extraction and analysed Sanger sequencing (figure 2C). We verified the fusion site in fusion transcripts on chromosome 1 exon18 of PUM1 and chromosome 14 exon10 of TRAF3.

We performed proximal ligation assay(PLA) to identify the presence and location of fusion protein on tissue slides of bile duct cancer patients. It can detect, quantify and determine cell localization of protein interactions and their modifications in a single experiment. It need to two primary antibodies from different species that recognize two target epitopes. PLA analysis was performed in patients with and without confirmed PUM1-TRAF3 fusion gene expression by conventional PCR. To perform PLA analysis, we used PUM1 mouse antibody and TRAF3 rabbit antibody and confirmed by fluorescence microscopy. We also perform DAPI (4′,6-diamidino-2-phenylindole) staining because DAPI is commonly used as a nuclear counterstain in fluorescence microscopy. We can detect fusion protein in fusion gene positive slide and it is located in the cytoplasm (figure 2D).

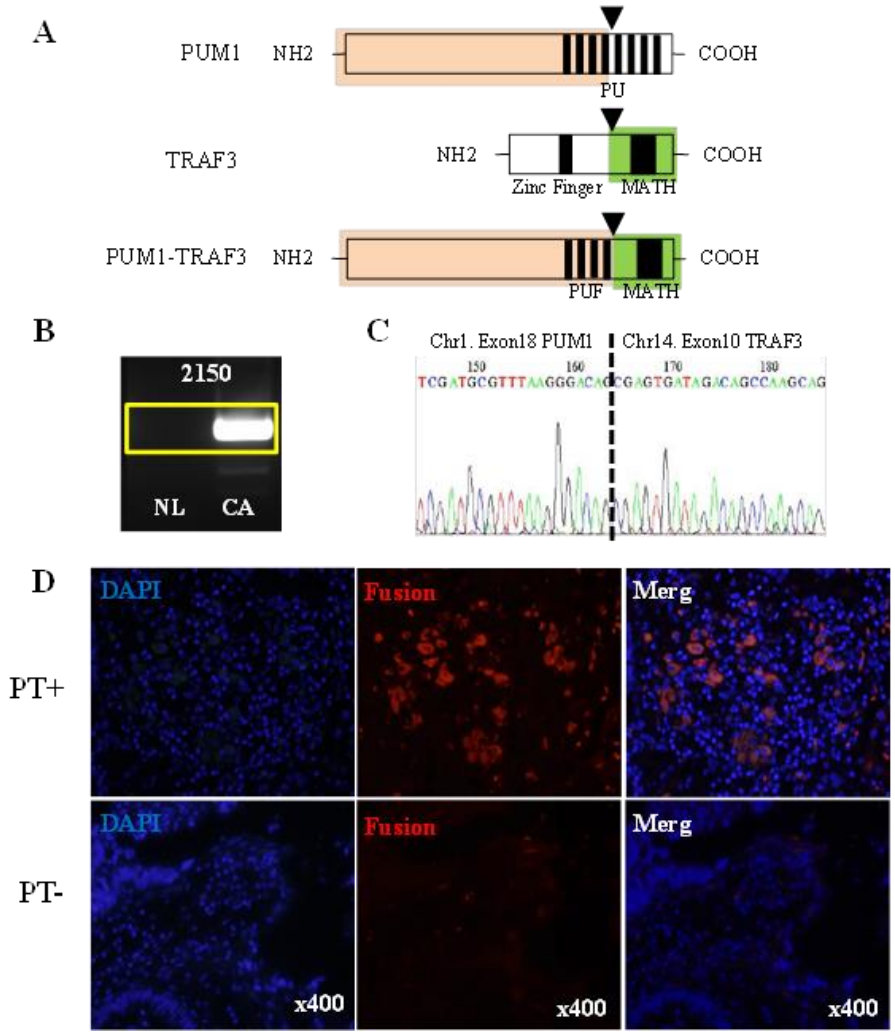

Figure 2. PUM1-TRAF3 is a novel fusion in cholangiocalcinoma. (A)Protein domain ideogram of PUM1-TRAF3. As a result of the gene fusion, PUM1 loses a part of puf region. TRAF3 loses the zinc finger domain but retains the MATH domain(MATH: Meprin And Traf-Homology). (B)RT-PCR of normal/cancer pairs of bile duct cancer with PUM1-TRAF3 fusion in patient tissue which was validated from RNA sequencing. (C)Representative Sanger sequencing of RT-PCR bands confirmed that it contains the expected PUM1-TRAF3 fusion junction. Fusion point between PUM1 and TRAF3 is indicated by black dashed line. (D)fusion protein expression in bile duct cancer patient specimens. (PT: patient with PUM1-TRAF3 fusion gene, PT-: patient without PUM1-TRAF3 fusion gene)

3. Recurrent PUM1-TRAF3 Fusion in Cholangiocarcinoma

To determine whether the PUM1-TRAF3 fusion gene is expressed specifically in cancer, we obtained 21 pairs(cancer tissues and its adjacent normal tissues) of bile duct cancer patient's tissues. We performed conventional PCR after extracted total RNA from 22 pairs of tissues and synthesized cDNA. As a result we found PUM1-TRAF3 fusion gene in 7 patients out of 21 patients and only detected in cancer tissue (figure 3A). However, Its signal was smeared. We need to find more delicate method to detect fusion transcript. Droplet digital PCR(ddPCR) is a method for performing digital PCR that is based on water-oil emulsion droplet system. PCR amplification of the template molecules occurs in each individual droplet. Prior to droplet generation, cDNA are prepared and using a real-time primer. We analyzed the 22 pairs of patient's tissue using ddPCR including a 2150 patient that was previously analyzed. We can find PUM1-TRAF3 fusion gene in 13 patients out of 22 patients and only detected in cancer tissue but not found in any normal samples (Figure 3B). We can detect a larger number of patients expressed PUM1-TRAF3 in ddPCR than conventional PCR. It is due to allow for the sensitive detection of rare targets.

Additionally we obtained formaline-fixed paraffin embedded (FFPE) cancer tissue slides of 33 bile duct cancer patients to determine the clinical significance of the fusion gene and performed ddPCR. As a result we found PUM1-TRAF3 fusion gene in 28 patients out of 55 patients. It was found in patients with bile duct cancer with a 50.9% frequency (Figure 3C).

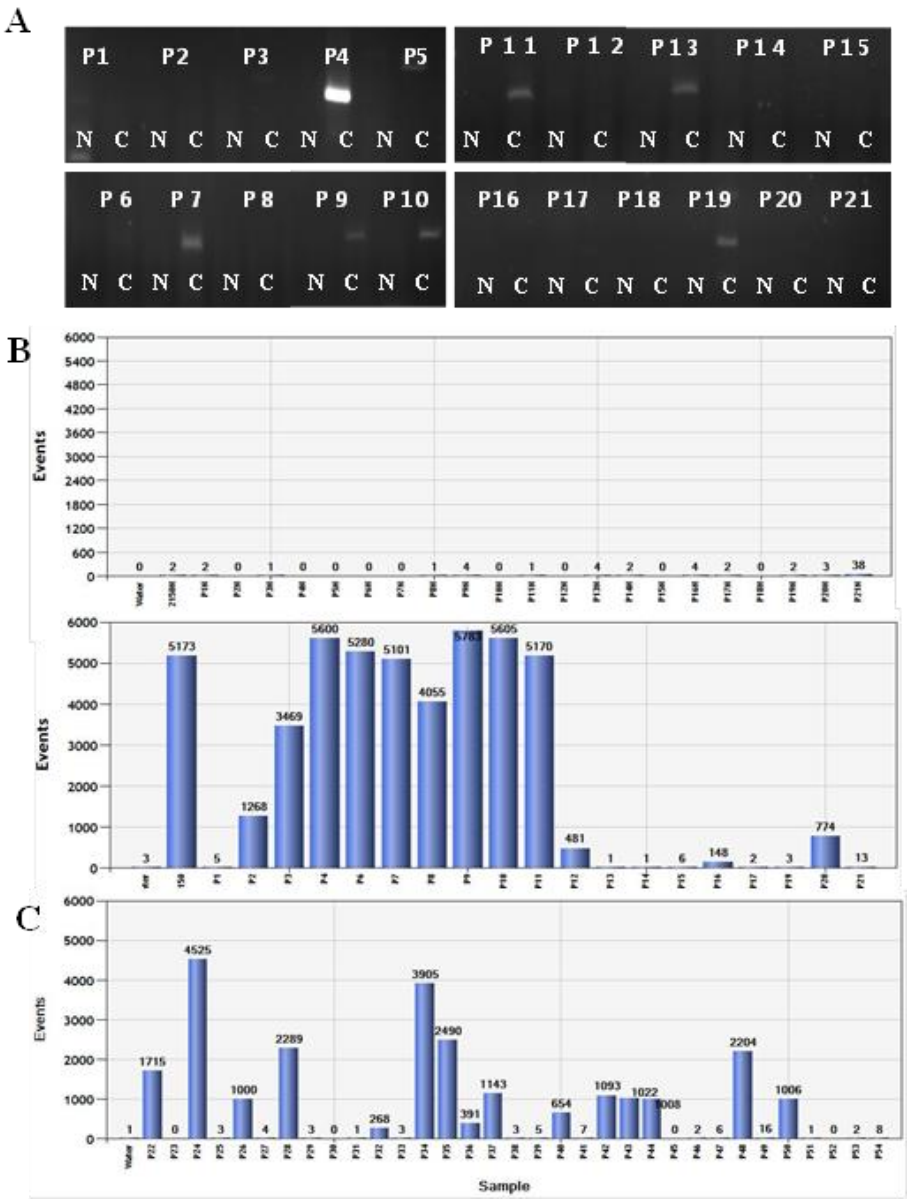

Figure 3. Identification of fusion genes in patient tissues. (A)Expression of PUM1-TRAF3 fusion gene in additional 22 bile duct cancer tissues using RT-PCR. (P: patient, N: adjacent normal tissue, C: cancer tissue) (B)22 FFPE bile duct cancer tissues using previous tissue sample and (C)additionally 33 FFPE bile duct cancer samples using digital droplet PCR(P22-P54).

4. PUM1-TRAF3 overexpression resulted in Epithelial-Mesenchymal Transition(EMT)

PUM1_TRAF3 is expressed at a high frequency in patients with bile duct cancer and we predicted that it is related to bile duct cancer. We investigated fusion mRNA expression by conventional PCR to confirm whether the fusion gene is expressed in 7 bile duct cancer cell line including SNU245, SNU308, SNU478, SNU869, SNU1079, SNU1196 and HUCCT1. No fusion gene was found in any cell line compared with house keeping gene, Glyceraldehyde 3-phosphate dehydrogenase(GAPDH) which is often used as control (Figure 4A).

We selected SNU1196 which was derived from Klatskin tumour arising in the hepatic duct bifurcation and mutations of p15, p16 and p53 genes was found in SNU1196. In order to predict the function of PUM1-TRAF3 in vivo, we synthesized the full sequence of PUM1-TRAF3 found in the #2150 patient by conventional PCR, inserted it into the vector including FLAG which is hydrophilic 8-amino acid peptide that is fused to the recombinant protein of interest, and transfected into SNU1196 to induce overexpression. As a result, we confirmed that the fusion protein was overexpressed by western blot using FLAG antibody which can detect to flag tag and TRAF3antibody which can detect C-terminus of TRAF3. We were able to identify a band of PUM1-TRAF3 fusion protein at 150kDa size and GAPDH is used as control (Figure 4B).

To determine whether it is associated with PUM1 or TRAF3, we have examined protein levels associated with PUM1 such as cyclin B1, cyclin D3, PCNA and CDK2 and protein levels associated with TRAF3 such as NFkB. GAPDH is used as control. As a result, proteins including cyclinB1, D3 and CDK2 level related to PUM1 was not changed and NFkB related to TRAF3 was increased (Figure 4C). NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. Defects in NF-κB results in increased susceptibility to apoptosis leading to increased cell death.[14, 15]

To evaluate whether the phenotypic changes induced by PUM1-TRAF3 reflected a EMT, we investigated the expression of various EMT markers by

western blot. As a result, expression of PUM1-TRAF3 fusion protein leads to upregulation of EMT markers including N-cadherin and Slug. Cell adhesion associated protein, ILK, was downregulated in the group transfected fusion gene (Figure 4D), suggesting that this fusion gene leads to an epithelial to a mesenchymal transition.

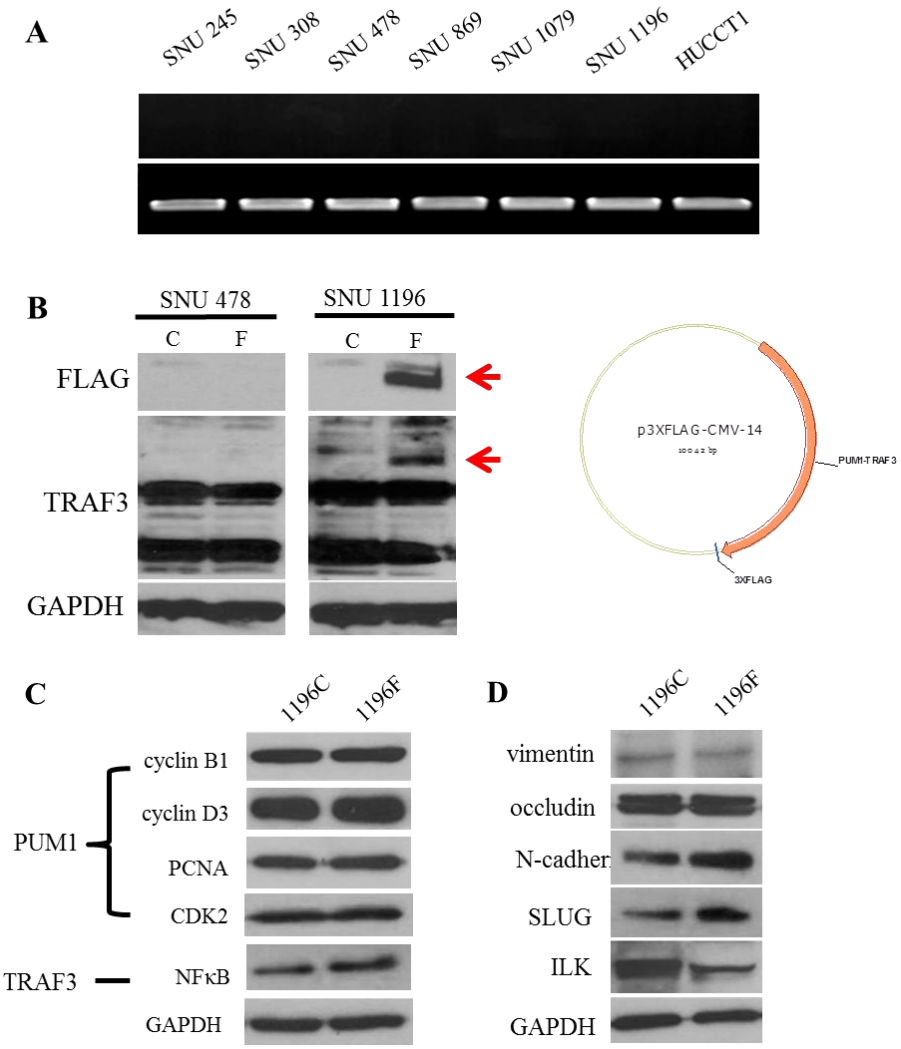

Figure 4. PUM1-TRAF3 overexpression in bile duct cancer cell line. (A)Expression of fusion gene mRNA in bile duct cancer cell line. (B)Western blot analysis of SNU478 and SNU 1196 after transient transfection of PUM1-TRAF3. (Upper)Western blot using FLAG antibody. (middle)Western blot using TRAF3 antibody. GAPDH is used as loading control. (C)Expression levels of proteins related to the front and back gene (D)Expression of various EMT markers using western blot. GAPDH is used as loading control. (1196 C: control vector trasfected cells, 1196 F: PUM1_TRAF3-FLAG vector transfected cells)

5. PUM1-TRAF3 increases cell motility.

We expected that PUM1-TRAF3 fusion gene can cause to increase cell mobility after confirming change in the EMT markers. To investigate whether the change induced by PUM1-TRAF3, we performed proliferation assay using EZ-CYTOX which is to measure the amount of living cells using tetrazolium salt. The WST of EZ-CYTOX is reduced by succinate-tetrazolium reductase which is dehydrogenase, which is active only in living cells, to produce formazan. Therefore, as the number of living cells in the sample increases, the dehydrogenase activity of the mitochondria increases, and the formazan production increases and the absorbance increases. As a result, a group of transfected with PUM1-TRAF3 slightly increased proliferation (Figure 5A).

To investigate whether the cell motility changes induced by PUM1-TRAF3, we performed Migration and invasion. As a result, migration and invasion were markedly increased in PUM1-TRAF3 transfected cells. We performed migration, and invasion assays several times in order to ensure the reliability of the experiment. The number of cells was counted and we present it as a graph with error bars. Cell migrated with the PUM1-TRAF3 transfected group was approximately1.5-fold greater than that of the control group. Invasion in the PUM1-TRAF3 transfected cells was also approximately 7-fold greater than that of the control (Figure 5B).

We performed clonogenic assay to investigate the ability of a single cell to grow into a colony. Transient transfected SNU1196 cells were cultured in soft agar and increased number of colonies was observed in PUM1-TRAF3 transfected cells compared to the control group. The number of cells was also counted and we present it on a graph with error bars (Figure 5C). Overexpression of PUM1-TRAF3 increases migration, invasion and colony formation, indicating that PUM1-TRAF3 expression result in serious consequences including metastasis and tumorigenicity.

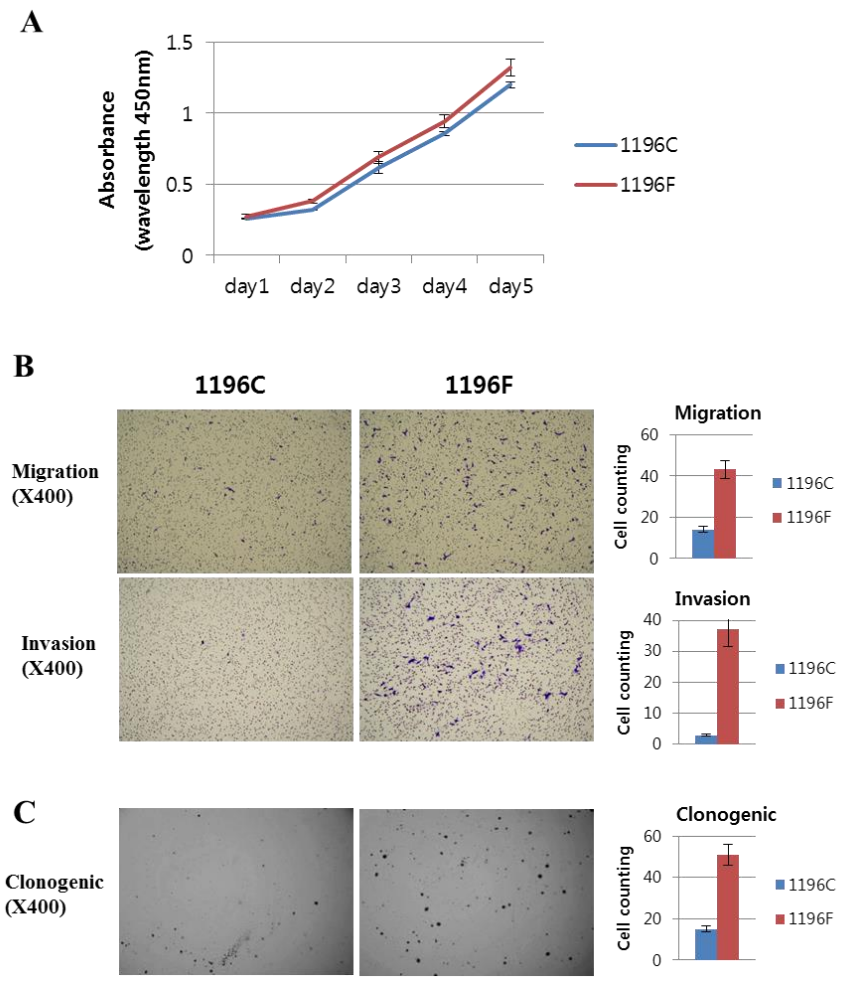

Figure 5. Effect of PUM1-TRAF3 on proliferation and cell motility. (A)Increased cell proliferation in fusion-expressing SNU1196 cell. (B)SNU1196 cell expressing fusion gene increased the cell motility both in migration and invasion. (C)SNU1196 cells expressing fusion gene increased in clonogenic ability. (1196 C: control vector transfected cells, 1196 F: PUM1_TRAF3-FLAG vector transfected cells) The number of cells was counted and presented it as a graph with error bar.

6. Statistical analysis

Total 55 patients with bile duct cancer who underwent curative surgery were evaluated to investigate clinical significance of PUM1-TRAF3. There were 28 patients (50.1%) who expressed PUM1-TRAF3 by ddPCR. We also performed statistical analysis. There were 28 patients (50.1%) who expressed PUM1-TRAF3 by ddPCR. We collected information such as age, gender, origin of tumor, stage, differentiation, CA19-9, resection margin, recurrence, distant metastasis, palliative CTx and median survival. Patients with PUM1-TRAF3 presented higher rate of female gender (57.1 vs. 25.9, p=0.019), advanced stage (25% vs. 3.7%, p=0.025) and recurrence rate (64.7 vs. 48.1%, p=0.080) compared to patients without PUM1-TRAF3. Disease free survival (DFS) was 12.1 vs. 29.8 (p=0.078) and overall survival was 26.1 vs. 36.6 (p=0.239) between the patients with PUM1-TRAF3 and without PUM1-TRAF3. (Table 1). The expression of fusion gene was significantly significant in the gender and advanced stages, with a p-value≤0.05, and the remainder were not statistically significant.

Disease free survival(DFS) was analyzed comparing fusion gene negative (PT-) groups versus fusion gene positive (PT+) groups by Kaplan-Meier analysis for survivals. Fusion gene negative groups had disease free survival dates for 29.8 months in average whereas fusion gene positive groups lived for 12.1 months (p-value=0.078) (Figure 6A). Overall survival (OS) was also analyzed comparing two groups. Overall survival dates of fusion gene negative groups were 36.6 months whereas fusion gene positive were 26.1 months in average. (p-value=0.239) (Figure 6B).

We performed multivariate survival analysis using Cox-regression. As a result, the hazard ratio (HR) of fusion gene occurrence to the disease free survival dates was 2.86 with p value of 0.023, stage was 2.69 with p value of 0.041, CA19-9 was 1.00 with p value of 0.011 and resection margin(RM) was 4.57 with p value of 0.002. The patient with fusion gene (PT+) showed significant decrease in DFS compared to the fusion gene negative (PT-) groups (Table 2).

Table 1. Baseline characteristics

	Fusion gene (PT-) N=27 (49.1%)	Fusion gene (PT+) N=28 (50.1%)	P value†
Age at diagnosis			0.259
Mean (SD)	67.0 (±9.5)	68.7 (±7.2)	
Gender (%)			0.019
Female	7 (25.9%)	16 (57.1%)	
Male	20 (74.1%)	12 (42.9%)	
Origin of tumor			0.533
Intrahepatic CCC	2 (7.4%)	7 (25.0%)	
Extrahepatic CCC	22 (81.5%)	16 (57.1%)	
Perihilar CCC	2 (7.4%)	4 (14.3%)	
GB cancer	1 (3.7%)	1 (3.6%)	
Stage			0.285
I	6 (22.2%)	9 (32.1%)	
II	20 (74.1%)	12 (42.9%)	
III	1 (3.7%)	4 (14.3%)	
IVa	0 (0.0%)	3 (10.7%)	
Stage			0.025
I/II	26 (96.3%)	21 (75.0%)	
III/IVa	1 (3.7%)	7 (25.0%)	
Differentiation (%)			0.389
Well	4 (14.8%)	3 (10.7%)	
Moderate	15 (55.6%)	19 (67.9%)	
Poor	7 (25.9%)	5 (17.9%)	
Undiff.	1 (3.7%)	0 (0%)	
CA 19-9	997.1 (±2910.2)	311.4 (±865.2)	0.242

Resection margin (%)			0.469
R0	20 (74.1%)	23 (82.1%)	
R1	7 (25.9%)	5 (17.9%)	
Adj CTx			0.166
Yes	13 (48.1%)	18 (64.7%)	
Recurrence			**0.080**
Yes	11 (40.7%)	18 (64.3%)	
Distant metastasis*	9 (81.8%)	17 (94.4%)	0.324
Liver	7 (63.6%)	8 (42.1%)	0.369
Lung	1 (9.1%)	4 (21.1%)	0.364
Brain	1 (9.1%)	3 (15.8%)	0.562
Peritoneum	1 (9.1%)	6 (31.6%)	0.143
Palliative CTx			0.199
Yes	7 (50.0%)	15 (71.4%)	
Median Survival, mo			
DFS (range)	29.8 (1.4-110.3)	12.1 (0.8-58.2)	**0.078**
OS (range)	36.6 (1.8-110.4)	26.1 (1.2-59.6)	0.239

* The ratio in the patients with recurrence and multiple organ metastases were counted separately.

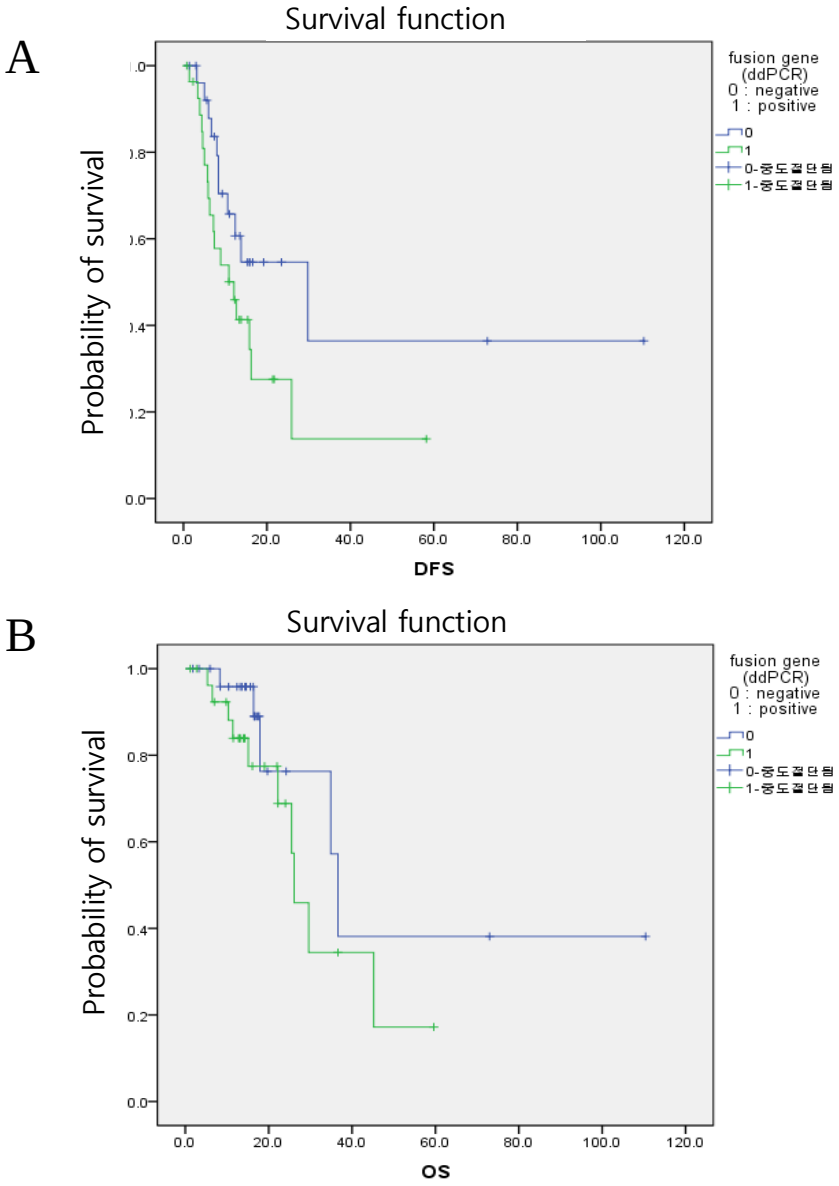

Figure 6. Kaplan-Meier analysis for survivals. (A)Disease free survival(DFS) was analyzed comparing fusion gene negative (PT-) groups versus fusion gene positive (PT+) groups. Fusion gene negative groups had disease free survival dates for 29.8 months in average whereas fusion gene positive groups lived for 12.1 months (p-value=0.078) (b) Overall survival (OS) was analyzed comparing two groups. Overall survival dates of fusion gene negative groups were 36.6 months whereas fusion gene positive were 26.1 months in average. (p-value=0.239)

Table 2. Cox-regression analysis for disease free survival

	Univariate		Multivariate	
	HR (95% CI)	**p value**	**HR (95% CI)**	**p value**
Fusion gene (yes vs. no)	1.94 (0.92~4.13)	0.084	2.86 (1.16-7.05)	**0.023**
Sex (female vs. male)	1.64 (0.79~3.41)	0.184		
Age	1.039 (0.99~1.09)	0.120		
Stage (III/IVa vs.I/II)	2.49 (1.05 ~5.89)	0.038	2.69 (1.04-6.93)	**0.041**
CA19-9	1.00 (1.00-1.00)	0.157	1.00 (1.00-1.00)	**0.011**
RM (positive vs. negative)	2.37 (1.04~5.39)	0.040	4.57 (1.77-11.77)	**0.002**
Adj CTx (yes vs. no)	2.19 (0.96~5.01)	0.061		

IV. DISCUSSION

Bile duct cancer is a relatively rare but highly lethal malignancy arising from the biliary tract epithelium, and an increasing incidence of Bile duct cancer has been reported worldwide in recent years[1]. The cure for Bile duct cancer is attainable by curative radical surgical resection or liver transplantation, but the difficulty in early diagnosis often results in inoperable cases and hence a poor outcome of the patients with a 5-year survival of less than 5% and a median survival period of 6 months in advanced cases[2]. There was a need to do study that helped in the diagnosis and treatment of bile duct cancer.

To identify bile duct cancer specific fusion genes in patient tissues we performed RNA sequencing. The RNA-seq data was analyzed using three fusion detection tools, including ChimeraScan, FusionCatcher and JAFFA. As a result, a list of 52 candidates results were obtained. We performed convestional PCR to confirm the expression of the fusion gene in bile duct cancer tissues. However, except for two which is ASH1L-DOCK7 and PUM1-TRAF3, they were found in normal tissues and excluded. The reason for the expression of the fusion gene in normal tissues is expected to be that the tissue has been removed and mixed with cancer tissue during the process. The remaining two fusions ASH1L-DOCK7 and PUM1-TRAF3 was not expressed adjacent normal tissue. We focused on PUM1-TRAF3 fusion gene due to fact that ASH1L-DOCK7 fusion gene is too long to study functions.

In order to investigate the function of PUM1-TRAF3 fusion gene, FLAG tagged PUM1-TRAF3 was transiently transfected into SNU1196 cells. To confirm the expression of PUM1-TRAF3 fusion gene and changes of EMT markers and proteins related to front and back genes, we performed western blot. We used antibodies tagged with FLAG because we did not have antibodies of detecting fusion protein. However, we have confirmed that TRAF3 antibody can detect fusion protein as well. We predict that the PUM1-TRAF3 fusion gene will be associated with TRAF3, because protein level related to PUM1 was not changed and NFkB related to TRAF3 was increased.

We obtained Bile duct cancer tissues and tissue slides. A bile duct cancer tissue slide was more useful to confirm PUM1-TRAF3 expression. Because cancer tissues contain many normal tissues, they could not be detected sensitively when performing

ddPCR. However, tissue slides can be detected sensitively because they are used by scratching only the cancerous part while observing the microscope. PUM1-TRAF3 fusion was expressed in 28 samples among independent 55 bile duct cancer samples, with 50.9% frequency. Among the baseline characteristics, the reason for the high of p-value in recurrence, Disease free survival, and overall survival associated with the patient's prognosis seems to be due to the insufficient number of patients. In multivariate survival analysis, the presence of the fusion gene has a statistically significant effect on DFS, suggesting that the fusion gene may be a factor affecting recurrence. However, the OS did not show any significant difference, which is probably due to the fact that various factors such as the palliative CTx influence the OS, while the fallow-up period of the majority of patients is still about one year or less.

V. CONCLUSION

We present a new concept of inferring the oncogenic potential of novel fusion genes identified in tumor samples. This study showed that the PUM1-TRAF3 in tumor samples were expressed compared with normal tissues and functions of PUM1-TRAF fusion gene. To identify bile duct cancer fusion, we performed RNA-seq analysis with five fresh-frozen bile duct cancer samples and we identified bile duct cancer specific fusion gene, PUM1-TRAF3. This fusion is novel and has never been reported in cancers. We verified the fusion point in fusion transcripts on chromosome 1 exon18 of PUM1 and chromosome 14 exon 10 of TRAF3. Fusion protein expression site is in the cytoplasm.

In order to predict the function of PUM1-TRAF3 in vivo, PUM1-TRAF3 was transfected into SNU1196 to induce overexpression. Protein level related to PUM1 was not changed and NFkB related to TRAF3 was increased. Expression of PUM1-TRAF3 fusion gene lead to upregulation of EMT markers and cell adhesion associated protein was downregulated, suggesting that this fusion gene leads to an epithelial to a mesenchymal transition. Overexpression of PUM1-TRAF3 fusion gene increases cell proliferation, migration, invasion and clonogenicity indicating that PUM1-TRAF3 expression increase cell tumorigenicity. This study confirmed that PUM1-TRAF3 fusion as a potent oncoprotein in Bile duct cancer.

We found PUM1-TRAF3 fusion gene in 28 patients out of 55 patients. It was found in patients with bile duct cancer with a 50.9% frequency. A high expression PUM1-TRAF3 fusions in Korean Bile duct cancer patients was identified in our study, is more meaningful than in previous studies. This discovery will bring about a dramatic change in Bile duct cancer diagnosis.

This study confirmed that PUM1-TRAF3 fusion as a potent oncoprotein in Bile duct cancer. Specifically, overexpression of PUM1-TRAF3 fusion gene increases cell proliferation, migration, invasion and clonogenicity, indicating that PUM1-TRAF3 expression increase cell tumorigenicity. PUM1-TRAF3 fusion gene can serve as prognosis indicators, meaning if the patient harbors that certain gene fusion in a specific type of cancer the presence of the fusion can be used as early diagnosis or prognosis of bile duct cancer. In other words PUM1-TRAF3 fusion genes can be used biomarkers because of their high frequency in bile duct cancer.

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<ABSTRACT(IN KOREAN)>

담도 암에서의 새로운 fusion gene(융합유전자) 동정과 기능연구

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담도 암은 예후가 매우 불량한 대표적인 암으로 5 년 생존율이 매우 낮고 기존의 진단 방법들과 상용되는 마커들은 환자의 암조기 발병 진단에 어려움을 갖고 있다. 암의 발생과 진행과정 중 Somatic mutation 으로 인해 서로 다른 위치에 존재하는 두 유전자가 유전적 변이에 의해 나란히 배열됨으로 fusion gene 이 발생하게 되며 oncogenic 한 특성을 갖게 된다. 본 연구에서는 담도 암 환자에서 특이적으로 발현되는 fusion gene 을 동정하기 위해 담도 암 환자의 암조직과 이와 인접한 정상조직에서 RNA 를 추출하여 RNA sequencing 을 진행하여 도출된 sequencing 결과를 토대로 fusion gene 을 검출할 수 있는 ChimeraScan, Jaffa 그리고 Fusionchacher 라는 툴을 이용하여 PUM1-TRAF3 를 선별하게 되었다. 우리는 55 명의 담도암환자와 담도암 세포주에서 fusion gene 이 발현되는지 conventional PCR 과 ddPCR 을 통해 검증하였고 55 명의 환자중에서 28 명의 환자에서 PUM1-TRAF3 fusion gene 이 발견되었으며 그 빈도는 50.9%이다. 담도암 세포주에서는 해당 fusion gene 이 발현되지 않았으며 그중 SNU1196 에 PUM1-TRAF3 를 형질주입시켜 fusion protein 의 과발현을 유도하였다. 그 결과 PUM1-TRAF3 는 EMT marker 들의 증가와 세포 증식력과 이동성 및 클론형성력이 증가하였으며 이러한 발견은 담도 암 환자들의 예후를 예측할 수 있는 하나의 마커로의 가능성을 시사하고 있다.

핵심 되는 말 : 융합유전자, pum1, traf3, rna seq