Molecular mechanism of ANO/TMEM16-mediated cell growth in pancreatic cancer

Hyung Soon Park

Department of Medical Science

The Graduate School, Yonsei University

Molecular mechanism of ANO/TMEM16-mediated cell growth in pancreatic cancer

Directed by Professor Min Goo Lee

The Doctoral Dissertation submitted to the
Department of Medical Science,
The Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Hyung Soon Park

December 2015

This certifies that the Doctoral Dissertation of Hyung Soon Park is approved.

_____

Thesis Supervisor: Min Goo Lee

_____

Thesis Committee Member #1: Hei-Cheul Jeung

_____

Thesis Committee Member #2: Jae Young Choi

_____

Thesis Committee Member #3: Ho-Geun Yoon

_____

Thesis Committee Member #4: Wan Namkung

The Graduate School

Yonsei University

December 2015

ACKNOWLEDGEMENTS

박사 과정 동안 많은 분들의 도움으로 학위 과정을 무사히 마칠 수 있었습니다. 먼저 부족한 저에게 학문적으로 또 인격적으로 한 단계 성장 할 수 있게 이끌어주신 이민구 선생님께 진심으로 감사드립니다. 또한 저의 학위 논문에 대해 같이 고민해주시고 부족한 부분을 채워주신 정희철 선생님께 감사드립니다. 바쁘신 와중에 저의 학위 논문을 검토해주시고, 심사해주신 남궁완 선생님, 윤호근 선생님 그리고 최재영 선생님께 감사드립니다. 또한 약리학을 전공 하게 허락해주시고, 약리학 박사로서 발을 들일 수 있게 지도해주신 김경환 교수님, 안영수 교수님, 김동구 교수님, 박경수 교수님, 김철훈 교수님, 김주영 교수님, 김형범 교수님 그리고 헌영이 형께도 감사드립니다.

박사 과정 동안 함께 생활 하면서 많은 도움주신 약리학 교실 선생님들, 임종수 선생님, 민선자 선생님, 여정이 누나, 오빈이형, 손한길 선생님, 정호, 동우, 임영신 선생님께 감사드립니다. 그리고 제가 랩에 처음 들어와서 사수로서 여러 가지 많은 것을 가르쳐 주신 김소원 선생님에게 감사를 전하며, 김연정 선생님, 정남누나, 현우형, 진세형, 노신혜 선생님, 정우영 선생님, 박준희 선생님, 익현이, 정우, 학이, 수민이, 지윤이, 희원이, 혜민이, 운이, (엄)소원이, 지연,

동희, 경지, 모두 고맙습니다. 또한 Physician-Scientist Course로 같이 기초학 교실에 들어와 동고동락했던 홍인이형, 은석이, 호진이, 정환이에게 감사하며, Cancer Biology Study Team 용훈이, 한상이에게도 고맙단 말 전하고 싶습니다.

또한 저의 가족들, 저를 항상 믿어주시고 지지해주시고, 또 걱정해주시는 저희 아버지, 어머니, 그리고 아버님, 어머님에게 진심으로 감사드립니다. 저를 어릴 때부터 정말 예뻐해주시고 키워주셨던 할머니, 형으로서 많이 못 챙겨 줘서 미안한 동생 영순이, 주희 그리고 처남에게도 감사의 말을 전하며, 저를 따뜻한 눈으로 지켜 봐주신 친척 분들, 작은 아버지네, 고모님네, 외삼촌네, 그리고 이모님네 모두 감사합니다. 마지막으로 부족한 저를 항상 아껴주고 사랑해주는 아내와 눈에 넣어도 아프지 않은 이제 7살이 되는 너무 예쁜 우리 딸 진서에게 고맙고 사랑한다는 말을 전하고 싶습니다.

2015.12.17

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Molecular mechanism of ANO/TMEM16-mediated cell growth in pancreatic cancer

Hyung Soon Park

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Min Goo Lee)

Pancreatic cancer has a very poor prognosis and search for druggable target is unmet medical needs. TMEM16 family genes consist of 10 genes that range from *TMEM16A* to *TMEM16K*. Of these genes, *TMEM16A*, *TMEM16B*, and *TMEM16F* are known as ion channels. In addition *TMEM16C*, *TMEM16D*, *TMEM16F*, *TMEM16G*, and *TMEM16J* are reported as phospholipid scramblase, but their exact physiological function is not yet known. *TMEM16A* is most well known gene in TMEM16 family and is over-expressed in various cancer types. However, the role of *TMEM16A* in gastrointestinal cancer, such as pancreatic cancer, has not been revealed. Here we show that *TMEM16A* promotes cell proliferation in pancreatic cancer cell lines and low chloride status induced by *TMEM16A* is important mechanism for cell proliferation. In addition, we find that *TMEM16J* is over-expressed in pancreatic cancer and it has a role of promoting cell

proliferation via EGFR over-expression and MAPK pathway activation. Furthermore, pancreatic cancer patients with low expression of *TMEM16A* and *TMEM16J* show prolonged survival outcomes than patients with high expression of *TMEM16A* and *TMEM16J*. These results indicate that *TMEM16A* and *TMEM16J* can be new druggable targets for anti-cancer therapy and have a role as a prognostic factor for survival outcomes in pancreatic cancer. It enables accurate patient risk stratification and may aid in treatment selection.

Key words: TMEM16A, TMEM16J, proliferation, pancreatic cancer, chloride, MAPK, EGFR

Molecular mechanism of ANO/TMEM16-mediated cell growth in pancreatic cancer

Hyung Soon Park

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Min Goo Lee)

I. INTRODUCTION

Pancreatic cancer is the eighth leading cause of cancer death and is difficult to treat because clinical presentation is often late, and the disease is resistant to conventional chemotherapy.[1,2] About 57% of pancreatic cancer patients are diagnosed at distant metastasis status and five-year survival rate is only 2%.[3] Although recently FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) and gemcitabine plus nanoparticle albumin bound paclitaxel are considered standard treatments for patients with good performance status, median survival is 11.1 months and 8.5 months, respectively.[4,5] More than 90% of pancreatic cancer has activating mutations in *KRAS* and recent exome sequencing studies have identified additional mutations in several kinds of genes such as *AIRD1A*, *ARID1B*, *SMARCA1*.

However, most of mutational genes are tumor suppressor genes and there are no effective druggable target in pancreatic cancer.[1] Therefore, search for new druggable target is unmet medical need.

The ANO/TMEM16 family, also known as anoctamins, plays key roles in a variety of physiological functions that range from ion transport, to phospholipid scramblase, and to regulation of other ion channels.[6,7] The topology of ANO/TMEM16 homologues is still unclear but given their high amino acid identity, ANO1/TMEM16A and ANO2/TMEM16B appear as closely related members belonging to the same subfamily. TMEM16C/D/J, and TMEM16E/F may form two separate subgroups and TMEM16G/H/K may for another group.[8,9]

The first family member, ANO1/TMEM16A, has been shown to be a calcium-activated chloride channel that is expressed in diverse tissues, including airway epithelia, smooth muscles, and sensory neurons.[9-11] In addition, ANO1/TMEM16A has been found in many tumor types including gastrointestinal stromal tumors (GIST), breast cancer, and head and neck cancer.[12-15] Its over-expression is also correlated with poor prognosis of breast cancer and head and neck cancer patients.[12,13] Regarding mechanisms of ANO1/TMEM16A related proliferation, ANO1/TMEM16A has been found to promote tumorigenesis via activating the mitogen-activated protein kinase (MAPK) signaling pathway. Moreover, ANO1/TMEM16A has been reported to promote cancer progression by the activation of epithelial growth factor receptor (EGFR).[12,13] However, proliferation mechanism of ANO1/TMEM16A as a chloride channel has been less investigated. Therefore, the first aim of this study is to reveal the proliferation mechanism related to chloride, which is modulated by ANO1/TMEM16A.Furthermore, expression and function of ANO1/TMEM16A in pancreatic cancer remain

unclear. Thus, we investigated the expression and proliferation effect of ANO1/TMEM16A in different pancreatic cancer cell lines and evaluated as a potential prognostic marker in pancreatic cancer patients.

ANO/TMEM16 family is involved in diverse functions such as chloride channel and phospholipid scramblase. It is also involved in various disease including cervical dystonia, muscular dystrophy, scott syndrome and cancer.[6] However, ANO/TMEM16 family other than ANO1/TMEM16A has not been investigated for their expression and functional role in cancer. Previously, ANO7/TMEM16G was reported that it is up-regulated in prostate cancer, but the clinical and biological role of ANO7/TMEM16G in prostate cancer was not investigated.[16] ANO6/TMEM16F is suggested that it is associated with metastatic potential of breast cancer and cell migration.[17] However, the mechanism of ANO6/TMEM16F related cancer progression and migration has not been investigated. ANO9/TMEM16J which is also known as TP53I5 (tumor protein p53 inducible protein 5) is expressed in human colorectal, lung, and breast cancer but the clinical and biological meanings have not been investigated in cancer.[18] Therefore, we hypothesized that unknown ANO/TMEM16 family genes could be over-expressed in cancer and have a role in cancer biology. The secondary aim of this study is to screen ANO/TMEM16 gene family in pancreatic cancer cell and investigate the role and related mechanisms in pancreatic cancer.

From this study, we demonstrate that ANO1/TMEM16A and ANO9/TMEM16J are over-expressed in pancreatic cancer and increase cell viability. In addition, we find that low chloride status and increased EGFR along with MAPK pathway activation are important mechanisms for cell viability induced by ANO1/TMEM16A and ANO9/TMEM16J, respectively. Therefore, both ANO1/TMEM16A and ANO9/TMEM16J can be possible

druggable targets using ANO1/TMEM16A inhibitor and EGFR inhibitor. Finally, we show that ANO1/TMEM16A and ANO9/TMEM16J have role as prognostic factors for survival outcomes in pancreatic cancer.

II. MATERIALS AND METHODS

1. Cell culture, plasmids and antibodies

PANC-1 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). ASPC-1 and BXPC-3 cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin. hTERT-HPNE cell line was obtained from ATCC and maintained in 75 % DMEM and 25 % Medium M3 Base (Incell Corp. Cat# M300F-500) supplemented with 5 % fetal bovine serum, 10 ng/ml human recombinant EGF and 750 ng/ml puromycin. Cells were grown at 37 °C in a 5 % CO_2 incubator.

The coding regions of human ANO1/TMEM16A (ac isoform) sub-cloned into the pEGFP-N1 mammalian expressible plasmid and pLVX-AcGFP-N1 without a C-terminal GFP using BamH1 and Xho1 restriction enzymes. The coding regions of ANO9/TMEM16J sub-cloned into the pCMV-Myc vector with N-terminal myc-tag using EcoR1 and Xho1 restriction enzymes. In addition, the coding regions of ANO9/TMEM16J sub-cloned into the pTripZ vector using Age1 and EcoR1 restriction enzymes for lentiviral system. Triple mutant yellow fluorescent protein (YFP, H148Q/I152L/F46L) was kindly provided by W. Namkung at Yonsei University College of Pharmacy.

Antibodies against ANO1/TMEM16A (Western blot: Santa Cruz Biotechnology, CA, USA; Immunohistochemistry (IHC): LifeSpan BioSciences, Seattle, WA, USA), ANO9/TMEM16J (Western blot: Santa Cruz Biotechnology, CA, USA; Immunohistochemistry (IHC): LifeSpan

BioSciences, Seattle, WA, USA), aldolase A (Santa Cruz Biotechnology, CA, USA), epithelial growth factor receptor (EGFR), phospho-EGFR, extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, AKT1, phospho-AKT1 (Cell Signaling Technology, Beverly, MA, USA) were obtained from commercial sources.

2. Chemicals, transfection and transduction

ANO1/TMEM16A inhibitor was provided by W. Namkung at Yonsei University College of Pharmacy. Zero chloride DMEM and RPMI were manufactured by Welgene Inc and doxycycline, ouabain, hexadimethrine bromide (polybrene) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Louis, MO, USA). ANO9/TMEM16J siRNA (ON-TARGET plus as SMARTpool) was purchased commercially (GE Healthcare Dharmacon Inc.). CellTiter 96^{R} AQueous One Solution Cell Proliferation assay kit was purchased from Promega.

Plasmids or siRNA were transiently transfected into PANC-1 or BXPC-3 cell using TransIT-X2^{R} reagent (Mirus Bio Corporation, Madison, WI, USA). For virus generation, lentiviral vectors expressing the ANO1/TMEM16A or ANO9/TMEM16J gene, together with psPAX2 packaging and pMD2.G envelope plasmid DNA were transfected into 293T cells and collected the supernatants after 24 hr. Before we start transduction, PANC-1 cell was treated with polybrene for 4 hr, and then virus supernatants were added to medium. For stable cell line generation, puromycin (5 μg/ml) was used and cell lines which are resistant to puromycin were selected.

3. Real time PCR analysis

Total RNA was extracted from PANC-1, ASPC-1, BXPC-3 and hTERT-HPNE cells by using GeneAll Hybrid-R RNA purification kit (GeneAll, Korea) according to the manufacturer's instructions. The RNA samples were then subjected to the RT-PCR reaction with RNA to cDNA EcoDry Premix (Clontech, Mountain View, CA, USA). The real-time PCR was performed with the Applied Biosystems StepOne^{TM} system (Applied Biosystems, Foster city, CA). The reaction was measured by detecting the binding of fluorescent dye SYBR Green to double-strand DNA. Reactions were set up in a total volume of 20 μl using 2 μl of cDNA and 10 μl of 2X SYBR Premix Ex Taq II (Takara Bio, Mountain View, CA, USA). Amplification was performed following cycling condition: 95°C for 10 min; 49 cycles of 95°C for 15 sec, 60°C for 1 min. Specificity of the PCR products was confirmed by analysis of the dissociation curve. According to the comparative Ct method, gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the delta Ct value.

4. Immunoblotting

For immunoblotting, PANC-1 cells were grown and transfected with ANO1/TMEM16A or ANO9/TMEM16J. After 48 to 72 hr, cells were lysed with lysis buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (v/v) sodium deoxycholated, and complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). After centrifugation, protein samples were suspended in a 5X sodium dodecyl sulfate (SDS) sample buffer and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane and blotted with appropriated secondary antibodies. Protein bands were detected by

enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and the densities of the immunoblots were analyzed by using imaging software (Multi Gauge ver. 3.0, Fujifilm).

5. Whole-cell patch clamping

Anion channel activities were measured in PANC-1 cells by using whole-cell patch clamp techniques. Briefly, cells were transferred into the bath mounted on a stage with an inverted microscope (IX-70; Olympus). The bath solution was perfused at 10 mL/min. The voltage and current recordings were performed at room temperature (22~25 ^{o}C). Patch pipettes with a free-tip resistance of 2-5 MOhm were connected to the head stage of a patch-clamp amplifier (Axopatch-700B; Axon Instruments). The current reversal potential (E_{rev}) was measured in current clamp or voltage clamp experiments. To determine the current-voltage (I-V) relationship during zero-current clamp recordings, the clamp mode was shifted to the voltage clamp mode, and the I-V curve was obtained by applying ramp pulses from -100 to 100 mV or step pulses from -100 to 100 mV.

6. Cell viability assay and colony formation assay

For viability analysis, we used cells with transient transfection or stable cell lines. In transient transfection model, cells were plated in 24-well optical plates at 25% confluency for 24 hr and transfected with plasmid or siRNA. Three days after transfection, the CellTiter 96^{R} AQueous One Solution Cell Proliferation assay kit was used for measuring cell viability. In stable cell line model, cells were plated in 24-well optical plates at 12.5% confluency and viability was measured three day after plating using CellTiter 96^{R} AQueous One Solution Cell Proliferation assay kit. For colony formation assay, 300 cells were plated in each well of 6 well plate, and

colonies were counted 2 wk after plating using crystal violet.

7. Flowcytometry for cell cycle analysis

Cells were treated with ANO1/TMEM16A inhibitor for 3 days, then were detached using trypsin and resuspended in growth medium. Cells were washed with PBS and fixed with 66% ethanol at 4 ºC. After washing twice with PBS, the cells were stained with a solution containing 50 μg/ml of propidium iodide (PI) and 550U/ml RNase A (Abcam flow cytometry kit, ab139418, Abcam, Cambridge, MA, USA) for 30 min at 37 ºC in the dark. The stained cells were analyzed by flow cytometry (BD biosciences).

8. Measurement of intracellular chloride concentration

Measurement of intracellular chloride concentration in PANC-1 cells with or without ANO1/TMEM16A was performed with a chloride sensing fluorescent, triple mutant YFP (H148Q/I152L/F46L). Cells were transiently transfected with triple mutant YFP, together with EGFP-N1 mock plasmid or ANO1/TMEM16A for 24-48 hr. Cells were perfused with HEPES-buffered solution and YFP fluorescence was recorded at the excitation wavelengths 480 nm and 440 nm at a resolution of 2 /sec on a recording setup (Delta Ram; PTI Inc, Birmingham, New Jersey, USA). Chloride calibration was performed with zero chloride (150 mM K-Gluconate) and 40 mM chloride solution (made by 150 mM K-Gluconate and 150 mM KCl) with 5 μM nigericin ionophore and 10 μM tributyltin chloride (Sigma-Aldrich, Louis, MO, USA).

9. Patients samples

From August 1998 to May 2012, a total of 78 pancreatic cancer patients were enrolled at Gangnam Severance Hospital, Seoul, Korea. Inclusion criteria for further analysis were as follows: (1) age >18 year, (2) histologically confirmed diagnosis of pancreatic ductal adenocarcinoma, (3) curative resection of pancreatic cancer, and (4) available electronic medical records including treatment information. Exclusion criteria were as follows; (1) unresectable disease who received systemic chemotherapy, (2) other histology types such as cystic neoplasms, neuroendocrine tumors, lymphomas, or (3) synchronous malignancies. The following baseline data were recorded at the time of diagnosis; age, sex, tumor differentiation (grade), T stage, N stage, TNM stage, lymphovascular (LV) invasion, perineural (PN) invasion, and operation types.

10. Tissue microarray and Immunohistochemistry

Paraffin block containing representative pancreatic cancer samples were selected by reviewing all of the hematoxylin and eosin-stained slides. Tissue cores with a diameter of 1.5 mm were extracted from each donor block, and precisely arrayed into a new paraffin recipient block. Sections were obtained from formalin-fixed and paraffin-embedded tissue microarray (TMA) blocks, mounted on poly-L-lysine-coated glass slides, and used for immunohistochemistry (IHC). Sections were deparaffinized with xylene, rehydrated. Antigen retrieval was performed by autoclaving the slides in 10mM citric acid buffer. Sections were incubated with primary anti-bodies against ANO1/TMEM16A and ANO9/TMEM16J. The staining intensity was scored as negative (0), mild (1+), moderate (2+), and strong (3+). Positive staining in less than 5% of tumor cells was considered negative.

11. Statistical analysis

The results were presented as the mean ± SEM and statistical analysis was performed with student *t*-tests. The characteristics of patients with or without ANO1/TMEM16A and ANO9/TMEM16J were compared using the chi-square or Fisher's exact test. Disease-free survival (DFS) was defined as the time from surgery to the first documented recurrence or death from any cause. Overall survival (OS) was calculated from the date of surgery to death from any cause. Time to event endpoint was analyzed using the Kaplan-Meier method, and DFS and OS were compared between subgroups using the log-rank test. We performed univariate analysis to determine the influence of prognostic factors on DFS and OS. Multivariate analysis was also performed to determine independent prognostic factors for survival using the Cox proportional hazard regression model. Hazard ratios (HR) and 95% confidence intervals (CI) were obtained for all regressions. A *P*-value threshold of 0.05 was considered statistically significant. The SPSS Statistics 18.0 software (SPSS Inc. Chicago, Ill) was used for all statistical analyses.

III. RESULTS

1. ANO1/TMEM16A and ANO9/TMEM16J are over-expressed in pancreatic cancer

It is known that ANO1/TMEM16A is over-expressed in several kinds of cancer including breast cancer, head and neck cancer, and prostate cancer. To see whether ANO/TMEM16 family genes are over-expressed in pancreatic cancer, we examined the expression level of ANO/TMEM16 family genes using quantitative PCR in 3 pancreatic cancer cell lines (ASPC-1, BXPC-3, PANC-1) and 1 normal immortalized pancreas cell line (hTERT-HPNE). As shown in Figure 1A, ANO1/TMEM16A was over-expressed in ASPC-1, BXPC-3 compared to normal pancreatic cancer cell lines, hTERT-HPNE. In contrast, PANC-1 had low expression level of ANO1/TMEM16A. In addition, ANO9/TMEM16J was over-expressed in ASPC-1, BXPC-3 and PANC-1 compared to hTERT-HPNE which is normal pancreatic cancer cell lines. Then protein level of ANO1/TMEM16A was evaluated by western blot in 4 pancreatic cancer cell lines. In Figure 1B, ANO1/TMEM16A was over-expressed in ASPC-1 and BXPC3, but HPNE and PANC-1 had a little amount of endogenous ANO1/TMEM16A.

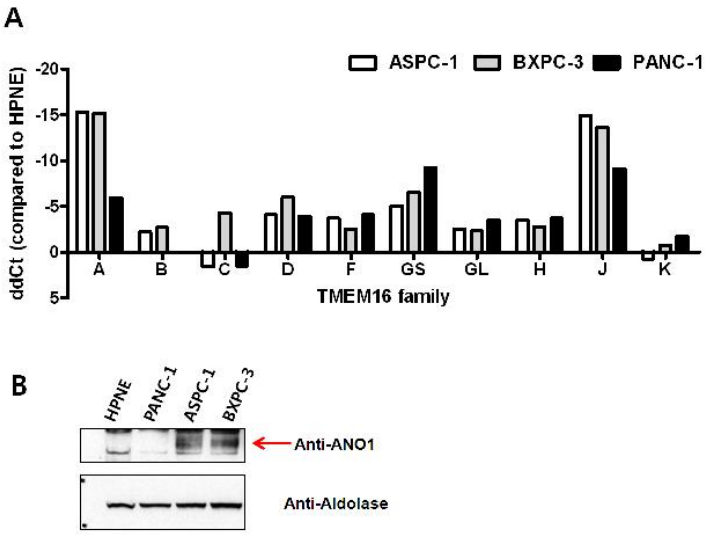


Figure 1. Expressions of TMEM16 family genes in pancreatic cancer cell lines. (A) ANO1/TMEM16A and ANO9/TMEM16J are over-expressed in ASPC-1 and BXPC-3 compared to hTERT-HPNE. (B) In western blot, ASPC-1 and BXPC-3 had a large amount of endogenous ANO1/TMEM16A.

2. Calcium activated chloride channel (CACC) currents is blocked by ANO1/TMEM16A inhibitor

To validate functional expression of the ANO1/TMEM16A channel in the pancreatic cancer cell lines, we performed whole-cell patch clamp recordings of PANC-1 with or without ANO1/TMEM16A. As shown in Figure 2A, PANC-1 with ANO1/TMEM16A showed currents in response to the voltage step of stimuli in the presence of 1 μM calcium. We also added ANO1/TMEM16A specific inhibitor (T16Ainh-A01) to PANC-1 with ANO1/TMEM16A, and calcium activated chloride channel (CACC) currents were decreased by 10 μM T16Ainh-A01 (Figure 2B). After wash out (WO) of T16Ainh-A01, CACC currents in PANC-1 with ANO1/TMEM16A were recovered.

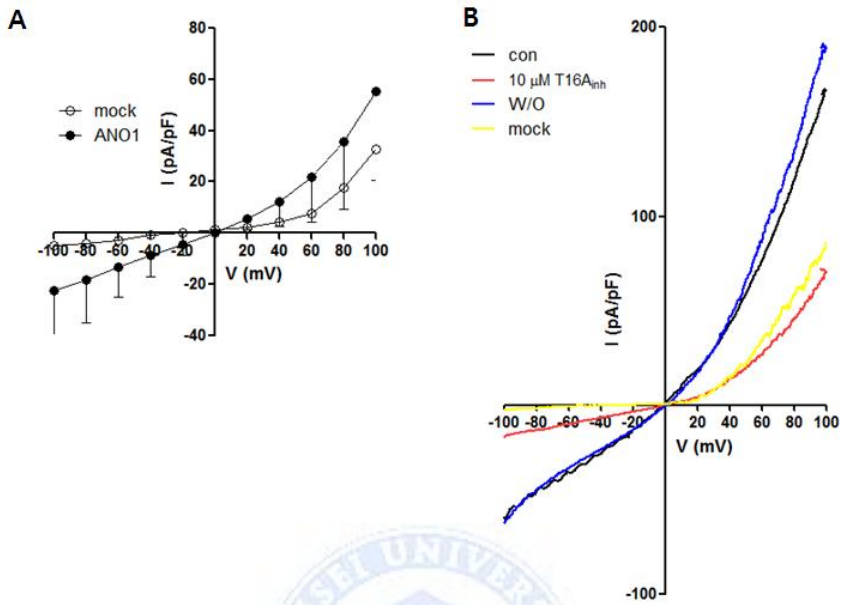


Figure 2. Calcium activated chloride channel (CACC) currents was blocked by T16Ainh-A01. (A) PANC-1 cells were transiently transfected with mock or ANO1/TMEM16A. Two days after transfection, CACC currents were measured using whole cell patch clamp recordings. (B) T16Ainh-A01 block the CACC currents in PANC-1 with ANO1/TMEM16A and CACC currents was recovered after wash out (W/O) of T16Ainh-A01.

3. ANO1/TMEM16A is necessary and sufficient factor for cell viability and MAPK pathway is involved in ANO1/TMEM16A related cell viability

ANO1/TMEM16A was examined to see whether it is essential for cell proliferation. Firstly, PANC-1 and BXPC-3 were treated with ANO1/TMEM16A inhibitor (T16Ainh-A01). As shown in Figure 3A, BXPC-3, which has large amount of endogenous ANO1/TMEM16A, showed more sensitivity to ANO1/TMEM16A inhibitor than PANC-1 with

low amount of ANO1/TMEM16A. Then we conducted FACS (Fluorescence-Activated Cell Sorting) to find which cell cycle is blocked by T16Ainh-A01. BXPC-3 cell was treated with T16Ainh-A01 (10 μM) for 3 days, and FACS was conducted. As shown in Figure 3B, G0/G1 phase was significantly increased in inhibitor treated group compared to DMSO treated group; inhibitor treated group had shortened S phase.

Furthermore, we examined the role of ANO1/TMEM16A as a sufficient factor for cell viability. PANC-1 was transiently transfected with mock plasmid or ANO1/TMEM16A plasmid, and cell viability was compared between these two group using MTS assay (CellTiter 96^{R} AQueous One Solution Cell Proliferation Assay). When ANO1/TMEM16A is over-expressed in PANC-1 cell, cell viability was significantly increased (Figure 3C). These result showed that ANO1/TMEM16A is important factor for cell viability and is a potential druggable target for anti-cancer therapy.

To reveal the molecular mechanisms of ANO1/TMEM16A related cell viability, we conducted immunoblot of proliferation pathway such as mitogen-activated protein kinase (MAPK) and AKT pathway, in ANO1/TMEM16A knock-down or over-expression experiments. Firstly, BXPC-3 was treated with DMSO or ANO1/TMEM16A inhibitor, and expression of ANO1/TMEM16A was decreased in inhibitor treated group. In addition, phospho-ERK1/2 level was decreased according to the decreased ANO1/TMEM16A level in inhibitor treated BXPC-3 (Figure 3D). For over-expression experiments, we made a stable cell line using lenti-virus system. We found that phospho-ERK1/2 was increased in ANO1/TMEM16A over-expressed PANC-1 stable cell lines (Figure 3E).

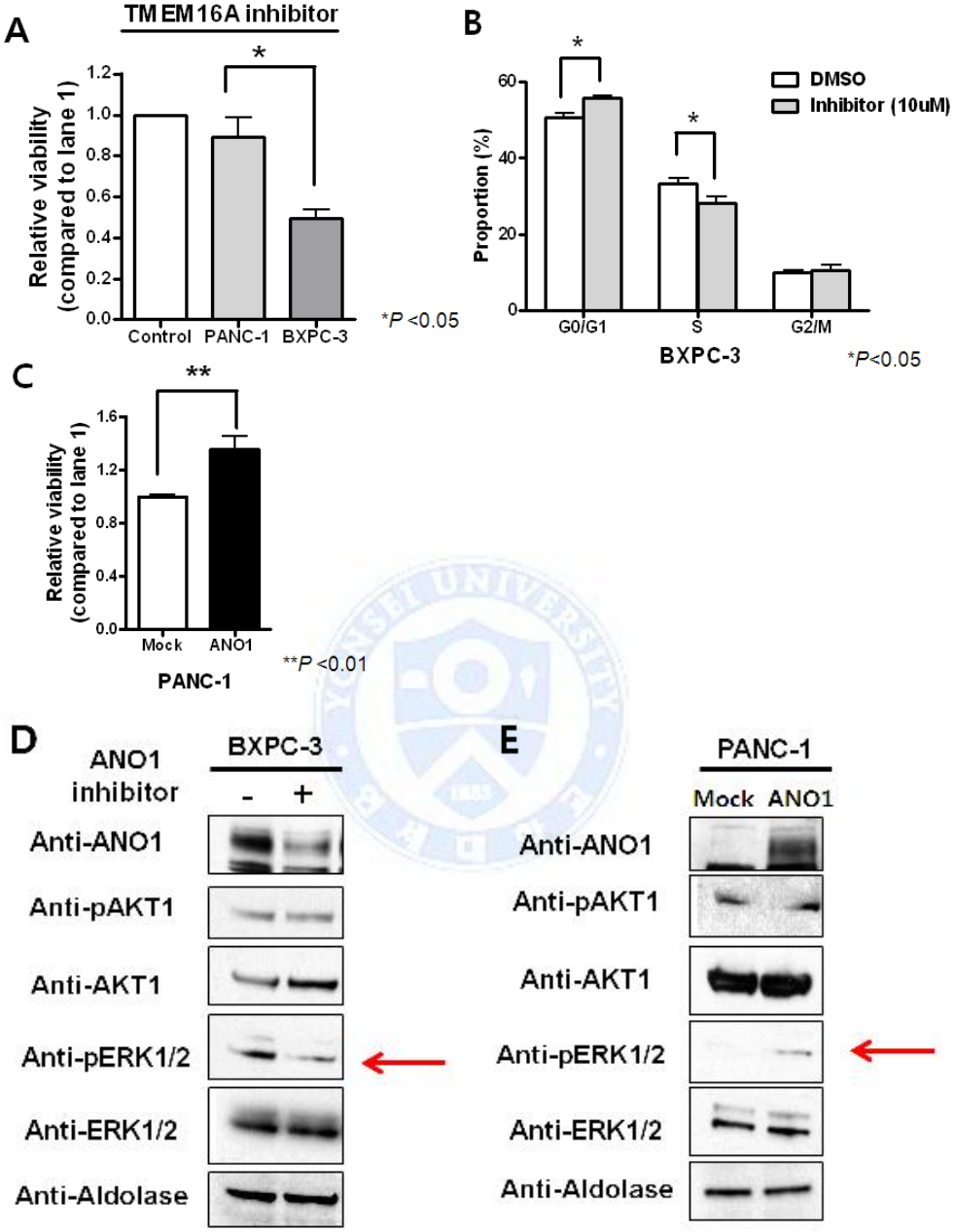

A
TMEM16A inhibitor
Relative viability (compared to lane 1)
Control
PANC-1
BXPC-3
*P <0.05
B
DMSO
Inhibitor (10uM)
Proportion (%)
G0/G1
S
G2/M
BXPC-3
*P<0.05
C
Relative viability (compared to lane 1)
Mock
ANO1
PANC-1
**P <0.01
D
ANO1 inhibitor
BXPC-3
Anti-ANO1
Anti-pAKT1
Anti-AKT1
Anti-pERK1/2
Anti-ERK1/2
Anti-Aldolase
E
PANC-1
Mock
ANO1
Anti-ANO1
Anti-pAKT1
Anti-AKT1
Anti-pERK1/2
Anti-ERK1/2
Anti-Aldolase

Figure 3. Cell viability according to ANO1/TMEM16A inhibitor treatment or over-expression. (A) ANO1/TMEM16A inhibitor had a large effect on BXPC-3 which had a high level of ANO1/TMEM16A compared to PANC-1. (B) Using FACS, cell cycle analysis was conducted after T16Ainh-A01 in BXPC-3. G0/G1 phase is increased by T16Ainh-A01. (C) ANO1/TMEM16A over-expression increases cell viability compared to mock transfection in PANC-1 cell. (D) BXPC-3 which was treated with ANO1/TMEM16A inhibitor showed decrease in phoshpo-ERK1/2 level compared to BXPC-3 treated with DMSO. (E) PANC-1 with ANO1/TMEM16A showed increased phopho-ERK1/2 level compared to PANC-1 without ANO1/TMEM16A.

4. Intracellular chloride level is lowered by ANO1/TMEM16A and it is important mechanisms for ANO1/TMEM16A related cell viability

ANO1/TMEM16A is an important factor for cell viability in several kinds of cancer types. However, the mechanism of cell viability related to ANO1/TMEM16A has not been revealed. Therefore, we studied the mechanism of ANO1/TMEM16A related cell viability and hypothesized that chloride is important for TMEM16A related cell viability. Since ANO1/TMEM16A is calcium activated chloride channel (CACC) and mutation of pore lesion is known to be important for cell viability, we measured intracellular chloride level according to mock plasmid or ANO1/TMEM16A plasmid transfection using triple mutant YFP (H148Q/ I152L/F46L). As shown in Figure 4A/B, 0 mM chloride solution and 40 mM chloride solution was used to calibrate the intracellular chloride level. Intracellular chloride level of PANC-1 without TMEM16A over-expression was about 35 mM, but when TMEM16A is transfected to PANC-1, chloride

level was reduced to about 20 mM (Figure 4C). Then, we thought that low chloride level induced by ANO1/TMEM16A might be important for cell viability. Therefore, we made different kinds of chloride concentration medium (0 mM, 60 mM, 90 mM, 120 mM), and cultured in each medium for 3 days. As shown in Figure 4D, 60 mM chloride medium was most effective for cell proliferation, and this result may suggest that there is a precise range of relatively low chloride concentrations those are suitable for cell viability.

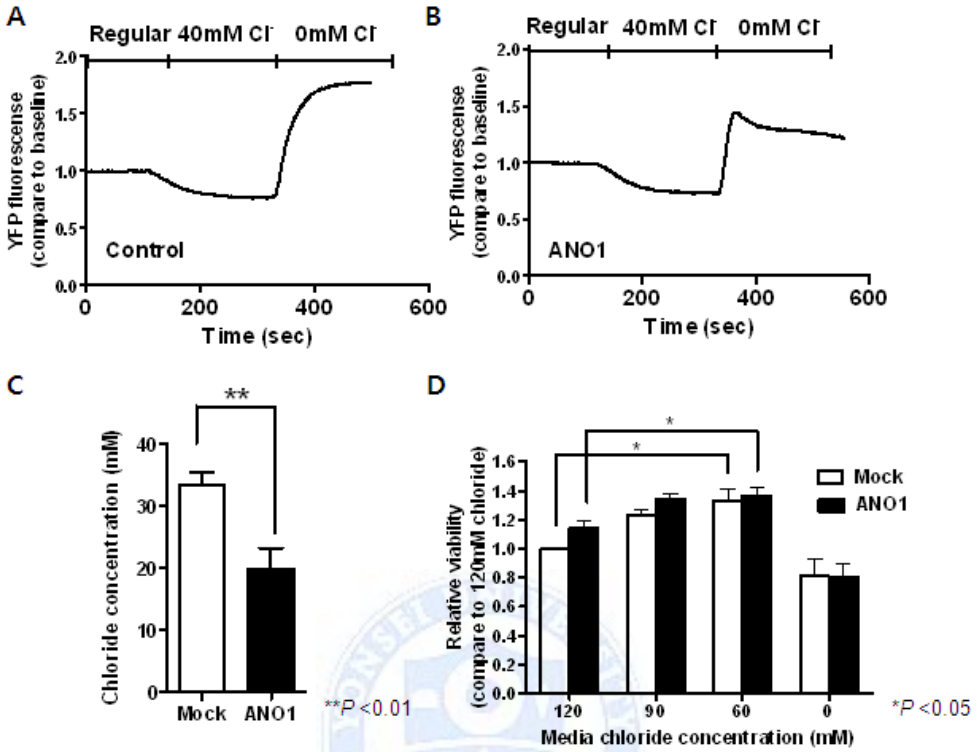


Figure 4. Baseline chloride levels in PANC-1 with or without ANO1/TMEM16A and the role of chloride concentration on cell viability. (A/B) Trace of triple mutant YFP fluorescence in PANC-1 with or without ANO1/TMEM16A for chloride concentration measurement. (C) ANO1/TMEM16A over-expressed PANC-1 have a low concentration of chloride compared to PANC1 without ANO1/TMEM16A. (D) Low chloride medium such as 60 mM promotes cell proliferation regardless of ANO1/TMEM16A.

5. Depolarization induced by ANO1/TMEM16A is not an important factor for cell viability

Resting membrane potential (RMP) is also known to be an important factor for cell viability. We hypothesized that resting membrane potential can be changed according to ANO1/TMEM16A expression and be an important factor for ANO1/TMEM16A related cell viability. Using membrane potential dye (DiBac, Life technology), resting membrane potential was measured in PANC-1 cell with or without ANO1/TMEM16A transfection. When ANO1/TMEM16A is over-expressed in PANC-1 cell, resting membrane potential was changed to depolarized status (Figure 5A). Chloride concentration of culture medium was reduced from 120 mM to 0 mM, thus degree of depolarization was increased (Figure 5B). PANC-1 with ANO1/TMEM16A over-expression was more depolarized than PANC-1 without ANO1/TMEM16A over-expression in every chloride concentrations (Figure 5B). To evaluate the role of depolarization induced by ANO1/TMEM16A in cell viability, we induced depolarization in the cell by using two different methods. First, different concentrations of K-Gluconate were added to growth media to increase potassium level, and cell viability was measured. However, depolarization induced by K-Gluconate could not increase the proliferation rate (Figure 5C). Then, we used ouabain to make the cell depolarized, but it also could not increase the cell viability (Figure 5D). Therefore, we thought that chloride concentration is more important factor for ANO1/TMEM16A related cell viability than resting membrane potential.

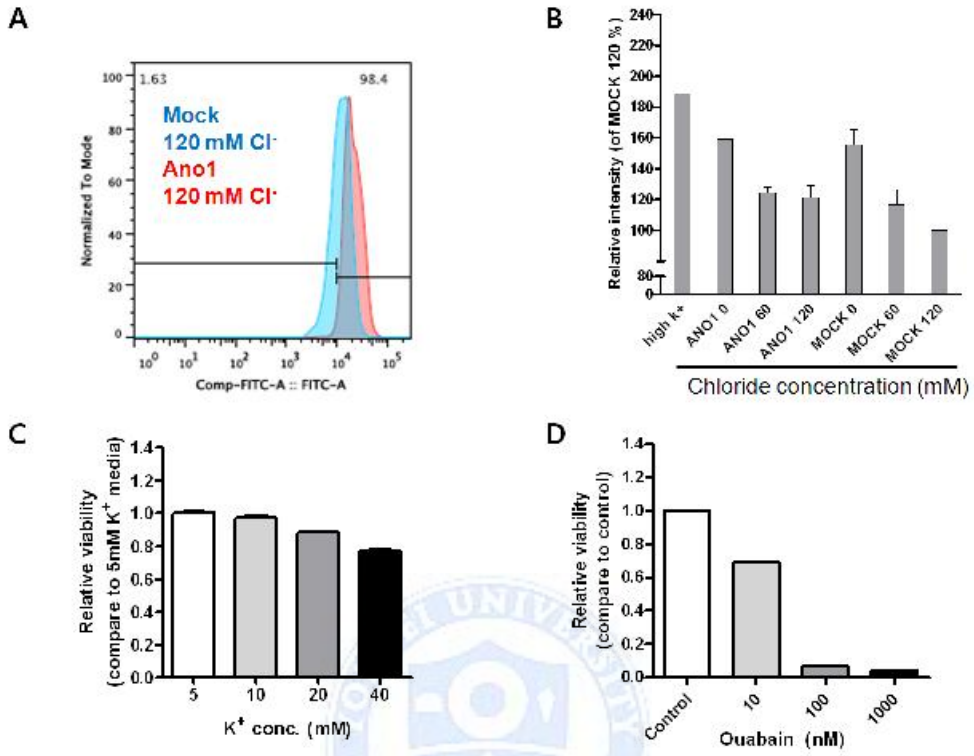


Figure 5. Resting membrane potential (RMP) in PANC-1 with or without ANO1/TMEM16A and the role of resting membrane potential on cell viability. (A) Flow cytometry analysis using DiBac dye in PANC-1 with or without ANO1/TMEM16A for resting membrane potential measurement. (B) PANC-1 with ANO1/TMEM16A shows more depolarized status than PANC1 without ANO1/TMEM16A. And low chloride medium induces cell depolarization regardless of ANO1/TMEM16A. (C) Depolarization induced by K-Gluconate has no positive effects on cell viability. (D) Ouabain which acts by inhibiting the Na^+/K^+-ATPase sodium-potassium ion pump affect the membrane voltage of the cell, but depolarization induced by ouabain has no effect on viability of PANC-1.

6. ANO1/TMEM16A is significant prognostic factor for pancreatic cancer

The prognostic role of ANO1/TMEM16A has not been studied in pancreatic cancer patients. We evaluated the prognostic role of ANO1/TMEM16A in resected pancreatic cancer patients using immunohistochemistry (IHC) stain. A total of 78 patients were enrolled, and the baseline characteristics were listed in Table 1. The median age was 64 year (range, 37-83 year) and 39 (50%) of patients were men. Patients with TNM stage 1, stage 2, and stage 3 were 5 (6.4), 69 (88.5%), and 4 (5.1%), respectively. There was no significantly different baseline characteristic between ANO1 low and high expression group. Although they were not statistically different, ANO1 high expression group showed more advanced T stage than ANO1 low expression group (P=0.13).

Forty six patients (59%) showed 0 or 1+ expression of TMEM16A (Figure 6A) and 32 (41%) patients showed 2+ or 3+ expression level (Figure 6B). As previously reported, the localization of ANO1/TMEM16A was noted in plasma membrane. Survival analysis was conducted and patients with high expression of ANO1/TMEM16A showed poor disease free survival (DFS) than patients with low expression (DFS, median survival time 12.2 *vs.* 9.7 mo, P=0.143, Figure 6C). In overall survival, patients with high expression of ANO1/TMEM16A showed inferior survival outcomes than patients with low expression of ANO1/TMEM16A (OS, median survival time 39.2 *vs.* 21.7 mo, P=0.037, Figure 6D).

Univariate and multivariate analysis were performed to identify the prognostic factors associated with DFS and OS (Table 2). In univariate analysis, tumor grade was significantly associated with DFS, and ANO1/TMEM16A was borderline significant for DFS (P = 0.146, HR =

1.461, 95% CI = 0.877 – 2.437). Multivariate analysis revealed tumor grade as independent risk factors for recurrence. Regarding OS, T stage and ANO1/TMEM16A expression were notable prognostic factors in univariate analysis. However, there was no significant independent factor for OS in multivariate analysis. From these results, we suggest that ANO1/TMEM16A might be a possible prognostic factor for survival outcome in pancreatic cancer.

Table 1. Patient characteristics by ANO1/TMEM16A expression

Characteristics		ANO1-Low		ANO1-High		Total		*P*
		N	%	N	%	N	%	
Age (n, %)	<65	26	56.5	14	43.8	40	51.3	0.27
	≥65	20	43.5	18	56.3	38	48.7	
Sex (n, %)	Male	23	50	16	50	39	50	1
	Female	23	50	16	50	39	50	
Tumor grade (n, %)	Well	8	18.6	5	15.6	13	17.3	0.83
	Moderate	28	65.1	23	71.9	51	68.0	
	Poor	7	16.3	4	12.5	11	14.7	
T stage	1-2	7	15.2	1	3.1	8	10.3	0.13
	3-4	39	84.8	31	96.9	70	89.7	
N stage	0	16	34.8	11	34.4	27	34.6	0.97
	1	30	65.2	21	65.6	51	65.4	
TNM Stage (n, %)	I	5	10.9	0	0	5	6.4	0.09
	II	38	82.6	31	96.9	69	88.5	
	III	3	6.5	1	3.1	4	5.1	
Perineural invasion	No	6	15.8	3	12	9	14.3	1.00
	Yes	32	84.2	22	88	54	85.7	
Lymphovascular invasion	No	16	42.1	9	33.3	25	38.5	0.47
	Yes	22	57.9	18	66.7	40	61.5	
Operation type (n, %)	Whipple	6	13	1	3.1	7	9	0.39
	PPPD	22	47.8	19	59.4	41	52.6	
	Distal	14	30.4	11	34.4	25	32.1	
	Total	4	8.7	1	3.1	5	6.4	

N: number; PPPD: pylorus preserving pancreatoduodenectomy; Distal: distal pancreatectomy; Total: total pancreatectomy; *P*: p-value

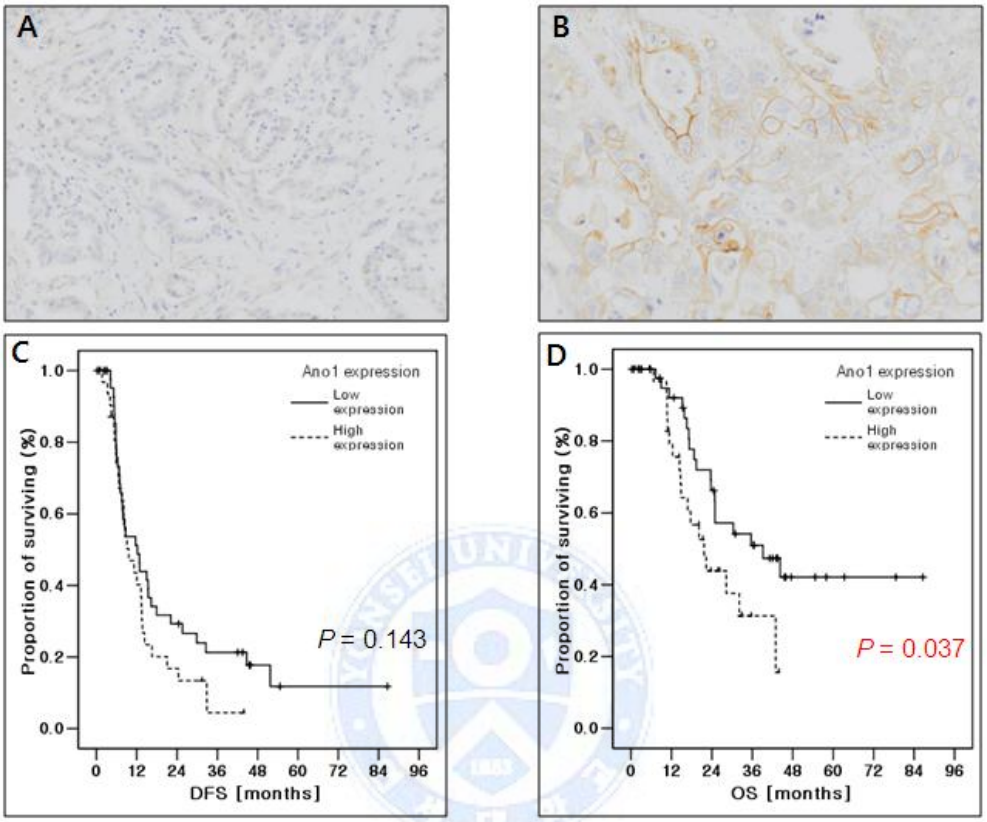


Figure 6. IHC (immunohistochemistry) stain of ANO1/TMEM16A and survival analysis according to ANO1/TMEM16A expression in pancreatic cancer patients. (A/B) Example of low and high expression of ANO1/TMEM16A in pancreatic cancer patient. (C/D) Kaplan-Meier survival curves for disease free survival (DFS) and overall survival (OS) according to ANO1/TMEM16A expression in pancreatic cancer patients.

Table 2. Univariate and multivariate analysis of prognostic factors including ANO1/TMEM16A for disease-free survival and overall survival

	Disease-free survival (DFS)				Overall survival (OS)			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)		0.66				0.49		
<65	1				1			
≥65	0.89 (0.53-1.49)				0.80 (0.41–1.54)			
Sex		0.81				0.16		
Male	1				1			
Female	1.07 (0.65-1.75)				1.60 (0.83–3.10)			
Tumor grade		0.006		0.003		0.25		
Well	1		1		1			
Moderate	2.48 (1.16-5.30)		2.29 (1.06-4.92)		2.10 (0.80-5.53)			
Poor	4.55 (1.81-11.49)		5.02 (1.97-12.77)		2.60 (0.78-8.61)			

(continued)

	Disease-free survival (DFS)				Overall survival (OS)			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
T stage		0.28				0.04		0.07
1-2	1				1		1	
3-4	1.68 (0.66-4.32)				7.78 (1.06-56.99)		6.48 (0.86-48.62)	
N stage		0.88				0.54		
0	1				1			
1	0.96 (0.57-1.62)				1.24 (0.62-2.48)			
TNM Stage		0.37				0.59		
I	0.43 (0.08-2.17)				0.00 (0.00 -)			
II	1.01 (0.31-3.25)				0.53 (0.16-1.77)			
III	1				1			
Perineural invasion		0.24				0.40		
No	1				1			
Yes	1.68 (0.71-3.98)				1.58 (0.55-4.54)			

(continued)

	Disease-free survival (DFS)				Overall survival (OS)			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Lymphovascular invasion		0.40				0.34		
No	1				1			
Yes	1.28 (0.72-2.29)				1.44 (0.68-3.05)			
Operation type		0.37				0.82		
Whipple	1				1			
PPPD	0.48 (0.21-1.10)				0.96 (0.33-2.79)			
Distal pancreatectomy	0.50 (0.21-1.22)				0.73 (0.23-2.33)			
Total pancreatectomy	0.49 (0.15-1.57)				0.57 (0.10-3.14)			
ANO1/TMEM16A		0.15		0.08		0.04		0.19
Low	1		1		1		1	
High	1.46 (0.88-2.44)		1.63 (0.94-2.81)		2.00 (1.03-3.89)		1.57 (0.81-3.08)	

7. ANO9/TMEM16J is related to cell viability and activates the MAPK pathway

We evaluated the biological role of ANO9/TMEM16J in pancreatic cancer. First, we examined whether ANO9/TMEM16J is essential for cell viability. Knock-down experiment with specific siRNA for ANO9/TMEM16J in BXPC-3 cell, which had relatively large amount of endogenous ANO9/TMEM16J, revealed that ANO9/TMEM16J is necessary factor for cell viability (Figure 7A). When PANC-1 cell was transfected with ANO9/TMEM16J, cell viability was increased by 20% compared to PANC-1 cell with mock plasmid (Figure 7B). This suggests that ANO9/TMEM16J could be a sufficient factor for increased cell viability.

To find mechanism of ANO9/TMEM16J related cell viability, we evaluated the expression level of MAPK and AKT pathway in both BXPC-3 cell with specific ANO9/TMEM16J siRNA, and PANC-1 cell with ANO9/TMEM16J transfection. Notably, phospho-ERK1/2 level was decreased in BXPC-3 with specific siRNA and phospho-ERK1/2 was increased when ANO9/TMEM16J was over-expressed (Figure 7C/D). Thus, it could be explained that ANO9/TMEM16J increased cell viability using MAPK pathway activation.

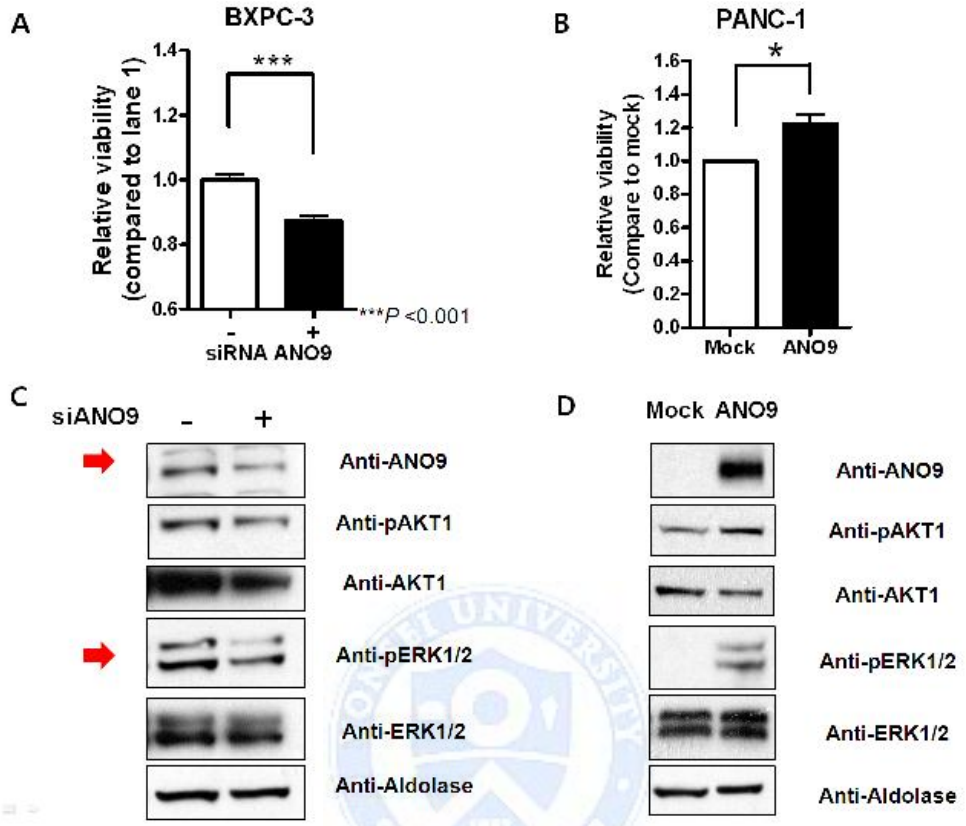


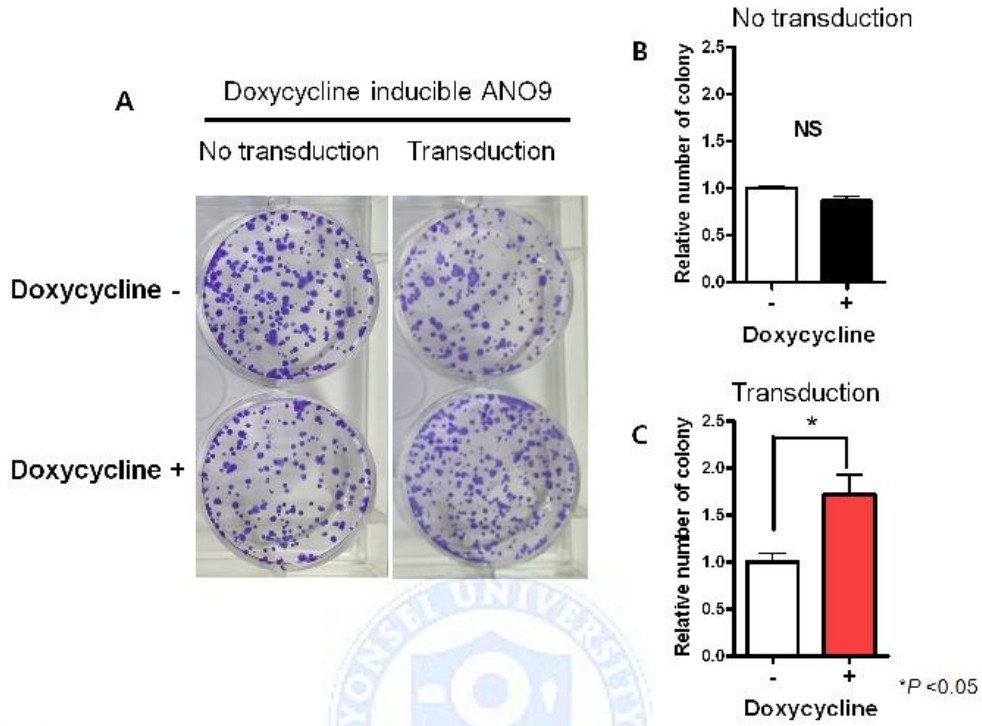
Figure 7. Cell viability according to ANO9/TMEM16J knock-down and over-expression in pancreatic cancer cell lines. (A) In BXPC-3 cell, ANO9/TMEM16J knock-down decreases cell viability. (B) ANO9/ TMEM16J over-expression increases cell viability compared to mock transfection in PANC-1 cell. (C) ANO9/TMEM16J knock-down decreased phosphor-ERK1/2 in BXPC-3. (D) ANO9/TMEM16J activate MAPK pathway in PANC-1.

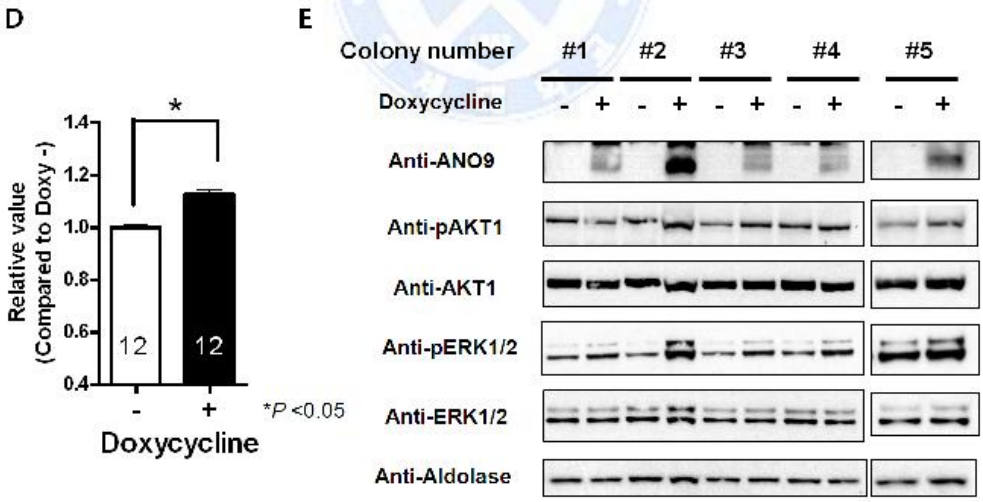
8. ANO9/TMEM16J increases colony formation capacity

For colony formation assay, we made a PANC-1 with stably expressed ANO9/TMEM16J. Doxycycline inducible lentivirus plasmid, pTripZ, was used for generation of stable cell lines and puromycin selection

(5 μg/ml) was conducted after transduction. To activate stable cell line with doxycycline inducible plasmid, we add doxycycline (3 μg/ml) to growth medium. After generation of PANC-1 based ANO9/TMEM16J stable cell lines, we compared the colony formation capacity according to ANO9/TMEM16J expression. As shown in Figure 8A, colony formation capacity was not affected by doxycycline treatment in PANC-1 without transduction (negative control), but colony formation was increased by doxycycline treatment in PANC-1 with ANO9/TMEM16J transduction. Number of colony was counted after crystal violet stain and colony number was significantly increased in ANO9/TMEM16J over-expressed cell line compared to PANC-1 without ANO9/TMEM16J (Figure 8B/C). Viability assay using MTS was also conducted in PANC-1 with stably expressed ANO9/TMEM16J. As shown in Figure 8D, cell viability was significantly increased in doxycycline treated group after 3 day treatment of doxycycline.

To confirm our finding on the mechanisms of ANO9/TMEM16J related cell viability, we evaluated the expression of MAPK and AKT pathway in PANC-1 cell with stably expressed ANO9/TMEM16J. As expected, phospho-ERK1/2 was increased when ANO9/TMEM16J was over-expressed (Figure 8E). Quantification of AKT and MAPK immunoblots revealed that only phosphor-ERK1/2 was increased in PANC-1 with ANO9/TMEM16J and these results imply that ANO9/TMEM16J increases cell viability through activation of MAPK pathway (Figure 8F/G/H/I).


A
Doxycycline inducible ANO9
No transduction
Transduction
Doxycycline -
Doxycycline +
B
No transduction
Relative number of colony
NS
Doxycycline
C
Transduction
Relative number of colony
*
*P <0.05
Doxycycline


D
Relative value
(Compared to Doxy -)
12
12
*
*P <0.05
Doxycycline
E
Colony number
#1
#2
#3
#4
#5
Doxycycline
Anti-ANO9
Anti-pAKT1
Anti-AKT1
Anti-pERK1/2
Anti-ERK1/2
Anti-Aldolase

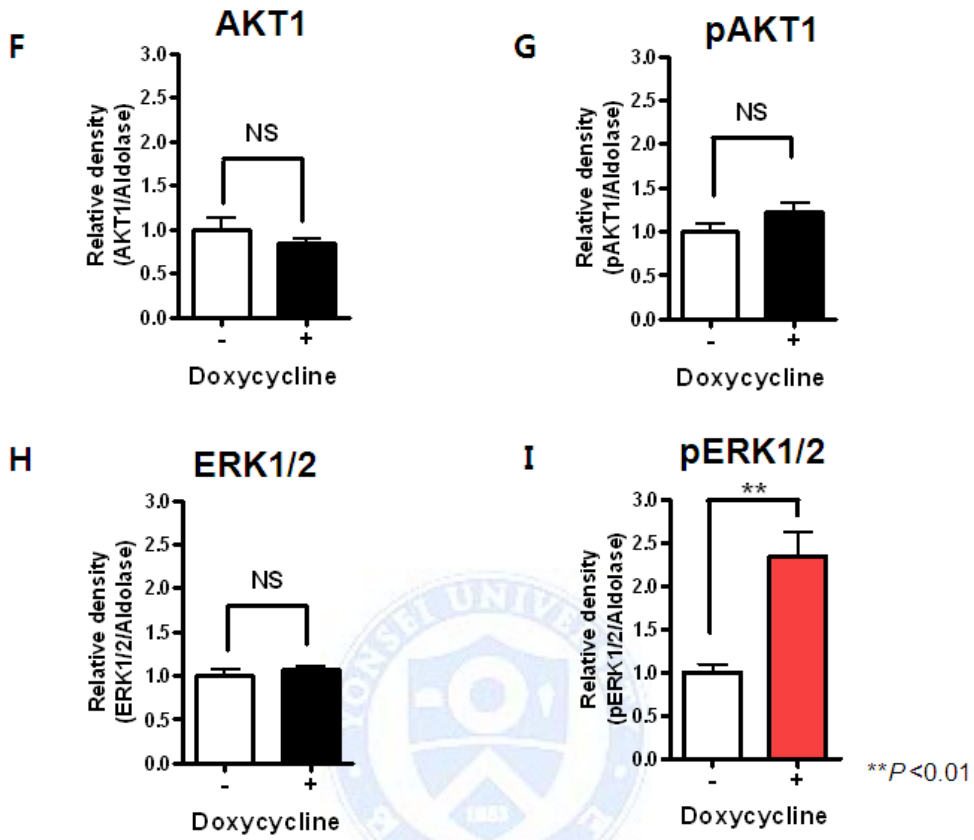

F
AKT1
Relative density (AKT1/Aldolase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NS
-
+
Doxycycline
G
pAKT1
Relative density (pAKT1/Aldolase)
NS
Doxycycline
H
ERK1/2
Relative density (ERK1/2/Aldolase)
NS
Doxycycline
I
pERK1/2
Relative density (pERK1/2/Aldolase)
**
Doxycycline
**P<0.01

Figure 8. ANO9/TMEM16J increases colony formation and cell viability through MAPK pathway activation. (A) PANC-1 with or without ANO9/TMEM16J transduction is plated on 6 well plate and maintained for 2 weeks. Doxycycline treated group shows increased colony formation in ANO9/TMEM16J transduction groups, but groups without ANO9/ TMEM16J transduction shows no difference in cell viability between doxycycline treated and untreated group. (B/C) Counting number of colonies confirms that ANO9/TMEM16J over-expression group induced by doxycycline treatment increases colony formation. (D) PANC-1with stably expressed doxycycline inducible ANO9/TMEM16J shows increased cell viability after 3 day treatment of doxycycline. (E) After 7 day treatment of doxycycline, MAPK and AKT immunoblots were conducted using 5 independent stable cell lines. Regardless of any kind of stable cell lines, phospho-MAPK is increased by doxycycline treatment. (F/G/H/I) Quantification of AKT1, phospho-AKT1, MAPK and phospho-MAPK was conducted, and phospho-MAPK (ERK1/2) was significantly increased in doxycycline treated group.

9. EGFR expression is regulated by ANO9/TMEM16J

To investigate the mechanisms of ANO9/TMEM16J in the regulation of cell viability, EGFR and phospho-EGFR expression were compared using immunoblot assay between doxycycline treated and untreated group. We found that both EGFR and phosphor-EGFR was increased in ANO9/TMEM16J over-expression group (Figure 9A). Quantification of EGFR and phospho-EGFR immunoblot revealed that both EGFR and phospho-EGFR were significantly increased in PANC-1 with ANO9/TMEM16J and these results suggest that ANO9/TMEM16J can increase cell viability through the activation of EGFR (Figure 9B/C). This is very interesting and important finding because EGFR is currently available drug target.

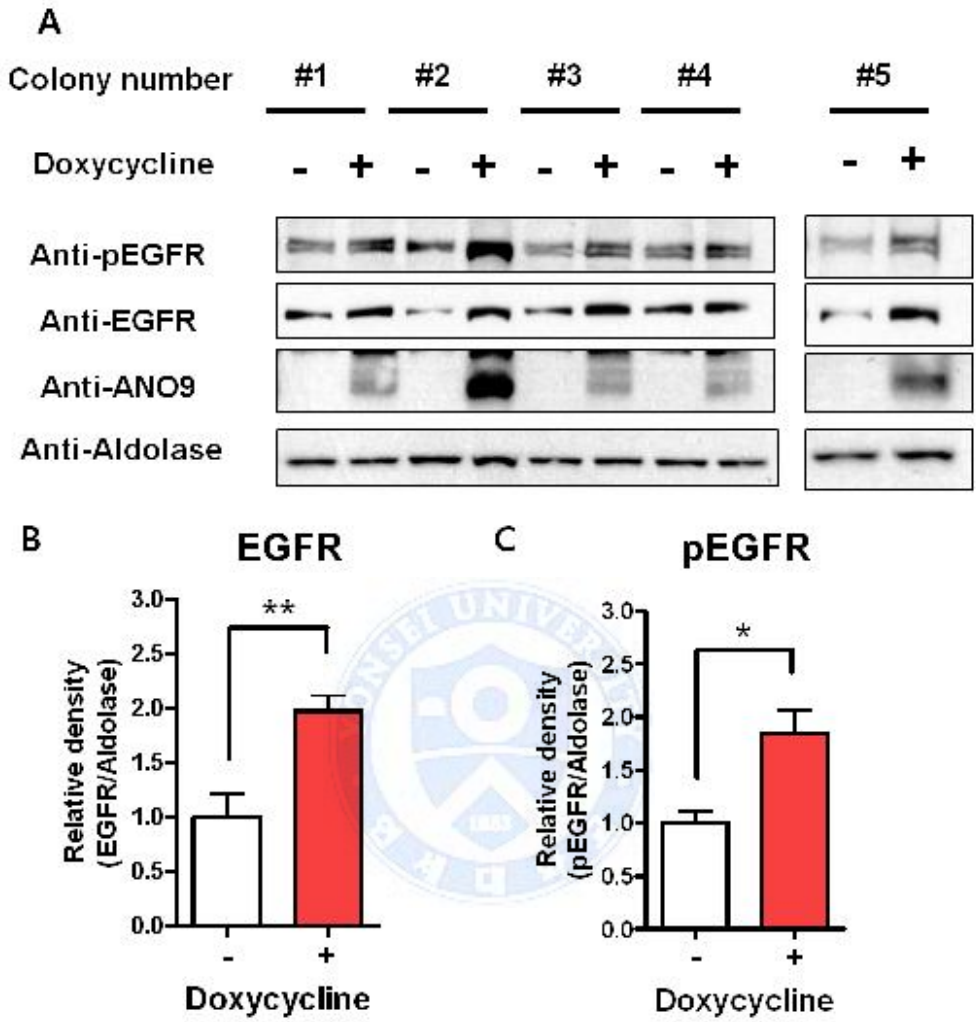


Figure 9. ANO9/TMEM16J regulates EGFR expression. (A) PANC-1 cells with stably expressed doxycycline inducible plasmid were treated with doxycycline for 7 days. EGFR and phospho-EGFR expressions were increased in all independent stable cell lines. (B/C) Quantification of EGFR and phospho-EGFR were conducted, and both EGFR and phospho-EGFR were significantly increased in doxycycline treated group. * $P < 0.05$, ** $P < 0.01$

10. ANO9/TMEM16J is significant prognostic factor for pancreatic cancer

The functional and clinical role of ANO9/TMEM16J has not been studied and the prognostic impact on pancreatic cancer is also unknown. Like ANO1/TMEM16A, 78 patients were enrolled to evaluate the ANO9/TMEM16J expression using immunohistochemistry (IHC) stain; low (0 or 1+) and high expression (2+ or 3+) was observed in 70.5% and 29.5% of patients, respectively (Figure 10A/B). Baseline characteristics were listed in Table 3. Patients with high expression of ANO9/TMEM16J tended to have poorly differentiated cancer and perineural invasion (P= 0.08 and P=0.05, respectively).

Survival analysis was conducted, and patients with high expression of ANO9/TMEM16J showed poor disease free survival (DFS) than patients with low expression (DFS, median survival time 13.3 *vs.* 7.5 mo, P = 0.042, Figure 10C). In overall survival, patients with high expression of ANO9/TMEM16J showed inferior survival outcomes than patients with low expression of ANO9/TMEM16J (OS, median survival time 30.4 *vs.* 20.2 mo, P = 0.128, Figure 10D).

Univariate and multivariate analysis were performed to identify the prognostic factors associated with DFS and OS (Table 4). On univariate analysis, tumor grade and ANO9/TMEM16J were significantly associated with DFS. Multivariate analysis revealed that tumor grade is the only independent risk factor for recurrence. Regarding OS, T stage was significantly associated with DFS, and ANO9/TMEM16J was borderline significant for OS (P = 0.13, HR = 1.68, 95% CI = 0.85 – 3.32). In multivariate analysis, both T stage and ANO1/TMEM16A were significant prognostic factors for OS. From these results, we suggest that

ANO9/TMEM16J might be a potential prognostic factor for survival outcome in pancreatic cancer.

Table 3. Patient characteristics by ANO9/TMEM16J expression

Characteristics		ANO9-Low		ANO9-High		Total		*P*
		N	%	N	%	N	%	
Age (n, %)	<65	26	47.3	14	60.9	40	51.3	0.27
	≥65	29	52.7	9	39.1	38	48.7	
Sex (n, %)	Male	25	45.5	14	60.9	39	50	0.21
	Female	30	54.5	9	39.1	39	50	
Tumor grade (n, %)	Well	12	23.1	1	4.3	13	17.3	0.08
	Moderate	34	65.4	17	73.9	51	68.0	
	Poor	6	11.5	5	21.7	11	14.7	
T stage (n, %)	1-2	4	7.3	4	17.4	8	10.3	0.23
	3-4	51	92.7	19	82.6	70	89.7	
N stage (n, %)	0	20	36.4	7	30.4	27	34.6	0.62
	1	35	63.6	16	69.6	51	65.4	
TNM Stage (n, %)	I	2	3.6	3	13	5	6.4	0.03
	II	52	94.5	17	73.9	69	88.5	
	III	1	1.8	3	13	4	5.1	
Perineural invasion	No	9	20	0	0	9	14.3	0.05
	Yes	36	80	18	100	54	85.7	
Lymphovascular invasion	No	17	37	8	42.1	25	38.5	0.70
	Yes	29	63	11	57.9	40	61.5	
Operation type (n, %)	Whipple	6	10.9	1	4.3	7	9	0.10
	PPPD	24	43.6	17	73.9	41	52.6	
	Distal	20	36.4	5	21.7	25	32.1	
	Total	5	9,1	0	0	5	6.4	

N: number; PPPD: pylorus preserving pancreatoduodenectomy; Distal: distal pancreatectomy; Total: total pancreatectomy; *P*: p-value

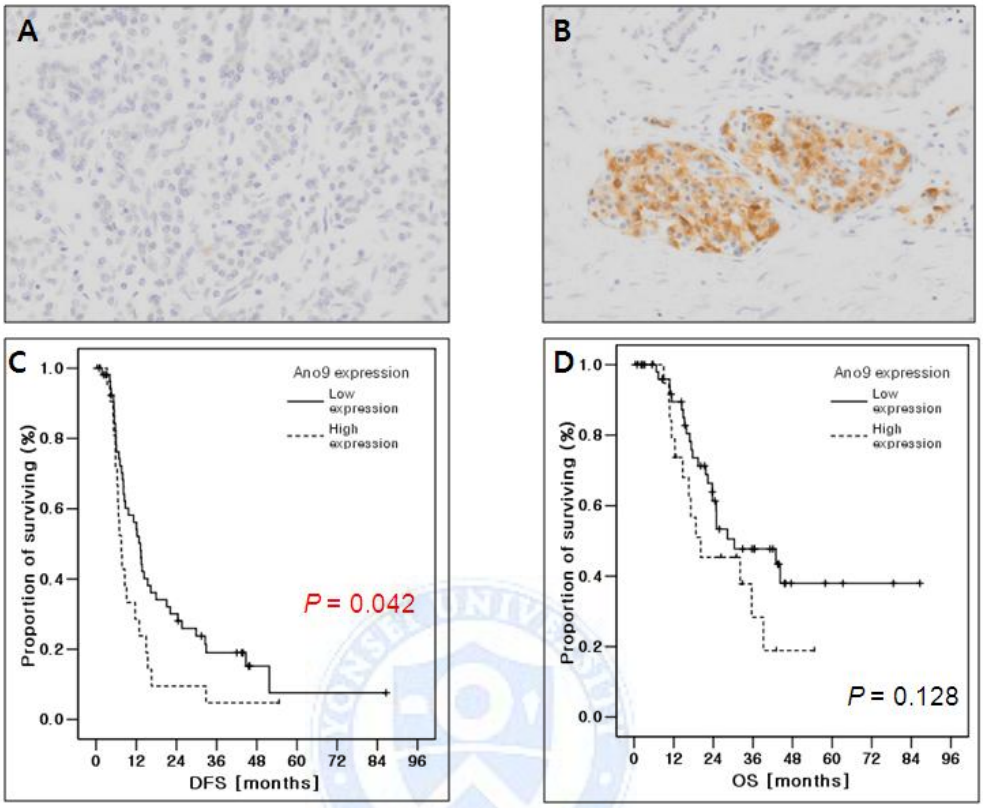


Figure 10. IHC (immunohistochemistry) stain of ANO9/TMEM16J and survival analysis according to ANO9/TMEM16J expression in pancreatic cancer patients. (A/B) Example of low and high expression of ANO9/TMEM16J in pancreatic cancer patient. (C/D) Kaplan-Meier survival curves for disease free survival (DFS) and overall survival (OS) according to ANO9/TMEM16J expression in pancreatic cancer patients.

Table 4. Univariate and multivariate analysis of prognostic factors including ANO9/TMEM16J for disease-free survival and overall survival

	Disease-free survival (DFS)				Overall survival (OS)			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Tumor grade		0.006		0.008		0.25		
Well	1		1		1			
Moderate	2.48 (1.16-5.30)		2.19 (1.01-4.79)		2.10 (0.80-5.53)			
Poor	4.55 (1.81-11.49)		4.33 (1.71-10.97)		2.60 (0.78-8.61)			
T stage		0.28				0.04		0.027
1-2	1				1		1	
3-4	1.68 (0.66-4.32)				7.78 (1.06-56.99)		9.66 (1.30-71.76)	
N stage		0.88				0.54		
0	1				1			
1	0.96 (0.57-1.62)				1.24 (0.62-2.48)			

(continued)

	Disease-free survival (DFS)				Overall survival (OS)			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Perineural invasion		0.24				0.40		
No	1				1			
Yes	1.68 (0.71-3.98)				1.58 (0.55-4.54)			
ANO9/TMEM16J		0.04		0.108		0.13		0.033
Low	1		1		1		1	
High	1.74 (1.02-2.99)		1.59 (0.90-2.79)		1.68 (0.85-3.32)		2.13 (1.06-4.24)	

IV. DISCUSSION

Pancreatic cancer represents an unmet therapeutic challenge. Pancreatic cancer is addicted to the activity of the mutated KRAS oncogene which is considered so far an undruggable therapeutic target. Therefore, exome sequencing was conducted in various groups and KRAS, TP53, CDKN2A, SMAD4, MLL3, TGFBR2, ARID1A, SF3B1 along with genes involved in chromatin modification, DNA damage repair and other mechanisms were mutated in pancreatic cancer.[1,19, 20] Despite all these efforts, effective drug target in pancreatic cancer has not been suggested.

Since ANO1/TMEM16A was first identified in 2008,[7,10,21] many studies have been performed to examine the functional, biological and clinical meaning of ANO/TMEM16 family.[9,22-26] ANO3/TMEM16C facilitates sodium activated potassium currents in rat primary sensory neuron and regulates pain processing.[27] ANO5/TMEM16E is reported that recessive mutation of ANO5/TMEM16E cause proximal limb girdle muscular dystrophy (LGMD2L) and distal non-dysferlin Miyoshi myopathy (MMD3) muscular dystrophy.[28] ANO6/TMEM16F is known as calcium activated cation channel required for lipid scrambling in platelets during blood coagulation.[29] NGEP, also known as ANO7/TMEM16G is expressed in normal prostate and prostate cancer, but the biological and clinical role of NGEP has not been studied yet.[16] ANO10/TMEM16K has been reported to be associated with autosomal-recessive cerebellar ataxia.[30]

Of ANO/TMEM16 family, ANO1/TMEM16A is the most well studied protein about the functional and clinical role; over-expression in several types of cancer such as breast cancer, head and neck cancer, colorectal cancer and prostate cancer was reported.[12-15] ANO1/TMEM16A is

located in the 11q13 amplicon, one of the most frequently amplified chromosomal regions in human cancer.[12] Fine mapping of 11q13 in HNSCC led to the identification of ANO1/TMEM16A in this amplicon.[12] Interestingly, ANO1/TMEM16A expression is associated with reduced survival and increased metastatic potential in several cancer types. Previous studies identified that MAPK pathway activation is involved in ANO1/TMEM16A related cell viability.[12,13] However, the underlying cell viability related mechanism of ANO1/TMEM16A as a chloride channel is poorly understood.

The present study demonstrates that ANO1/TMEM16A affects cell viability in pancreatic cancer cells. In addition, MAPK activation was also noted in PANC1- with ANO1/TMEM16A compared to PANC-1 without ANO1/TMEM16A. A novel finding of this study is that ANO1/TMEM16A lower the intracellular chloride concentration in pancreatic cancer cells. In addition, we observed that depolarization of resting membrane potential (RMP) was induced by ANO1/TMEM16A expression.

We evaluated whether low chloride concentration or depolarization status is important for cell viability, which is increased by ANO1/TMEM16A. Firstly we made a low intracellular chloride level using low chloride medium to mimic ANO1/TMEM16A over-expression. This experiment shows that 60 mM chloride medium was most effective for cell viability, and this result may suggest that there is a precise range of relatively low chloride concentrations those are suitable for cell viability. Next, we made cell depolarization status to mimic ANO1/TMEM16A. However, depolarization induced by adding a potassium or ouabain can not increase cell viability. From these results, we conclude that important factor for cell viability, which is associated with ANO1/TMEM16A, is not depolarization of resting

membrane potential but low intracellular chloride level.

Then we enrolled the 74 pancreatic cancer patients. They received curative resection for primary pancreatic cancer, and TNM stage 1, 2 and 3 were included. Although ANO1/TMEM16A is known as important prognostic factor for diverse types of cancer, the clinical role of ANO1/TMEM16A has not been reported in pancreatic cancer. To evaluate the role of ANO1/TMEM16A in pancreatic cancer, we first evaluated the protein expression level using immunohistochemistry (IHC). As a result, 41% of pancreatic cancer patients showed high expression (2+ or 3+) of ANO1/TMEM16A. Furthermore, this study identified the ANO1/TMEM16A as a prognostic factor for survival outcome of pancreatic cancer. As shown in Figure 6C/D, patients with high expression of ANO1/TMEM16A showed poor overall survival outcomes compared to patients with low expression of ANO1/TMEM16A. Patients with high expression of ANO1/TMEM16A had poorly differentiated cancer, which probably means that the underlying biology of the disease might be closely related to ANO1/TMEM16A expression. It enables accurate patient risk stratification and may aid in treatment selection. The most important implication is that ANO1/TMEM16A inhibitor is ready to be used for treatment of cancer, although clinical trial will be needed.

Secondly, this study focuses on the role of ANO9/TMEM16J in pancreatic cancer. ANO9/TMEM16J is rarely reported and has not been revealed its expression and function in cancer field, including pancreatic cancer. ANO9/TMEM16J is firstly reported as TP53 inducible protein 5,[18] and this previous article based on in silico analysis reported that TP53I5 mRNA was expressed in human cancer, including colorectal, lung and breast cancer.

In this study, we first identified the expression of ANO9/TMEM16J in pancreatic cancer. In addition this study demonstrates that ANO9/TMEM16J affects cell viability and colony formation capacity in pancreatic cancer. Then, we searched for molecular mechanism of ANO9/TMEM16J mediated cell viability, by knock-down or over-expression experiments. Both knock-down and transient transfection system revealed that ANO9/TMEM16J does not activate AKT1 pathway but activates MAPK (ERK) pathway. Activation of MAPK pathway was also observed in stable cell line using doxycycline inducible lenti-virus plasmid.

Then we evaluated the interacting molecules between ANO9/TMEM16J and MAPK pathway. Previous studies reported that ANO1/TMEM16A increases cell viability through the regulation of EGFR expression.[12] Based on this previous report, we hypothesized that EGFR can be a key component of ANO9/TMEM16J related cell viability. Thus we compared EGFR and phospho-EGFR expression using immunoblot between PANC-1 with and without ANO9/TMEM16J and confirmed the elevated EGFR and phospho-EGFR in PANC-1 with ANO9/TMEM16J. EGFR regulation by ANO9/TMEM16J is very interesting and important findings, because EGFR is currently available target; EGFR over-expression is a known predictive marker for erbitux (EGFR monoclonal antibody) therapy in colorectal cancer and advanced non-small cell lung cancer.[31,32] Therefore, ANO9/TMEM16J may be suggested as a potential predictive marker in response to EGFR inhibitor in pancreatic cancer.

To date, there is no study on the clinical role of ANO9/TMEM16J. Similar to ANO1/TMEM16A study, 74 pancreatic cancer patients were enrolled in ANO9/TMEM16J study. In immunohistochemistry (IHC) stain of ANO9/TMEM16J, 29.5% of pancreatic cancer patients showed high

expression (2+ or 3+) of ANO9/TMEM16J. Patients with high expression of ANO9/TMEM16J have more advanced T stage and perineural invasion which might affect survival outcomes. As expected, in survival analysis, ANO9/TMEM16J was a significant prognostic factor for pancreatic cancer. As shown in Figure 10C/D, disease free survival (DFS) was significantly longer in patients with low expression of ANO9/TMEM16J. Although overall survival did not show significant differences between patients with high and low ANO9/TMEM16J, patients with high level of ANO9/TMEM16J tend to show poor overall survival outcomes compared to patients with low expression of ANO9/TMEM16J. In multivariate analysis, ANO9/TMEM16J was revealed to be an independent prognostic factor for overall survival. For further studies, ANO9/TMEM16J needs to be characterized of its localization and physiological functions such as channel activity and lipid scramblase activity.

Along with these findings, present study strongly suggests that ANO1/TMEM16A and ANO9/TMEM16J may be the novel regulators of cell viability. Furthermore, they can be potential prognostic factors and novel druggable targets for pancreatic cancer.

V. CONCLUSION

To date, the expression of TMEM16 family in pancreatic cancer has not been revealed. In addition, the mechanism of cell viability regulation by ANO1/TMEM16A and the biological and clinical role of ANO9/TMEM16J have not been fully examined. The present study contributes to the area of ANO/TMEM16 family in pancreatic cancer, demonstrating that:

1. ANO1/TMEM16A and ANO9/TMEM16J is over-expressed in pancreatic cancer.
2. ANO1/TMEM16A and ANO9/TMEM16J increases cell viability through MAPK pathway.
3. ANO1/TMEM16A lower intracellular chloride concentration and it is important factor for cell viability.
4. ANO9/TMEM16J regulates EGFR expression and activation.
5. ANO1/TMEM16A and ANO9/TMEM16J are possible prognostic factors for pancreatic cancer patients.

These results demonstrate that ANO/TMEM16 is a novel regulator of cell viability and can be novel druggable targets for anti-cancer therapy. In addition, ANO/TMEM16 have a role as a prognostic factor for survival outcomes in pancreatic cancer and it enables accurate patient risk stratification and may aid in treatment selection.

REFERENCES

1. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med 2014;371:2140-1.

2. Stocken DD, Hassan AB, Altman DG, Billingham LJ, Bramhall SR, Johnson PJ, et al. Modelling prognostic factors in advanced pancreatic cancer. Br J Cancer 2008;99:883-93.

3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015;65:5-29.

4. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011;364:1817-25.

5. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013;369:1691-703.

6. Pedemonte N, Galietta LJ. Structure and function of TMEM16 proteins (anoctamins). Physiol Rev 2014;94:419-59.

7. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, et al. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 2008;322:590-4.

8. Wang Y, Alam T, Hill-Harfe K, Lopez AJ, Leung CK, Iribarne D, et al. Phylogenetic, expression, and functional analyses of anoctamin

homologs in Caenorhabditis elegans. Am J Physiol Regul Integr Comp Physiol 2013;305:R1376-89.

9. Huang F, Rock JR, Harfe BD, Cheng T, Huang X, Jan YN, et al. Studies on expression and function of the TMEM16A calcium-activated chloride channel. Proc Natl Acad Sci U S A 2009;106:21413-8.

10. Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 2008;455:1210-5.

11. Rock JR, O'Neal WK, Gabriel SE, Randell SH, Harfe BD, Boucher RC, et al. Transmembrane protein 16A (TMEM16A) is a Ca2+-regulated Cl- secretory channel in mouse airways. J Biol Chem 2009;284:14875-80.

12. Britschgi A, Bill A, Brinkhaus H, Rothwell C, Clay I, Duss S, et al. Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling. Proc Natl Acad Sci U S A 2013;110:E1026-34.

13. Duvvuri U, Shiwarski DJ, Xiao D, Bertrand C, Huang X, Edinger RS, et al. TMEM16A induces MAPK and contributes directly to tumorigenesis and cancer progression. Cancer Res 2012;72:3270-81.

14. Simon S, Grabellus F, Ferrera L, Galietta L, Schwindenhammer B, Muhlenberg T, et al. DOG1 regulates growth and IGFBP5 in gastrointestinal stromal tumors. Cancer Res 2013;73:3661-70.

15. Liu W, Lu M, Liu B, Huang Y, Wang K. Inhibition of Ca(2+)-activated Cl(-) channel ANO1/TMEM16A expression suppresses tumor growth and invasiveness in human prostate carcinoma. Cancer Lett 2012;326:41-51.

16. Bera TK, Das S, Maeda H, Beers R, Wolfgang CD, Kumar V, et al. NGEP, a gene encoding a membrane protein detected only in prostate cancer and normal prostate. Proc Natl Acad Sci U S A 2004;101:3059-64.

17. Dutertre M, Lacroix-Triki M, Driouch K, de la Grange P, Gratadou L, Beck S, et al. Exon-based clustering of murine breast tumor transcriptomes reveals alternative exons whose expression is associated with metastasis. Cancer Res 2010;70:896-905.

18. Katoh M, Katoh M. Identification and characterization of human TP53I5 and mouse Tp53i5 genes in silico. Int J Oncol 2004;25:225-30.

19. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 2012;491:399-405.

20. Zorde Khvalevsky E, Gabai R, Rachmut IH, Horwitz E, Brunschwig Z, Orbach A, et al. Mutant KRAS is a druggable target for pancreatic cancer. Proc Natl Acad Sci U S A 2013;110:20723-8.

21. Schroeder BC, Cheng T, Jan YN, Jan LY. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 2008;134:1019-29.

22. Almaca J, Tian Y, Aldehni F, Ousingsawat J, Kongsuphol P, Rock JR, et al. TMEM16 proteins produce volume-regulated chloride currents that are reduced in mice lacking TMEM16A. J Biol Chem 2009;284:28571-8.

23. Hartzell HC, Yu K, Xiao Q, Chien LT, Qu Z. Anoctamin/TMEM16 family members are Ca2+-activated Cl- channels. J Physiol 2009;587:2127-39.

24. Schreiber R, Uliyakina I, Kongsuphol P, Warth R, Mirza M, Martins JR, et al. Expression and function of epithelial anoctamins. J Biol Chem 2010;285:7838-45.

25. Milenkovic VM, Brockmann M, Stohr H, Weber BH, Strauss O. Evolution and functional divergence of the anoctamin family of membrane proteins. BMC Evol Biol 2010;10:319.

26. Duran C, Qu Z, Osunkoya AO, Cui Y, Hartzell HC. ANOs 3-7 in the anoctamin/Tmem16 Cl- channel family are intracellular proteins. Am J Physiol Cell Physiol 2012;302:C482-93.

27. Huang F, Wang X, Ostertag EM, Nuwal T, Huang B, Jan YN, et al. TMEM16C facilitates Na(+)-activated K+ currents in rat sensory neurons and regulates pain processing. Nat Neurosci 2013;16:1284-90.

28. Bolduc V, Marlow G, Boycott KM, Saleki K, Inoue H, Kroon J, et al. Recessive mutations in the putative calcium-activated chloride channel Anoctamin 5 cause proximal LGMD2L and distal MMD3 muscular dystrophies. Am J Hum Genet 2010;86:213-21.

29. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, et al. TMEM16F forms a Ca2+-activated cation channel required for lipid scrambling in platelets during blood coagulation. Cell 2012;151:111-22.

30. Vermeer S, Hoischen A, Meijer RP, Gilissen C, Neveling K, Wieskamp N, et al. Targeted next-generation sequencing of a 12.5 Mb homozygous region reveals ANO10 mutations in patients with autosomal-recessive cerebellar ataxia. Am J Hum Genet 2010;87:813-9.

31. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006;66:3992-5.

32. Pirker R, Pereira JR, von Pawel J, Krzakowski M, Ramlau R, Park K, et al. EGFR expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. Lancet Oncol 2012;13:33-42.

ABSTRACT (IN KOREAN)

ANO/TMEM16 에 의한 췌장암세포 증식 분자기전

<지도교수 이민구>

연세대학교 대학원 의과학과

박형순

췌장암은 예후가 매우 불량하며 효과적인 약물 치료가 없어 표적 발굴은 미충족 의료 수요이다. TMEM16 유전자 군은 10개의 유전자로 이루어져 있고, TMEM16A 부터 TMEM16K 로 구성된다. 이 유전자들 중에 TMEM16A, TMEM16B 와 TMEM16F 는 이온 채널로 알려져 있으며, TMEM16C, TMEM16D, TMEM16F, TMEM16G 와 TMEM16J 의 경우 인지질 scramblase 로 알려져 있으나 정확한 생리적 기능은 아직 확인되지 않았다.

TMEM16A 의 경우 TMEM16 유전자 군에서 가장 잘 알려진 유전자이며 여러 종류의 암에서 과발현이 보고 되었다. 하지만 췌장암 같은 소화기암에서는 발현이나 임상적 역할이 알려져 있지 않아 본 연구에서는 TMEM16A 가 췌장암의 성장에 관여하며, TMEM16A 에 의해 유도되는 저 클로라이드 농도가 세포 성장에 중요한 인자임을 보였다. 또한 본 연구에서는 TMEM16J 가

췌장암에서 과발현 하며 EGFR 의 조절과 MAPK pathway 활성화를 통해 세포 성장에 관여하는 것을 입증하였다.

추가적으로 췌장암 환자들을 대상으로 TMEM16A 와 TMEM16J 의 발현 여부에 따라 무병 생존기간 및 전체 생존기간의 증가 또는 감소를 확인 함으로 TMEM16A 와 TMEM16J 가 췌장암에서 중요한 예후 인자임을 밝혔다.

이번 연구 결과들을 통해 TMEM16A 억제제의 활용과 TMEM16J 와 같은 EGFR 을 조절할 수 있는 새로운 타겟을 제안함으로 미충족 의료 수요인 췌장암 치료의 새로운 가능성을 제시할 수 있었고, TMEM16A 와 TMEM16J 가 췌장암의 예후 인자로서 환자의 재발 또는 생존 위험성을 예측 하는데 활용 할 수 있기에 큰 의미를 가진다고 할 수 있다.

핵심되는 말: TMEM16A, TMEM16J, 세포 성장, 췌장암, 클로라이드, MAPK, EGFR

PUBLICATION LIST

1. **Park HS**, Jung M, Kim HS, Kim HI, An JY, Cheong JH, et al. Proper timing of adjuvant chemotherapy affects survival in patients with stage 2 and 3 gastric cancer. Ann Surg Oncol 2015;22(1):224-31.

2. **Park HS**, Jung M, Shin SJ, Heo SJ, Kim CG, Lee MG, et al. Benefit of adjuvant chemotherapy after curative resection of lung metastasis in colorectal cancer. Ann Surg Oncol 2015 Oct 29.

3. **Park HS**, Lim SM, Cho A, Shin JG, Lee MG, Kim HR, et al. Pharmacogenetic analysis of advanced non-small-cell lung cancer patients treated with first-line paclitaxel and carboplatin chemotherapy. Pharmacogenet Genomics 2015 Dec 4.