Identification of tumorigenesis related signaling pathways in the tumors with nuclear β-catenin overexpression

Minhee Park

Department of Medical Science

The Graduate School, Yonsei University

Identification of tumorigenesis related signaling pathways in the tumors with nuclear β-catenin overexpression

Directed by Professor Hoguen Kim

The Doctoral Dissertation

submitted to the Department of Medical Science,

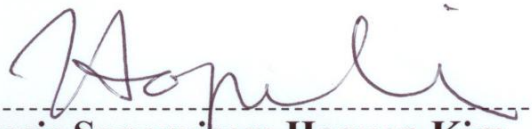
the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

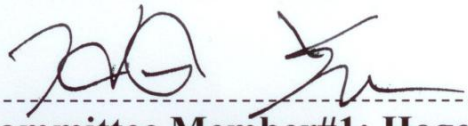
Minhee Park

December 2015

This certifies that the Doctoral Dissertation of Minhee Park is approved.

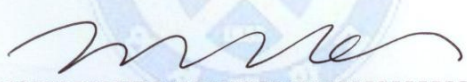


Thesis Supervisor: Hoguen Kim

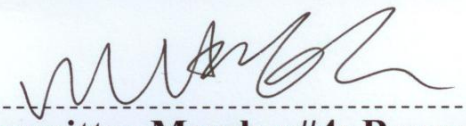


Thesis Committee Member#1: Hogeun Yoon

Thesis Committee Member#2: Kang-Yell Choi



Thesis Committee Member#3: Kyung-Hee Chun



Thesis Committee Member#4: Byung-so Min

The Graduate School
Yonsei University

December 2015

Acknowledgements

지난 5년간은 정말 제 인생에 있어서 큰 밑거름이 될 시간이었습니다. 정말 많은 잊지 못할 일들이 있었고 그럴 때마다 제 스스로의 부족함을 느끼며 과연 내가 박사과정을 무사히 마칠 수 있을까 끊임없이 자문하는 시간이었던 것 같습니다. 항상 초심처럼 모든 일에 성실하게 임하자고 스스로 다짐하고 노력했지만, 돌이켜 보면 제가 충분히 열심히 했는지에 대해 부끄러운 생각이 듭니다. 처음 실험실에 들어왔을 때 아무것도 모르던 학부생 시절이 아직도 생생한데, 어느덧 박사 졸업을 앞두고 있다는 사실에 기쁘면서도 또 한편 아직도 많이 부족하다는 생각에 걱정이 됩니다. 계속된 실험 속에서 수많은 시행착오를 겪으면서도 저의 능력보다 많은 일들을 할 수 있었던 이유는, 제가 힘들고 어려운 시기에 항상 저를 지지해주고 도와주셨던 감사한 분들 덕분이었다고 생각합니다. 그분들 덕분에 아직도 많이 부족하지만, 감사한 마음으로 바람직한 연구자의 자세에 대해 배울 수 있었던 것 같습니다.

먼저 부족한 저를 제자로 받아주시고, 지난 5년간 항상 과학자로서 필요한 조언과 격려를 아끼지 않으시고 지도해주신 김호근 교수님께 깊은 감사를 드립니다. 바쁘신 와중에도 논문 지도에 시간을 할애해주신 최강열 교수님, 윤호근 교수님, 전경희 교수님, 민병소 교수님께도 깊은 감사를 드립니다.

실험실 선배로서 많은 조언과 격려를 아끼지 않고 바람직한

과학자상에 대해 많은 생각을 하게해준 김원규 선배님, 그리고 권유진, 윤성주, 실험실 사람들 모두 고맙습니다. 그리고 같은 학교는 아니지만 학위 과정 동안 여러 번 정말 많은 도움을 주셨던 김민형 선생님께도 진심으로 감사의 말을 전하고 싶습니다. 같은 실험실은 아니지만, 많은 도움을 준 혜영이, 또 제가 학위 기간을 하는 동안에 제 곁에서 항상 응원을 보내준 도영이, 나리, 정은, 규리 언니에게도 진심으로 고맙다고 말하고 싶습니다. 각자 다른 길을 걷고 있지만 든든한 06 동기들, 항상 공감해주고 응원해주는 미선이, 소연에게도 감사합니다. 너무 많은 분들에게 도움을 받아 이곳에 일일이 다 감사의 말씀을 적지 못해 죄송합니다. 저에게 따끔한 충고와 조언뿐만 아니라 응원을 해주신 선후배, 친구들 모두에게 감사합니다.

마지막으로 부족한 딸이지만 항상 최고라 말해주시고 학위 기간 동안 저의 곁에서 항상 변함없는 모습으로 용기를 북돋아 주시고 응원해주시는 어머니 감사하고 사랑합니다. 또 저를 자랑스럽게 생각해주고 멋진 앞날을 위해 노력하고 있는 동생에게도 고마움과 사랑을 전합니다. 앞으로 주어진 위치에서 겸손한 마음으로 항상 감사하며 성실히 임하겠습니다. 관심과 사랑으로 지켜봐 주시고 응원해주시길 바랍니다. 감사합니다.

박민희 올림.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Identification of tumorigenesis related signaling pathways in the tumors with nuclear β-catenin overexpression

Minhee Park

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Hoguen Kim)

Mutations of *β-catenin*, *CTNNB1*, have been reported in various cancers, including colorectal cancer, breast cancer and lung cancer. Most *β-catenin* mutations are located in exon 3 of *β-catenin* genes, which lead to stabilization of β-catenin by blocking the destruction complex of β-catenin. In colorectal cancers, *APC* mutations are detected in 70-80% of colon cancers and *β-catenin* mutations are mutually exclusive and detected in about 5% of the colorectal cancers. Although the involvement of *APC* mutations in the altered Wnt/β-catenin pathway activity is well documented, the functional relevance between the deregulated Wnt/β-catenin signaling pathway and *β-catenin* mutations has not yet been fully understood in colon cancers. Also, the molecular significances of *β-catenin* mutations in the other

cancers are not fully understood. Unlike colorectal carcinomas, nearly all of solid-pseudopapillary neoplasm (SPN) exhibit somatic mutation in exon3 of *β-catenin*, which results in abnormal nuclear accumulation (overexpression) of β-catenin. Although *β-catenin* mutation and activation of the Wnt/β-catenin signaling pathway have been implicated in the pathogenesis of SPN, the molecular regulatory networks remain poorly understood.

To identify the altered pathways by nuclear overexpression of β-catenin in tumors, colon cancer and SPN were selected as study models. *β-catenin* mutation is responsible for the development of a small subset of colon cancers, while development of SPN is solely driven by *β-catenin* mutation. The molecular significances of nuclear β-catenin overexpression were compared in colon cancer as a model of nuclear β-catenin overexpression by altered genes involved in β-catenin degradation, and SPN as a model of nuclear β-catenin overexpression by β-catenin mutation. Since nuclear β-catenin shows heterogeneous expressions in colon cancers, the colorectal tumors were classified by percentage of nuclear β-catenin and identified a subset of colon cancers by comparing gene expression profiles in the colon cancers showing heterogeneous expression of nuclear β-catenin. As a result, it was found that overexpressed nuclear β-catenin activates genes involved in Wnt/β-catenin signaling, Notch signaling, Hedgehog signaling and ECM-receptor interaction in colon cancer.

To identify gene subsets associated with nuclear β-catenin overexpression by β-catenin mutation, mRNA expression profiles of SPN and other pancreatic tumors (pancreatic adenocarcinomas and neuroendocrine tumors) were performed. All SPNs harbor *β-catenin* mutation, while the other pancreatic tumors harbor no *β-catenin* mutation. Unsupervised clustering

analysis of mRNA expression distinguished SPNs as a distinct type of pancreatic tumor. Analysis of differentially expressed genes in SPN demonstrated that genes involved in Wnt/β-catenin, Hedgehog and androgen receptor signaling pathways as well as epithelial-mesenchymal transition were activated in solid-pseudopapillary neoplasms.

Finally, the altered expression level of genes involved in three activated signaling pathways was confirmed by using cells transfected with *β-catenin* constructs. Transfection of mutant *β-catenin* constructs resulted in the translocation of androgen receptor into the nucleus and up-regulated several molecules involved in Wnt/β-catenin, Notch and Hedgehog signaling.

In conclusion, overexpression of nuclear β-catenin commonly or selectively affects the signaling pathways of the Wnt/β-catenin, Notch, Hedgehog and androgen receptor, and contributes to the tumorigenesis of colon cancers and SPN.

Key words : β-catenin, colon cancer, solid-pseudopapillary neoplasm (SPN), gene expression, Wnt/β-catenin signaling, Notch signaling, Hedgehog signaling

Abbreviation : APC; Adenomatous polyposis coli, GSK3β; Glycogen synthase kinase 3 beta, AR; androgen receptor SPN; Solid-pseudopapillary neoplasm, PCA; Pancreatic adenocarcinoma, NET; Neuroendocrine tumor, DEG; Differentially expressed gene, EMT; Epithelial mesenchymal transition

Identification of tumorigenesis related signaling pathways in the tumors with nuclear β-catenin overexpression

Minhee Park

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Hoguen Kim)

I. INTRODUCTION

Tumorigenesis is a complicated process, comprising multiple cascades of genetic aberrations that promote or inhibit the cell cycle, proliferation, and apoptosis. In normal cells, proto-oncogenes regulate the cell cycle and differentiation. However, if mutated or overexpressed, these oncogenes promote excessive cell proliferation. Typically, single oncogene is involved in only one specific step in tumorigenesis, such that multiple oncogenes are responsible for the entire tumorigenesis process. One oncogene known to be relevant to the development of various cancers is β-catenin, a component of the cell adhesion complex, including E-cadherin, and a key molecule in the Wnt/β-catenin signaling pathway.[1,2]

In general, Wnt/β-catenin signaling is important to cell proliferation, differentiation, and embryonic development.[1,3] Regulation of the Wnt/β-catenin pathway depends on the interaction between the frizzled receptor and Wnt ligand. In the absence of Wnt, the destruction complex composed of adenomatous polyposis

coli (APC), Axin, glycogen synthase kinase (GSK) and casein kinase 1 (CK1) binds to and phosphorylates β-catenin at Ser-33, Ser-37, Thr-41, and Ser-45, making it recognizable by the ubiquitin ligase, β-TrCP. Degradation of β-catenin is then carried out by the ubiquitin-proteasome system. Meanwhile, in the presence of Wnt, activated frizzled receptor phosphorylates the dishevelled protein, stabilizing β-catenin and allowing it to accumulate in the cytoplasm.[4] Once accumulated, β-catenin binds to LEF/TCF transcription factors, and this complex is translocated into nucleus, where it leads to the upregulation of Wnt target genes, such as *c-myc* and *cyclinD1*.[5,6]

The Wnt/β-catenin signaling pathway, itself, plays key roles in multiple biological processes. However, it can contribute to tumorigenesis and stem cell maintenance in combination with other pathways, such as Notch and Hedgehog signaling pathways: briefly, increased JAG1 expression by β-catenin induces activation of the Notch pathway, which can also be activated upon JAG2 stimulation by Gli molecules.[7,8]

In addition to mutations in *APC* genes, mutation of *β-catenin* mutation can also activate abnormal Wnt/β-catenin signaling. *β-catenin* mutations have been reported in various cancers, including colorectal cancer, breast cancer, lung cancer ,and glioblastoma.[9,10] Most *β-catenin* mutations are reportedly detected in exon 3 of *CTNNB1* genes that encode β-TrCP binding regions, blocking degradation of β-catenin and resulting in constant activation of Wnt/β-catenin signaling pathway.[5,6] In colorectal cancers, dysregulation of the Wnt/β-catenin signaling pathway caused by *APC* mutations is detected in 70-80% of colorectal cancers, while *β-catenin* mutations are detected in about 50% of tumors with wild-type *APC*.[11-14] Although the frequency of mutations in *β-catenin* is about 5% for all colon cancers, varying expression levels of nuclear β-catenin are observed in colon cancer tissues (0-100%).[15]

Most previous studies on Wnt/β-catenin signaling in cancer have used indirect mouse models, including *APC* Min mice or *β-catenin* exon 3 deletion mice, to study the effects of activated Wnt/β-catenin signaling. [7,16] However, the regulatory mechanisms of β-catenin proposed by these studies remain controversial: for example, indirect mouse models of *APC* mutations alone or Wnt-induced stabilization of β-catenin failed to show explicit nuclear accumulation of β-catenin.[17-20] Moreover, although the involvement of *APC* mutations in altered Wnt/β-catenin signaling is well documented, the functional relevance of the altered Wnt/β-catenin signaling and *β-catenin* mutations has not yet been fully elucidated in colon cancers. Also, the molecular impact of nuclear accumulation of β-catenin in other cancers is not fully understood.

Solid-pseudopapillary neoplasms (SPN) are characterized by mutations in exon 3 of *β-catenin* and abnormal β-catenin nuclear accumulation.[21-23] The molecular genetics of SPN has recently become one of the best understood among all human tumors.[24] SPN is classified as a distinct phenotype due to its characteristic clinicopathologic features and distinct immunophenotype, compared to other pancreatic tumors that show no mutations in *β-catenin*.[25,26] Although *β-catenin* mutation and activation of the Wnt/β-catenin signaling pathway have been implicated in the pathogenesis of solid-pseudopapillary neoplasm, the molecular regulatory networks of this tumor remain poorly understood.

To elucidate the pathways altered by overexpression of nuclear active β-catenin in tumors, colon cancer and SPN were selected as study models. Since *β-catenin* mutation is responsible for the development of a subset of colon cancers and since most abnormal nuclear β-catenin overexpression is induced by altered genes, especially *APC* mutation, colon cancers were selected as a model of heterogeneous overexpression of nuclear β-catenin caused by alteration in genes involved in β-catenin degradation.

Meanwhile, SPN was chosen as a model of homogenous overexpression of nuclear

β-catenin caused by mutation in *β-catenin*. In the present study, colorectal tumors were classified according to levels of nuclear β-catenin overexpression and pancreatic tumors according to *β-catenin* mutation. Then, mRNA expression profiles for these tumors were analyzed. Although the colon cancers showing high nuclear overexpression of β-catenin is not included in specific one cluster of grouped samples, overexpressed nuclear β-catenin activates genes involved in Wnt/β-catenin signaling, Notch signaling, and ECM-receptor interactions. Additional analysis of differentially expressed genes in SPNs harboring *β-catenin* mutation revealed that Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways, as well as genes involved in epithelial-mesenchymal transition (EMT), to be activated in SPNs.

II. MATERIALS AND METHODS

1. Tissue samples and RNA preparation

The 101 Colorectal cancer tissue samples, 35 matched normal colon tissues, 14 solid-pseudopapillary neoplasms (SPN), six pancreatic adenocarcinomas (PCA), six neuroendocrine tumors (NET), and five non-neoplastic pancreatic tissue samples were used in this study. The specimens were obtained from the archives of the Department of Pathology, Yonsei University, Seoul, Korea, and from the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korean Science and Engineering Foundation of the Ministry of Science and Technology. Patient data were collected retrospectively from hospital records. All colon cancer patients had undergone colorectal resection between 2006-2012 and pancreatic tumor patients had undergone pancreatic resection between 2001-2011 and fresh snap-frozen samples were obtained immediately at the time of surgery. All carcinoma samples comprised B70% tumor cells, and none of the patients had received neo-adjuvant chemotherapy. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University of College of Medicine.

Total RNA was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturers' protocol. After DNase digestion and other clean-up procedures, RNA samples were quantified, aliquoted, and stored at -80℃ until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, measurement of the A260/280 ratio, and analyzed using the 2100 bioanalyzer (Agilent Technologies). For all samples, the RNA integrity number scores were >9.5.

2. Gene expression Analysis

For DNA microarray hybridization, RNA was pooled by mixing equal amounts of total RNA. Biotin-labeled cRNA targets were synthesized starting from 1.5 μg of total RNA. Double-stranded cDNA synthesis was performed using the Illumina TotalPrep RNA Amplification Kit (Illumina), while biotin-UTP-labeled antisense

RNA was transcribed *in vitro* using Ambion's Kit (Ambion Life Technologies). All steps of the labeling procedure were performed according to the manufacturers' protocol. Microarray experiments were conducted on the HumanHT-12 v4 Sentrix Expression BeadChip (Illumina). Hybridization of labeled cRNA to the BeadChip, washing, and scanning were performed according to the Illumina Bead Station 500X manual.

3. mRNA expression data preparation and statistical analysis

Raw data were extracted using the software provided by the manufacturer (Illumina Genome Studio v2011.1 (Gene Expression Module v1.9.0)). Expression intensities were normalized using quantile normalization techniques.[27] Using the normalized intensities, differentially expressed genes between normal and tumor tissues were determined using the integrated statistical method previously reported.[28] Briefly, (1) two independent tests were performed: Student's t-test and $\log_2$–median-ratio test; (2) adjusted *P*-values from each test were computed using an empirical distribution of the null hypothesis that the means of the genes were not different, which was obtained from random permutations of the samples; (3) the *P*-values from the two tests were combined to compute the overall *P*-values using Stouffer's method[29] and (4) differentially expressed genes were selected as those with $p<0.05$ or 0.01 and a fold change >1.5. Finally, functional enrichment analysis of the differentially expressed genes was performed using DAVID software[30] to identify GO biological processes and KEGG pathways represented by the genes in individual clusters with statistical significance.

4. TMT Labeling, LC-MS/MS Analysis and Peptide identification

Denaturation and reduction of the sample was performed in 8M urea, 5mM DTT and 25mM NH4HCO3 (pH 8.0) for over 30min. The urea was diluted to a conc. 1M with NH4HCO3 and then digested with modified trypsin. (37°C, 16hr) Each 100μg sample in 20μl 500mM TEAB was reduced, alkylated, digested, and labeled with

TMT reagents. Nano-high-performance liquid chromatography (nano-LC) analyses were performed using an Easy n-LC 1000 system. A C18 nanobore column (150mm × 0.1mm, 3mm pore size) was used for peptide separation. LTQ-Orbitrap MS was used for either identification or quantification of peptides. The Xcalibur (v2.1) used to generate peak lists. Proteome Discoverer software(v1.4) was used for protein identification and quantification. (with UniProt and by calculating the ratio between the peak areas of the TMT reporter groups) All quantitative results were normalized using medians (minimum protein count: 20). Reverse sequences were used for evaluation of the false discovery rate (FDR<1%).

5. Cell culture and plasmids transfection

For the transfection assay analysis, HEK293 and RKO cells were used. Cells were maintained in DMEM, or RPMI (HyClone) containing 10% FBS (HyClone) and 1% penicillin/streptomycin at 37℃ in a 5% CO_2 incubator according to ATCC guidelines. The *β-catenin* wild-type and mutant (S33A, S37A, T41A and S45A) construct were used in this study. 293T and RKO cells were cultured in 60-mm dishes and were transiently transfected with 3ug of each specific plasmid using Lipofectamine 2000 according to the manufacturer's protocol. Cells were harvested 48 hours later and were used for RNA extraction and protein purification.

6. Construction of expression plasmid vectors

The *β-catenin* gene including CDS was cloned into a pLECE3 vector conjugated with EGFP. For evaluating the effect of *β-catenin* mutant, *β-catenin* S33A, S37A, T41A and S45A mutant constructs were generated by substitution mutagenesis at each 33^{th}, 37^{th}, 41^{th} and 45^{th} amino acid of wild-type (WT) *β-catenin* construct.

7. RT-PCR and real-time PCR

Total RNAs were isolated from cells using illustra RNAspin Mini kits (GE Healthcare, Fairfield, CT, USA) and reverse transcription was performed with 2μg

of RNA. Real-time PCR was performed using the ABI PRISM 7500 Sequence Detector (Applied Biosystems) and SYBR Premix Ex TaqII (TaKaRa), according to the manufacturers' guidelines. The mRNA expression levels are presented as means ± S.D. from three independent experiments. Melting curves of PCR products were assessed for quality control. The sequences of the primers used are listed in Table 1.

Table 1. Primers used for RT-PCR and qRT-PCR

Gene	Direction	Sequence
CTNNB1	Forward	5'- AGCTTCCAGACACGCTATCAT
	Reverse	5'- CGGTACAACGAGCTGTTTCTAC
Axin2	Forward	5'- CAACACCAGGCGGAACGAA
	Reverse	5'- GCCCAATAAGGAGTGTAAGGACT
GLI2	Forward	5'- CCCCTACCGATTGACATGCG
	Reverse	5'- GAAAGCCGGATCAAGGAGATG
CDH1	Forward	5'- CGAGAGCTACACGTTCACGG
	Reverse	5'- GGGTGTCGAGGGAAAAATAGG
CDH2	Forward	5'- AGCCAACCTTAACTGAGGAGT
	Reverse	5'- GGCAAGTTGATTGGAGGGATG
AR	Forward	5'- GACGACCAGATGGCTGTCATT
	Reverse	5'- GGGCGAAGTAGAGCATCCT
18S ribosomal	Forward	5'- GTAACCCGTTGAACCCCATT
	Reverse	5'- CCATCCAATCGGTAGTAGCG
GAPDH	Forward	5'- AAGGTGAAGGTCGGAGTCAAC
	Reverse	5'- GGGGTCATTGATGGCAACAATA

8. Western blot

Total proteins were prepared using passive lysis buffer (Promega) and 40 μg of each protein sample were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with TBST containing 5% skim milk, blots were

incubated for 1hr at room temperature with primary antibodies against WIF-1, GSK3β (Santa Cruz biotechnology), GLI2, N-cadherin (Abcam), β-catenin, AR, E-cadherin (BD Bioscience), Axin, phospho-GSK3β, CyclinD1, β-actin (Cell signaling), phospho-β-catenin (Millipore) and GAPDH (Trevigen, Gaithersburg, MD, USA). HRP-conjugated secondary antibody (Santa Cruz technology) was used.

9. Immunohistochemistry

Paraffin-embedded tissue blocks were cut into 4-μm sections. Immunohistochemical analysis was performed using a Ventana XT automated stainer (Ventana) with antibodies against β-catenin (diluted 1:100, BD Biosciences), WIF-1 (diluted 1:50, Santa Cruz biotechnology), GLI2 (diluted 1:100, Abcam), androgen receptor (prediluted; Roche), E-cadherin for extra-cellular domains (diluted 1:100, Dako) and intracellular domains (diluted 1:200, BD Biosciences) and N-cadherin (diluted 1:50, Abcam).

10. Immunofluorescence microscopic analysis

Subcellular localization of mutant β-catenin was analyzed by immunofluorescence staining. The cells attached to glass coverslips were rinsed with PBS followed by fixation with 4% paraformaldehyde for 15min, and permeabilization in 0.3% Triton X-100 in PBS. Upon the removal of Trixon X-100, cells were again rinsed. Nonspecific sites were blocked with 2% bovine serum albumin for 1hr. After blocking, the medium was replaced with the respective primary antibodies, and cells were incubated overnight. Cells were then washed and incubated for 1hr with the appropriate fluorescently labeled secondary antibodies. All images were obtained using an LSM700 confocal microscope (Carl Zeiss).

III. RESULTS

1. Identification a subset of colon cancers with nuclear β-catenin overexpression

Nuclear overexpression of β-catenin was identified in colorectal tumors, especially at tumor budding cells. In the normal colon mucosa, β-catenin expression was localized to the membrane of normal colonocytes, while β-catenin expression was increased or accumulated in nucleus in some of colon adenocarcinomas. Nuclear β-catenin was not uniformly expressed, expressed in a wide range between 0 (negative) and 60%, especially high nuclear expression was verified in over 50% of tumor budding cells (Table 2 and Figure 1). To identify altered gene subsets and pathway

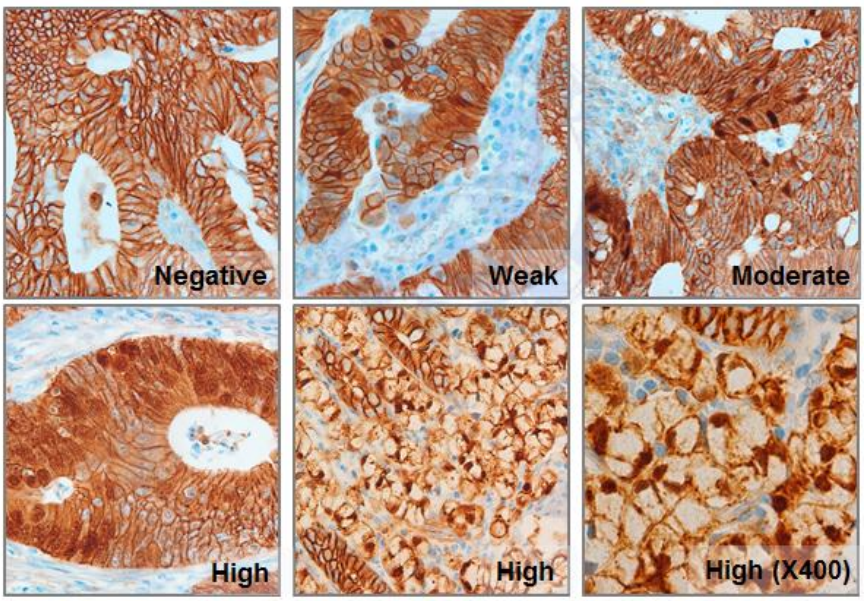


Figure 1. Representative immunohistochemical analysis of β-catenin expression. Tumors showed negative β-catenin expression in the nucleus of tumor cells, weak (1-3%), moderate (5-25%) and high nuclear expression of β-catenin (>30%). Figures are X200 and X400. Negative shows staining of β-catenin only at the membrane, but no staining in the nucleus. Weak, moderate or high show staining of β-catenin in the nucleus.

induced by nuclear β-catenin overexpression, colon cancer samples are classified by percentage of β-catenin nuclear overexpression and subsequently analyzed based on the classified sample groups. Normal colon epithelium showed only membranous expression with β-catenin. In the colorectal cancers, however, three distinct expression patterns were seen: i) negative expression similar to normal tissue showing only membranous expression without nuclear expression, ii) weak nuclear expression of β-catenin (1-3% of tumor cells), iii) moderate nuclear expression of β-catenin (5-25%) and iv) high nuclear expression of β-catenin with 30% as the high expression cutoff.

2. Overexpression of nuclear β-catenin correlates with frequent metastasis after operation

To identify the altered pathways by nuclear β-catenin overexpression, gene expression analysis of colorectal cancer tissues were performed as a model with heterogeneous nuclear β-catenin expression. As a result, I found that colon samples were nicely clustered into normal versus tumor group (Figure 2A). I also performed *β-catenin* mutation analysis using about 100 cases of colon cancers tissues and

Table 2. Number and proportion of colorectal cancers showing nuclear overexpression of β-catenin

β-catenin nuclear accumulation (IHC result)	Number	%
Negative	33	32.7
Weak (1-3%)	23	22.8
Moderate (5-25%)	30	29.7
High (≥30%)	15	14.9
Total cases	101 cases	100 %

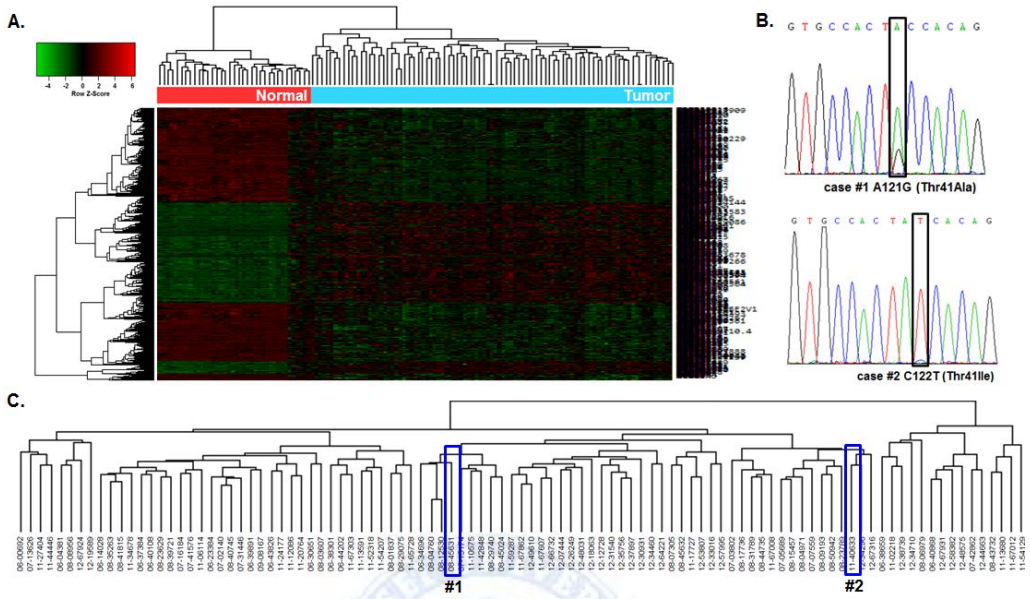


Figure 2. Hierarchical Clustering of gene expression profiles of colon cancers and *β-catenin* mutation analysis. Unsupervised hierarchical clustering analysis of gene expression. Red and green indicate transcript levels above and below the sample median, respectively. Complete separation of 35 normal colon tissues (red) and 101 colon cancers (skyblue) were evident based on gene expression profiles of colon cancers (A). The results of sequencing result of tumors with β-catenin mutation. β-catenin codon 41 mutations were detected in two cases (B). Two cases with β-catenin mutation were not positioned in the same complete linkage group (C).

found only 2 cases have *β-catenin* mutation in exon3 (Figure 2B). These two cases harboring *β-catenin* mutation were not positioned in the same group clustered by complete linkage analysis (Figure 2C and 3A). Except for 2 cases with mutations, further analysis was conducted to study the effect of nuclear overexpression of β-catenin driven not only by *β-catenin* mutations, but also by indirect activation such as *APC* mutations.

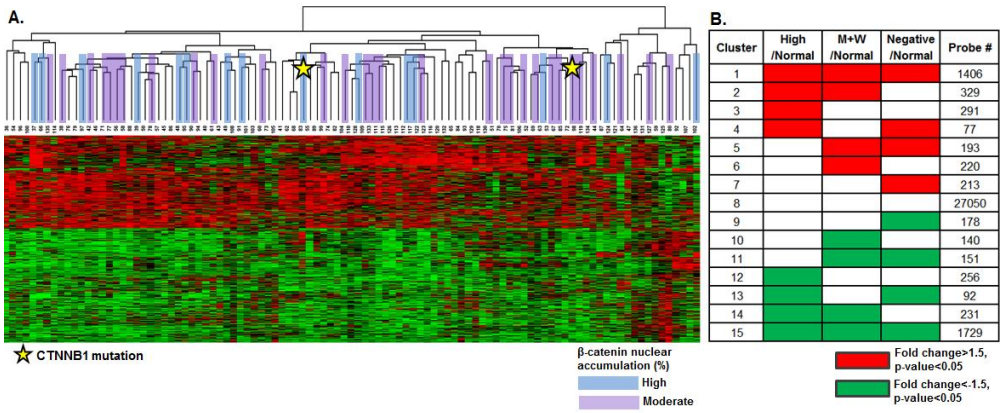


Figure 3. Hierarchical clustering analysis of mRNA expression profiles of colorectal tumors and clusters of differentially expressed genes. Red and green indicate transcript levels above and below the sample median, respectively. Blue and purple indicate colorectal tumors that show high and moderate nuclear expression of β-catenin, respectively (A). Clusters of differentially expressed genes showing all combination of differential expression in the three comparisons: High expression of nuclear β-catenin versus normal colon, Moderate and weak expression of nuclear β-catenin versus normal colon, and negative expression of nuclear β-catenin versus normal colon. Red and green denote up- and down-regulated genes (|fold change|>1.5 and p-value<0.05 as cut-off) (B).

Hierarchical cluster analysis using complete linkage did not distinguish tumors showing highly expressed nuclear β-catenin as tumors with weak or negative expression of nuclear β-catenin. Tumors with high nuclear overexpression of β-catenin (>30%) is widely located in the clustered samples. (Figure 3A) Based on our analysis of dysregulated mRNAs in colorectal tumors showing more than 30% nuclear overexpression of β-catenin, I identified 291 up-regulated genes and 256 down-regulated genes compared to tumors with no nuclear expression of β-catenin (Figure 3B). Additionally the biological significance of nuclear β-catenin

overexpression was related to the frequent post-operative metastasis (Figure 4A). The proportion of nuclear β-catenin level in colon tumor cells was higher in tumors with metastasis after operation than in tumors with no metastasis after operation (Figure 4B).

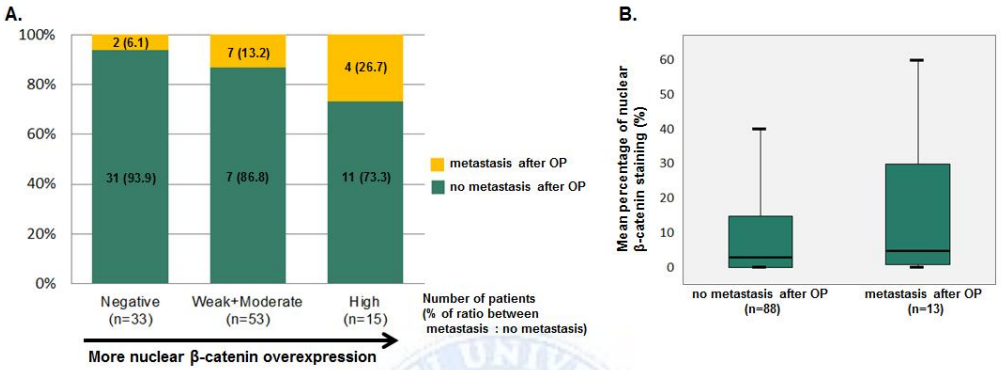


Figure 4. Correlation between nuclear β-catenin and metastasis. Ratio between patients with metastasis and patients with no metastasis according to nuclear β-catenin expression. Tumors with high nuclear β-catenin expression show more frequent metastasis after curative resection (A). Colon cancer patients with metastasis after curative resection showed high nuclear β-catenin staining compared to patients with no metastasis (B).

3. Nuclear β-catenin plays roles in Wnt/β-catenin signaling as well as regulation of cell development, cell adhesion and ECM-receptor interaction

To identify the signaling pathways associated with high nuclear β-catenin overexpression, I analyzed which categories of gene ontology are associated with specifically upregulated or down-regulated genes in the cases with high expression of nuclear β-catenin by performing an enrichment analysis of GO biological process and KEGG pathways using DAVID software. GO biological process analysis demonstrated that 291 up-regulated genes are involved in Wnt/β-catenin signaling

Table 3. Functional categories with differentially up-regulated genes in high, moderate nuclear expression of β-catenin compared to negative nuclear expression of β-catenin

Category	Term	Count	%	p-value	Benjamini
GOTERM_BP_FAT	cell cycle	45	7.7	0.000052	0.022
GOTERM_BP_FAT	RNA processing	45	7.7	3.9E-09	0.0000084
GOTERM_BP_FAT	cellular response to stress	35	6	0.00013	0.039
GOTERM_BP_FAT	DNA metabolic process	35	6	0.000014	0.0099
GOTERM_BP_FAT	cell cycle process	34	5.8	0.00028	0.058
GOTERM_BP_FAT	response to DNA damage stimulus	30	5.1	0.0000039	0.0042
GOTERM_BP_FAT	chromosome organization	26	4.4	0.0076	0.45
GOTERM_BP_FAT	DNA repair	24	4.1	0.000021	0.011
GOTERM_BP_FAT	mRNA metabolic process	24	4.1	0.001	0.12
GOTERM_BP_FAT	mitotic cell cycle	23	3.9	0.0023	0.23
GOTERM_BP_FAT	mRNA processing	23	3.9	0.00035	0.067
GOTERM_BP_FAT	chromatin organization	22	3.7	0.0061	0.41
GOTERM_BP_FAT	RNA splicing	22	3.7	0.00017	0.045
GOTERM_BP_FAT	cell division	21	3.6	0.00074	0.11
GOTERM_BP_FAT	regulation of cell cycle	21	3.6	0.0029	0.26
GOTERM_BP_FAT	mitosis	15	2.6	0.0077	0.44
GOTERM_BP_FAT	translational elongation	13	2.2	0.000057	0.02
KEGG_PATHWAY	Axon guidance	7	2.2	0.039	0.55
KEGG_PATHWAY	Purine metabolism	7	2.2	0.077	0.74
KEGG_PATHWAY	Spliceosome	7	2.2	0.035	0.6
GOTERM_BP_FAT	**Wnt receptor signaling pathway**	11	1.9	0.0076	0.44
KEGG_PATHWAY	Cell cycle	6	1.9	0.096	0.68
KEGG_PATHWAY	Basal cell carcinoma	5	1.6	0.022	0.89
GOTERM_BP_FAT	tRNA processing	9	1.5	0.0022	0.23
GOTERM_BP_FAT	**Notch signaling pathway**	8	1.4	0.00096	0.12
GOTERM_BP_FAT	protein amino acid lipidation	7	1.2	0.0053	0.38
KEGG_PATHWAY	Base excision repair	4	1.2	0.03	0.64
KEGG_PATHWAY	**Hedgehog signaling**	4	1.2	0.095	0.72

	pathway				
KEGG_PATHWAY	Pentose phosphate pathway	3	0.9	0.084	0.72

as well as Notch signaling, Hedgehog signaling, regulation of cell development, cell adhesion and ECM-receptor interaction (Table 3), while 242 down-regulated genes are involved in focal adhesion, cell adhesion and MAPK signaling pathway.

4. All of SPN have mutation in exon3 of *CTNNB1* and nuclear overexpression of β-catenin

Mutation of SPN was detected by direct sequencing of PCR products. All of 14 SPNs have mutation in exon3 of *CTNNB1*, while the pancreatic adenocarcinomas and neuroendocrine tumors have no mutation in exon3 of *CTNNB1* (Table 4). Immunohistochemistry analysis was performed to verify localization of β-catenin, and confirmed that strong β-catenin nuclear expression in all 14 SPNs, whereas no nuclear β-catenin expression was found in pancreatic adenocarcinomas or neuroendocrine tumors. (Figure 7C)

Table 4. Mutation status of solid-pseudopapillary neoplasm

	Case no.	Mutation status		Case no.	Mutation status		Case no.	Mutation status
SPN	1	C97T	PCA	1	No mutation	NET	1	No mutation
	2	C85T		2	No mutation		2	No mutation
	3	G81A		3	No mutation		3	No mutation
	4	G81T		4	No mutation		4	No mutation
	5	G88T		5	No mutation		5	No mutation
	6	C109T		6	No mutation		6	No mutation
	7	G87A						
	8	A82G						
	9	C97G						
	10	C85G						
	11	G81A						
	12	C97T						
	13	C97G						
	14	G87A						

5. Unsupervised clustering analysis of mRNA expression distinguishes SPN as a distinct type of pancreatic tumor

As a first step in the molecular analysis of SPN, I examined whether the mRNA expression profiles are distinct among the different pancreatic tumors examined. The mRNA expression profiling was performed on 14 SPNs, two types of other 12 pancreatic tumors without β-catenin mutation [six pancreatic adenocarcinomas (PCA), six neuroendocrine tumors (NET), and five non-neoplastic pancreatic tissues] by using the Human HT-12 v4 Expression Bead Chip. The chip contains 47,231 probes representing 31,332 annotated genes. Unsupervised hierarchical clustering analysis based on the 6,777 differentially expressed genes was performed by using the 6,777 differentially expressed genes relative to non-neoplastic samples. mRNA expression values were divided by the median value of five non-neoplastic pancreatic tissues. The clustering result represents that 26 tumor samples into separate three clusters according to the tumor type, SPN, PCA and NET compared to non-neoplastic pancreas, suggesting that these tissue types could be characterized by unique differential gene expression (Figure 5A).

6. Identification of characteristic signaling pathways associated with SPN

I then examined whether the SPN-specific up- and downregulated genes are associated with signaling pathways by performing an enrichment analysis of GO biological processes and KEGG pathways using DAVID software. GO biological processes enrichment analysis demonstrated that the SPN-specific upregulated genes represent several signaling pathways, including the Wnt/β-catenin, Hedgehog, and androgen receptor signaling cascades (Table 5). KEGG pathway enrichment analysis also revealed an association with the Wnt/β-catenin and Hedgehog signaling pathways in addition to identifying other pathways represented by the upregulated genes (eg, ubiquitin-mediated proteolysis). GO biological process

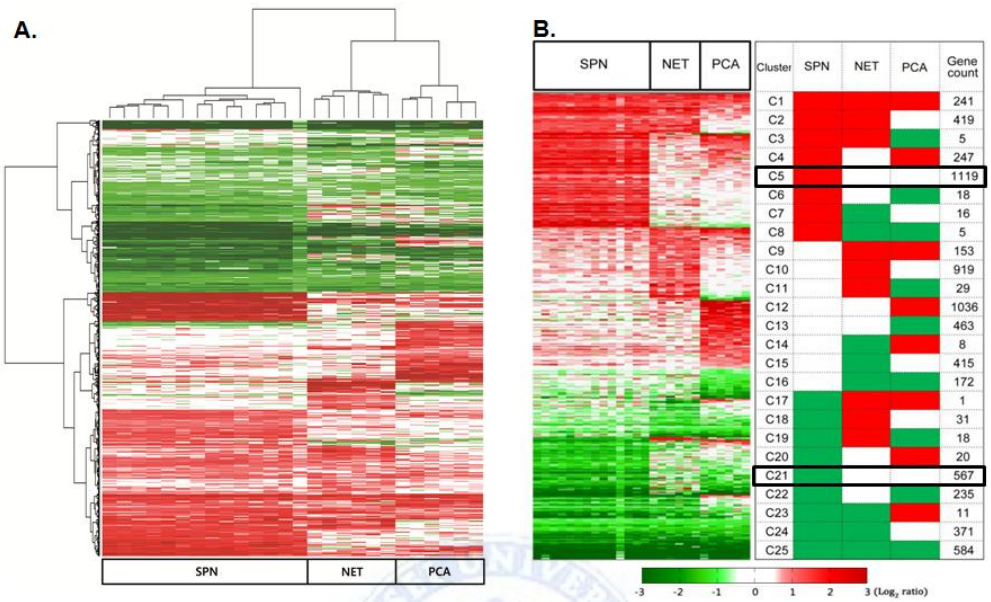


Figure 5. Unsupervised hierarchical clustering analysis of mRNA expression profiles of pancreatic tumors and clusters with DEGs. Clustering analysis using the filtered 6777 differentially expressed genes in 26 pancreatic tumors composed of six pancreatic adenocarcinomas, 14 solid-pseudopapillary neoplasms, and six neuroendocrine tumors. Each mRNA expression value was divided by the median value of five non-neoplastic pancreatic tissues. Color bar gradient represents log2-fold changes in each pancreatic tumor compared to non-neoplastic pancreas (A). The 25 clusters of differentially expressed genes (B). KEGG pathway enrichment analysis with SPN-specific up-regulated genes represent several signaling pathways, including the Wnt/β-catenin, Hedgehog, and androgen receptor signaling cascades (C). GO biological process analysis of up-regulated genes also demonstrated these three activated signaling pathways (D).

analysis of the downregulated genes demonstrated their association with cell motility, migration, and blood vessel development. Moreover, the KEGG pathways

represented by the downregulated genes included fructose and mannose metabolism, the MAPK signaling pathway, and endocytosis (Figure 6A and 6C). Notably, comparison of the GO biological process and KEGG pathways found in the 25 differentially expressed genes clusters demonstrated that the genes specifically upregulated in SPNs were exclusively related to the Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways (cluster 5 in Figure 5B and Figure 6). I selected *CTNNB1* and *Axin2* to assess the Wnt/β-catenin signaling pathway, *GLI2* for the Hedgehog pathway, and androgen receptor for the androgen receptor signaling pathway. E-cadherin (*CDH1*) and N-cadherin (*CDH2*) were chosen as representative genes of epithelial-mesenchymal transition. mRNA levels of these selected genes were verified by quantitative RT-PCR. (Figure 7A). Marked upregulation of *CTNNB1* was found in SPN, while only a slight increase was observed in pancreatic adenocarcinoma (PCA) and neuroendocrine tumor (NET) compared with non-neoplastic pancreatic tissues (Figure 6). Expression of *Axin2*, *GLI2*, and *androgen receptor* were markedly higher in SPN relative to PCA, NET, and non-neoplastic pancreatic tissues. E-cadherin, an epithelial marker, was downregulated, while N-cadherin was significantly upregulated in SPN relative to non-neoplastic pancreatic tissues and PCA. I also validated β-catenin, WIF-1, GLI2, androgen receptor, E-cadherin, and N-cadherin expression by western blotting (Figure 7B) and immunohistochemistry (Figure 7C). Both western blotting and immunohistochemical analysis revealed increased levels of β-catenin, WIF-1, GLI2, androgen receptor, and N-cadherin, along with decreases in E-cadherin in SPNs. All SPNs showed increased β-catenin expression, whereas PCAs and NETs showed slightly increased β-catenin expression compared with non-neoplastic pancreas by western blot. WIF-1 protein was also highly expressed in SPN tumor tissues relative to matched normal pancreatic tissues by western blot. GLI2 was highly expressed in all SPNs and three out of six PCAs, but was not expressed in NETs and non-neoplastic pancreas according to western blot. Androgen receptor was highly expressed in all SPNs, but not expressed in PCAs, NETs, or non-neoplastic

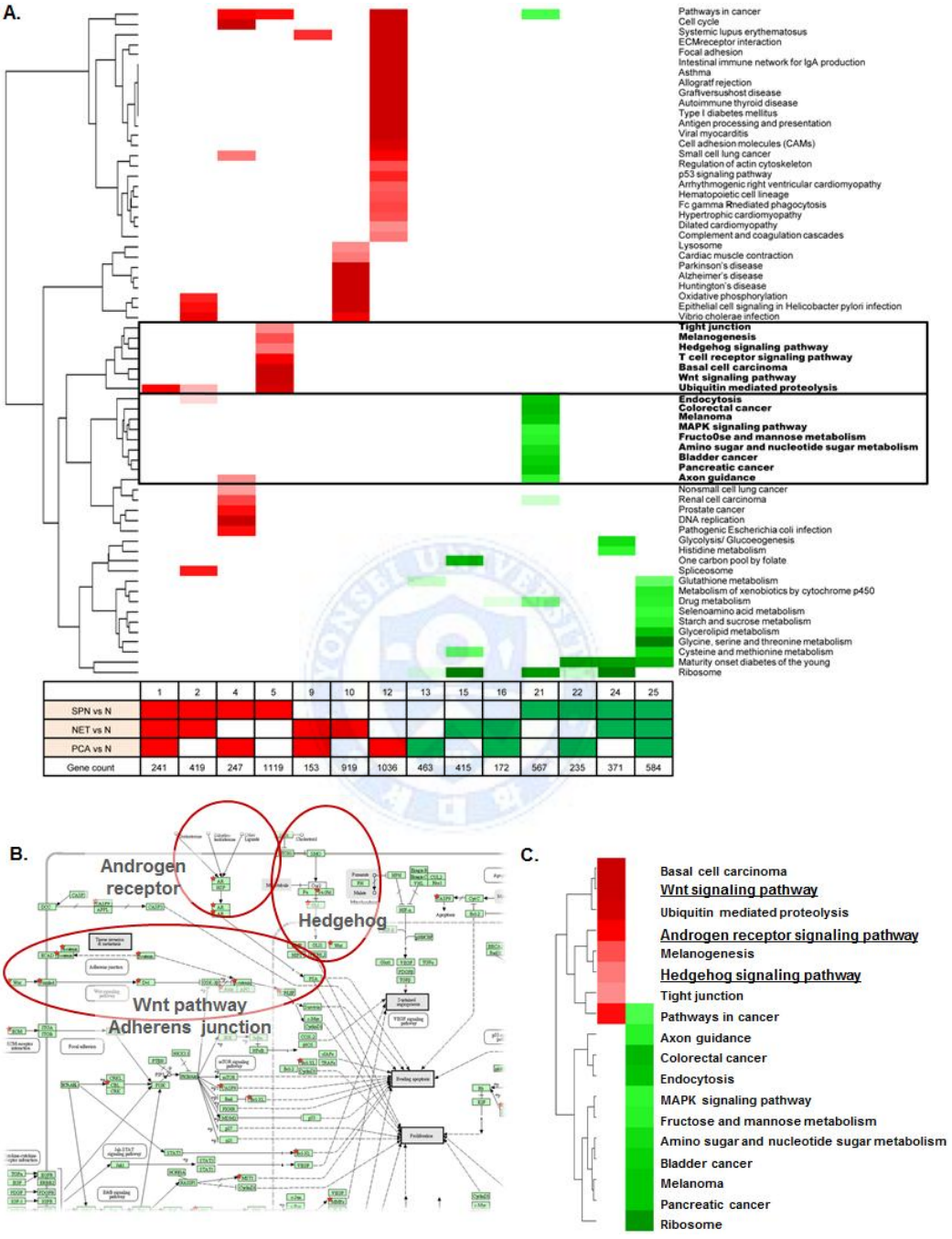


Figure 6. Gene ontology biological process (GOBP) and KEGG pathways represented by differentially expressed genes. Results of GOBP and KEGG

analysis. Red and green indicate the significance of GOBP enrichment by the up- and down-regulated genes, respectively in SPN, NET, or PCA compared to non-neoplastic pancreas. Color bar represent the gradient of $-\log_{10}(P)$ where P indicates enriched P values calculated by the DAVID software. The dendrogram was generated by cluster analysis of $-\log_{10}(P)$ values (A). KEGG pathway enrichment analysis with SPN-specific up-regulated genes represent several signaling pathways, including the Wnt/β-catenin, Hedgehog, and androgen receptor signaling cascades (B). GO biological process analysis of up-regulated genes also demonstrated these three activated signaling pathways (C).

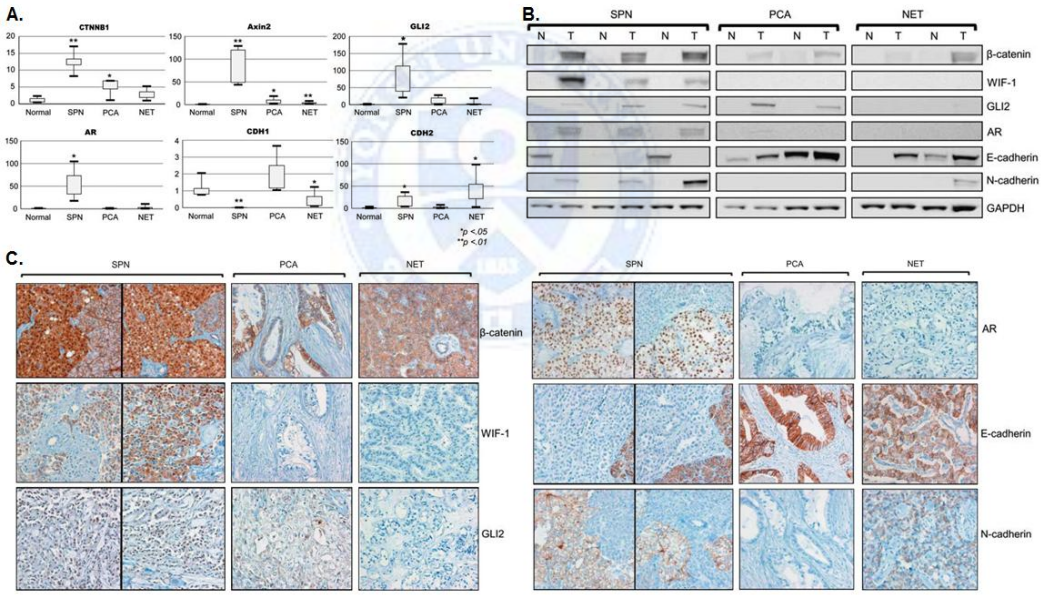


Figure 7. Validation of differentially expressed genes in solid-pseudopapillary neoplasm by quantitative RT-PCR, western blotting and immunohistochemistry. mRNA levels of *CTNNB1* (β-catenin), *Axin2, GLI2*, androgen receptor (AR), *CDH1* (E-cadherin) *and CDH2* (N-cadherin) were verified by qRT-PCR. The expression level of each mRNA was normalized to that of 18s rRNA and arbitrarily set to 1. *p<0.05; **p<0.01 based on the Student's t-test (A).

Protein levels of components of the Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways were assessed in solid-pseudopapillary neoplasm (SPN), non-neoplastic pancreatic tissues, pancreatic adenocarcinomas (PCA), and neuroendocrine tumors (NET) by western blot (B) and immunohistochemistry (C).

Table 5. List of Wnt/β-catenin, Hedgehog, and AR signaling-related genes up-regulated in SPN

Official symbol	Gene name	Fold change	*P* value
Wnt/β-catenin signaling pathway			
DKK4	dickkopf homolog 4 (Xenopus laevis)	300.66	3.7E-07
WIF1	WNT inhibitory factor 1	74.77	1.3E-05
NKD1	naked cuticle homolog 1 (Drosophila)	64.91	4.9E-06
AXIN2	axin 2	31.44	1E-05
FZD7	frizzled homolog 7 (Drosophila)	14.74	0.00011
WNT2B	wingless-type MMTV integration site family, member 2B	11.49	0.00017
RUVBL1	RuvB-like 1 (E. coli)	9.34	0.00022
WNT5A	wingless-type MMTV integration site family, member 5A	8.12	0.00094
NKD2	naked cuticle homolog 2 (Drosophila)	5.75	0.00329
MAP3K7	mitogen-activated protein kinase kinase kinase 7	5.63	0.00035
TCF7	transcription factor 7 (T-cell specific, HMG-box)	5.45	0.00063
PPARD	peroxisome proliferator-activated receptor delta	5.29	0.00029
FZD2	frizzled homolog 2 (Drosophila)	4.69	0.00049
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	4.04	0.00117
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	3.92	0.00331
CCND3	cyclin D3	2.69	0.00264
SMAD3	SMAD family member 3	2.62	0.00661
SKP1	S-phase kinase-associated protein 1	2.54	0.00148
SIAH1	seven in absentia homolog 1 (Drosophila)	2.46	0.00875
DVL2	dishevelled, dsh homolog 2 (Drosophila)	2.31	0.00201
BTRC	beta-transducin repeat containing	2.18	0.00881
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	2.02	0.0108
Hedgehog signaling pathway			
BMP7	bone morphogenetic protein 7	9.89	0.00021

ZIC2	Zic family member 2 (odd-paired homolog, Drosophila)	5.87	0.00298
GLI2	GLI family zinc finger 2	3.84	0.00438
GLI3	GLI family zinc finger 3	2.99	0.00925
Sufu	suppressor of fused homolog (Drosophila)	1.99	0.00651
Androgen receptor signaling pathway			
AR	androgen receptor	9.89	0.000486
MED30	mediator complex subunit 30	2.63	0.001934
MED17	mediator complex subunit 17	2.04	0.009345
DAXX	death-domain associated protein	1.83	0.010927
THRAP3	thyroid hormone receptor associated protein 3	1.73	0.016438

pancreas on western blot. Of the epithelial-mesenchymal transition related markers, E-cadherin expression was detected in 1 case of SPNs, 3 out of 6 NETs, and 5 out of 6 PCAs by using E-cadherin. N-cadherin expression was found in 8 cases of SPNs, 4 out of 6 NETs. None of the six PCAs were assessed by western blot. Immunohistochemical analysis revealed characteristic β-catenin nuclear accumulation exclusively in all 14 SPNs, whereas no nuclear β-catenin expression was found in PCAs or NETs. Strong cytoplasmic WIF-1 expression, and strong nuclear expressions of GLI2 and androgen receptor were demonstrated in all 14 SPNs (Figure 7C). In contrast to rare membranous E-cadherin expression in all of the 14 SPNs, N-cadherin expression was found in 12 ouf of SPNs. In 3 ouf of 6 NETs and all of 6 PCAs, E-cadherin expression was detected by immunohistochemistry.

7. Distinct protein expression profiles of SPN

Additionally, protein expression profiling of solid-pseudopapillary neoplasm was performed by TMT labeling and LC-MS/MS (Figure 8A), and I integrated protein expression results with those of the mRNA expression. First, I identified 551 proteins with high confidence (FDR<1%). Among them, 329 proteins (150 up-regulated and 179 down-regulated) were further selected for quantitative analysis

(Figure 8B). I chose 329 differentially expressed proteins with an SPN/normal ratio of 1.5 as cut-off, since fold ratio of β-catenin was 1.68. Among the identified 551 proteins, 150 proteins were upregulated in the proteomics data, and 87 (58.0%) proteins were up-regulated in both the proteome and the mRNA expression analyses (Figure 8C). We found that proteins that are involved in Wnt/β- catenin signaling [DKK4 (8.06-fold), and CTNNB1 (1.68-fold)] or proteins that bind to β-catenin [FN1 (5.9-fold), SELENBP1 (4.99-fold), DDX5 (3.09-fold), YWHAZ (2.24-fold), NONO (1.89-fold), BGN (1.80-fold), hnRNPM (1.71-fold) and FUS (1.51-fold)] were up-regulated (Table 6). Furthermore, among the up-regulated proteins, 24 (4.36% of 551 identified proteins) overlapped with the genes that were specifically up- regulated in SPNs on the mRNA array. I could not find specific signaling

Table 6. List of dysregulated proteins in SPNs

A) Up-regulated proteins in SPN

Gene Symbol	Protein SPN_T/N*	mRNA _SPN	interact	Gene Symbol	Protein SPN_T/N	mRNA _SPN	interact
CYB5B	8.93	4.71	-	NPNT	2.47	4.47	-
NOTUM	8.49	52.98	-	DLD	2.21	-1.39	-
DKK4	8.06	329.26	-	RAN	2.17	6.46	AR
PKM2	6.81	10.10	-	HSP90AA1	1.90	1.79	-
CAPS	6.49	5.10	-	NONO	1.89	1.30	-
GSN	5.93	3.28	AR**	MATR3	1.84	1.98	-
ENO2	5.25	21.11	-	PSME2	1.84	2.34	-
SELENBP1	4.99	13.47	β***	HNRNPA1	1.81	2.75	AR
PDXK	4.82	12.12	-	SFPQ	1.78	1.01	-
ACSS3	3.80	4.90	-	HNRNPA2B1	1.74	1.02	β
MFAP2	3.77	3.03	-	CTNNB1	1.68	4.38	AR
LSAMP	3.75	11.35	-	NPM1	1.67	1.07	-
HK1	3.37	7.01	-	TPM4	1.64	1.98	-
IGFBP7	3.21	4.69	-	NOV	1.63	50.86	AR
SLC25A13	3.20	3.29	-	PDHB	1.61	1.10	-
DDX5	3.09	2.53	-	ALDH7A1	1.61	1.42	-

PGK1	3.07	2.67	-	GPI	1.59	1.60	-
NUMA1	3.00	1.33	-	PAICS	1.55	4.51	-
CTSB	2.81	3.84	-	FUS	1.51	2.19	β/AR
TUBB	2.79	3.09	-				

B) Down-regulated proteins in SPN

Gene Symbol	Protein SPN_T/N	mRNA _SPN	interact	Gene Symbol	Protein SPN_T/N	mRNA _SPN	interact
GCG	0.03	-85.00	β	IDH2	0.39	-3.99	-
TSC22D1	0.04	6.04	β	HBB	0.41	-1.85	AR
GSTA1	0.05	-126.01	AR	YBX1	0.43	1.35	AR
INS	0.09	-19.92	-	HBA1	0.46	-3.00	AR
P4HB	0.11	-4.79	β	AARS	0.47	-2.08	β
KRT8	0.12	-8.42	β/AR	CALR	0.53	-1.24	AR
KRT7	0.13	-2.20	AR	BLVRB	0.54	-2.50	AR
TPSAB1	0.14	-3.69	AR	RPL22	0.57	-1.74	-
KRT18	0.16	-3.44	β/AR	PGM1	0.63	1.43	-
FGG	0.16	-1.13	AR	MSN	0.63	1.14	AR
HSPA5	0.25	-3.81	AR	LDHB	0.63	-1.56	AR
DDT	0.32	-1.48	AR	IQGAP1	0.63	1.31	β
ADH1B	0.33	-1.88	AR	COL1A1	0.64	-1.45	-
TXNDC5	0.37	-2.98	β	C4A	0.64	-1.05	AR
CYB5A	0.38	-9.94	β	PDIA3	0.66	-1.05	β
RPN1	0.38	-1.10	-				

*tumor/normal, **androgen receptor and *** β-catenin

pathways that were activated in SPNs according to the proteome data, however, I identified many proteins that interact to the key molecules of Wnt/β-catenin, AR, Notch and Hedgehog signaling pathways are up-regulated in our proteomic analysis, although key molecules involved in these activated pathways was not detected proteomic analysis. (Figure 8D).

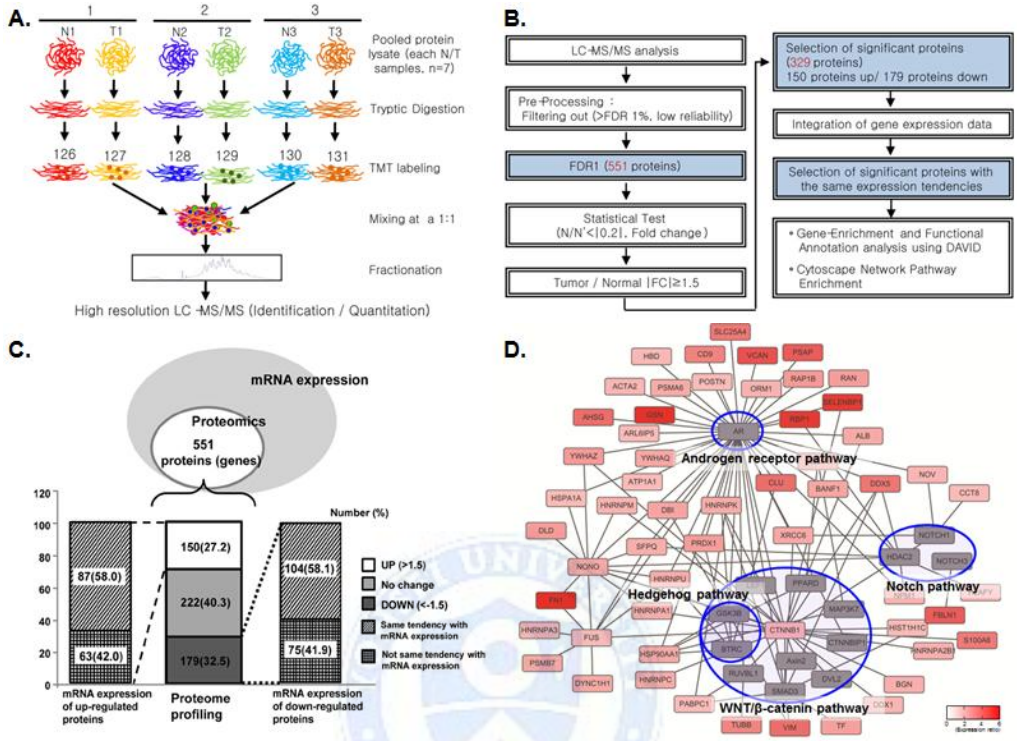


Figure 8. Proteomics analysis of solid-pseudopapillary neoplasm. Workflow overview of proteome identification and quantification (A), and workflow of proteomics data analysis (B). Venn diagrams showed overlap between the identified proteins and the mRNA expression data. Among 551 proteins matched with mRNA data, 150 proteins were up-regulated, and 179 proteins were downregulated. Among the 150 up-regulated proteins, 87 proteins (58.0%) showed the same tendency with mRNA expression. Among the 179 down-regulated proteins, 104 proteins (58.1%) showed the same tendency with mRNA expression (C). Schematic of protein expression profile of SPN for the genes known to interact to the key genes of the Wnt/β-catenin, androgen receptor, Notch and Hedgehog signaling pathways. The key genes of the activated signaling pathways in SPNs are marked with blue circular area. The intensity of red color in the box represents expressional value compared with those of non-neoplastic tissues. The grey color in the box denotes

that protein was not identified by our proteomics analysis. Among the 150 up-regulated proteins in SPNs, we selected 45 proteins that are known to interact to the 15 key molecules of four activated signaling pathways (D).

8. Overexpression of β-catenin mutants activates several molecules involved in Wnt/β-catenin signaling, Hedgehog signaling and androgen receptor signaling pathway

Mutant *β-catenin* vectors (S33A, S37A, T41A and S45A) resistant to GSK3β and CK1-dependent phosphorylation, which is essential to β-catenin degradation, were generated and used to identify gene subsets altered by constant activation of β-catenin. HEK293 cells and RKO cells were transfected with each *β-catenin* expression vector (wild-type, S33A, S37A, T41A, and S45A), and similar mRNA levels of *β-catenin* were confirmed by qRT-PCR (Figure 9A). Protein expression level of wild-type β-catenin, however, was lower than expression levels of other β-catenin mutants, because of proteasome-dependent degradation of wild-type β-catenin. We also validated protein expression levels of phosphorylated β-catenin mutants (S33, S37, T41 and S45) to verify whether or not these β-catenin mutants were phosphorylated. Phosphorylation of β-catenin at Ser-33, Ser-37, or Thr-41 was detected only in HEK293 and RKO cells transfected with wild-type *β-catenin* and a single *β-catenin* mutant (S45A), whereas phosphorylation of β-catenin at Ser-45 was detected in HEK293 cells transfected with *β-catenin* mutants S33A, S37A and T41A (Figure 9B). Since nuclear localization of β-catenin is important to the action of activated β-catenin, localization of β-catenins from each construct was also visually validated by immunofluorescence microscopic analysis (Figure 9C).

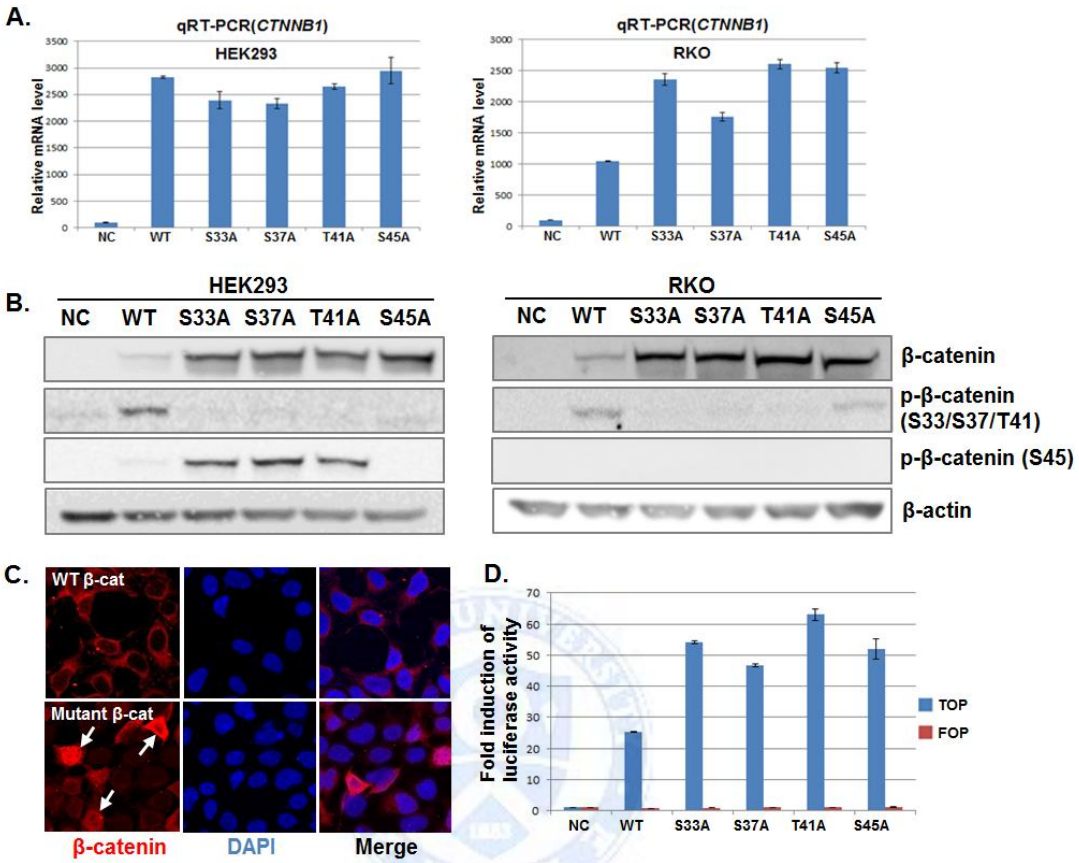


Figure 9. Mutants of β-catenin resistant for phosphorylation increase Wnt activity compared to wild-type β-catenin. HEK293 cells and RKO cells were transfected with each *β-catenin* expression vectors (wild-type, S33A, S37A, T41A and S45A) and similar mRNA levels of *β-catenin* were confirmed by qRT-PCR (A). Total β-catenin and phosphorylated β-catenin were validated by western blot analysis (B). Localization of β-catenins from each construct was validated by immunofluorescence microscopic analysis. Wild-type β-catenin is localized in the cytoplasm, while β-catenin mutants are localized in the nucleus (C). To confirm if Wnt/β-catenin signaling activity is induced by mutant β-catenin, TOP-Flash (blue) or FOP-Flash (red) was used to measure luciferase activity. Luciferase activity in HEK293 transfected with *β-catenin* mutants was much higher than luciferase activity in HEK293 transfected with wild-type *β-catenin* (D).

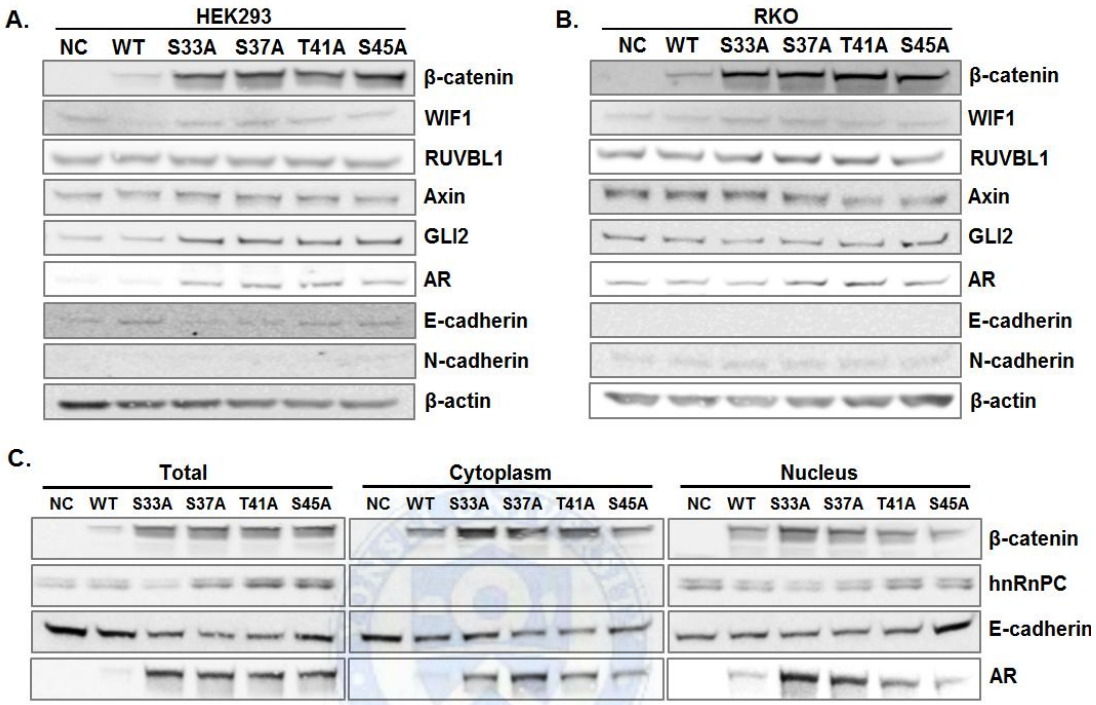


Figure 10. Overexpression of mutant β-catenin increased expression of molecules involved in Wnt/β-catenin signaling, Hedgehog signaling, androgen receptor signaling and epithelial-mesenchymal transition. Expression of WIF1, GLI2 and androgen receptor was increased, whereas E-cadherin was slightly reduced in HEK293 cells transfected with *β-catenin* mutants compared to the control (wild-type *β-catenin*). According to the mutation type of β-catenin, expression of WIF1 and androgen receptor in HEK293 cells transfected with mutant S45A were distinctively detected compared to the expression WIF1 and androgen receptor in HEK293 cells transfected with other *β-catenin* mutants (S33A, S37A and T41A) (A), whereas altered expressions of WIF1, androgen receptor and E-cadherin were not detected in RKO transfected with wild-type or mutant types of *β-catenin* (B). *β-catenin* mutants were similarly detected at total protein level, but expression of β-catenin were differently detected in cytoplasmic and nuclear

fraction. Expression of S33A mutant β-catenin was much higher than S45A mutant β-catenin in nuclear fraction, and expression of androgen receptor in HEK293 cells transfected with S33A was higher than in HEK293 cells transfected with S45A. Expression of E-cadherin was reduced in HEK293 cells transfected with *β-catenin* mutants (S33A, S37A and T41A), while nuclear E-cadherin increased in HEK293 cells transfected with mutant *β-catenin* (S45A) compared to wild-type *β-catenin* and the other *β-catenin* mutants (S33A, S37A and T41A) (C).

To confirm whether Wnt/β-catenin was induced by mutant β-catenin, TOP-Flash (a reporter construct that has eight copies of the consensus Lef/Tcf binding site) or FOP-Flash (a reporter construct that has eight copies of the mutated consensus Lef/Tcf binding site) was co-transfected into HEK293 cells with *β-catenin*. Luciferase activity was increased greatly in HEK293 cells transfected with mutant *β-catenin*, compared to empty vector. Also, luciferase activity in HEK293 cells transfected with *β-catenin* mutants was much higher than luciferase activity in HEK293 cells transfected with wild-type *β-catenin* (Figure 9D). Next, the expression levels of proteins altered by transfected *β-catenin* mutants were evaluated, including in those involved in Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways (WIF1, RUVBL1, Axin, GLI2, androgen receptor, E-cadherin, and N-cadherin). The expressions of WIF1, GLI2, and androgen receptor were increased, whereas E-cadherin expression was slightly reduced in HEK293 cells transfected with *β-catenin* mutants, compared to the control (wild-type *β-catenin*). Meanwhile, the expressions of WIF1 and androgen receptor in HEK293 cells transfected with mutant S45A differed from those in HEK293 cells transfected with other *β-catenin* mutants (S33A, S37A, and T41A) (Figure 10A). The expressions of WIF1, androgen receptor, and E-cadherin were not altered in RKO cells transfected with wild- type or mutant *β-catenin* (Figure 10B).

To measure nuclear forms of β-catenin, HEK293 cells transfected with wild-type

or mutant *β-catenin* were fractionated into cytoplasmic and nuclear fractions, and β-catenin levels were determined by western blotting (Figure 10C). Although β-catenin mutants showed similar total protein levels, the expressions of β-catenin mutants differed in cytoplasmic and nuclear fractions. In the nucleus, the expression of S33A mutant β-catenin was much higher than that for S45A mutant β-catenin. Also, the expression of androgen receptor in HEK293 cells transfected with S33A was greater than that in HEK293 cells transfected with S45A, demonstrating that mutant *β-catenin* induces greater nuclear androgen receptor expression than wild-type β-catenin.

IV. DISCUSSION

β-catenin is a key molecule in Wnt/β-catenin signaling, which plays crucial roles in tumorigenesis. In a various types of tumors, a high frequency of mutations in the genes controlling Wnt/β-catenin signaling has been reported. Mutations in APC are commonly found in colorectal cancers, and lead to the stabilization and accumulation of β-catenin in the cell. In this study, the mRNA expression profiles of colorectal cancers and pancreatic tumors, including solid-pseudopapillary neoplasms, were assessed to identify signaling pathways altered by accumulation of nuclear β-catenin. Colon cancer was selected as a model of nuclear β-catenin overexpression resulting from alterations in genes involved in β-catenin degradation, and SPNs were chosen as a model of nuclear β-catenin overexpression induced by *β-catenin* mutation. In unsupervised clustering samples, analysis of expression profiles for colorectal tumors indicated that tumors with high nuclear β-catenin (>30%) are randomly distributed. Therefore, the present study attempted to identify differentially expressed genes (DEGs) exclusively in tumors showing nuclear β-catenin expression, compared to tumors showing negative expression of nuclear β-catenin. As nuclear accumulation of β-catenin can be achieved with *β-catenin* mutation and mutations in genes involved in β-catenin degradation, such as *APC*. *β-catenin* mutation analysis was performed to verify mutation status of *β-catenin* in colon cancer samples from about 100 cases. In doing so, only two cases showed *β-catenin* mutation in exon 3, and these two cases were not positioned in the same group clustered by complete linkage analysis. Thus, by excluding these two cases, the present study was able to analyze differential expression of genes in colon cancers related to nuclear accumulation of β-catenin driven by inhibition of β-catenin degradation, such as that associated with *APC* mutations, not by mutation in *β-catenin*. The results thereof revealed 15 different groups of DEGs involved in Wnt/β-catenin, Notch, and Hedgehog signaling pathway, as well as extracellular matrix (ECM)-interactions.

In analysis of nuclear β-catenin staining and metastasis in colon cancer tissues,

patients with metastasis showed higher percentages of nuclear β-catenin staining. Moreover, tumors with the greater expression of nuclear β-catenin showed the greater metastatic potential. Interestingly, a previous study on colorectal tumors identified DEGs in the invasive front of tumors (related to nuclear expression of β-catenin), compared to the tumor center (related to cytoplasmic expression of β-catenin). The identified DEGs were further found to be related to cell adhesion, invasion, and EMT.[31] Although the present study did not distinguish between the invasive front and tumor center, the DEGs noted in tumors with high nuclear β-catenin expression were found to be involved in ECM-interaction and cell-cell adhesion.

Nevertheless, even if analysis of gene expression profiles in the colorectal cancers exhibiting nuclear β-catenin expression were to show up-regulated genes to be involved in several signaling pathways, the amounts of nuclear β-catenin expression are not high enough to explain the tumorigenesis of colon cancers, since the frequency of *β-catenin* mutation is about 5% and colorectal tumorigenesis is a complicated mechanism affected by mutations of other genes, such as *APC* and *KRAS*.[32,33]

Solid-pseudopapillary neoplasm of the pancreas is a rare pancreatic tumor, which has an activating *β-catenin* mutation, making it a good model for identifying gene subsets and signaling pathways induced by *β-catenin* mutation. Unsupervised hierarchical clustering analysis of DEGs in 31 microarrays grouped non-neoplastic tissues, SPNs and other pancreatic tumors (neuroendocrine tumors and pancreatic adenocarcinomas). Groups were separated according to tumor types, which suggested that tumors with *β-catenin* mutation could be characterized by unique gene expression patterns. Although expression of β-catenin was slightly increased in neuroendocrine tumors and pancreatic adenocarcinomas, compared to non-neoplastic tissues, gene expression profiles and the immunophenotypes of SPNs were clearly different from other pancreatic tumors. This result suggests that SPN-specific mRNA is affected by nuclear β-catenin, not merely β-catenin

overexpression.

In accordance with results in this study, previous studies on SPN have reported activating *CTNNB1* mutations and abnormal β-catenin nuclear accumulation due to inhibition of its degradation. Consequently, activation of downstream β-catenin targets was expected. *CTNNB1* mutation in SPN is heterozygous and many genes encoding components of the Wnt/β-catenin signaling pathway have been shown to be upregulated in SPN by this study and others.[34] Specifically, studies have found that mRNA levels of *CTNNB1* are approximately three to four-fold upregulated in SPNs and that β-catenin protein accumulates in nucleus. The proportion of mutant to wild-type β-catenin protein in the nucleus and its effects on downstream targets should be further studied.

The present study compared gene expression profiles of SPN with non-neoplastic pancreas or other pancreatic tumors. As expected, Wnt/β-catenin signaling pathway was activated in SPNs due to *β-catenin* mutations and abnormal nuclear accumulation of β-catenin. Besides the Wnt/β-catenin pathway, SPNs also exhibited upregulation of genes involved in the activation of the Hedgehog and androgen receptor signaling pathways. Based on the analysis of upregulated genes in SPN, I propose that SPNs can be characterized by activation of the Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways. At present, only one report has attempted to outline the gene expression profiles of SPNs. The study showed that the Wnt/β-catenin and Notch signaling pathways are involved in SPN.[34] Consistent with this, the present study demonstrated that genes in the Notch signaling pathway are differentially expressed in SPNs, compared to non-neoplastic pancreatic tissues. However, because these genes were also differentially expressed in pancreatic adenocarcinomas and/or neuroendocrine tumors, the Notch signaling pathway was not regarded as being distinctively activated in SPNs and, therefore, not related to β-catenin mutation. Instead, the Hedgehog signaling pathway was significantly activated in SPNs alone. The Notch and Hedgehog signaling pathways are closely related to the Wnt/β-catenin pathway, with many components in

common.[35,36] The Notch signaling pathway is also activated in colorectal cancers exhibiting high amounts of nuclear β-catenin expression. Previous studies have demonstrated that nuclear accumulation of β-catenin induces the expression of *hes1*, which plays an important role in Notch signaling and is associated with the Notch1 intracellular domain (N1IC).[37] This suggests that activation of Notch signaling may be associated with β-catenin overexpression, not with *β-catenin* mutation.

As a novel finding, activation of the androgen receptor signaling pathway was discovered in SPNs. Indeed, increased androgen receptor (AR) expression was noted at the transcriptional and translational levels. Studies have shown that AR binds to the armadillo repeat domain of β-catenin and enhances the nuclear localization thereof.[38-40] As well, increased AR-dependent transcriptional activity has been reported in prostatic tumors with *CTNNB1* mutations.[41] However, no direct relationship between activation of Wnt/β-catenin and AR signaling pathways has been reported. For the first time, a high level of nuclear AR expression was noted in SPNs (n=14) in this study. In addition, increased nuclear androgen receptor was detected in cells transfected with mutant *β-catenin* relative to cells transfected with wild-type *β-catenin*. Crosstalk between AR and β-catenin has been well documented in prostate cancers.[42,43] Some studies have also reported that AR induces nuclear localization of β-catenin and activates β-catenin-mediated transcription, while the others have demonstrated that interaction between AR and β-catenin is necessary for co-translocation. In this study, nuclear localization of AR was found to be affected by overexpression of β-catenin in the nucleus as a result of *β-catenin* mutation.

Additionally, genes specifically altered in SPNs were found to be linked to EMT. Marked down-regulation of E-cadherin (CDH1) and up-regulation of N-cadherin (CDH2) and vimentin in SPNs were validated by western blotting. These findings are in accordance with the rare epithelial differentiation observed in SPN tumor cells. Interestingly, up-regulation of the EMT regulator genes *TWIST2* and *ZEB2* was also observed. The expression of EMT-related genes is closely linked with activation of the Wnt/β-catenin, Notch, and Hedgehog signaling pathways.[36,44-48]

Wnt signaling promotes EMT, which is associated with cancer progression via β-catenin-mediated transcription.[49] In Hedgehog signaling, ligand binding to patched receptor activates glioma (GLI) transcription factors, and subsequently induces EMT-related gene expression and loss of E-cadherin. Additionally, the Notch intracellular domain has been considered as an EMT regulator, such as Snail and Slug,[50,51] although some studies suggest that Notch signaling is not sufficient to completely induce EMT.[52] Accordingly, SPN can be characterized by activation of Wnt/β-catenin, Hedgehog, and androgen receptor signaling pathways, as well as EMT.

To evaluate the effect of β-catenin *in vitro*, four mutant *β-catenin* constructs (S33A, S37A, T41A and S45A) were generated, and the expression levels of selected genes activated in SPNs after transfection with the *β-catenin* constructs were assessed. In doing so, the protein expressions of WIF1, GLI2, AR, and E-cadherin were found to be dysregulated in cells transfected with the mutant *β-catenin* constructs, compared to cells transfected with wild-type *β-catenin*. Interestingly, transfected *β-catenin* mutants showed different effects on the translocation of AR into the nucleus. Cells transfected with mutant constructs S33A, S37A, and T41A showed higher nuclear AR expression than cells transfected with the wild-type *β-catenin* construct. Meanwhile, cells transfected with mutant *β-catenin*-S45A showed lower AR expression in nucleus compared to cells transfected with other mutant constructs of *β-catenin*. Considering β-catenin S45A is not phosphorylated by CK1, β-catenin would be expected to be stabilized, as serial phosphorylation at codons 33, 37, and 41 by GSK3β would be inhibited. Analysis of gene expressions in HEK293 cells expressing each *β-catenin* construct demonstrated that *β-catenin* mutantion activates genes involved in Wnt/β-catenin, and Hedgehog signaling pathway, as well as cell-cell adhesion. Biological processes, including cell-cell adhesion and migration, were also activated in colon cancers with high nuclear β-catenin expression. In HEK293 cells, Notch signaling was not distinctly activated by *β-catenin* mutants alone, as it was also activated by wild-type β-catenin. Thus, activation of Notch

signaling may not be SPN-specific, because it is also found to be activated in neuroendocrine tumors and pancreatic adenocarcinomas in which (cytoplasmic) β-catenin expression is up-regulated, compared to non-neoplastic tissue without *β-catenin* mutation. Notch signaling was also detected as an activated pathway in colon cancers showing high β-catenin expression in the nucleus. Thus, activation of Notch signaling in colorectal cancer and SPN is more likely caused by overexpression and accumulation of β-catenin, rather than *β-catenin* mutation.

Studies suggest that several genes involved in a specific pathway can also play additional roles in another pathway.[53-57] By combining the results of the present study, which evaluated colon cancer, SPNs and HEK293 cells transfected with *β-catenin* constructs, several intermediate genes that connect Wnt/β-catenin signaling with Hedgehog, Notch, and androgen receptor signaling can be selected. I finally selected about 30 genes that could be expected to connect one of these pathways with another.

For future study, selected genes should be narrowed down based on the hypothesis that nuclear β-catenin expression leads to the activation of Notch and Hedgehog signaling pathway via genes concomitantly involved in multiple-pathways. Also, the effects of regulating master genes on the Notch and Hedgehog pathways should also be investigated in various disease models.

V. CONCLUSION

In conclusion, nuclear β-catenin overexpression affects the signaling pathways of Wnt/β-catenin, Notch, Hedgehog and androgen receptor signaling pathway according to the disease model.

In my study, I found;

1. Nuclear β-catenin overexpression plays roles in Wnt/β-catenin signaling pathway.

2. Notch and Hedgehog signaling pathways are identified as novel pathways relevant with nuclear β-catenin overexpression in both colon cancers and SPN.

3. Androgen receptor signaling pathway is identified as characteristic and up-regulated pathway in SPN.

In summary, nuclear β-catenin commonly or selectively affects the signaling pathways such as Wnt/β-catenin, Notch, Hedgehog and androgen receptor signaling pathway according to the disease model.

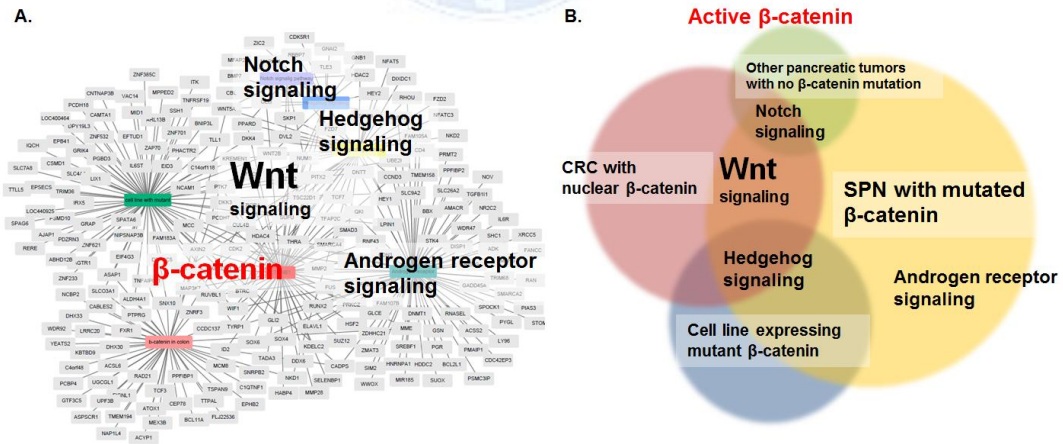


Figure 11. A schematic model of the nuclear β-catenin-related network describing potential intermediate genes associated with the Wnt/β-catenin, Notch, Hedgehog, and androgen receptor signaling pathways. With β-catenin

located in the center, genes up-regulated in SPN, colon cancer or in vitro model are shown around Wnt/β-catenin, Notch, Hedgehog, and androgen receptor signaling pathways (A). Simplified version of networks including activated pathways in colon cancer, pancreatic tumors and cell lines expressing mutant β-catenin (B).

REFERENCES

1. Barker N, Clevers H. Catenins, Wnt signaling and cancer. Bioessays 2000;22:961-5.
2. Hlsken J, Behrens J. The Wnt signalling pathway. J Cell Sci 2000;113 (Pt 20):3545.
3. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781-810.
4. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 2004;5:691-701.
5. Barth AI, Näthke IS, Nelson WJ. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. Curr Opin Cell Biol 1997;9:683-90.
6. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997;275:1787-90.
7. Rodilla V, Villanueva A, Obrador-Hevia A, Robert-Moreno A, Fernández-Majada V, Grilli A, et al. Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. Proc Natl Acad Sci U S A 2009;106:6315-20.
8. Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. Stem Cell Rev 2007;3:30-8.
9. Polakis P. Wnt signaling and cancer. Genes Dev 2000;14:1837-51.
10. Lustig B, Behrens J. The Wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol 2003;129:199-221.
11. Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000;103:311-20.
12. Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.
13. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-67.
14. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the

APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998;58:1130-4.

15. Network CGA. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330-7.
16. Leedham SJ, Rodenas-Cuadrado P, Howarth K, Lewis A, Mallappa S, Segditsas S, et al. A basal gradient of Wnt and stem-cell number influences regional tumour distribution in human and mouse intestinal tracts. Gut 2013;62:83-93.
17. Städeli R, Hoffmans R, Basler K. Transcription under the control of nuclear Arm/beta-catenin. Curr Biol 2006;16:R378-85.
18. Phelps RA, Chidester S, Dehghanizadeh S, Phelps J, Sandoval IT, Rai K, et al. A two-step model for colon adenoma initiation and progression caused by APC loss. Cell 2009;137:623-34.
19. Krieghoff E, Behrens J, Mayr B. Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. J Cell Sci 2006;119:1453-63.
20. Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F. Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 2008;133:340-53.
21. Abraham SC, Klimstra DS, Wilentz RE, Yeo CJ, Conlon K, Brennan M, et al. Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal adenocarcinomas and almost always harbor beta-catenin mutations. Am J Pathol 2002;160:1361-9.
22. Tanaka Y, Kato K, Notohara K, Hojo H, Ijiri R, Miyake T, et al. Frequent beta-catenin mutation and cytoplasmic/nuclear accumulation in pancreatic solid-pseudopapillary neoplasm. Cancer Res 2001;61:8401-4.
23. Aoki M, Hecht A, Kruse U, Kemler R, Vogt PK. Nuclear endpoint of Wnt signaling: neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. Proc Natl Acad Sci U S A 1999;96:139-44.
24. Bosman FT, World Health Organization., International Agency for Research on Cancer. WHO classification of tumours of the digestive system. 4th ed. Lyon: International Agency for Research on Cancer; 2010.
25. Gerdes B, Ramaswamy A, Simon B, Pietsch T, Bastian D, Kersting M, et al.

Analysis of beta-catenin gene mutations in pancreatic tumors. Digestion 1999;60:544-8.

26. Hervieu V, Lepinasse F, Gouysse G, Guillaud O, Barel C, Chambonniere ML, et al. Expression of beta-catenin in gastroenteropancreatic endocrine tumours: a study of 229 cases. J Clin Pathol 2006;59:1300-4.
27. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
28. Lee HJ, Suk JE, Patrick C, Bae EJ, Cho JH, Rho S, et al. Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. J Biol Chem 2010;285:9262-72.
29. Hwang D, Rust AG, Ramsey S, Smith JJ, Leslie DM, Weston AD, et al. A data integration methodology for systems biology. Proc Natl Acad Sci U S A 2005;102:17296-301.
30. Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44-57.
31. Hlubek F, Brabletz T, Budczies J, Pfeiffer S, Jung A, Kirchner T. Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer. Int J Cancer 2007;121:1941-8.
32. Fodde R. The APC gene in colorectal cancer. Eur J Cancer 2002;38:867-71.
33. Hsieh JS, Lin SR, Chang MY, Chen FM, Lu CY, Huang TJ, et al. APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am Surg 2005;71:336-43.
34. Cavard C, Audebourg A, Letourneur F, Audard V, Beuvon F, Cagnard N, et al. Gene expression profiling provides insights into the pathways involved in solid pseudopapillary neoplasm of the pancreas. J Pathol 2009;218:201-9.
35. Jiang J, Hui CC. Hedgehog signaling in development and cancer. Dev Cell 2008;15:801-12.
36. Katoh Y, Katoh M. Hedgehog signaling, epithelial-to-mesenchymal

transition and miRNA (review). Int J Mol Med 2008;22:271-5.

37. Shimizu T, Kagawa T, Inoue T, Nonaka A, Takada S, Aburatani H, et al. Stabilized beta-catenin functions through TCF/LEF proteins and the Notch/RBP-Jkappa complex to promote proliferation and suppress differentiation of neural precursor cells. Mol Cell Biol 2008;28:7427-41.

38. Yang F, Li X, Sharma M, Sasaki CY, Longo DL, Lim B, et al. Linking beta-catenin to androgen-signaling pathway. J Biol Chem 2002;277:11336-44.

39. Mulholland DJ, Cheng H, Reid K, Rennie PS, Nelson CC. The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. J Biol Chem 2002;277:17933-43.

40. Pawlowski JE, Ertel JR, Allen MP, Xu M, Butler C, Wilson EM, et al. Liganded androgen receptor interaction with beta-catenin: nuclear co-localization and modulation of transcriptional activity in neuronal cells. J Biol Chem 2002;277:20702-10.

41. Truica CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60:4709-13.

42. Song LN, Gelmann EP. Interaction of beta-catenin and TIF2/GRIP1 in transcriptional activation by the androgen receptor. J Biol Chem 2005;280:37853-67.

43. Yang X, Chen MW, Terry S, Vacherot F, Bemis DL, Capodice J, et al. Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. Oncogene 2006;25:3436-44.

44. Bolós V, Grego-Bessa J, de la Pompa JL. Notch signaling in development and cancer. Endocr Rev 2007;28:339-63.

45. Jiang YG, Luo Y, He DL, Li X, Zhang LL, Peng T, et al. Role of Wnt/beta-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1alpha. Int J Urol 2007;14:1034-9.

46. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 2005;24:7443-54.

47. Moustakas A, Heldin CH. Signaling networks guiding epithelial-

mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007;98:1512-20.

48. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15:740-6.

49. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 2004;6:931-40.

50. Timmerman LA, Grego-Bessa J, Raya A, Bertrán E, Pérez-Pomares JM, Díez J, et al. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev 2004;18:99-115.

51. Niessen K, Fu Y, Chang L, Hoodless PA, McFadden D, Karsan A. Slug is a direct Notch target required for initiation of cardiac cushion cellularization. J Cell Biol 2008;182:315-25.

52. Garg M. Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer. World J Stem Cells 2013;5:188-95.

53. Yuan X, Cai C, Chen S, Yu Z, Balk SP. Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. Oncogene 2014;33:2815-25.

54. Liu XH, Wu Y, Yao S, Levine AC, Kirschenbaum A, Collier L, et al. Androgens up-regulate transcription of the Notch inhibitor Numb in C2C12 myoblasts via Wnt/β-catenin signaling to T cell factor elements in the Numb promoter. J Biol Chem 2013;288:17990-8.

55. Ye F, Chen Y, Hoang T, Montgomery RL, Zhao XH, Bu H, et al. HDAC1 and HDAC2 regulate oligodendrocyte differentiation by disrupting the beta-catenin-TCF interaction. Nat Neurosci 2009;12:829-38.

56. Kioussi C, Briata P, Baek SH, Rose DW, Hamblet NS, Herman T, et al. Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 pathway mediating cell-type-specific proliferation during development. Cell 2002;111:673-85.

57. Javelaud D, Pierrat MJ, Mauviel A. Crosstalk between TGF-β and hedgehog signaling in cancer. FEBS Lett 2012;586:2016-25.

ABSTRACT (IN KOREAN)

핵내 베타카테닌 과발현에 의한 암 발생 신호전달체계 규명

<지도교수 김 호 근>

연세대학교 대학원 의과학과

박 민 희

베타카테닌의 핵내 과발현과 돌연변이는 대장암, 유방암, 폐암 등의 다양한 암에서 보고되어왔다. 대부분의 베타카테닌 돌연변이는 *CTNNB1* 유전자의 3 번 exon 에 위치하고 있으며, 이 돌연변이는 베타카테닌의 분해 복합체를 억제함으로써 베타카테닌의 안정화를 유발한다. 대장암에서, APC 돌연변이는 대장암의 70-80%에서 발견되고 베타카테닌 돌연변이는 APC 유전자와 상호 배타적으로 약 5%의 대장암에서 발견된다. 변화된 Wnt 신호전달체계에 APC 돌연변이가 관련되어 있다는 연구는 많이 보고되었지만, 대장암에서 변화된 Wnt 신호전달체계와 베타카테닌의 돌연변이 사이의 기능적 연관성은 아직 완전히 설명되지 않았다. 또한 다른 종양에서의 베타카테닌 돌연변이의 분자생물학적 중요성 역시 아직 완전히 이해되지 않았다. 췌장 고형성 가성유두상 종양 (solid-pseudopapillary neoplasm, SPN)은 거의 모든 예에서 베타카테닌 3 번 exon 에 체세포

돌연변이를 갖고 있으며, 이는 비정상적인 베타카테닌의 핵내 과발현을 유발한다. 그러나 베타카테닌의 돌연변이와 Wnt 신호전달체계의 활성화는 SPN 발생의 원인으로 알려져 있지만 분자생물학적 조절 연관성에 대한 연구는 미비하다. 종양에서 베타카테닌의 핵내 축적에 의해 변하는 신호전달체계를 동정하기 위해서 연구모델로 대장암과 SPN 을 선택하였다. 베타카테닌 돌연변이는 대장암 일부의 발생에 관여하는 반면, SPN 의 발생은 베타카테닌 돌연변이에 의해서 유발되기에 대장암을 핵내 베타카테닌 과발현이 베타카테닌을 분해하는 유전자의 이상으로 초래된 불균질 모델로, SPN 은 베타카테닌 돌연변이에 의해 초래되는 균질모델로 정하고 실험을 진행하였다. 대장암에서 핵에 위치한 베타카테닌이 일정하게 발현하지 않기 때문에, 우선 대장암을 핵에 위치한 베타카테닌의 비율을 기준으로 나누고 비율에 따라 부분집합으로 대장암을 분류하였다. 베타카테닌의 핵발현이 높은 (30% 이상) 대장암의 mRNA 발현 특성을 분석한 결과 핵에 위치한 베타카테닌은 대장암에서 Wnt, Notch 및 Hedgehog 신호전달계 뿐만 아니라 상피-간엽 전환 (Epithelial-Mesenchymal Transition. EMT) 수용체와의 상호작용에 관여하는 유전자를 활성화시켰다. 거의 모든 종양세포에서 베타카테닌이 핵에 발현하는 질병 모델에서 관련한 유전자 집합을 발굴하기 위해, SPN 과 다른 종류의 췌장암 (췌장 선암종, 췌장내분비 종양)에 대해 유전자 발현 분석을 수행한 결과 mRNA 발현의 집단화 분석은 SPN 과 다른 종류의 췌장암을 구별하였다. 전 예에서 베타카테닌 돌연변이와 핵 발현을 보이는 SPN 에서 발현차이를

보이는 유전자들은 Wnt, Hedgehog 그리고 androgen receptor 신호전달체계와 관련이 있었고, 아울러 EMT 와 관련 유전자들이 활성화되어 있다.

마지막으로 돌연변이형 베타카테닌에 의해 직접 변화되는 신호전달체계와 유전자들을 동정하기 위하여 두가지 세포주를 이용하여 여러 종류의 돌연변이형 베타카테닌 벡터를 직접 도입한 결과, androgen receptor 가 핵으로 이동하는 것과 Wnt, Notch, Hedgehog 신호전달계에 속한 유전자들의 발현이 증가 하는 사실을 확인하였다.

본 실험을 통하여 나는 베타카테닌의 돌연변이에 의한 베타카테닌의 핵내 축적은 Wnt, Notch, Hedgehog 그리고 androgen receptor 신호전달체계의 활성화를 시켜 대장암 일부와 SPN 종양의 발생과 진행에 관여한다고 결론지었다.

핵심되는 말 : 베타카테닌, 대장암, 췌장 고형성 가성유두상 종양, 유전자 발현, Wnt 신호전달체계, Notch 신호전달체계, Hedgehog 신호전달체계

PUBLICATION LIST

1. Lee H, Park MH, Shin N, Kim GM, Kim YG, Shin JS, et al. High mobility group box-1 is phosphorylated by protein kinase C zeta and secreted in colon cancer cells. Biochem Biophys Res Commun 2012; 28;424(2):321-6.

2. Kim WK, Park M, Park MH, Kim YJ, Shin N, Kim H, et al. Identification and selective degradation of neopeptide-containing truncated mutant proteins in the tumors with high microsatellite instability. Clin Cancer Res 2013;19:3369-82.

3. Park M, Kim WK, Song M, Park MH, Kim H, Nam HJ, et al. Protein kinase C-δ-mediated recycling of active KIT in colon cancer. Clin Cancer Res 2013;19:4961-71.

4. **Park MH**, Kim M, Hwang D, Kim WK, Kim SK, Shin J, et al. Characterization of gene expression and activated signaling pathways in solid-pseudopapillary neoplasm of pancreas. Mod Pathol 2014;27:580-93.

5. **Park MH**, Lim J, Lee HJ, Na K, Lee MJ, Kang CM, et al. Distinct Protein Expression Profiles of Solid-Pseudopapillary Neoplasms of the Pancreas. J Proteome Res 2015; 10;14(8):3007-14.