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의학박사 학위논문

Pathogenetic role of Wnt signaling pathway and the function of Dickkopf-1 in thyroid cancers

갑상선암에서 Wnt signaling pathway 의 병인론적 역할과 Dickkopf-1 이 미치는 영향

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서울대학교 대학원

의학과 분자유전체의학 전공

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December, 2012

The Department of Medicine,

Seoul National University

College of Medicine

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Pathogenetic role of Wnt signaling pathway and the function of Dickkopf-1 in thyroid cancers

by

Eun Jung Lee

A Thesis Submitted to the Department of Medicine in partial Fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine

(Molecular Genomic Medicine)

at the Seoul National University College of Medicine

December, 2012

Approved by Thesis Committee:

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서울대학교총장 귀하

Abstract

Pathogenetic role of Wnt signaling pathway and the function of Dickkopf-1 in thyroid cancers

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Wnt/β-catenin signaling plays a role in tumorigenesis of human papillary thyroid cancer (PTC). Dickkopf (Dkk) -1 is a known inhibitor of Wnt/β-catenin signaling, the therapeutic potential of which is undetermined. In this study, we investigated the therapeutic potential of Dkk-1 in human PTC.

In order to evaluate the clinical significance of Wnt/β-catenin pathway, we investigated the expression of Wnt in relation to downstream molecules including β-catenin, cyclin D1, E-cadherin in papillary thyroid cancer. In addition we investigated the anti-tumoral effect of Dkk-1 on two different

PTC cell lines, *in vitro,* to determine the therapeutic potential of Dkk-1 in PTC.

A paired comparison between human PTC and normal thyroid tissues from PTC patients revealed that *Wnt3* gene expression and total β-catenin proteins were up-regulated in human PTC tissues. Tissue microarray data revealed that 9.5% of PTC and 100.0% of anaplastic thyroid cancer had aberrant cytoplasmic staining of β-catenin, while membranous staining was found in all normal tissue. Aberrant locations of β-catenin in PTC tissues were correlated with the loss of membranous E-cadherin expression. Dkk-1 treatment inhibited PTC cell survival via a pro-apoptotic action. Dkk-1 reversed the aberrant expression of β-catenin from nucleus to membrane and inhibited basal levels of TCF/LEF–dependent transcriptional activities. Furthermore, Dkk-1 inhibited cell viability in dose dependent manners and adenoviral transduction of constitutively active β-catenin blocked these effects, suggesting that an anti-tumoral effect of Dkk-1 is mediated by Wnt/β-catenin signaling. In addition, Dkk-1 also recovered the loss of membranous E-cadherin expression with consequent inhibition of cell migration and invasion in both cell lines.

Our results suggest that Dkk-1 inhibited human PTC survival and migration by regulating Wnt/β-catenin signaling and E-cadherin expression. Thus, the interruption of this signaling may therapeutically be useful in thyroid cancer.

Key words : Dkk-1, Wnt, β-catenin, E-cadherin, Papillary thyroid cancer

Student number : 2007-30566

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PTC papillary thyroid cancer

TSH thyroid stimulating hormone

AJ adherens junction

LRP lipoprotein-receptor-related protein

GSK-3β glycogen synthase kinase-3β

APC adenomatous polyposis coli

TCF/LEF T cell factor/lymphoid enhancer factor

Dkk-1 Dickkopf-1

SD standard deviation

Introduction

Papillary thyroid cancer (PTC) is the most common thyroid cancer representing about 80 % of all thyroid cancers [1, 2]. The conventional multimodality treatment for patients with PTC is surgical excision (a near-total thyroidectomy or a total thyroidectomy), radioactive iodine ablation and/or thyroid hormone supplementation to suppress thyroid-stimulating hormone (TSH). Although the biological mechanisms of PTC have been robustly studied during the past decade, there is still no effective treatment for PTC. Numerous studies suggest that activation of the Wnt/β-catenin signaling pathway plays an important role in human tumorigenesis [3-5]. Therefore, many components of this signaling pathway may serve as rational targets of cancer drug development. Given the complexity of Wnt/β-catenin signaling pathway, it is conceivable that potential cancer drugs can be developed by targeting the different nodal points of this signaling pathway.

The term "Wnt" was coined from a combination of the Drosophila segment polarity gene *Wingless* and the mouse proto-oncogene *Int-1*. Wnt gene family encodes the multi-functional signaling glycoproteins that are involved in the regulation of cell proliferation, survival, and fate [6, 7]. Wnt signaling cascades can be divided into a Wnt/β-catenin dependent canonical pathway and β-catenin independent noncanonical pathway which can be further divided in to the Planar Cell Polarity and the Wnt/Ca^{2+} pathway [8-11]. The signaling events of β-catenin-independent, noncanonical Wnt signaling are

poorly defined. In the Wnt/Ca^{2+} pathway, specific Wnt and frizzled receptors homologues acting through heterotrimeric GTP-binding proteins trigger intracellular calcium release, which activates the calcineurin and the calcium sensitive kinases calcium/calmodulin-dependent kinase II and protein kinase C. The downstream signaling pathways activated by such ligand-receptor interactions have yet to be delineated. β-catenin is a key modulator of canonical Wnt signaling [12]. β-catenin is a multifunctional protein with an important role in cell adhesion and signal transduction. In normal resting cells in the absence of Wnt activation, β-catenin is localized to the adherens junctions (AJs) on the cell membrane and free cytoplasmic β-catenin levels are very low because β-catenin is rapidly destroyed by ubiquitin-proteasome degradation. Upon binding of Wnt lignads to Frizzled receptors and low-density lipoprotein-receptor-related protein (LRP) coreceptors, a multiprotein complex composed of Axin, glycogen synthase kinase-3β (GSK-3β), and tumor suppressor adenomatous polyposis coli (APC) phosphorylates β-catenin, leading to degradation [13, 14]. In the canonical pathway, Wnt ligands bind to Frizzled receptors resulting in inactivation of GSK-3β, inhibition of β-catenin phosphorylation and degradation, and accumulation of stabilized β-catenin in the cytoplasm. This elevated level of β-catenin diverted to the nucleus where it stimulates T cell factor/lymphoid enhancer factor (TCF/LEF) and target genes such as *cyclin D1*,*c-myc* and *matrilysin* [15-17]. Several lines of evidence show that aberrant activation of Wnt/β-catenin signaling might play a role in thyroid tumorigenesis [18, 19]. In addition, *C-*

myc and *cyclin D1* genes are amplified in sporadic differentiated thyroid. Although a reduction of β-catenin bound to the cell surface has been recently demonstrated in thyroid carcinoma, the biological and clinical relevance of β-catenin dysregulation in thyroid neoplasia is primarily unknown.

The adhesion of cells to their neighbors determined cellular and tissue morphogenesis and regulates major cellular processes including motility, growth, differentiation, and survival. Cell to cell AJs, the most common type of intercellular adhesions, are important for maintaining tissue architecture and cell polarity and can limit cell movement and proliferation. AJs assemble via homophillic interactions between the extracellular domains of calcium-dependent cadherin receptors on the surface of neighboring cells. The cytoplasmic domains of cadherins bind to the submembranal β-catenin. The interaction between the cytoplasmic tail of cadherins with the catenins is critical for the establishment of stable and functional AJs. Malignant transformation is often characterized by major changes in the organization of the cytoskeleton, decreased adhesion, and aberrant adhesion-mediated signaling. Disruption of normal cell-cell adhesion in transformed cells may contribute to the cells' enhanced migration and proliferation, leading to invasion and metastasis. This disruption can be achieved by down regulating the expression of cadherin. The importance of the major epithelial cell cadherin, E-cadherin, in the maintenance of normal cell architecture and behavior is underscored by the observation that hereditary predisposition to gastric cancer. Loss of E-cadherin expression eliminates AJ formation and is

associated with the transition from adenoma to carcinoma and acquisition of metastatic capacity. A major route for signal transduction by AJs involves the regulation of β-catenin–TCF/LEF signaling. The interaction of β-catenin with cadherins and TCF/LEF family members is mediated by the same domainon the β-catenin molecule, these interactions are mutually exclusive. Loss of E-cadherin that courses disruption of cell adhesion and polarity allows tumor cell metastasis, while the translocation of β-catenin into the nucleus might be required to induce the expression of genes that promote cell proliferation and invasion.

Dickkopf-1 (Dkk-1), a secretory protein originally described as an endogenous canonical Wnt antagonist. Dkk-1 interacts with the Wnt co-receptor, LRP 5/6, and blocks Wnt/β-catenin signaling by preventing the formation of Wnt–Frizzled–LRP5/6 receptor complexes [20, 21]. Several studies have shown that the dysregulation of *Dkk-1* gene is related to tumor cell growth or invasive potential in human cancers [22-24]. However, there are only a few studies demonstrating the therapeutic potential of Dkk-1 [25-27] .

In order to evaluate the clinical significance of Wnt/β-catenin pathway, we investigated the expression of Wnt in relation to downstream molecules including β-catenin, cyclin D1, E-cadherin in papillary thyroid cancer. In addition we investigated the anti-tumoral effect of Dkk-1 on two different PTC cell lines, *in vitro*, to determine the therapeutic potential of Dkk-1 in PTC.

Materials and Methods

Study subjects and samples

Thyroid tissue samples were obtained at the time of surgery from patients who had undergone thyroidectomy. Fresh tissue samples from 6 PTC patients were snap-frozen in liquid nitrogen in the operating room. Frozen sections were prepared from each sample and reviewed by a single pathologist for a confirmatory histological diagnosis. For tissue microarray, 228 tissue samples were examined, and were obtained from the surgical pathology files of the Department of Pathology, Seoul National University Boramae Hospital and the Department of Pathology, Seoul National University Hospital from January 1993 to December 2003. These tissue samples comprised, samples from patients with a normal thyroid (n=5), adenomatous goiter (n=58), PTC (n=148), and anaplastic thyroid cancer (n=18). Hematoxylin and eosin stained slides were reviewed and one appropriate paraffin block was selected for each case. All experiments were conducted in accordance with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving humans and all experiments were approved by the Institutional Review Board of Seoul National University Bundang Hospital.

Reagents and antibodies

Total β-catenin antibody against the C-terminal portion (amino acids 680–781) of human β-catenin, cleaved caspase-3 and E-cadherin antibody were

purchased from Cell Signaling Technologies (Beverly, MA). Human cyclin D1, GAPDH, Na/K-APTase and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human Dkk-1 was purchased from R&D system (Minneapolis, MN). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

Cell cultures

The human papillary thyroid carcinoma cell line, SNU-790 with heterozygous $BRAF^{V600E}$ and B-CPAP containing heterozygous $BRAF^{V600E}$ cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and grown at 37°C in a humidified 5% CO_2 atmosphere. SNU-790 with heterozygous $BRAF^{V600E}$ was purchased from the Korean cell Line Bank (Seoul) [28], and B-CPAP containing heterozygous $BRAF^{V600E}$ was kindly provided by Dr. Minho Shong (Chungnam National University, Daejon, South Korea

Tissue microarrays

Representative core tissue sections (2mm in diameter) were taken from individual paraffin embedded thyroid tissues (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Super Biochips Laboratories, Seoul). Each tissue array block contained up to 60 cores, and total 4 tissue microarray blocks were made. Four micrometer-thick sections were cut from the completed array blocks and transferred to

silanized glass slide

Immunohistochemical assays

We defaraffined 4 mm sections form the tissue microarray blocks in xylene and rehydrated them in graded ethanol. The succeeding steps were performed automatically at 37 using the BenchMark® Slide Staining System Specifications (Ventana Medical Systems, Tucson, AZ, USA). Endogenous peroxidases were quenched with 1% H_2O_2 for 4 min. The section were incubated with the rabbit polyclonal antibody for (H-89, Santa Cruz, dilution 1:100), the mouse monoclonal antibody for β-catenin (5H10, Zymed Laboratorys, dilution 1:1000), the mouse monoclonal antibody for cyclin D1 (SP4, Thermo Fisher Scientific, dilution 1:200), the mouse monoclonal antibody for E-cadherin (SPM471, Thermo Fisher Scientific, dilution 1:1000). The secondary biotinylated antibody was incubated for 8 min. the slides were stained using a diaminobenzidine (DAB) detetion kit and counterstained with haematoxylin.

For the expression of cyclin D1, the percentage of tumor cells with nuclear staining of cyclin D1 were counted and more than 10% of nuclear staining were regarded as positive. For the expression of E-cadherin, showing more than 10% loss of membranous immunostaining was regarded as aberrant. For analyzing β-catenin expression, tumors having more than 90% of membranous staining were considered normal while others including

decreased membranous staining and/or cytoplasmic staining and nuclear staining were considered abnormal according to a previous study [29]. Tumors having membranous expression of E-cadherin were considered normal while the cases showing more than 10% loss of membranous immunostaining were regarded as aberrant. For cyclin D1, the percentage of tumor cells with nuclear staining of cyclin D1 were counted and more than 10% of nuclear staining were regarded as positive.

RNA extraction and RT-PCR analysis

Total RNA was extracted from frozen tissue using the Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription System kit (Promega, Madison, WI). PCR was performed using 100 ng of cDNA, 20 pmol of each primer (synthesized by Bioneer Corp., Chungwon, Korea), 2.5mM of dNTPs,1mM of $MgCl_2$, and 1 U of Taq polymerase in a 50 μl reaction volume containing 1×Taq polymerase buffer using a Perkin-Elmer Gene Amp PCR System 9600. The PCR primer sets were as follows: for *Wnt 3* (193bp), forward primer 5'-TGCTGGACAAAGCTACCAGG-3', reverse primer 5'-ACCATCCCACCAAACTCGAT-3'; for *Wnt 10B* (256bp), forward primer 5'- ACTGTCCCGAGGCAAGAGTT-3', reverse primer 5'-GCCATGACACTTGCATTTCC-3'; for *Cyclin D1* (434bp), forward primer 5'- CTGGAGCCCGTGAAAAAGAGC-3', reverse primer 5'-CTGGAGAGGAAGCGTGTGAGG-3'.

Western blot analysis

Cells were lysed in 50 mM Tris (pH 7.5), 150 mM NACl, and 1 % Triton X-100 supplemented with a protease inhibitor mixtures (Sigma, added at a dilution of 1:100). Lysates were size-separated by SDS-PAGE gel, and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ). Blots were blocked with 5% mil for 1 h at 4℃, and then incubated with appropriate primary and secondary antibodies for 24 h and 1 h, respectively. Blots were then washed (three times for 20 min each) and proteins were visualized using enhanced chemiluminexcence kit (Amersham, Piscataway, NJ). Protein (30 ug) from thyroid tissue or cells was subjected to western blot analysis. Total β-catenin, cyclin D1, E-cadherin, caspase-3 and β-actin antibodies were used after 1:1000 dilution. Secondary antibodies conjugated with horseradish peroxidase were used after 1:5000 dilution. Subcellular fractionation was performed with the Qproteome cell compartment kit according to the manufacturer's protocol (Qiagen, Valencia, CA).

Construction of Adenoviral Vectors Expressing Triple Mutant β-Catenin

Adenoviruses expressing the triple mutant β-catenin (Ad-β-catenin), containing three mutations in phosphorylation sites (S33Y/S37F/T41A) with

green fluorescence protein (GFP)-tag, was a kind gift from Dr. Hyo-Soo Kim (Seoul National University, Seoul, South Korea). Adenovirus encoding GFP (Ad-GFP) was used as control. Successful transfection of Ad-β-catenin into the cells was determined by visualizing green fluorescence from the coexpression of GFP under microscope. Transduction efficacy was more than 90% both in Ad-β-catenin and Ad-GFP group.

Immunofluorescent staining

Cells were grown on glass coverslips and treated with Dkk-1 (20nM) or vehicle for 48hr. Cells were fixed with 4% of paraformaldehyde after washing with ice-cold PBS and permeabilized with 0.05% Triton X-100 for 15 min. Cells were then blocked with 5% goat serum/1% bovine serum albumin at room temperature fallowed by a-1hr incubation with fluorescein anti-mouse β-catenin antibody. Nuclear binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was used for nuclear staining. Cells were visualized under a confocal scanning laser microscope using 1000x objectives.

Cell viability assay

The viability of SNU-790 and B-CPAP cells were measured by a 3-(4,5-dimethyl thiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT was dissoloved in PBS at a concentration of 0.5 mg/dl and sterilized by passage through a 0.22 μM filter. The SNU-790 and B-CPAP cells, seeded on

a 96 well plate (2 x 10^4 cells per well) in 50 μl if RPMI containing 10 % FBS. 24 hrs later, media were removed and rinsed with PBS, and 100 μl of DMEM containing 0.1 % BSA and various concentirations of Dkk-1 (0 to 100 nM) were treated for 48 hrs. , MTT solution (Sigma-Aldrich Corporation, St. Louis, MO) was added to cells in 96 well plates to the final concentration fo 0.5 mg/dl, and cells were incubation at 37℃ for 4hrs. After removing culture media, 30 μl of dimethyl sulfoxide (DMSO) was added, and mixed thoroughly to lyse the cells and dissolve the dark blue crystals for 5 min. The absorbance was read by and ELISA reader (ThermoMax; Scientific Surplus, Hillsborough, NJ) at a wavelength of 540 nm.

Assessment of apoptosis

Cells were seeded into 24-well plates at 5 x 10^4 cells/well. After 12 hr of serum starvation, a 1hr-pretreatment of Dkk-1 (10 or 20 nM) or vehicles was followed by etoposides treatment (10 nM, Sigma-Aldrich) to induced cell apoptosis. After 48hr, the extent of apoptosis was quantified by assessing the characteristic nuclear chromatin condensation and nuclear fragmentation with DAPI staining under fluorescence microscopy.

Cell migration assay

Cells migration was performed with the Transwell system (Corning, NY, USA), which allows cells to migrate through 8-μm pore size polycarbonate

membrane. Polycarbonate membranes with pores were coated with a 10 µg/well gelatin solution in starvation medium and dried. Inserts containing cells were placed into a 24-well plate in starvation medium. Cells were trypsinized, washed, and resuspended in starvation medium. This suspension ($2x10^4$ cells) was added to the upper chamber of transwells. The lower chamber was filled with 500 µl by addition of 50nM of Dkk-1. After 24 hr, the surface of the upper membrane was swabbed with a cotton-tipped applicator to remove non-migrating cells. Inserts were fixed in methanol for 30 min and stained with 1% Crystal Violet for 2 hr.

Luciferase reporter assay

Cells were seeded into 24-well plates at 5×10^4 cells/well and 12 hrs later, three optimal (TOP Flash) or mutated (FOPflash) copies of TCF/LEF sites upstream of a thymidine kinase promoter – luc reporter vectors were transiently transfected for 4 hrs in reduced serum and antibiotics free OptiMEN with Lipofectamine 2000.

Cell lysates were prepared using the Promega Luciferase assay system, and activity was measured using a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany). All the transient transfections were performed in triplicate and all luciferase activity values were normalized for transfection efficiency against the β-galactosidase activity from the cotransfected pCMV-β-gal plasmid. All values were expressed relative to basal promoter activity as a fold induction.

Statistical analysis

For continuous variables, means and standard errors were calculated. After distributions were determined for normality using the Kolmogorov-Smirnov test, the Kruskal-Wallis test with the Mann-Whitney U test or one-way ANOVA with post-hoc analysis were used. For categorical variables, frequencies and percentages were reported. Proportions were compared using the χ^2 test or Fisher's exact test. Statistical analysis was performed using SPSS version 12.0 (version 12.0, SPSS Inc., Chicago, Illinois). All *P* values are two-sided, and a value of less than 0.05 is considered statistically significant.

Results

Increased Wnt/β-catenin signaling in human thyroid cancer tissues

To verify the status of Wnt/β-catenin signaling in human PTC tissues, a paired comparison of endogenous gene expression of *Wnt* was evaluated between PTC tissues and normal thyroid tissues from 6 PTC patients. The mRNA expression of *Wnt 3* was strongly up-regulated in cancer tissues compared to normal tissues (Fig. 1A). Protein levels of total β-catenin were also increased in cancer tissues compared to normal tissues (Fig. 1B). We next performed a tissue microarray analysis in a panel of PTC (n=148), anaplastic thyroid cancer (ATC) (n=18), adenomatous goiters (n=58), and normal thyroid tissues (n=5). Table 1 summarizes the results of β-catenin immunohistochemical staining of the tissue microarray. β-catenin immunoactivity was localized in the cell membrane in all normal thyroid tissues (5/5), 96.5% (56/58) of adenomatous goiter tissues, and 90.5% (134/148) of PTC tissues. On the other hand, 9.5% of PTC (14/148) showed aberrant immunoactivity of β-catenin including decreased or discontinuous membranous staining with cytoplasmic staining of β-catenin (Fig. 2B) and cytoplasmic dominant staining (Fig. 2C) and/or nuclear staining of β-catenin (Fig. 2D).

To evaluate the effect of this aberrant localization of β-catenin on downstream targets, we further performed immunohistochemial staining of cyclin D1 and E-cadherin using the same tissue microarray samples. In terms

of cyclin D1, all normal thyroid tissues showed negative nuclear staining while 39.7% of adenomatous goiter (23/58), 97.3% of PTC (144/148) and 83.3% ATC (15/18) showed positive nuclear staining (Fig. 3). Cytoplasmic dominant staining of β-catenin was not significantly correlated with the degree of positive nuclear staining of cyclin D1 in adenomatous goiter or PTC (Table 1). Immunohistochemical staining of membranous E-cadherin was absent in 15.5% of adenomatous goiter (9/58), 8.8% of PTC (13/148) and all of ATC (18/18), whereas E-cadherin expression was strong in the membranes of all normal thyroid tissues (Fig. 4). On the contrary to cyclin D1, loss of membranous E-cadherin immunoreactivity was observed more in tissues with cytoplasmic dominant staining of β-catenin than in those with normal membranous staining of β-catenin both in adenomatous goiter and in PTC (Table 1).

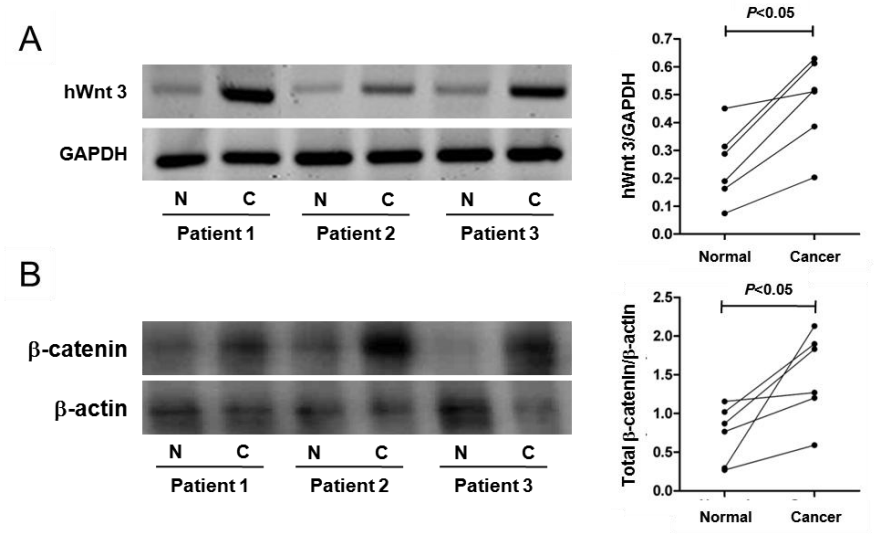

Figure. 1. Wnt/β-catenin signaling in human thyroid tissues.

Endogenous gene expression of *Wnt 3* and *β-catenin* was evaluated between papillary thyroid cancer (PTC) tissues and normal thyroid tissues from 6 PTC patients. The mRNA expression of *Wnt 3* was strongly up-regulated in PTC tissues compared to normal thyroid tissues. Representative images (left) and densitometric analysis (right) of human *Wnt3* mRNA expression (A) and total β-catenin protein expression in human PTC and paired normal tissues. The intensity of bands was quantified using densitometry unit from Kodak Digital Science. *P*- value < 0.05

Table 1. Association between localization of β-catenin and cyclinD1/E-cadherin in human thyroid pathology .

	Adenomatous goiter			Papillary thyroid cancer			Aplastic thyroid cancer		
calization of β-catenin	Normal membranous	Cytoplasmic dominant	*P*-value	Normal membranous	Cytoplasmic dominant	*P*-value	Normal membranous	Decreased Membranous/ Cytoplasmic	Cytoplasmic dominant
	N=56	N=2		N=134	N=14		N = 0	N=17	N=1
CyclinD1			*P*=0.75			*P*=0.42			
(-)	34 (60.7)	1 (50.0)		4 (3.0)	0			3 (17.6)	0
(+)	22 (39.3)	1 (50.0)		130 (97.0)	14 (100.0)			14 (82.4)	1 (100)
E-cadherin			*P*=0.02			*P*<0.001			
Normal	49 (87.5)	0		132 (98.5)	3 (21.4)			0	0
Loss (aberrant)	7 (12.5)	2 (100.0)		2 (1.5)	11 (78.6)			17 (100)	1 (100)

This table summarizes the results of β-catenin, cyclin D1, E-cadherin immunohistochemical staining of the tissue microarray. β-catenin immunoactivity was localized in the cell membrane in all normal thyroid tissues (5/5), 96.5% (56/58) of adenomatous goiter tissues, and 90.5% (134/148) of PTC tissues. On the other hand, 9.5% of PTC (14/148) showed aberrant immunoactivity of β-

catenin including decreased or discontinuous membranous staining with cytoplasmic staining of β-catenin (Fig. 2B) and cytoplasmic dominant staining and/or nuclear staining of β-catenin.

Cyclin D1, all normal thyroid tissues showed negative nuclear staining while 39.7% of adenomatous goiter (23/58), 97.3% of PTC (144/148) and 83.3% ATC (15/18) showed positive nuclear staining. Cytoplasmic dominant staining of β-catenin was not significantly correlated with the degree of positive nuclear staining of cyclin D1 in adenomatous goiter or PTC .

Membranous E-cadherin was absent in 15.5% of adenomatous goiter (9/58), 8.8% of PTC (13/148) and all of ATC (18/18), whereas E-cadherin expression was strong in the membranes of all normal thyroid tissues. Loss of membranous E-cadherin immunoreactivity was observed more in tissues with cytoplasmic dominant staining of β-catenin than in those with normal membranous staining of β-catenin both in adenomatous goiter and in PTC.

For the expression of cyclin D1, the percentage of tumor cells with nuclear staining of cyclin D1 were counted and more than 10% of nuclear staining were regarded as positive. For the expression of E-cadherin, showing more than 10% loss of membranous immunostaining was regarded as aberrant. For analyzing β-catenin expression, tumors having more than 90% of membranous staining were considered normal while others including decreased membranous staining and/or cytoplasmic staining and nuclear staining were considered abnormal according to a previous study

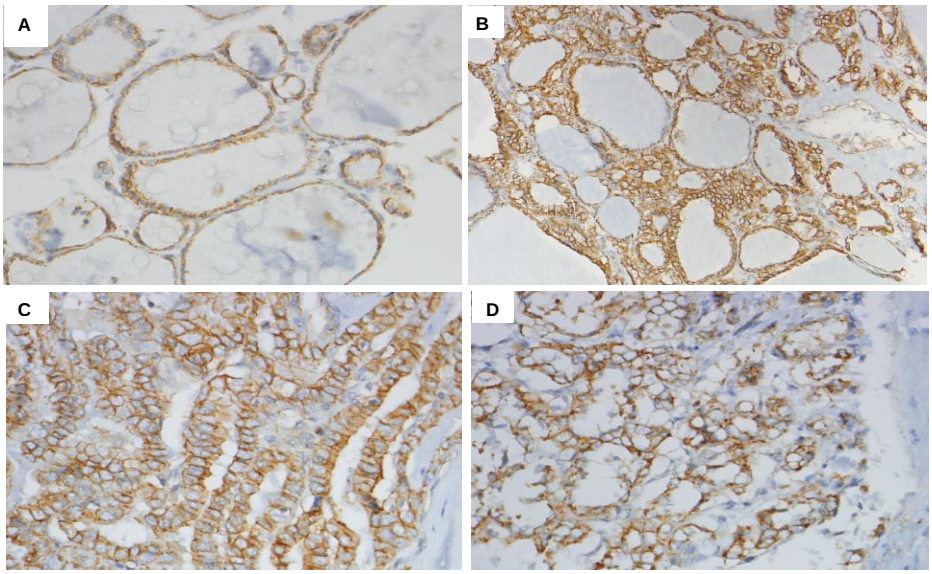

Figure 2. Immunohistochemical staining of β-catenin in human PTC.

β-catenin immunoactivity was localized in the cell membrane in normal thyroid tissues (A). On the other hand, 9.5% of pTC (14/148) showed aberrant immunoactivity of β-catenin including decreased or discontinuous membranous expression with cytoplasmic granular staining, (B) cytoplasmic granular staining (C), and nuclear staining (D). Original magnification, ×400.

For analyzing β-catenin expression, tumors having more than 90% of membranous staining were considered normal while others including decreased membranous staining and/or cytoplasmic staining and nuclear staining were considered abnormal.

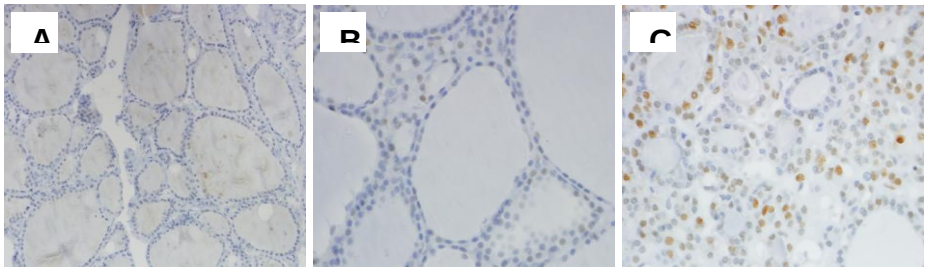

Figure 3. Immunohistochemical staining of cyclin D1 in human PTC.

Cyclin D1 immunoactivity showed negative nuclear staining in all normal thyroid tissues (A), while 39.7 % of adenomatous goiter (B), 97.3 % of PTC (C) showed positive nuclear staining.

For the expression of cyclin D1, the percentage of tumor cells with nuclear staining of cyclin D1 were counted and more than 10% of nuclear staining were regarded as positive.

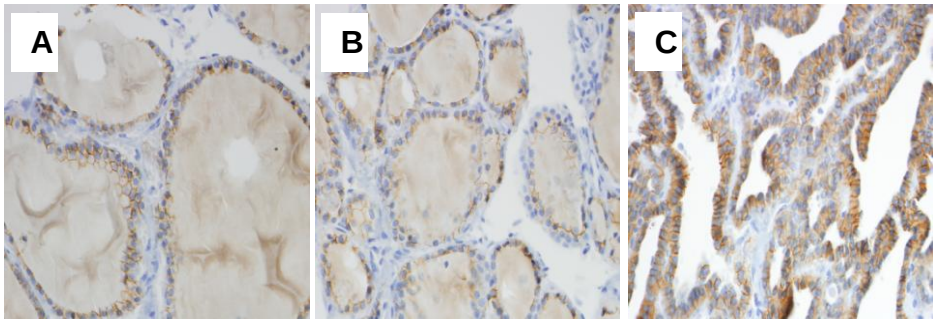

Figure 4. Immunohistochemical staining of E-cadherin in human PTC.

Immonohistochemical stainng of membranous E-cadherin was absent in 15.5 % of adenomatous goiter (B) and 8.8 % of PTC (C), whereas E-cadherin expression was strong in the membranes of all normal thyroid tissues (A). For the expression of E-cadherin, showing more than 10% loss of membranous immunostaining was regarded as aberrant.

Effect of Dkk-1 on Wnt/β−catenin signaling in human PTC cells

To verify the effect of Dkk-1 on Wnt/β−catenin signaling in human PTC cells, we first evaluate β-catenin expression in SNU−790 cells with or without Dkk-1 treatment. As shown in Figure 5A, treatment of Dkk-1 (20 nM) relocated β−catenin (green fluorescence) from the nucleus to the cytoplasm and/or plasma membrane, indicating that blocking Wnt signaling could rescue the aberrant expression of β-catenin in human PTC cells. B−CPAP cells showed similar results (Figure 5B).

We then measured the effect of Dkk-1 on TCF/LEF−dependent transcriptional activities, a nuclear target of Wnt/β-catenin signaling. Transient transfection of TOPflash showed 3.5~4.5−fold increases of transactivation compared with FOPflash transfected cells and treatment of Dkk-1 (50nM) for 48hrs significantly reduced this activities by ~59% (P<0.05) in SNU−790 (Fig. 6A) and ~52% (P<0.05) in B−CPAP (Fig. 6B) cells. Taken together, Dkk-1 inhibited aberrant activation of Wnt/β-catenin signaling in human PTC cells.

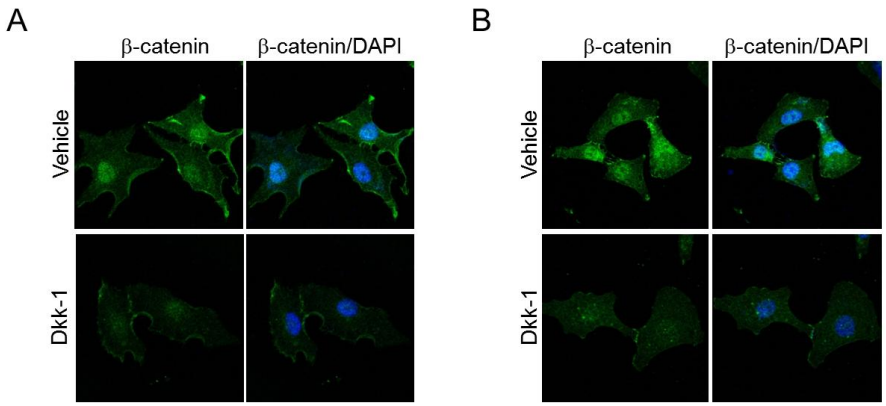

Figure 5. Effects of Dkk-1 onWnt/β-catenin signaling in human PTC cells. Immunofluorescence images of β-catenin localization in SNU-790 cells. Treatment of Dkk-1 (20 nM) in PTC cells shifted the intracellular localization of β-catenin (green fluorescence) form the nucleus to the cytoplasm and/or plasma membrane. Forty-eight hrs after treatment of Dkk-1 (20 nM), β-catenin was stained with appropriate Alexa Fluor® 488 Conjugated anti-mouse antibody and DAPI counter staining to label cell nuclei was performed (original magnification, ×1000). SNU-790 cells were grown on glass coverslips and treated with Dkk-1 (20nM) or vehicle for 48hr. Cells were fixed with 4% of paraformaldehyde after washing with ice-cold PBS and permeabilized with 0.05% Triton X-100 for 15 min. Cells were then blocked with 5% goat serum/1% bovine serum albumin at room temperature followed by an-1hr incubation with fluorescein anti-mouse β-catenin antibody. Nuclear binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was used for nuclear staining. Cells were visualized under a confocal scanning laser microscope using 1000x objectives.

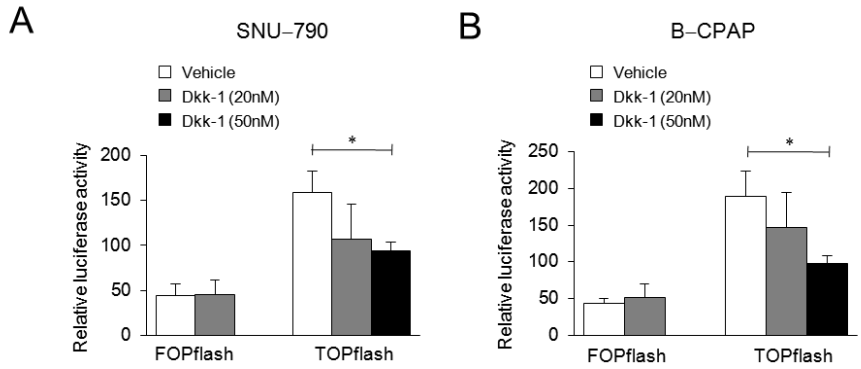

Figure 6. Effects of Dkk-1 on Wnt/β-catenin signaling in human PTC cells.

Transient transfection of TOPflash showed 3.5~4.5–fold increases of transcription compared with FOPflash transfected cells and treatment of Dkk-1 (50nM) for 48hrs significantly reduced this activities by ~59% (P<0.05) in SNU–790 (A) and ~52% (P<0.05) in B–CPAP (B) cells. A reporter construct containing three optimal (TOP Flash) or mutated (FOP Flash) copies of the TCF/LEF-binding site directing transcription of a luciferase gene were transfected following 48hrs treatment of Dkk-1 (0, 20, or 50 nM). Luciferase activity was measured and normalized by β-galactosidase activity from the cotransfected pCMV-β-gal plasmids. All data are expressed as mean ± SEM of at least two independent experiments performed in triplicate. $^{*}P$<0.05.

Inhibitory effect of Dkk-1 on cell survival in human PTC cells

To investigate the effect of Dkk-1 on human PTC cell survival, SNU−790 and B−CPAP cells were treated with Dkk-1 (0, 20, 50, and 100 nM) and cell proliferation was assessed by the Brdu incorporation assay. Treatment of Dkk-1 induced a slight decrease of cell proliferation with Dkk-1 treatment by ~10% ($p<0.05$) in SNU-790 (Fig. 7A) and ~ 16% ($p<0.01$) in B-CPAP (Fig. 7B) cells.

To further delineate whether the inhibitory role of Dkk-1 in PTC cell survival was mediated by β-catenin or not, we performed MTT assay with SNU−790 cells containing constitutively active β-catenin-TCF/LEF signaling. Triple mutant Ad-β-catenin, which is resistant to GSK3β-mediated protein degradation resulting constitutively active Wnt/β-catenin signaling [30], was transduced into SNU−790 cells. As a result, Dkk-1 treatment (20 nM for 48 hr) reduced cell viability by ~30% in Ad-GFP group, similarly to the null cells, whereas it had no effect on the Ad-β-catenin group (Fig. 8), demonstrating that the inhibitory effects of Dkk-1 on cell viability in SNU-790 cells were induced by blocking β-catenin-TCF/LEF signaling. Collectively, Dkk-1 inhibited PTC cell survival in a Wnt/β-catenin dependent manner.

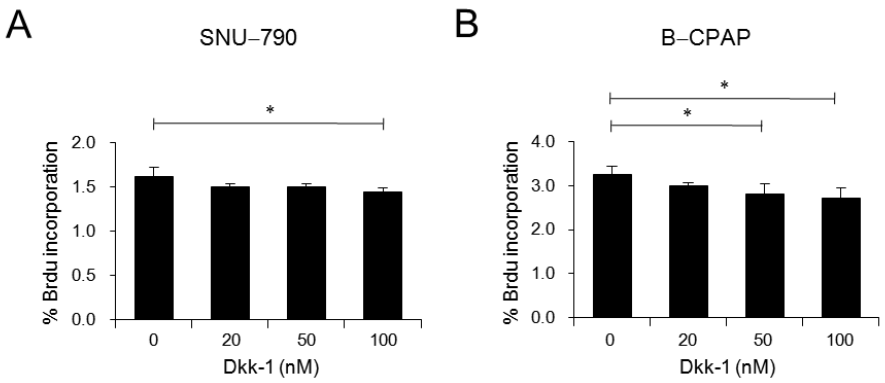

Figure 7. Effects of Dkk-1 on cell proliferation of human PTC cells.

The BrdU incorporation assay was performed at 48 hrs after treatment with vehicle or Dkk01 (20, 50, and 100 nM) in SNU-790 (A) and B-CPAP cells (B). The BrdU incorporation assay showed a slight decrease of cell proliferation with Dkk-1 treatment by ~10% ($p<0.05$) in SNU-790 (A) and ~ 16% ($p<0.05$) in B-CPAP (B) cells. $^{*}P<0.05$.

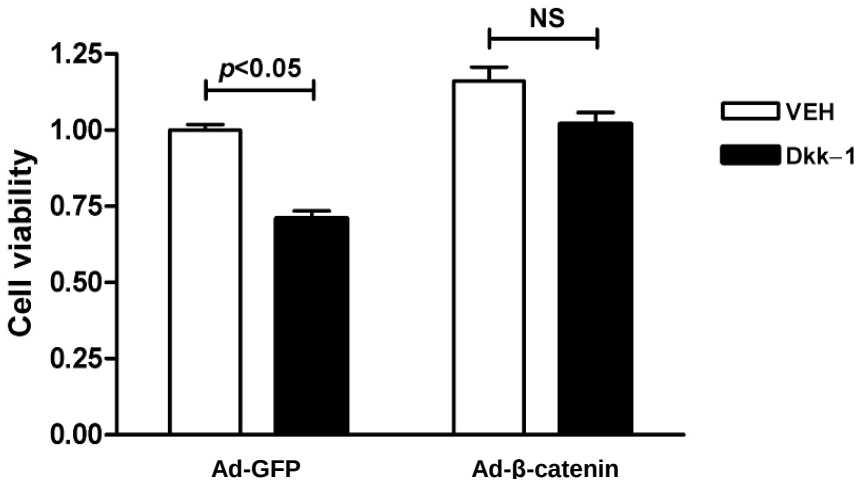

Figure 8. Effects of Dkk-1 on cell viability in human PTC cells.

We performed MTT assay with SNU−790 cells containing constitutively active β-catenin-TCF/LEF signaling. Triple mutant Ad-β-catenin, which is resistant to GSK3β-mediated protein degradation resulting constitutively active Wnt/β-catenin signaling, was transduced into SNU−790 cells. Dkk-1 treatment (20 nM for 48 hr) reduced cell viability by ~30% in Ad-GFP group, similarly to the null cells, whereas it had no effect on the Ad-β-catenin group, demonstrating that the inhibitory effects of Dkk-1 on cell viability in SNU-790 cells were induced by blocking β-catenin-TCF/LEF signaling. All data are expressed as mean±SEM of at least two independent experiments performed in triplicate.

Effect of Dkk-1 on the cell proliferation and apoptosis of human PTC cells

To further investigate the molecular mechanism of the inhibitory effect of Dkk-1 on PTC cell survival, we evaluated the cell proliferation and apoptosis in Dkk-1 treated PTC cells. Although the protein levels of cyclin D1 was decreased with Dkk-1 (50 nM) treatment in both SNU-790 (Fig. 9A) and B-CPAP (Fig. 9B) cells. Meanwhile, measurement of apoptotic cells with DAPI staining showed that Dkk-1 treatment significantly stimulated etoposide-induced cell apoptosis in a dose-dependent manner by upto 86% ($p<0.01$) in SNU-790 (Fig. 10A) and 51% ($p<0.01$) in B-CPAP cells (Fig. 10B). Dkk-1 (20nM) also up-regulated cleaved caspase-3 by ~40% ($p<0.01$) in SNU-790 (Fig. 11A) and ~20% ($p<0.05$) in B-CPAP (Fig. 11B) cells.

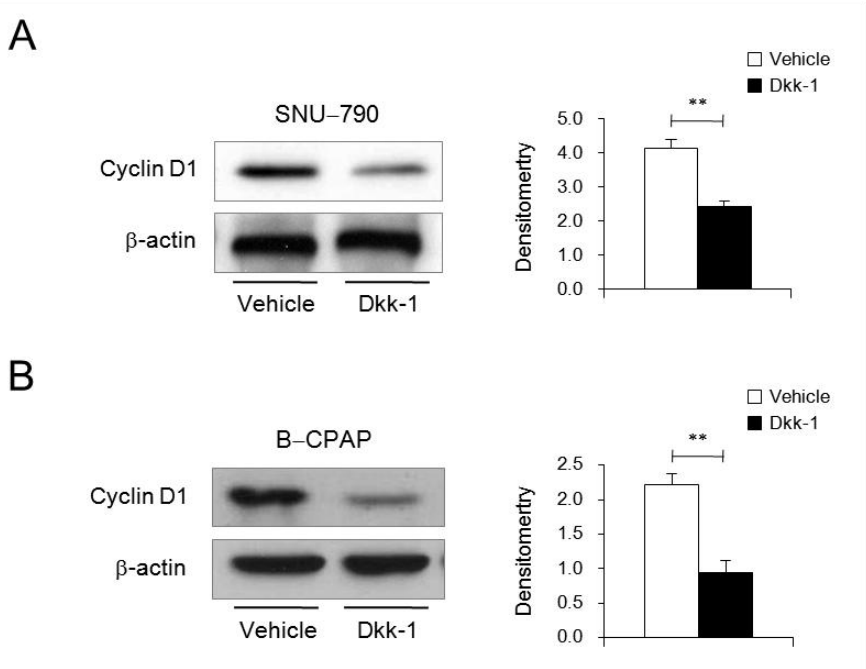

Figure 9. Effects of Dkk-1 on cyclin D1 expression in human PTC cells.

Representative images (left) and densitometric analysis (right) of cyclin D1 protein levels. The protein levels of cyclin D1 was decreased with Dkk-1 (50 nM) treatment in both SNU-790 (A) and B-CPAP (B). SNU−790 and B−CPAP cells were treated with vehicle or Dkk-1 (50nM) for 48hrs and Western blot analysis was performed. $^{**}P<0.01$.

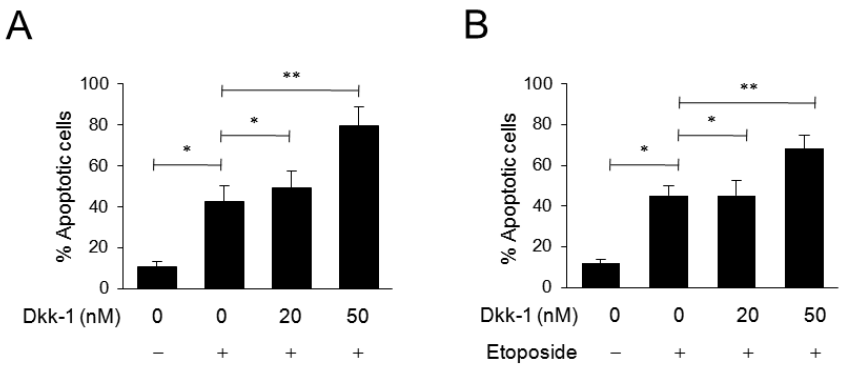

Figure 10. Effects of Dkk-1 on cell apoptosis in human PTC cells.

SNU−790 and B−CPAP cells were pretreated with vehicle or Dkk-1 (20 and 50 nM) for 1hr and treated with etoposide (10nM). Forty-eight hours later, cell apoptosis was quantified by DAPI staining and fluorescence microscopy. Percentages of apoptotic cells in (A) SNU−790 and (B) B−CPAP cells. DAPI staining for the quantification of apoptotic cells showed that Dkk-1 treatment significantly stimulated Etoposide-induced cell apoptosis in a dose-dependtdent manner by upto 86 % ($p<0.01$) in SNU-790 (B) and 51% ($p<0.01$) in B-CPAP (B) cells. Each bar represents mean±SEM of at least two independent experiments performed in quadruplicate. $^{*}P<0.05$;$^{**}P<0.01$.

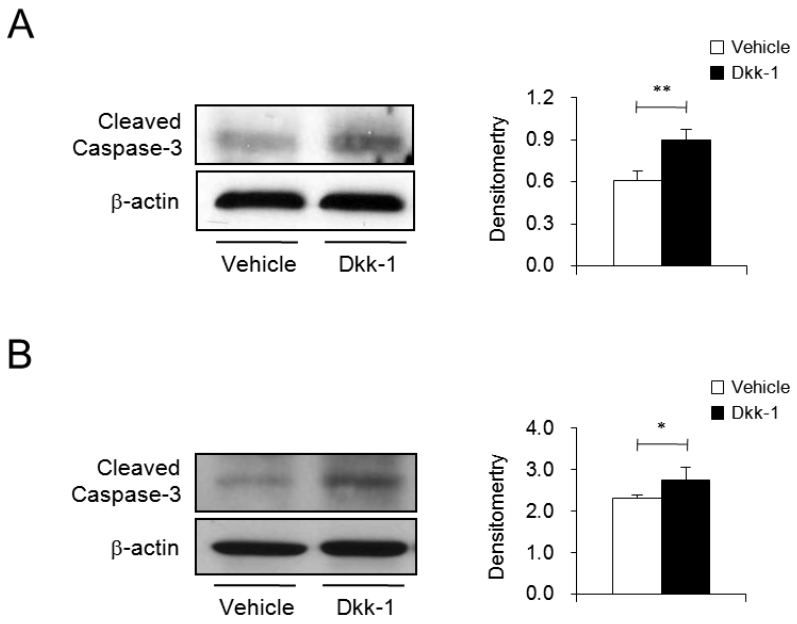

Figure 11. Effects of Dkk-1 on cleaved caspase-3 expression in human PTC cells.

Representative images (left) and densitometric analysis (right) of cleaved caspase-3 protein levels. Treatment of Dkk-1 (20nM) resulted in a respectable increase in cleaved caspase-3 by ~40% ($p<0.01$) in SNU-790 (A) and ~20% ($p<0.05$) in B-CPAP (B) cells.

Dkk-1 inhibits cell migration through β-catenin/E-cadherin in human PTC cells

Since the human tissue array data showed an association between the loss of E-cadherin expression and aberrant β-catenin immunoreactivity in PTC patient (Table 1), we evaluated the effect of Dkk-1 on E-cadherin expression and cell migration in PTC cells. Western blot analysis and immunofluorescence staining showed that loss of membranous E-cadherin expression was markedly rescued by Dkk-1 treatment in both SNU-790 (Fig. 12A) and B-CPAP cells (Fig. 12B). To elucidate the effect of Dkk-1 on cell invasiveness of PTC cells, the transmigration assay using an ECM-like membrane was performed. As shown in Figure13, cell invasiveness was significantly inhibited in both SNU-790 and B-CAP cells at 48hr after Dkk-1 treatment. Furthermore, 48 hrs-treatment with Dkk-1 (50nM) also inhibited cell migration activity by 70 % in SNU-790 and 68% in B-CPAP cells (Fig.13). These findings suggests that Dkk-1 inhibited cell migration/invasion of human PTC cells by functional rescures of membranous E-cadherin.

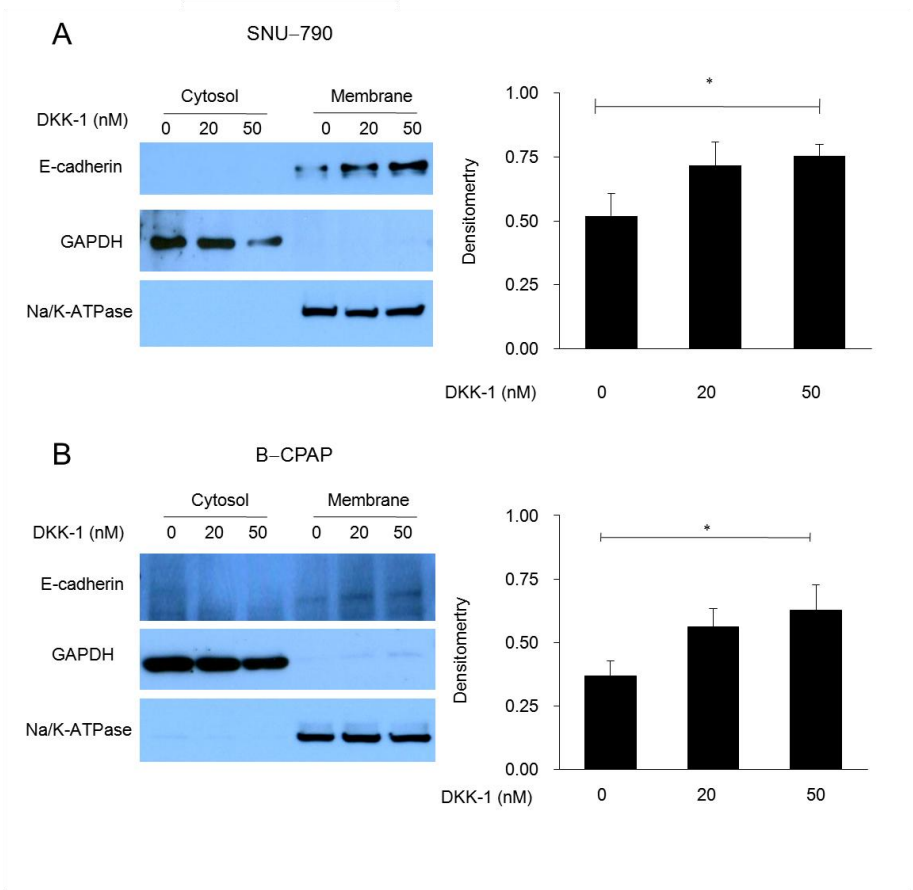

Figure 12. Effect of Dkk-1 on E-cadherin expression in human PTC cells. Cells were treated with Dkk-1 (0, 20, or 50 nM) for 48hrs following subcellular fractionation and Western blot analysis. Representative images (left) and densitometric analysis (right) of cytoplasmic or membranous expression of E-cadherin in (A) SNU–790 and (B) B–CPAP cells. Each bar represents mean±SEM of more than three independent experiments. $^{*}P<0.05$.

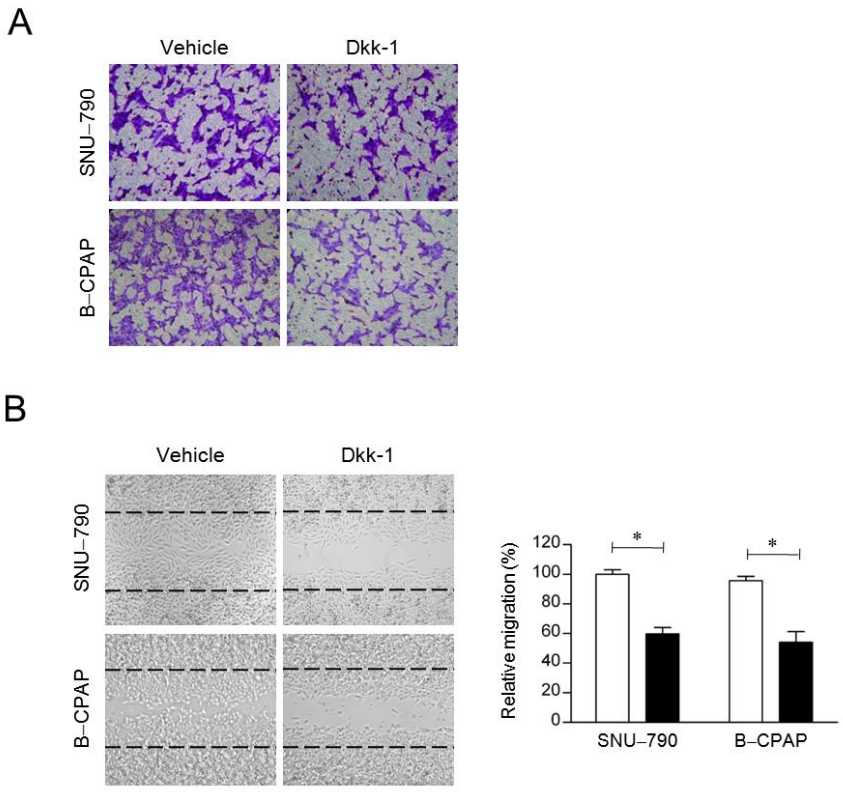

Figure 13. Effect of Dkk-1 on cell migration potential in human PTC cells.

Cell migration assay was performed using the Transwell system, which allows cells to migrate through an 8-μm pore size polycarbonate membrane. SNU-790 or B-CPAP cells were treated with vehicle or Dkk-1 (50nM) for 48hrs and migrated cells were stained with Crystal Violet (A). Wound healing assay in SNU-790 or B-CPAP cells. Cells were cultured up to 100% confluency, then the cell monolayer was wounded with a p10 tip (width=10mm). Vehicle or Dkk-1 (50nM) was treated immediately after scratching, incubated at 37℃, and observed at 24 and 48 hr. Representative images are captured at 24 hr and 48 hr (B, left). The length of migration was

quantified by measuring the total distance between the edge of the wound (shadow of pre-designed line) and the closest cell to the center at 24 hr and 48hr (B, right). All experiments were performed at least 2 times with duplication within each individual experiment. Data are expressed as mean±SEM of the length. $^{*}P<0.05$.

Discussion

In this study, Dkk-1 inhibited tumor cell growth by restoring the aberrant expression of β-catenin from the cytoplasm to the membrane in PTC. In this way, Dkk-1 reversed the loss of membranous expression of E-cadherin, inhibiting cell migration and invasion. To our knowledge, this is the first study showing that Dkk-1 rescued the loss of membranous E-cadherin expression in human cancer cells.

A growing body of evidence indicates the relationship between Wnt/β-catenin signaling and thyroid cancers. Several studies with immunohistochemical staining revealed that the translocation of β-catenin from membrane to cytoplasm was observed in both follicular and papillary thyroid cancer [31, 32]. Ishigaki et al. reported that cytoplasmic dominant expression of β-catenin was observed in 67% of human PTC, while the incidence was ~10% in the current study despite the similar clinicopathologic characteristics such as age, sex, and tumor size between the two studies [19]. The reason for this discordance is currently unknown. Since a recent study showed that $BRAF^{V600E}$ mutation was associated with Wnt signaling [33], the different incidence or status of $BRAF^{V600E}$ mutation would be one of the possible explanations. Indeed, recent meta-analysis showed a markedly higher incidence of $BRAF^{V600E}$ mutation in our Korean population *vs.* the Japanese or other western populations [34]. Further study is needed to validate the relationship between β-catenin and the $BRAF^{V600E}$ mutation in PTC

pathogenesis. Additionally, in contrast to the previous study [19], we found no significant relationship between dislocation of β-catenin and overexpression of cyclin D1. This could be explained by the relatively low incidence of aberrant expression of β-catenin (~10%) in this study. Another possible explanation is that alternative signaling, such as the ras-MAPK pathway, might be involved in activation of cyclin D1 [35]. Intriguingly, in contrast to the nuclear shifting of cyclin D1, loss of E-cadherin correlated significantly with the dislocation of β-catenin in PTCs.

Wnt signaling is well characterized in playing a role not only in oncogenesis but also in organogenesis (reviewed in ref [36]). Accordingly, Dkk-1 treatment for therapeutic purposes in various human pathophysiologic conditions has been challenged [37-39]. Antibody-based inhibition of Dkk-1 is being developed for several human diseases such as osteoporosis [37], rheumatic disease [38, 39], and cancer related bone disease [40, 41]. However, the evidence for the therapeutic potential of Dkk-1 in primary tumors is still limited [25, 26, 42]. The diverse functions of Dkk-1 in human cancers restrict its therapeutic application. Dkk-1 was significantly up-regulated in pancreatic [42], esophageal and non-small cell lung cancers [43], while it was down-regulated in colon cancer [23] and hematocellular carcinoma [44].

In the present study, treatment of Dkk-1 clearly inhibited cell survival in two different PTC cell lines by relocating β-catenin from the cytoplasm to cell membranes. The inhibitory effect of Dkk-1 on PTC cells was due to the promotion of cell apoptosis rather than the inhibition of cell proliferation. This

pro-apoptotic effect of Dkk-1 on cancer cells was supported by previous studies conducted using breast carcinoma cells [45], Hela cells [46], methotherlioma cells [24] and brain glioma cells [26]. Since PTC is well-known to be a representative slow-growing cancer, pro-apoptotic characteristics could be more beneficial than anti-proliferative characteristics in the therapeutic application of Dkk-1.

One of the novel findings in this study is that Dkk-1 rescued the loss of membranous expression of E-cadherin in PTC cell lines. In addition to Wnt/β-catenin, E-cadherin is a major cell-cell adhesion molecule (reviewed in [47]). In differentiated thyroid cancers, loss of E-cadherin expression has been reported in correlation with tumor invasion or metastasis [48-50]. However, the effect of Dkk-1 on E-cadherin expression has remained elusive. Recently, Kuphal et al. showed that over-expression of Dkk-3 rescued the loss of E-cadherin expression in malignant melanoma cells [23]. A disrupted interaction between E-cadherin and β-catenin in normal thyroid epithelial cells has been reported to lead to a papillary structural transformation, and sequential cytoplasmic translocation of β-catenin inducing cell proliferation [19]. In this aspect, the rescuing effect of Dkk-1 on E-cadherin expression might suggest a therapeutic potential in blocking the early oncogenic process of thyroid cancers.

In conclusion, our study provides a novel therapeutic potential for the anti-tumoral activity of Dkk-1 through Wnt/β-catenin signaling. Treatment of Dkk-1 effectively inhibited the cell survival in PTC cell lines via the Wnt/β-

catenin pathway and also inhibited cell migration by rescuing membranous expression of E-cadherin. This study suggests that Dkk-1 could be a new therapeutic option for PTC patients, and paves the way for further investigations to explore.

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국문초록

Wnt/β-catenin 신호전달체계는 배아 발생에 중요한 조절인자로, 갑상선암을 포함하여 여러 종류의 암에서 활성화 되어 있다고 알려져 있다. E-cadherin의 비정상적인 발현은 암의 침윤성 또는 전이성과 관련이 있는 것으로 보고된 바 있다. Dickkopf (Dkk) -1 은 Wnt/β-catenin 신호전달체계의 억제제로 갑상선암에서의 치료효과는 아직 알려진 바가 많지 않다. 본 연구에서는 갑상선암에서 Wnt/β-catenin 신호전달체계 관련 유전자의 활성화를 분석하고, Dkk-1의 치료적 효과에 대하여 규명하고자 하였다. 갑상선 암 조직에서 Wnt/β-catenin 신호 관련 단백질 wnt3, β-catenin의 발현 여부를 RT-PCR로 확인하였으며, tissue microarray를 제작하여 면역화학염색을 시행하여 β-catenin, cyclin D1과 E-cadherin을 관찰하였다. 두 종류의 갑상선암 세포주를 통해 Dkk-1 처리 전후 변화관찰을 통해 치료적 효과를 확인하였다. 본 연구 결과 갑상선암 조직에서 wnt3와 β-catenin 의 과발현이 관찰되었으며, tissue microarray 에서는 β-catenin 가 비정상적인 위치에서 관찰되었으며, 이는 E-cadherin의 발현 저하와 관련이 있었다. 갑상선암세포에 Dkk-1을 처리하였을 때 갑상선암세포의 세포사멸이 촉진되었으며, β-catenin의 비정상적인 위치가 정상화되었으며, E-cadherin의 발현도 회복되었다. Wnt/β-catenin 신호전달체계는 갑상선암에서

활성화되어 있음을 확인하였으며, Dkk-1은 wnt/β-catenin 신호전달체계와 E-cadherin 발현을 조절함으로써 갑상선암세포의 생존과 이주를 억제하였다. 향후 갑상선암의 치료에서 Dkk-1을 이용한 Wnt/β-catenin 차단이 새로운 방법을 제시할 수 있을 것으로 생각되며 추후 기전에 대한 지속적인 연구가 필요할 것으로 생각한다.

주요어 : Dkk-1, Wnt, β-catenin, E-cadherin, Papillary thyroid cancer

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