Therapeutic Potential of Dickkopf-1 in Wild-Type BRAF Papillary Thyroid Cancer via Regulation of β-Catenin/E-cadherin Signaling

Sun Wook Cho,* Young A. Kim,* Hyun Jin Sun, Hwa Young Ahn, Eun Kyung Lee, Ka Hee Yi, Byung-Chul Oh, Do Joon Park, Bo Youn Cho, and Young Joo Park

Department of Internal Medicine (S.W.C., H.Y.A., D.J.P., B.Y.C., Y.J.P.), Seoul National University College of Medicine, Seoul 110-744, Korea; Department of Internal Medicine (S.W.C., H.J.S.), National Medical Center, Seoul 110-799, Korea; Departments of Pathology (Y.A.K.) and Internal Medicine (K.H.Y.), Seoul National University Boramae Medical Center, Seoul 156-707, Korea; Department of Internal Medicine (H.Y.A., B.Y.C.), Chung-Ang University Hospital, Seoul 156-755, Korea; Lee Gil Ya Cancer and Diabetes Institute (B-C.O.), Gachon University Graduate School of Medicine, Incheon 405-760, Korea; and Center for Thyroid Cancer (E.K.L.), National Cancer Center, Gyeonggi-do 410-769, Korea

Background: Aberrant activation of the Wnt/β-catenin pathway is a common pathogenesis of various human cancers. We investigated the role of the Wnt inhibitor, Dkk-1, in papillary thyroid cancer (PTC).

Methods: Immunohistochemical β-catenin staining was performed in tissue microarray containing 148 PTCs and five normal thyroid tissues. In vivo effects of Dkk-1 were explored using ectopic tumors with BHP10–3SC cells.

Results: In 27 PTC patients, 60% of patients showed β-catenin up-regulation and *Dkk-1* down-regulation in tumor vs normal tissues. Tissue microarray analysis showed that 14 of 148 PTC samples exhibited cytoplasmic-dominant β-catenin expression compared to membranous-dominant expression in normal tissues. Aberrant β-catenin expression was significantly correlated with higher rates of the loss of membranous E-cadherin expression and poor disease-free survival than that in the normal membranous expression group over a median follow-up period of 14 years. Implantation of Dkk-1-overexpressing BHP10–3SC cells revealed delayed tumor growth, resulting from the rescue of membranous β-catenin and E-cadherin expressions. Furthermore, tissue microarray analysis demonstrated that $BRAF^{WT}$ patients had higher rates of aberrant expressions of β-catenin and E-cadherin than $BRAF^{V600E}$ patients. Indeed, the inhibitory effects of Dkk-1 on cell survival were more sensitive in $BRAF^{WT}$ (BHP10–3SC and TPC-1) than in $BRAF^{V600E}$ (SNU-790 and BCPAP) cells. Overexpression of $BRAF^{V600E}$ in normal thyroid epithelial (H tori) cells also reduced the effects of Dkk-1 on cell survival.

Conclusion: A subset of PTC patients showed aberrant expression of β-catenin/E-cadherin signaling and poor disease-free survival. Dkk-1 might have a therapeutic role, particularly in $BRAF^{WT}$ patients. **(*J Clin Endocrinol Metab* 99: E1641–E1649, 2014)**

Papillary thyroid cancer (PTC) is the most prevalent endocrine cancer (1), and the incidence of newly diagnosed PTC has recently increased worldwide (2). Although the overall survival rate of PTC exceeds 90% and has been increasing over recent decades, there is still a subset of PTC patients that shows limited response to con-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received December 19, 2013. Accepted May 7, 2014.
First Published Online May 21, 2014

* S.W.C. and Y.A.K. contributed equally to this work.

Abbreviations: APC, adenomatous polyposis coli; Dkk-1, Dickkopf-1; GFP, green fluorescent protein; PTC, papillary thyroid cancer; WT, wild-type.

ventional treatment modalities such as surgery, radioiodine therapy, and TSH-suppressive T_4 therapy (2, 3). Currently, there are no effective treatment strategies for persistent or metastatic PTC that is resistant to radioactive iodine. There are ongoing trials using redifferentiation agents such as retinoids (4) or using lithium (5) or tyrosine kinase inhibitor (6) treatments for radioactive iodine-resistant PTC; however, their therapeutic effects are limited. Thus, novel treatment strategies for PTC are needed.

The Wnt/β-catenin pathway is an extensively studied human oncogenic signaling system (7), and β-catenin is well-characterized as a major player in canonical Wnt signaling. In the absence of Wnt protein, β-catenin localizes at the plasma membrane, creates a complex with α-catenin and E-cadherin, and regulates cell–cell adhesion (8). Unbounded cytosolic β-catenin is rapidly degraded by adenomatous polyposis coli (APC)/glycogen synthase kinase-3β/Axin complexes, resulting in low levels of β-catenin in cytoplasm. Once activated, β-catenin translocates to the nucleus and stimulates transcription of target genes such as *cyclin D1* and *c-myc* (7). Aberrant activation of Wnt/β-catenin signaling related to diverse mutations in *β-catenin*, *APC*, or *Axin* genes has been demonstrated in human colorectal cancers (9), hepatocellular carcinomas (10), and thyroid cancers (11).

Dickkopf (Dkk)-1 is an extracellular protein that was originally characterized as a Wnt inhibitor. Binding to the lipoprotein receptor-related protein (LRP) 5/6 receptor, Dkk-1 inhibits the formation of Wnt–Frizzled–LRP5/6 receptor complexes and blocks Wnt/β-catenin signaling (12). Loss of Dkk-1 expression has been associated with advanced stages and poor prognoses in several human cancers including gynecological (13), malignant melanoma (14), and mesothelioma (15). However, Dkk-1 overexpression has been reported to inhibit cell tumorigenicity (16). A previous study showed that aberrant expressions of β-catenin and E-cadherin in human PTC cell lines could be rescued by Dkk-1 treatment and resulted in decreased cell survival and cell migration, suggesting a therapeutic potential of Dkk-1 treatment in human PTC (17).

Recently, several reports have suggested that there is a possible association between the BRAF and Wnt signaling pathways (18, 19) and that *BRAF* mutation is a common source of oncogenic signaling in thyroid cancer (20). Therefore, the inhibitory effects of Dkk-1 on thyroid cancer cells might differ according to the mutational status of *BRAF*; thus, an accurate description of the BRAF–Dkk-1 relationship would be very important for clinical applications.

The aim of this study was to investigate β-catenin/E-cadherin expressions in human PTC and determine the therapeutic effects of Dkk-1 in ectopic tumor models in vivo, with a special focus on *BRAF* mutational status.

Subjects and Methods

Study subjects, construction of tissue microarrays, immunohistochemical staining, and RT-PCR analysis

Thyroid tissue samples were obtained at the time of surgery from patients who had undergone thyroidectomy. For tissue microarray purposes, we examined 228 tissue samples obtained from the January 1993 to December 2003 surgical pathology files of the departments of pathology at Seoul National University Boramae Medical Center and Seoul National University Hospital. Those tissue samples comprised samples from patients with normal thyroid (n = 5), PTC (n = 148), and anaplastic thyroid cancer (n = 18). For the quantitative RT-PCR experiment, we obtained 27 PTC tissues paired with normal thyroid tissues from surgical pathology files of the center for thyroid cancer at the National Cancer Center of Korea.

All experimental procedures were conducted in accordance with the guidelines involving humans proposed in The Declaration of Helsinki (http://www.wma.net). Moreover, all experiments were approved by the institutional review boards of Seoul National University Hospital (1107-060-369), Seoul National University Boramae Medical Center (06-2010-176), and the National Cancer Center (NCCNCS-13-770). More details are included in the Supplemental Materials and Methods.

Animal studies

Athymic nu/nu mice (6-wk-old, female) were purchased from Japan SLC. All animal experiments were performed under an approval from the Institutional Animal Care and Use Committee of Seoul National University. To obtain the ectopic tumor model, BHP10–3SC overexpressing Dkk-1 or control cells (1×10^7/100 μL PBS) were mixed with growth factor-reduced Matrigel (70 μL, 4°C; BD Biosciences), followed by sc injection into four different points along the dorsal skinfold in nude mice. Injected mice were regularly monitored for tumor development during the following 4 weeks, and tumors were measured at least twice a week. Tumor size was measured with a caliper and calculated as a volume by using the equation: volume = $\frac{1}{2} \times a \times b^2$, where a = long tumor diameter and b = short tumor diameter (21). After 5 weeks, at least two measurable tumors were observed in each mouse (seven mice/group). Mice were killed, and tumors were surgically removed and fixed with 10% formalin. Among them, one tumor section per mouse (total of seven tumor sections per group) were selected, and immunohistochemical staining was performed with antihuman β-catenin and antihuman E-cadherin antibodies.

Cell cultures, transduction, viability study, and Western blot analysis

Several PTC cells were treated with 20 nM Dkk-1, WIF-1, SFRP-1, or vehicle for 48 hours, and cell viability was assessed. H tori/$BRAF^{V600E}$ or H tori/green fluorescent protein (GFP) cells were treated with Dkk-1 (20 nM) for 48 hours, after a cell viability test or Western blot analysis for Erk phosphorylation.

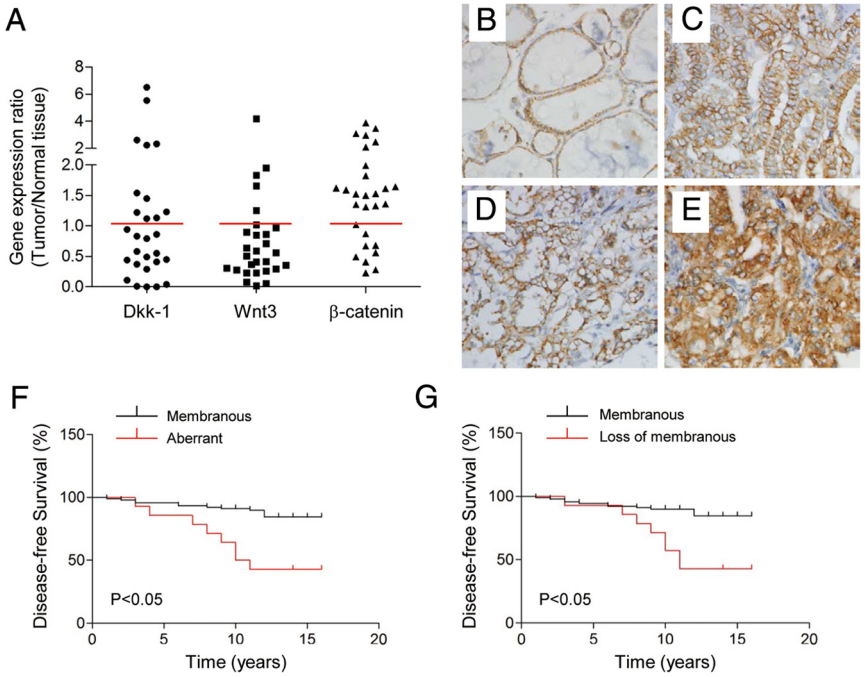

Figure 1. Wnt/β-catenin signaling in human thyroid tissues and disease-free survival for PTC patients according to the β-catenin/E-cadherin signaling status. A, mRNA expression ratios of human *Dkk-1*, *Wnt 3*, and *β-catenin* in tumor and paired normal thyroid tissues from PTC patients (n = 6). B–E, Immunohistochemical staining of β-catenin: membranous staining of normal thyroid tissue (B) and PTC (C); decreased or discontinuous membranous expression with cytoplasmic granular staining (D); and cytoplasmic granular staining of PTC (E). Original magnification, ×400. F and G, Cumulative disease-free survival for the patients with PTC according to the β-catenin (F) and E-cadherin expressions (G).

More details are included in Supplemental Materials and Methods.

Statistical analysis

For analysis of continuous variables, means and SE values were calculated. After distribution normality was determined by using the Kolmogorov-Smirnov test, the Kruskal-Wallis test with the Mann-Whitney *U* test or a one-way ANOVA with post hoc analysis was applied to the data. For analysis of categorical variables, frequencies and percentages were determined. Proportions were compared using the χ^2 test or Fisher's exact test. Statistical analysis was performed by using SPSS version 12.0 (SPSS, Inc) or GraphPad Prism version 5 (GraphPad Software). All *P* values are two-sided, and a $P < .05$ was considered statistically significant.

Results

Aberrant expression of β-catenin and E-cadherin in human thyroid tissues

To verify the status of Wnt/β-catenin signaling in human PTC tissues, we first investigated the endogenous gene expression of Wnt-related genes, *Dkk-1*, *Wnt3*, *Wnt10B*, and *β-catenin* in tumor vs normal paired thyroid tissues from 27 PTC patients. The age of diagnosis was 45.8 ± 10.6 years, and the average size of the biggest tumor was 1.7 ± 1.1 cm. Among them, 16 (64%) and 19 (76%) patients showed down-regulation of *Dkk-1* and *Wnt3*, respectively, whereas 15 (60%) patients showed up-regulation of *β-catenin* (Figure 1A). There were no significant correlations between *Dkk-1* and *Wnt3* or *β-catenin* (Supplemental Figure 1). The expressions of *Wnt10B* were undetectable in both normal and cancer tissues. Interestingly, tumor size was significantly bigger in PTC with the down-regulated Dkk-1 group than the up-regulated Dkk-1 group, whereas extrathyroidal invasion or lymph node metastasis showed no difference between groups (Supplemental Table 1).

Next, to check the downstream signal activities of Wnt proteins, we performed immunohistochemical staining of β-catenin in the tissue microarray blocks containing normal thyroid (n = 5) and PTC (n = 148) tissues. Representative images of various patterns of β-catenin immunostaining activity are shown in Figure 1, B–E. All normal thyroid tissues (Figure 1B) and approximately 90% of PTC tissues (Figure 1C) showed normal β-catenin membranous immunostaining activity, whereas 9.5% of PTC samples showed aberrant β-catenin immunostaining activity (Figure 1, D and E). Eight of the 148 PTC tissue samples (5.4%) showed low or discontinuous membranous staining with cytoplasmic staining (Figure 1D), whereas six of those 148 (4.1%) showed a predominance of cytoplasmic and/or nuclear staining (Figure 1E).

To evaluate the effect of this aberrant localization of β-catenin on downstream targets, we performed immunohistochemical analysis of E-cadherin and cyclin D1 activity in the tissue microarray samples. Loss of membranous E-cadherin expression was significantly correlated with cytoplasmic translocation of β-catenin in PTC samples, but was not correlated with positive cyclin D1 nuclear staining (Table 1).

Clinical characteristics of PTC in relation to β-catenin expression

Table 2 summarizes the clinicopathological characteristics of PTC as indicated by the β-catenin/E-cadherin expression patterns of normal membranous vs aberrant or lost membranous immunostaining. Age at diagnosis, sex, tumor size, extrathyroidal invasion, and lymph node me-

Table 1. Association Between Localization of β-Catenin and Cyclin D1/E-cadherin in PTC Subjects

	β-Catenin			
	Normal Membranous	Decreased Membranous	Cytoplasmic and/or Nucleus	*P* Value
n	134	8	6	
E-cadherin				<.001
Normal	132 (98.5)	2 (25)	1 (16.7)	
Loss	2 (1.5)	6 (75)	5 (83.3)	
Cyclin D1				.210
Negative	4 (3)	0 (0)	0 (0)	
Positive	130 (97)	8 (100)	6 (100)	

Data are expressed as number (percentage).

tastasis showed no significant difference between the different β-catenin expression pattern groups; however, the rate of locoregional recurrence or distant metastasis was significantly higher in the aberrant β-catenin group than in the normal group (46 vs 12%; $P = .013$; Table 2). Similarly, although clinicopathological characteristics showed no significant difference between the different E-cadherin expression groups, the group exhibiting a loss of E-cadherin had a higher rate of locoregional recurrence or distant metastasis than that in the normal expression group (50 vs 12%; $P = .007$; Table 2). Moreover, the disease-free survival rate for PTC patients clearly revealed that patients with aberrant β-catenin expression (Figure 1F) or loss of membranous E-cadherin expression (Figure 1G) had poor disease-free survival rates. Five- and 10-year recurrence rates were 1 vs 6% and 2 vs 10%, respectively ($P < .001$ from log-rank test), with 14.2 ± 0.4 years of the mean follow-up duration.

Therapeutic effects of Dkk-1 overexpression on xenograft of human PTC in vivo

A previous study demonstrated that Dkk-1 inhibited cell survival and cell migration by regulating both Wnt/β-catenin signaling and E-cadherin expression in several human PTC cell lines (17). To investigate whether the effects of Dkk-1 on PTC cells were owing to the direct inhibition of Wnt molecules or not, other Wnt inhibitors such as WIF-1 or SFRP-1 that directly quench Wnt molecules and inhibit the signaling were tested. As shown in Figure 2A, WIF-1 or SFRP-1 showed no effects on PTC cell viability, whereas Dkk-1 inhibited PTC cell survival up to 35%.

To verify further the therapeutic potential of Dkk-1 overexpression on human PTC, an in vivo ectopic tumor experiment using athymic nude mice was performed. By using a retroviral system, we initially established a stable cell line: BHP10–3SC cells overexpressing Dkk-1 (BHP10–3SC/Dkk-1). BHP10–3SC cells containing pMSCV-HA-GFP (BHP10–3SC/GFP) were used as a control. Flow cytometric analyses showed that the transduction efficacy of a retroviral system was more than 90% (Figure 2B). From day 21 to day 35, BHP10–3SC/Dkk-1 exhibited slower tumor growth than that in BHP10–3SC/GFP (Figure 2, C and D), indicating that Dkk-1 overexpression inhibits tumor growth. Moreover, tumors in the BHP10–3SC/GFP showed a predominance of cytoplasmic expression of β-catenin and a loss of membranous expressions of E-cadherin, whereas tumors in BHP10–3SC/Dkk-1 showed a predominance of membranous β-catenin expression and a relatively strong expression of membranous E-cadherin (Figure 2E). Collectively, overexpression

Table 2. Correlation Between β-Catenin/E-cadherin Staining and Various Clinicopathological Factors in PTC Subjects

	β-Catenin			E-cadherin		
	Normal Membranous	Aberrant	*P* Value	Normal Membranous	Loss of Membranous	*P* Value
n	134	14		135	13	
Age at diagnosis, y	50.3 ± 15.9	48.6 ± 18.5	.702	49.7 ± 15.6	53.1 ± 19.8	.062
Female gender	96 (71.6)	10 (62.5)	.674	95 (71.4)	11 (64.7)	.326
Tumor size, cm	2.4 ± 1.4	2.6 ± 1.0	.412	2.4 ± 1.5	2.5 ± 1.0	.489
Extrathyroidal invasion	64 (66.7)	11 (78.6)	.457	61 (64.9)	14 (87.5)	.572
Lymph node metastasis	68 (50.7)	7 (50.0)	.435	67 (49.6)	8 (61.5)	.275
Recurrence or distant metastasis	8 (11.8)	5 (45.5)	.013	8 (11.6)	5 (50.0)	.007

Data are expressed as mean ± SD or number (percentage).

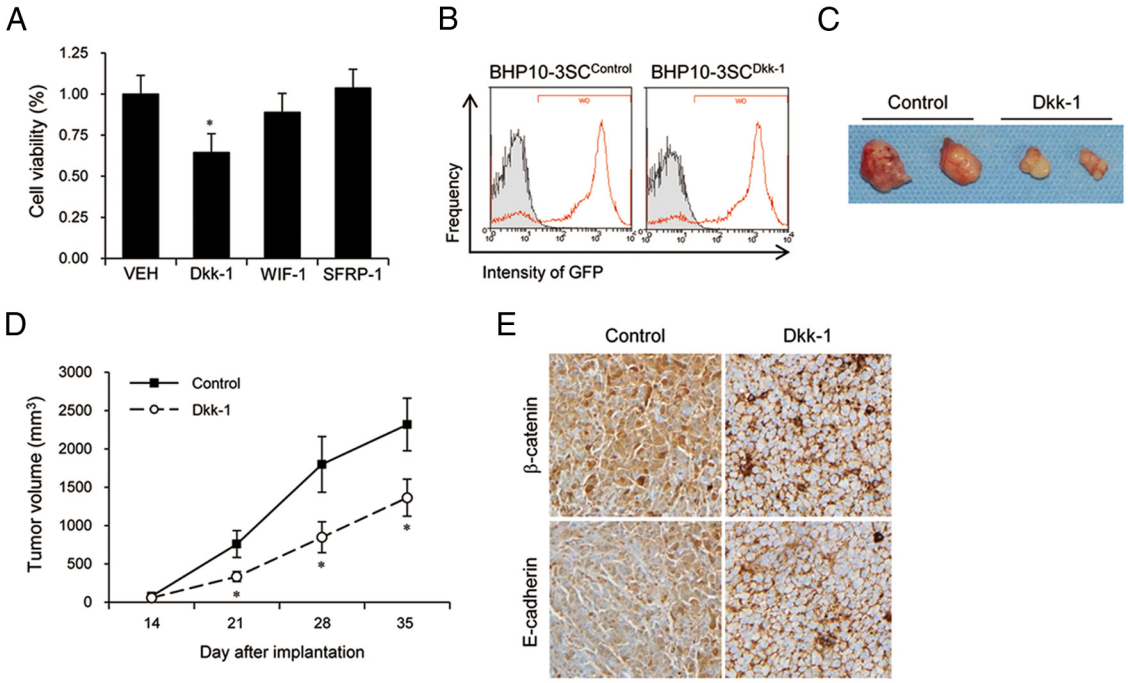

Figure 2. Effects of Dkk-1 overexpression on PTC tumor growth in vivo. A, BHP10–3SC cells were treated with 20 nM of Dkk-1, WIF-1, or SFRP-1 for 48 hours, and cell viabilities were analyzed by CCK-8 assay. B, BHP10–3SC cells transduced with pMSCV-Dkk-1-HA-GFP (BHP10–3SC/Dkk-1) or pMSCV-HA-GFP (BHP10–3SC/GFP) containing retrovirus. GFP-positive cells were measured by flow cytometry. C and D, BHP10–3SC/Dkk-1 (Dkk-1) or BHP10–3SC/GFP (control) cells were sc injected (1×10^7 cells/tumor) into nude mice (female, 6 wk old; n = 7/ group). C, Tumors from control and Dkk-1 groups were surgically dissected on the same day and photographed. D, Tumor growth curves of control and Dkk-1 groups from day 14 to day 35 (14 tumors from 7 mice/group). Data are expressed as mean ± SEM of two independent experiments. *, $P < .05$ vs control. E, Immunohistochemical staining of β-catenin (upper) and E-cadherin (lower) staining on tumors from control and Dkk-1 groups at day 35.

of Dkk-1 inhibited PTC tumor growth in vivo and regulated β-catenin/E-cadherin signaling, suggesting a potential therapeutic effect of Dkk-1 on human PTC and indicating that down-regulation of Dkk-1 in PTC is one of the main causes of aberrant expression of β-catenin and loss of membranous expression of E-cadherin.

Different β-catenin expression according to BRAF mutation status

To evaluate whether there was an association between *BRAF* mutation and Wnt signaling pathways in thyroid cancer, we analyzed β-catenin and E-cadherin expressions in two types of *BRAF*: wild-type (WT) and V600E-mutation type. Interestingly, aberrant expressions of both β-catenin and E-cadherin were significantly higher in $BRAF^{WT}$ than in $BRAF^{V600E}$ (18.2 vs 3.8%, $P = .032$; and 18.2 vs 6.3%, $P = .045$, respectively) (Table 3). In contrast, aberrant cyclin D1 expression was higher in $BRAF^{V600E}$ than $BRAF^{WT}$ (91.3 vs 81.8%; $P = .048$) (Table 3). Although, the clinicopathological characteristics showed no statistical difference between different BRAF mutational status in subgroups of different β-catenin/E-cadherin expressions, these subgroup analyses showed that the rate of recurrence or distant metastasis was significantly higher in loss of membranous E-cadherin groups only in the BRAF WT group (Supplemental Tables 2 and 3).

Different inhibitory effect of Dkk-1 according to BRAF mutation status

Human data showed low rates of aberrant β-catenin expression in PTC patients with $BRAF^{V600E}$ (Table 3). However, SNU-790 and BCPAP, PTC cell lines harboring BRAFV600E, have been reported to show an aberrant β-catenin nuclear expression, which is decreasing with Dkk-1 treatment (17). On the other hand, $BRAF^{WT}$

Table 3. Relationship Between BRAF Types and β-Catenin, E-cadherin, and Cyclin D1 Staining Results

	BRAF Wild-type	BRAF V600E	*P* Value
β-Catenin			.032
Normal membranous	36 (81.8)	76 (96.2)	
Decreased membranous	5 (11.4)	2 (2.5)	
Cytoplasmic and/or nuclear	3 (6.8)	1 (1.3)	
E-cadherin			.045
Normal	36 (81.8)	74 (93.7)	
Loss	8 (18.2)	5 (6.3)	
Cyclin D1			.048
Negative	8 (18.2)	5 (8.8)	
Positive	36 (81.8)	73 (91.3)	

Data are expressed as number (percentage).

BHP10–3SC cells showed decreased membranous β-catenin expression, but nuclear relocalization of β-catenin was not clear (Supplemental Figure 2). Treatment of Dkk-1 also rescued membranous expression of β-catenin in BHP10–3SC cells (Supplemental Figure 2). Collectively, expressions of β-catenin in PTC cell lines were distinctive according to *BRAF* mutational status.

To derive a mechanistic insight into the relationship between β-catenin and BRAF signaling in thyroid cancer, we compared the effects of Dkk-1, an inhibitor of Wnt/β-catenin signaling, on different thyroid cancer cell lines harboring either $BRAF^{WT}$ (BHP10–3SC and TPC-1) or mutant $BRAF^{V600E}$ (SNU-790 and BCPAP). The inhibitory effects of Dkk-1 on cell viability were greater in BHP10–3SC or TPC-1 cells than in the SNU-790 or BCPAP cells (Figure 3A). When we treated all PTC cell lines with PLX4720, a selective BRAF inhibitor, there was no significant effect on BHP10–3SC cells (Δ45% in both treated and untreated cells). In TPC-1 cells, PLX4720 inhibited cell viability by 18%, whereas there were no synergistic effects of Dkk-1 and PLX4720 co-treatment. However, inhibition of BRAF signaling augmented the Dkk-1 inhibitory effect on cell survival in the two $BRAF^{V600E}$ cell lines (SNU-790, increase from Δ20% to Δ47%; BCPAP, increase from Δ25% to Δ36%; Figure 3A). These findings suggest that $BRAF^{V600E}$ mutation has a negative role in the inhibitory effects of Dkk-1 in contrast to its effect on Wnt/β-catenin/E-cadherin signaling. Because BHP10–3SC cells contain a *RET/PTC* genetic rearrangement abnormality, we generated $BRAF^{V600E}$ thyroid cells by using H tori (a normal thyroid epithelial cell line) and then examined the effects of $BRAF^{V600E}$ mutational status on the treatment responses of Dkk-1. The H tori cells were stably transduced with mutant $BRAF^{V600E}$ (H tori/$BRAF^{V600E}$) or a GFP control (H tori/GFP) by using a lentiviral system. Subsequent treatment with Dkk-1 (20 nM) inhibited cell viability not only in the PTC cell lines, but also in the H tori cells. Moreover, the inhibitory effects of Dkk-1 were greater in H tori/GFP than in H tori/$BRAF^{V600E}$ cells (Δ27% vs Δ15%; $P < .01$; Figure 3B). Furthermore, the inhibitory effects of Dkk-1 on ERK phosphorylation, which is well-characterized as a downstream signaling molecule of BRAF, were lower in H tori/$BRAF^{V600E}$ than in H tori/GFP (Δ16% vs Δ37%; $P < .05$; Figure 3C). Our results showed that $BRAF^{WT}$ cells were more sensitive than mutant $BRAF^{V600E}$ cells to the inhib-

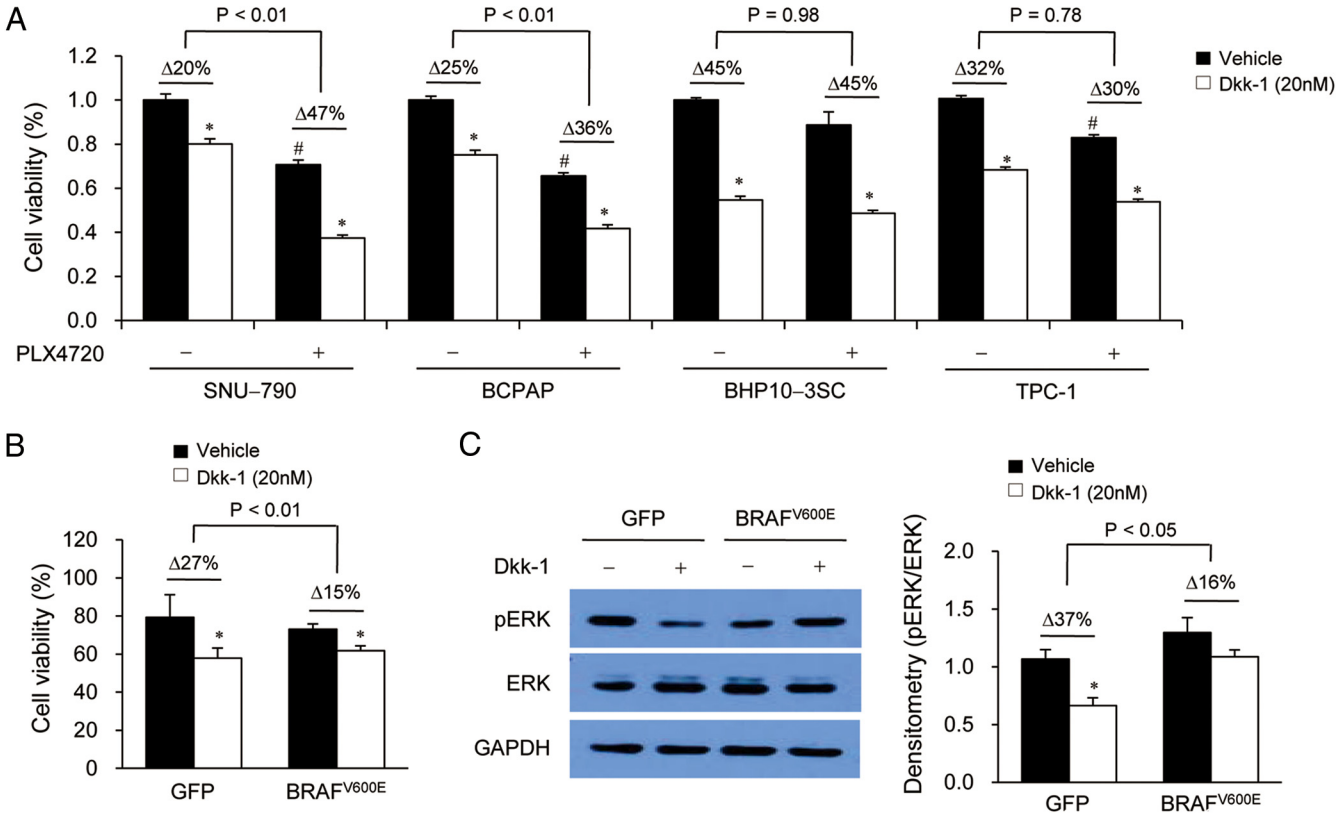

Figure 3. Effects of Dkk-1 on thyroid cells harboring $BRAF^{WT}$ or $BRAF^{V600E}$. A, PTC cells harboring $BRAF^{V600E}$ (SNU-790 and BCPAP) and $BRAF^{WT}$ (BHP10–3SC and TPC-1) were treated with Dkk-1 (20 nM) and/or PLX4720 (1 μg/mL) for 48 hours, and cell viabilities were analyzed by CCK-8 assay. B and C, H tori, a normal thyroid epithelial cell line, stably transduced with lentivirus containing pCAG-GFP (GFP) or pCAG-GFP-$BRAF^{V600E}$ ($BRAF^{V600E}$) were treated with Dkk-1 (20 nM) for 48 hours. B, Cell viabilities were analyzed by CCK-8 assay. C, Representative images and densitometry of Western blot analyses using anti-pERK, anti-ERK, and anti-GAPDH antibodies. All data are expressed as mean ± SEM of at least two independent experiments in triplicate. *, $P < .05$ vs vehicle; #, $P < .05$ vs vehicle-treated PLX4720(−) in each cell group.

itory effects of Dkk-1, indicating that the effects of Dkk-1 on thyroid cells are partly mediated by ERK signaling.

Discussion

Approximately 10% of the PTC patients in this study showed aberrant β-catenin and E-cadherin expressions and poor disease-free survival. The aberrant β-catenin/E-cadherin expressions were more frequent in $BRAF^{WT}$ patients than in $BRAF^{V600E}$ patients. Our ectopic tumor model results showed that Dkk-1 delayed tumor growth and resulted in relocation of aberrant β-catenin/E-cadherin expressions in vivo. Moreover, the inhibitory effects of Dkk-1 were more sensitive in PTC cells harboring $BRAF^{WT}$ than in those bearing $BRAF^{V600E}$ in vitro. The inhibitory effects of Dkk-1 were attenuated by co-treatment of a BRAF inhibitor in $BRAF^{V600E}$ PTC cells, suggesting a possible regulatory role of BRAF in Wnt/β-catenin signaling. Collectively, the data suggest that Dkk-1 might have therapeutic potential in a subset of PTC patients, in particular those with the $BRAF^{WT}$ genotype.

The aberrant expressions of Wnt/β-catenin signaling have been studied for more than a decade. Several mutations in Wnt component genes such as *β-catenin*, *APC*, and *Axin* have been described in anaplastic thyroid cancers, and the role of aberrantly activated β-catenin is well described (11). In the present study, we also analyzed 18 anaplastic thyroid cancer samples and confirmed that all of them showed cytoplasmic dominant expressions of β-catenin and near-complete loss of membranous expressions of E-cadherin (data not shown). However, the role of β-catenin in PTC remains to be described. Ishigaki et al (22) demonstrated that more than 50% of patients had aberrant cytoplasmic expression of β-catenin, whereas others reported no distinctive expression of β-catenin in PTC compared to that in normal or benign lesions (23). Another study reported no correlations among β-catenin, α-catenin, and E-cadherin expressions (24). The present study clearly demonstrated that aberrant β-catenin expression was significantly correlated with the loss of E-cadherin, but was not correlated with cyclin D1 expression. Furthermore, patients with aberrant β-catenin expression had poor disease-free survival compared to that in patients with normal membranous β-catenin expression. To our knowledge, this is the first study showing long-term clinical outcomes that were associated with β-catenin expression levels in PTC patients.

A previous study showed that Dkk-1, a Wnt inhibitor, inhibits PTC cell survival and cell migration by regulating β-catenin/E-cadherin signaling (17). In that study, treatment of Dkk-1 was shown to reduce nuclear expression of β-catenin and T-cell factor/lymphoid enhancer factor-dependent transcriptional activities. In addition, it restored membranous E-cadherin expression in PTC cell lines. Consistent with those in vitro data, the present study shows that Dkk-1 can significantly delay PTC tumor growth in vivo, and it can restore normal membranous expression of β-catenin and E-cadherin in ectopic PTC tumors. Furthermore, the present study demonstrated that the inhibitory effects on cell survival were specific to Dkk-1 compared with WIF-1 or SFRP-1, which directly quenches Wnt molecules. These data suggested that the major abnormality in PTC is a decrement of Dkk-1 rather than an increment of Wnt, and Dkk-1 has an independent role over inhibiting Wnt signaling.

In this study of a large PTC population (n = 148), we demonstrated that approximately 10% of patients had aberrant β-catenin expressions, a proportion that is less than that reported in other studies (22). This might be explained by the higher rate of $BRAF^{V600E}$ mutations in our study population, that is, 64% in this study and 15% to approximately 30% in study populations in other countries (25, 26); however, our study percentage is consistent with the reported range of $BRAF^{V600E}$ prevalence in Korea (60–85%) (27). The reason for the smaller frequency of aberrant expressions of β-catenin and E-cadherin in $BRAF^{V600E}$ mutated PTC is not clear. There is a report that anaplastic thyroid cancer comprises distinct subtypes of tumors that contain mutually exclusive mutations; for example, Wnt/β-catenin, p53, or activated phosphatidylinositol 3′-kinase/Akt signaling (28). In the same vein, aberrant Wnt/β-catenin signaling and the $BRAF^{V600E}$ mutation might be relatively exclusive to PTC, but that relationship has not been described.

On the other hand, studies of PTC cell lines in this study suggest possible interactions between the BRAF pathway and Wnt/β-catenin signaling. First, in $BRAF^{WT}$ PTC cells, there was a decrease in the expression of membranous β-catenin, which was rescued by Dkk-1 treatment. Second, the inhibitory effects of Dkk-1 on PTC cell survival were more potent in $BRAF^{WT}$ than in $BRAF^{V600E}$ cells. Similar results were obtained in $BRAF^{V600E}$-overexpressed normal thyroid epithelial cells. Furthermore, these Dkk-1 effects in $BRAF^{V600E}$ PTC cells could be augmented by inhibition of BRAF signaling, indicating a regulatory role of BRAF kinase in Wnt/β-catenin signaling in PTC. Previous studies showed that ERK signaling was activated by activation of the canonical Wnt/β-catenin pathway (29) and ERK is the major downstream molecule in the BRAF pathway; it is reasonable to deduce that the inhibitory effects of Dkk-1 on thyroid cell survival may be mediated, at least in part, by the Wnt-ERK pathway. Thus, we assessed the phosphorylation of ERK after Dkk-1

treatment, and, consistent with the effects on cell survivals, there was no reduction in ERK phosphorylation after Dkk-1 treatment in $BRAF^{V600E}$ cells.

Several recent studies suggested that RET-mediated oncogenesis is mediated through the β-catenin signaling in multiple endocrine neoplasia type 2 and in sporadic thyroid cancers (30, 31). BHP10–3SC and TPC-1, $BRAF^{WT}$ PTC cell lines, used in the present study, contained *RET*/PTC rearrangement which activate the transforming potential of RET kinase. In aspects of the interactions between RET and β-catenin signaling, the different effects of Dkk-1 between cells harboring $BRAF^{W}$/*RET*/PTC rearrangement and $BRAF^{V600E}$ might be related not only to the *BRAF* mutation status, but also to the *RET*/PTC mutation status. Further studies are needed.

Recently, several reports have shown relationships between $BRAF^{V600E}$ mutations and Wnt/β-catenin signaling in human melanoma. One such study showed that β-catenin is required for activation of the anticancer effects of the $BRAF^{V600E}$ inhibitor PLX4720, and a reduction of AXIN1, an antagonist of β-catenin signaling, was shown to enhance the therapeutic effects of PLX4720 (18). Those authors also suggested that BRAF signaling negatively regulates Wnt/β-catenin signaling. Using gene expression profiling and pathway analyses, others demonstrated that $BRAF^{V600E}$ clones exhibit lower Dkk-1 expression levels than those in $BRAF^{WT}$ cells and suggested possible associations between BRAF and Wnt/β-catenin signaling (32). Although we previously detected no difference in expression of Dkk-1 between $BRAF^{WT}$ and $BRAF^{V600E}$ cells (17), the current study results support the possibility of cross talk between the BRAF/ERK pathway and Wnt signaling in PTC.

Another study has demonstrated that sulindac, a nonsteroidal anti-inflammatory drug, reverses β-catenin activities in PTC cells with the $BRAF^{V600E}$ mutation but did not do so in $BRAF^{WT}$ cells (33). In addition, nonsteroidal anti-inflammatory drugs have been reported to enhance proteasomal degradation of cytoplasmic β-catenin in human cancers including colon (34), breast (35), and thyroid (33) cancers. Taken together, it would be valuable to consider therapeutic stratification of PTC patients based on molecular characteristics over histological subtypes.

In conclusion, a subset of PTC patients showed aberrant expressions of β-catenin/E-cadherin signaling and poor disease-free survival. Dkk-1 treatment inhibited PTC tumor growth in vivo, and the inhibitory effects of Dkk-1 on cell survival were greater in $BRAF^{WT}$ cells than in $BRAF^{V600E}$ cells, suggesting that Dkk-1 might have a therapeutic potential in PTC, especially in $BRAF^{WT}$ PTC patients.

Acknowledgments

Address all correspondence and requests for reprints to: Young Joo Park, MD, PhD, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yungun-dong, Chongno-gu, Seoul 110-744, Korea. E-mail address: yjparkmd@snu.ac.kr.

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A3007152).

Disclosure Summary: All authors have no conflict of interest.

References

1. **Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA.** High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Res*. 2003; 63:1454–1457.
2. **Cho BY, Choi HS, Park YJ, et al.** Changes in the clinicopathological characteristics and outcomes of thyroid cancer in Korea over the past four decades. *Thyroid*. 2013;23:797–804.
3. **Cho SW, Choi HS, Yeom GJ, et al.** Long-term prognosis of differentiated thyroid cancer with lung metastasis in Korea and its prognostic factors. *Thyroid*. 2014;24:277–286.
4. **Koh CS, Ku JL, Park SY, et al.** Establishment and characterization of cell lines from three human thyroid carcinomas: responses to all-trans-retinoic acid and mutations in the BRAF gene. *Mol Cell Endocrinol*. 2007;264:118–127.
5. **Liu YY, van der Pluijm G, Karperien M, et al.** Lithium as adjuvant to radioiodine therapy in differentiated thyroid carcinoma: clinical and in vitro studies. *Clin Endocrinol (Oxf)*. 2006;64:617–624.
6. **Perez CA, Santos ES, Arango BA, Raez LE, Cohen EE.** Novel molecular targeted therapies for refractory thyroid cancer. *Head Neck*. 2012;34:736–745.
7. **Polakis P.** Wnt signaling and cancer. *Genes Dev*. 2000;14:1837–1851.
8. **Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A.** The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest*. 2002;109: 987–991.
9. **Iwao K, Nakamori S, Kameyama M, et al.** Activation of the β-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. *Cancer Res*. 1998;58:1021–1026.
10. **Nhieu JT, Renard CA, Wei Y, Cherqui D, Zafrani ES, Buendia MA.** Nuclear accumulation of mutated β-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am J Pathol*. 1999;155:703–710.
11. **Garcia-Rostan G, Tallini G, Herrero A, D'Aquila TG, Carcangiu ML, Rimm DL.** Frequent mutation and nuclear localization of β-catenin in anaplastic thyroid carcinoma. *Cancer Res*. 1999;59: 1811–1815.
12. **Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA.** Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol*. 2001;3:683–686.
13. **Jiang T, Wang S, Huang L, Zhang S.** Clinical significance of serum DKK-1 in patients with gynecological cancer. *Int J Gynecol Cancer*. 2009;19:1177–1181.
14. **Kuphal S, Lodermeyer S, Bataille F, Schuierer M, Hoang BH, Bosserhoff AK.** Expression of Dickkopf genes is strongly reduced in malignant melanoma. *Oncogene*. 2006;25:5027–5036.
15. **Lee AY, He B, You L, et al.** Dickkopf-1 antagonizes Wnt signaling independent of β-catenin in human mesothelioma. *Biochem Biophys Res Commun*. 2004;323:1246–1250.

16. **Mikheev AM, Mikheeva SA, Rostomily R, Zarbl H.** Dickkopf-1 activates cell death in MDA-MB435 melanoma cells. *Biochem Biophys Res Commun*. 2007;352:675–680.
17. **Cho SW, Lee EJ, Kim H, et al.** Dickkopf-1 inhibits thyroid cancer cell survival and migration through regulation of β-catenin/E-cadherin signaling. *Mol Cell Endocrinol*. 2013;366:90–98.
18. **Biechele TL, Kulikauskas RM, Toroni RA, et al.** Wnt/β-catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAFV600E in human melanoma. *Sci Signal*. 2012;5:ra3.
19. **Conrad WH, Swift RD, Biechele TL, Kulikauskas RM, Moon RT, Chien AJ.** Regulating the response to targeted MEK inhibition in melanoma: enhancing apoptosis in NRAS- and BRAF-mutant melanoma cells with Wnt/β-catenin activation. *Cell Cycle*. 2012;11:3724–3730.
20. **Kim TH, Park YJ, Lim JA, et al.** The association of the BRAF(V600E) mutation with prognostic factors and poor clinical outcome in papillary thyroid cancer: a meta-analysis. *Cancer*. 2012;118:1764–1773.
21. **Tomayko MM, Reynolds CP.** Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol*. 1989;24:148–154.
22. **Ishigaki K, Namba H, Nakashima M, et al.** Aberrant localization of β-catenin correlates with overexpression of its target gene in human papillary thyroid cancer. *J Clin Endocrinol Metab*. 2002;87:3433–3440.
23. **Huang SH, Wu JC, Chang KJ, Liaw KY, Wang SM.** Expression of the cadherin-catenin complex in well-differentiated human thyroid neoplastic tissue. *Thyroid*. 1999;9:1095–1103.
24. **Kapran Y, Ozbey N, Molvalilar S, Sencer E, Dizdarolu F, Ozarmaan S.** Immunohistochemical detection of E-cadherin, α- and β-catenins in papillary thyroid carcinoma. *J Endocrinol Invest*. 2002;25:578–585.
25. **Stanojevic B, Dzodic R, Saenko V, et al.** Mutational and clinicopathological analysis of papillary thyroid carcinoma in Serbia. *Endocr J*. 2011;58:381–393.
26. **Carta C, Moretti S, Passeri L, et al.** Genotyping of an Italian papillary thyroid carcinoma cohort revealed high prevalence of BRAF mutations, absence of RAS mutations and allowed the detection of a new mutation of BRAF oncoprotein (BRAF(V599lns)). *Clin Endocrinol (Oxf)*. 2006;64:105–109.
27. **Park SY, Park YJ, Lee YJ, et al.** Analysis of differential BRAF(V600E) mutational status in multifocal papillary thyroid carcinoma: evidence of independent clonal origin in distinct tumor foci. *Cancer*. 2006;107:1831–1838.
28. **Sastre-Perona A, Santisteban P.** Role of the wnt pathway in thyroid cancer. *Front Endocrinol (Lausanne)*. 2012;3:31.
29. **Yun MS, Kim SE, Jeon SH, Lee JS, Choi KY.** Both ERK and Wnt/β-catenin pathways are involved in Wnt3a-induced proliferation. *J Cell Sci*. 2005;118:313–322.
30. **Tartari CJ, Donadoni C, Manieri E, et al.** Dissection of the RET/β-catenin interaction in the TPC1 thyroid cancer cell line. *Am J Cancer Res*. 2011;1:716–725.
31. **Castellone MD, De Falco V, Rao DM, et al.** The β-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. *Cancer Res*. 2009;69:1867–1876.
32. **Parisi F, Micsinai M, Strino F, et al.** Integrated analysis of tumor samples sheds light on tumor heterogeneity. *Yale J Biol Med*. 2012;85:347–361.
33. **Cho NL, Lin CI, Whang EE, Carothers AM, Moore FD Jr, Ruan DT.** Sulindac reverses aberrant expression and localization of β-catenin in papillary thyroid cancer cells with the BRAFV600E mutation. *Thyroid*. 2010;20:615–622.
34. **Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS.** Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-β-catenin signaling axis. *Science*. 2005;310:1504–1510.
35. **Basu GD, Pathangey LB, Tinder TL, Gendler SJ, Mukherjee P.** Mechanisms underlying the growth inhibitory effects of the cyclooxygenase-2 inhibitor celecoxib in human breast cancer cells. *Breast Cancer Res*. 2005;7:R422–R435.