DKK1’S POTENTIAL ROLE AS A BIOMARKER IN PANCREATIC ADENOCARCINOMA

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ABSTRACT

DKK1’S POTENTIAL ROLE AS A BIOMARKER IN PANCREATIC ADENOCARCINOMA

By ESEOSASERE A GRACE IGBINIGIE

‘Under the direction of JINPING LI, M.D., Ph.D.

Dickkopf-1 (Dkk1)’s dysregulation has been implicated in the pathogenesis of a variety of cancers. It is part of the Dkk family of proteins that includes Dkk2, Dkk3 and Dkk4. This family of secreted proteins shares similar conserved cysteine domains and inhibits the Wnt/β-catenin pathway by causing the degradation of β-catenin, thereby stopping cell proliferation. Dkk1 has also been previously shown to affect the CKAP/Akt pathway to increase Akt phosphorylation and promote cell proliferation. To determine the location and pathway that Dkk1 may regulate in pancreatic cancer cells, we performed immunofluorescence assays on Suit-2 cells. The results showed that Dkk1 is mainly located in the nucleus with a small percentage of the proteins in the cytoplasm. For Dkk1’s potential receptors, CKAP4 was found to have a similar staining to Dkk1 while Lrp6 was found to be evenly spread through the nucleus and cytoplasm. Further staining with the Wnt/β-catenin downstream protein, β-catenin, showed that it was colocalized with Dkk1 in the nucleus indicating that Dkk1’s presence did not inhibit its ability to translocate into the nucleus. Further studies into the cause of Dkk1’s inability to degrade and stop β-catenin’s translocation that causes increased cell proliferation is needed.
INTRODUCTION

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) currently accounts for 3% of all cancer diagnoses and is often diagnosed during Stage III or IV of the disease. The American Cancer Society estimates that in 2017 there will be approximately 53,670 people diagnosed and 43,090 deaths due to this disease [1]. PDAC survival rates are low, with an average of 13% for Stage I, 6% for Stage II, 3% for Stage III, 1% for Stage IV, and a five-year survival rate of 8% [1]. These low survival statistics are due to not only the late diagnosis but also due to the lack of efficient therapies at that point of the cancer’s progression. There are no significant early signs and the symptoms are nonspecific, including back pain, fatigue, weight loss, and jaundice. The current late stage therapy includes chemotherapy, surgery, radiation, and adjuvant therapy, all of which have limited effectiveness. There is a dire need for early detection methods for this disease and for a better understanding of its mechanisms to help facilitate creation of effective therapies.

PDAC’s pathogenesis involves the activation of various oncogenes such as KRAS, PAK4 and MYB as well as the inactivation of tumor suppressors such as TP53, SMAD4 and PTEN in respective signaling pathways [2,3]. KRAS is a GTPase that is mutated in over 90% of PDAC cases on codon 12 of the gene [4,5]. It is responsible for relaying signals for cell proliferation through the MAPK/ERK pathway where activated Raf activates Mek through phosphorylation and, in turn, Mek activates Erk through phosphorylation [6,7]. Active Erk is responsible for phosphorylating and activating
transcription factors such as c-JUN and c-myc, which are responsible for the progression of the cell cycle [6]. MYB is another oncogene that has been found to be upregulated in PDAC [8,9]. Like KRAS, MYB plays a role in promoting PDAC malignancy through cell growth, proliferation, and metastasis [8]. PAK4 is a serine/threonine-protein kinase that has also been linked to the cell cycle and apoptosis [10]. It is often overexpressed or upregulated in cancers, playing a role in cell proliferation and invasion [11–13]. Specifically, in PDAC, it has been confirmed that mutated KRAS activates PAK4, which aids in the acquisition of invasive properties of PDAC cells [13]. Tumor suppressor TP53 is commonly known to induce apoptosis in damaged cells and is often mutated in cancer cells leading to uncontrolled cell growth [14,15]. It has been shown to be mutated in multiple PDAC cells lines [16]. SMAD4 is also another tumor suppressor found to be inactivated in over 55% of PDAC tumors [17]. Many studies have shown a correlation between high SMAD4 levels and extended patient survival [18–20]. PTEN is not mutated in pancreatic tumors, which is a big contrast from other cancers in which it is often found to be mutated [20,21]. Its expression levels are confirmed to be reduced in both mice models and the Panc-1 cell lines by TGF-β1, a growth factor that is overexpressed in cancer cells [20,22]. Many of these molecules are currently being studied in hopes of better understanding the pathology of PDAC and discovering new biomarkers for early detection and treatment.

Multiple biomarkers are currently being studied for new methods of early detection. Some of these potential biomarkers include PAM4 (sensitivity = 64%; specificity = 96%), REG4 (sensitivity = 94.9%; specificity = 64%), Mesothelin (sensitivity = 68%; specificity = 91%), uPAR (sensitivity = 93.6%; specificity = 53.2%),
RCAS (sensitivity = 85%; specificity = 42%), miR-200a (sensitivity = 84.4%; specificity = 87.5%), miR-200b (sensitivity = 71.1%; specificity = 96.9%), glycolytic enzyme enolase (1/2) autoantibody (sensitivity = 62%; sensitivity = 97%) and miR-1246 [23–28]. In addition to these, Dkk1 is also being studied for use as a potential early detection biomarker [29]. It has been proven to be upregulated in gastric, breast, endometrial, ovarian and lung cancer and downregulated in colorectal cancer [30–33]. Recent studies show that Dickkopf-1 (Dkk1) is overexpressed in pancreatic cancer tissues and could be a potential biomarker for the disease, with a sensitivity of 89.3% and specificity 79.3% in PDAC [29].

* **Dkk1 and its Family of Proteins**

Dkk1 is part of the Dkk family of proteins that includes Dkk2, Dkk3 and Dkk4. This family of secreted proteins shares similar conserved cysteine domains and also inhibits the Wnt/β-catenin pathway [34,35]. Structurally, Dkk1, -2, and -4 are more similar to each other than to Dkk3, because the linker region between the two cysteine domains is much shorter in Dkk3 than in the others (Figure 1) [34]. Dkk1, -2 and -4 also map to chromosomal regions in the same genetic paralogy group while Dkk3 does not. Dkk1 maps to 10q11, Dkk2 maps to 4q25 and Dkk4 maps 8p11, while Dkk3 maps to 11p15 [36]. The major form of Dkk1 has been shown to have N- and O-linked glycosylations with a molecular weight of approximately 29.5 KDa while the minor form only has an O-linked glycosylation with a molecular weight of 27 KDa [37]. Dkk2 has a molecular weight of approximately 15-17KDa and Dkk3’s is near 38 KDa [34]. Dkk4 has been shown to have 3 different forms, the first with a molecular weight of 40 KDa,
the second with molecular weight between 30-32 KDa, and the third with a molecular weight between 15-17 KDa [34].

Dkk2 has been shown to be both an activator and inhibitor of the Wnt pathway with the Kremen2 receptor acting as its on/off switch [35]. It promotes invasiveness and metastatic spread in Ewing sarcoma as well as plays a role in osteoblast differentiation [38,39]. Dkk3 was not initially thought to affect Wnt signaling, however, more recent studies have shown that it does [40]. Dkk3 knockdown mice has been shown to decrease hemoglobin and hematocrit levels, decrease tidal volumes of lung ventilation, and increase levels of Immunoglobulin M and natural killer cells [41]. In addition, Dkk3 regulates cell proliferation, apoptosis, collagen synthesis and T-cell responses [42–44]. Dkk4 is the least studied protein of this family and has been shown to have a smaller inhibitory effect on the Wnt pathway [45].

Dkk1 was initially studied for its role in *Xenopus* head development by Glinka *et al.* in 1998 [46]. This group discovered that Dkk1 was secreted by the Spemann organizer, an embryonic cluster of cells responsible for neural plate and head formation from nearby ectodermal cells, that inhibit the Wnt signaling pathway in *Xenopus* embryos [47]. The Spemann organizer is initially activated by the Wnt signaling pathway during early embryogenesis, and during late embryogenesis it is deactivated by Wnt inhibitors such as Dkk1 [47,48]. Krupnik *et al.* later isolated a cloned sequence of Dkk1 from a human fetal kidney cDNA library [34].

Out of the Dkk protein family, Dkk1 has been studied the most and has been implicated in Alzheimer’s disease, bone formation and cancer [45,46,49]. In Alzheimer’s
Dkk1 has been shown to be overexpressed in the brain and induced by β-amyloid peptide toxicity [49,50]. Dkk1 is also expressed in cultured cortical neurons exposed to βAP as well as in neurons from autopic brain samples of Alzheimer’s patients. Additionally, Dkk1-expressing neurons have a decreased number of dendritic branches. Dkk1 regulates bone formation by blocking osteoblast bone maturation [51,52]. It does so through the Wnt and RANKL pathway which have been known to cross-talk. Through the RANKL pathway, Dkk1 increases RANL levels which increases osteoclast activity and leads to bone resorption. Expression of Dkk1 varies in cancerous tissues, increasing in some due to positive feedback and decreasing in others due to epigenetic silencing [30,53].

*Pathways of Dkk1*

Dkk1 is involved in cell apoptosis through the Wnt signaling pathway. The Wnt signaling pathway is important in cellular processes and has been implicated in various activities including skeletal development, cell differentiation, cell polarity, bone metabolism, and myogenesis [54–57]. There are three forms of the Wnt signaling pathway: the non-canonical, Wnt/Ca²⁺ and the canonical pathway. The Wnt non-canonical pathway, also known as the β-catenin-independent pathway, is involved in regulating the actin cytoskeleton and cell polarity [57–60]. The Wnt/Ca²⁺ pathway, a branch of the non-canonical β-catenin independent pathway, plays many roles in zebrafish and *Xenopus* embryogenesis such as dorsal axis and heart formation [58,61–63]. The roles of the Wnt non-canonical and Wnt/Ca²⁺ pathways are very diverse and are not fully understood. The Wnt canonical pathway, also known as the β-dependent or
Wnt/β-catenin pathway, has been studied more in relation to cancer and the Dkk1 protein.

In the active Wnt canonical pathway, secreted Wnt glycoprotein binds to a co-receptor complex consisting of LDL receptor-related protein 5 (Lrp5), Lrp6 and Frizzled (Fz) (Figure 1) [64–68]. Wnt proteins bind the cysteine-rich N-terminal of the Fz receptor to recruit and activate the Disheveled (Dvl) protein [67–69]. The binding of Wnt, through conformational changes from an oligomeric to an active monomeric state, causes phosphorylation of a serine residue on the intracellular PPPSP motif of the Lrp5/6 receptors by G-protein coupled receptor kinases 5/6 (GRK5/6) [64,70–73]. Active Dvl binds Fz through its PDZ domain, causing disruption of a destruction complex of proteins that includes Axin, Adenomatosis Polyposis Coli (APC), Casein kinase 1 (Ck1), and Glycogen Synthase Kinase 3 (GSK3) [66,74–76]. Axin is a scaffolding protein that has been shown to bind to phosphorylated Lrp5/6, with the phosphorylated PPPSP motif acting as a docking site [64,71,77]. Separation of Axin from the destruction complex allows it to bind to Dvl through the DIX domains on both proteins, which increases stability of its downstream effector, β-catenin [75]. With the destruction complex inactivated, β-catenin remains active and is translocated into the nucleus, where it binds to a complex of proteins (TCF, BCL9, C13P & Pygo) to activate the transcription of oncogenes, c-myc, cyclin D1 and Axin 2 (Figure 2) [78].

Dkk1 participates in the canonical Wnt (β-catenin dependent) pathway by acting as a competitive inhibitor of Wnt [34,79]. Dkk1 binds to, stabilizes and accumulates Lrp6 on cell surfaces [80]. The C-terminal of Dkk1 contains a colipase fold and is
especially necessary for the interaction between Dkk1 and Lrp6, Kremen1 and Kremen2 co-receptors [2,35,81–85]. Kremen2 binding initiates formation of a ternary complex with Dkk1 and Lrp6 that induces endocytosis or clearance of Lrp6 from the cell membrane, stopping further Wnt-Lrp5/6 interaction [80,86]. In the absence of Wnt, the destruction complex remains intact and Ck1, stimulated by Axin, phosphorylates β-catenin at its serine 45 initiating further phosphorylation of β-catenin by GSK3 [87,88]. This facilitates ubiquitin binding to β-catenin by Skp and β-TrCP and, therefore, subsequent degradation of β-catenin by a proteasome (Figure 3) [89,90]. This pathway is significant to cancer development because in the absence of Dkk1, the pathway is unregulated and leads to excessive cell proliferation through the upregulation of β-catenin.

Dkk1 has also been shown to affect the RANK/RANKL/OPG pathway for bone formation and resorption. Receptor activator of Nuclear Factor kappa-B ligand (RANKL), also known as Osteoprotegerin ligand (OPGL), is a cytokine that regulates bone resorption. The binding of RANKL to its receptor, RANK, causes multinucleation and maturation of osteoclast precursors into osteoclasts, leading to increased bone resorption [91]. Osteoprotegerin (OPG) inhibits the RANK/RANKL interaction, thereby preventing osteoclast formation, maturation, and activation. The Wnt/β-catenin pathway has been shown to cross-talk to with the RANK/RANKL/OPG pathway by upregulating osteoblasts and OPG and downregulating RANKL [92–94]. Dkk1 increases bone resorption by inhibiting Wnt [45,95,96], and has also been shown to decrease OPG and osteoblast levels as well as increase expression of RANKL [51,97,98].
Dkk1 as a Biomarker in Pancreatic Cancer

Currently, CA19-9 is the standard biomarker used for detection of pancreatic cancer, but has limited efficacy in detecting the disease at an early stage [99–104]. This is because CA19-9 serum levels not only increase in PDAC but also in pancreatitis, compromising its specificity for pancreatic cancer and therefore making it a poor biomarker [105–107]. CA19-9 has a sensitivity of 79-81% and a specificity of 82-90% for the diagnosis of PDAC [108–110].

Dkk1 has promise as a biomarker for early detection and progression of PDAC [29]. Han et al. measured serum Dkk1 levels in 140 PDAC patients at various stages of the PDAC, including before and after surgical treatment using ELISA and Immunohistochemistry. Their data showed that Dkk1 had significantly higher levels in PDAC serum compared to healthy controls, benign pancreatic tumors, and even chronic pancreatitis serum samples. Dkk1 serum levels also increased with the advancement of the disease from Stage 1 to Stage 4 [29]. However, the serum concentration levels of Dkk1 compared to that of CA19-9 was not found to be significantly different. The accuracy and AUC of the Dkk1 serum levels, determined using ROC curve analysis, was greater than that of CA19-9, suggesting that Dkk1 would be an excellent candidate as a biomarker for PDAC. The value of Dkk1 in monitoring prognosis before and after surgical treatment was also analyzed by monitoring the serum levels at both time points. They concluded that higher Dkk1 serum levels correlated with lower survival, supporting the utility of Dkk1 as a prognostic biomarker [29].
Dkk1 was initially found to be overexpressed in PDAC in 2010 by Takahashi et al. [111]. Several pancreatic cell lines were tested (such as Suit-2, Suit-4, AsPc-1, MIA PaCa-2, Panc-1, HPAF and BcPC3) and Dkk1 was found to be upregulated in these cell lines and in cells with good, moderate, and poor differentiation [111]. In well-differentiated cells, 50% of cases had high levels of Dkk1 as well as 66% in moderately-differentiated, and 100% of poorly-differentiated cells. While these results are very promising, it is important to mention that there was only a total of 23 cases. Dkk1 knockdown, Matrigel invasion assays, and cell scratch assay demonstrated a possible role of Dkk1 in cellular invasiveness of carcinogenic cells [111]. It was also confirmed that Dkk1 did not use the JNK pathway to affect changes in cells by knocking down Dkk1 and confirming that β-catenin, JNK phosphorylation and c-myc mRNA levels were not changed by this knockdown [111]. This left the need for further confirmation of the pathway Dkk1 in affecting cellular invasiveness in PDAC.

In 2011, transcription factor GATA6 was found to be a regulate Dkk1 in pancreatic cancer [112]. A variety of experiments were done to verify that GATA6 is amplified in the late stages of pancreatic cancer (more than 2 copies in PanIN-3 compared to 0 in PanIN-1 and PanIN-2), and that this amplification is highly correlated to increased nuclear β-catenin protein, which enhances cell proliferation [112].

Kimura et al. also investigated Dkk1’s activity in PDAC and found a receptor, cytoskeleton-associated protein 4 (CKAP4), which was upregulated in 66.1% of PDAC cases [113]. CKAP4 is a type II transmembrane protein [114,115] located on the cell surface in an MDCK cell line [113]. This group also found that co-expression of Dkk1
and CKAP4 is inversely related to prognosis and relapse-free 5-year survival. The co-expression was also found to have high levels of AKT, a proto-oncogene that has been implicated to be improperly regulated in cancer cells [113]. This study was helpful in discovering that Dkk1 binds to CKAP4 through its cysteine residues and knowledge that the upregulation of both proteins in PDAC is significant in helping better understand Dkk1’s mechanism of action as a biomarker, indicating immunotherapies could be developed to better target its action. Their initial experiment tested the efficiency of an anti-CAKP4 antibody on the S2-CP8 cell line and confirmed its effectiveness. The anti-CKAP4 antibody was injected, twice a week, in mice that were implanted with the S2-CP8 cell line. It was found that the antibody reduced volume, weight, Ki-67-positive cell number and AKT activity.

The latest work characterizing Dkk1 in PDAC was published in early 2016 from the D’Amico et al. group [116]. This group discovered a correlation between Dkk1 and myeloid derived suppressor cells (MDSCs) in PDAC [116]. D’Amico et al. used IHC in tissue microarray (TMA) slides obtained from patients who were diagnosed with invasive PDAC, had not received any neoadjuvant therapy, underwent a pancreaticoduodenectomy and then followed with adjuvant chemotherapy [116]. CD15+ leukocytes and Dkk1 expression levels were then analyzed and quantified from the TMA slides and the data collected confirmed that there is a correlation between high Dkk1 levels and high CD15+ levels in Stage 1 to Stage 2 PDAC (p < 0.05) [116]. A sub-group of MDSCs are CD15+ leukocytes often upregulated in several cancer tissues [117–119]. CD15+ is increased in the bone marrow and peripheral circulation of PDAC patients [119]. This work showed that Dkk1 regulated myeloid derived suppressor cells
(MDSCs) in PDAC tissues, which is insightful as MDSCs have been proven to advance PDAC tumor progression in mice models and patient tissues [120–122].
SPECIFIC AIMS

The two multipart aims are described with the over-arching hypothesis: “**Dkk1 has the potential to be used as a biomarker and is, in part, responsible for the progression of Pancreatic Ductal Adenocarcinoma.**” Two independent aims are proposed to advance our understanding of Dkk1’s function and to assess its potential as a biomarker by: (i) **(CLINICAL)** providing information concerning the serum and tissue concentrations the Dkk1, (ii) **(MECHANISM)** define the mechanism of action of Dkk1 in late stage PDAC. The current study will contribute to understanding Dkk1 in Pancreatic Ductal Adenocarcinoma and to direct its development as a potential screening and monitoring biomarker.

**Aim One, Hypothesis:** Examine Dkk1’s clinical application as a biomarker early and late metastatic stage PDAC.

(a) Analyzing Dkk1 staining in PDAC patient tissues from tissue microarray (TMA) to better understand Dkk1 pattern in normal pancreatic tissues and PDAC tissues in all cancer stages.

(b) Analyzing Dkk1 serum concentration in PDAC patient serum samples to understand the trend of Dkk1’s concentration in normal pancreatic tissues and PDAC serum in all cancer stages.

**Aim Two, Hypothesis:** Dkk1 is associated with PDAC’s progression from early stage to late stage cancer.

(a) Determine the role and mechanism of Dkk1 in late stage PDAC through the Suit-2 cell line.

(b) Determine the effect of chemotherapy on Dkk1 in late stage PDAC.
MATERIALS AND METHODS

**Cell Culture**

Suit-2 cells were maintained in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin (100X; Mediatech, Inc., Manassas, VA, USA) at 37°C and a 5% CO2 atmosphere.

**Paclitaxel Treatment**

Paclitaxel was obtained from Sigma-Aldrich Co. (Merck KGaA, Darmstadt, Germany), and diluted in DMSO. Suit-2 cells were seeded into 60mm plates to 70% confluency and after 24 hours, the cells were treated with 20 nM Pac.

**Transient Transfection**

Suit-2 cells were seeded into 35mm and 60mm plates and at 70% confluency, cells were transiently transfected with a pCMV-Tag2B Dkk1 plasmid according to the manufacturer’s instructions (Roche, Mannheim, Germany). After 48 hours, the total RNA of the 35mm plates were extracted and after 72 hours, the proteins of the 60mm plates were extracted.

**RNA extraction and Real-Time PCR Analysis of mRNA Levels**

Cultured cells were washed twice with cold PBS. Total RNA was extracted using a RNeasy plus mini kit (Cat. No. 74136; QIAGEN, Valencia, CA, USA). RNA concentration was measured with the use of NanoDrop 1000 spectrophotometer (Thermo
Fisher Scientific, Wilmington, DE, USA). 1µg of total RNA was used for cDNA synthesis with a High Capacity RNA-to-cDNA kit (Cat. No. 4387406; Applied Biosystems, Foster, CA, USA) in 20 µl reactions. cDNA was diluted to 100 µl in nuclease-free water. Real-time PCR was conducted in 12 µl reactions containing 2 µl diluted cDNA, 6 µl real-time PCR reaction mixture (Cat. No. 75600; VeriQuest SYBR-Green real-time PCR master mix with ROX; Affymetrix, Cleveland, OH, USA), 1 µl 10 µM forward primer, 1 µl 10 µM backward primer, and 2 µl nuclease-free water. Real-time PCR primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the following primers were selected: DKK1-Forward: 5’- CGG GCG GGA ATA AGT ACC AG; DKK1-Reverse: 5’- GGG ACT AGC GCA GTA CTC ATC; LRP6-Forward: 5’- AAC GCG AGA AGG GAA GAT GG; LRP6-Reverse: 5’- ATC GCA AGT CCC GTC TGT TT; CKAP4-Forward: 5’- TTT CTC GGG CTG GTG CGT C; CKAP4-Reverse: 5’- CAA AGA CTG CAC CTT CTG CTC G. Housekeeping gene GAPDH was used as an internal reference: GAPDH-Forward: 5’-ATG CAG CAG ATC CGC ATG T; GAPDH-Reverse: 5’-TCA TGG TGT TCT TGC CCA TCA. Reactions were carried out under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 secs, annealing and extension at 60°C for 1 min, using the ABI 7900HT fast Real-Time PCR system (Applied Biosystems). PCR products were resolved in 2% TAE agarose/ethidium bromide gels and specific amplification was indicated by a single band pattern to confirm the predicted size of PCR product. GAPDH was used as the internal reference gene for standardization of results. Relative changes in mRNA expression were calculated using the 2^ΔΔCt method (35) and presented as mean ± SD.
Western Blot Analysis

Cultured cells were washed twice with cold PBS (pH 7.4) and lysed on ice in cold lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40] supplemented with 1% Halt protease inhibitor cocktail (100X; Thermo Scientific, Rockford, IL, USA), 1 mM of phenylmethylsulfonyl fluoride (PMSF), 5 mM of sodium fluoride (NaF), and 1 mM of sodium vanadate (Na\textsubscript{3}VO\textsubscript{4}). Cell suspensions were kept on ice for 30 min before centrifugation at 1,400 x rpm at 4°C for 30 min. Supernatants were collected and stored at -80°C until use. 20µg of protein was mixed with SDS-loading buffer, boiled for 5 min, separated in 8 or 10% SDS-polyacrylamide gels, and transferred onto PVDF membranes. Non-specific reaction was blocked for 1 h at room temperature in TBS containing 5% non-fat milk and 0.1% Tween-20. Primary antibodies (anti-Dkk1 mouse monoclonal antibody, 1: 1,000, Cat. No. H00022943-m11, Abnova, Taipei, Taiwan; anti-beta catenin rabbit polyclonal antibody, 1: 3,000, Cat. No. ab16051; anti-CKAP4 rabbit polyclonal antibody, 1: 500, Cat. No. ab84712; anti-Cyclin D1 rabbit monoclonal antibody, 1: 400, Cat. No. ab16663; anti-LRP6 rabbit monoclonal antibody, 1: 1,000, Cat. No. ab134146; all from Abcam Cambridge, MA, USA) diluted in blocking solution were applied and incubation was carried out at room temperature for 1 h. Secondary antibodies (cat. no. 43R-IG099HRP, goat anti-Rabbit IgG (H + L) (HRP); Thermofisher, Waltham, MA USA) were diluted 1: 5,000 and incubation was performed at room temperature for 1 hr. The blots were developed with ECL system (Thermo Scientific) and exposed to X-ray film. Membranes were stripped and GAPDH was detected with anti-GAPDH rabbit monoclonal antibody.
Results of GAPDH detection served as protein loading controls.

**Immunofluorescence**

Suit-2 cells were grown to 70% confluence in complete 1640 medium in a 4-chamber plate (company, city, state) for 24 hours. The cells were then fixed with 4% Parafomaldehyde for 5 mins, washed 3x with PBS, then washed with 0.1% Triton-X (3x for 5mins each) and again washed 3x with PBS. The fixed cells were then incubated with mouse Dkk1 primary antibody (Abnova, Taipei, Taiwan; 1:100) for 1 hour at room temperature, washed with 0.1% Triton-X (3x5mins) and incubated with mouse secondary antibody (1:50) for 30 mins at room temperature in the dark. Slides were washed (4x5mins) and DAPI containing mounting medium was applied. Fluorescent images were obtained and processed.

**Immunohistochemistry**

Tissue array slides were deparaffinized with 3 changes of Xylene, each for 5 min. The slides were rehydrated by a sequential treatment with 100% ethanol, 2 changes for 5 min each; 95% ethanol, 2 changes for 5 min each; and 80% ethanol, 2 changes for 5 min each. Endogenous peroxidase activity was quenched with 3% H_2O_2 at room temperature for 30 min. After rinsing with distilled water twice, each for 2 min, the slides were incubated in Epitope Retrieval Buffer (IHC-101 IHC kit, Bethyl Laboratories, Inc., Montgomery, TX, USA) at 98 °C for 30 min. After cooling at room temperature for 30 min, Slides were exposed to blocking solution for 30 min. Slides were covered with diluted primary
antibody (anti-Dkk1 mouse monoclonal antibody, 1:500, Cat. No. H00022943-m11, Abnova, Taipei, Taiwan) at room temperature for 2 h. Following extensive washing with PBS, anti-rabbit secondary antibody was applied with 1:400 dilution for 1 h. Color development was performed with DAB Substrate (IHC-101, Bethyl Laboratories, Inc., Montgomery, TX, USA). Reactions were stopped and counterstaining was carried out with Gill’s Hematoxylin Solution (SC-24973, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The slides were dried and mounted with Organo/Limonene Mount medium (SC-45087, Santa Cruz Biotechnology, Dallas, TX, USA). Each specimen was scored based on the staining intensity (0-negative, 1-weak, 2-moderate, 3-strong) multiplied by the percentage of positive cells 0, 1 (1%–24%), 2 (25%–49%), 3 (50%–74%), and 4 (75%–100%). Tissue samples were classified as negative (score = 0), weak (score = 1–4), medium (score = 5–8), or strong (score = 9–12) according to the final score of individual tissue.

**ELISA**

Dkk1 serum concentrations were determined with the use of Human Dkk1 Immunoassay (DKK100, R&D Systems, Inc., Minneapolis, MN, USA). Measurement procedures were carried out following the manufacturers’ instructions and the values were read at recommended wave lengths of 450 nm and 560nm.

**Patient Samples**

Twenty-eight serum samples, including five chronic pancreatitis, seven benign tumor/cystic tumors, four neuroendocrine tumors, six pancreatic adenocarcinomas and
five metastasized pancreatic adenocarcinomas from Curtis & Elizabeth Anderson Cancer Center of Memorial Health. All the serum samples were stored at −80 °C until use.
RESULTS

1 – Dkk1 Expression in PDAC Tissues

To determine the expression of Dkk1 in PDAC tissues, immunohistochemistry (IHC) was carried out in a pancreatic tissue microarray (TMA) containing normal pancreatic tissues, pancreatic adenocarcinoma tissue, and normal tumor adjacent tissues (Figure 4). Each tissue core was then scored based on the percentage of Dkk1 staining observed – (0%), + (<25%), ++ (25-49%), +++ (50-74%), ++++ (75-100%) (Table 1). The representative micrographs are shown in Figure 4. Statistical analysis showed a significant decrease of Dkk1 expression in tumor tissues (p = 0.001). The PDAC tissues were further categorized by Dkk1 positive and negative cases (Table 2). A higher number of normal tissue cases were positively stained, and a higher number of cancer tissue cases were negatively/weakly stained with Dkk1.

2 – Dkk1 is Decreased in PDAC Blood Serum

To determine the diagnostic and disease monitoring value of Dkk1 in pancreatic adenocarcinoma, we calculated its sensitivity and specificity in metastasized tumor, non-metastasized tumor, and control patient serum samples. An ELISA assay was used to detect Dkk1 in 6 control/chronic pancreatitis, 13 early stage (Stage 1 & 2) PDAC, and 5 late stage (Stage 3 & 4) metastasized PDAC (Figure 5). The results showed that there is a significant decrease in Dkk1 serum concentrations from normal to early stage PDAC (p = 0.004) and from normal to late stage PDAC (p = 0.02). Lastly an increasing trend of Dkk1 concentration was seen from early stage to late stage PDAC (p = 0.4).
3 - Dkk1 Primarily Localized in the Nucleus

To establish the expression pattern of Dkk1 in PDAC, we performed immunofluorescence using antibodies to Dkk1. We used antibodies to Dkk1 and what we observed was that Dkk1 was mainly located in the nucleus of Suit-2 cells with faint staining in the cytoplasm (Figure 6A). With this information, we proceeded to perform a co-immunofluorescence with Dkk1 and NaATPase (to highlight the cell membrane) to confirm if the faint cytoplasm staining of Dkk1 extended to the cell membrane. Our results show that there is no Dkk1 on the membrane of the cell (Figure 6B). There is no overlapping yellow staining on the cell membrane.

4 - Dkk1 Colocalization with CKAP4 and LRP6

We also used the primary antibodies to Dkk1, CKAP4, and LRP6 in co-immunofluorescence experiments to determine the colocalization of Dkk1 with CKAP4 and Dkk1 with LRP6 (Figure 7 & 8). Our results showed that Dkk1 is colocalized with CKAP4 in the nucleus and lightly in the cytoplasm (Figure 7A, B) and it is also colocalized with LRP6 in the nucleus and cytoplasm like Dkk1 (Figure 8A, B).

5 - Endogenous Dkk1 expression in Pancreatic Cancer cell lines

In Suit-2 and Capan-1 pancreatic cancer cell lines, we tested the endogenous levels of Dkk1 through Western blot and ELISA. Our results showed that Dkk1 is present at a much higher level in Suit-2 cells than in Capan-1 (Figure 9).
6 – **Dkk1 is associated with CKAP4 but not LRP6 in Suit-2**

Using anti-IgG as a control, an Immunoprecipitation experiment was performed, and it was observed that Dkk1 binds to CKAP4 but not LRP6 (Figure 10).

7 – **Design of Dkk1 pCMB-Tag2B plasmid**

Mammalian expression vector, pCMV-Tag2B, was used to insert the Dkk1 sequence into the vector (Figure 11). Dkk1 was confirmed to be overexpressed at the mRNA and protein level through an agarose gel as well as qPCR and western blot analysis.

8 - **Dkk1 Overexpression Leads to Alteration of CKAP4 and LRP6**

Dkk1 has previously been implicated in Wnt/β-catenin and CKAP4/Akt signaling pathways. To determine which of these pathways through which Dkk1 affects, we ran a qPCR analysis to determine if there was a change in the protein levels of LRP6 and CKAP4 receptors. Our data showed that with a 4.5-fold increase in Dkk1 mRNA (p = 0.003), there was an approximately 43% decrease in LRP6 mRNA levels (p = 0.006), and an 11% increase in CKAP4 mRNA levels (p = 0.01). Additionally, western blot analysis showed an increase in Dkk1 protein levels, we saw a 40% decrease in LRP6 protein and a 68% increase in CKAP4 protein (Figure 12).
9 – *Upregulation of p-Akt and Cyclin D1 by Dkk1*

We used the primary antibodies to Dkk1 and β-catenin in co-immunofluorescence experiments to determine the colocalization of Dkk1 with β-catenin. Furthermore, our western blot analysis of Dkk1 overexpressed in Suit-2 cells showed a significant increase in Cyclin D1 but no significant change in β-catenin protein levels (Figure 13A, B). However, with p-Akt, we found that its protein levels increased 40% (p < 0.001).

10 – *Downregulation of Dkk1 Expression by Paclitaxel treatment*

To check on the implications of Paclitaxel treatment on Dkk1, Suit-2 cells were treated with 20nM Paclitaxel 24 hours after the cells were seeded. 24 hours after treatment, the Suit-2 cell RNA was extracted, and the samples were analyzed through RT-PCR and qPCR. The results showed that Paclitaxel treatment caused a decrease in the mRNA levels of Dkk1 compared to controls. Also, 72 hours after Paclitaxel treatment, the cells were lysated and analyzed through Western blot for Dkk1 protein levels. We also found a significant decrease in the amount of Dkk1 protein levels (Figure 14).

11 – *Dkk1 and HE4 are Colocalized in Suit-2 Cells*

We also used the primary antibodies to Dkk1, and HE4 in co-immunofluorescence experiments to determine the colocalization of Dkk1 with HE4 (Figure 15). Our results showed that Dkk1 is colocalized with HE4 in the nucleus and cytoplasm.
12 - Dkk1 is a Downstream Target of HE4

Previous studies from our lab has shown that HE4 is overexpressed in PDAC cells and serum [123]. With these results, we set out to determine if HE4’s overexpression influenced the expression levels of Dkk1. In our initial experiments, we transiently overexpressed HE4 with a pCDNA6.0 plasmid in Suit-2 cells and determined the effect this had on Dkk1’s protein levels. Our results showed that Dkk1’s protein levels are increased when HE4 is overexpressed (Figure 16).

13 – HE4 Rescues Paclitaxel Knockdown of Dkk1

In the presence of HE4 upregulation, we observe a 2.5-fold increase in Dkk1’s mRNA levels when compared to Paclitaxel treated samples (Figure 17).
DISCUSSION

There is currently a limited amount of knowledge on the mechanism of action of Dkk1 in the pancreas and pancreatic ductal adenocarcinoma. Some of the few available studies have shown low expression of Dkk1 in pancreatic cancer tissues via IHC while others have shown Dkk1 to be overexpressed compared to normal tissue via RT-PCR [111,112].

Our data showed that there was a decrease in Dkk1’s serum levels of pancreatic adenocarcinoma compared to normal (approx. 30% decrease) and chronic pancreatitis (approx. 40% decrease) patients. Interestingly, during metastasis Dkk1 is increased. This same trend is seen in the IHC Dkk1 staining of TMA slides. The average staining score drops between the normal patient and Stage 1 & Grade 1 of the disease and then it begins to increase with Stage and Grade increase. With these results, we decided to check on what pathway Dkk1 affects as it increases in the higher cancer stages.

Dkk1 has previously been shown to be involved in the Wnt signaling pathway in many cancers [34,79]. This pathway is significant to cancer development because in the absence of Dkk1, the pathway is unregulated and should lead to excessive cell proliferation through the upregulation of β-catenin. Due to this, Dkk1 is often known to act as a tumor suppressor, regulating cell proliferation by inducing apoptosis through the degradation of β-catenin. Degrading β-catenin in the cytoplasm, prevents it from accumulating in the nucleus and increasing cell proliferation by increasing Cyclin D1, c-Myc, and MMP-7 [34]. Our results, however, show that in pancreatic cancer Dkk1 is not effective in causing the degradation of β-catenin as we see there is no significant change
in β-catenin’s protein level. Additionally, Dkk1’s overexpression, which normally induces an increased expression of LRP6, its coreceptor, was proven to be decreased

With this information we took a further look into the other pathway through which Dkk1 has the potential to act through PDAC. Results showed that Dkk1 affects the Akt signaling pathway. As CKAP4 has previously been shown to be a potential receptor for Dkk1 in MDCK cells and our own IP western blot data showing the binding, we started by determining the effect of Dkk1 overexpression on CKAP4’s mRNA and protein levels. Our data showed an increase in both the protein and mRNA levels of CKAP4. We also saw that an increase in Dkk1 caused a significant increase in the protein levels of p-Akt, showing that in PDAC metastasis, Dkk1 aids in increased cell proliferation (Figure 18).

The summary of these results concludes that Dkk1 is ineffective in the Wnt pathway because of the increase in β-catenin. The reasoning for this could be due to a disconnect in other proteins that affect the Wnt pathway such as GSK3. Previous research has proven that p-Akt can inhibit GSK3, which is a part of the destruction complex in the Wnt pathway. Without an active GSK3 being active, then the destruction complex is incomplete leading to the continuous production of β-catenin. It is also interesting to note that active β-catenin cannot only lead to increased cellular proliferation but can increase Dkk1’s presence in the cell as well.

With our immunofluorescence experiments, we were also able to determine the location of these key proteins in these pathways. Dkk1 is primarily located in the nucleus with a lighter amount in the cytoplasm. CKAP4 also had a similar immunofluorescent
staining pattern with Dkk1, this could be because Dkk1 is bound to CKAP4. Dkk1 binding to CKAP4 has previously been shown to occur in PDAC cells by Han et al. [29]. While previously studies have assumed that CKAP4 and Dkk1 bind on the cell membrane, our studies show that that may not be the case. As CKAP4 is a transmembrane protein, it has been posed that it’s glycine rich domain could act as a nuclear localization sequence (NLS) for unrelated proteins, which in this case could be Dkk1 [124,125]. The co-immunofluorescence with LRP6 was also showed that Dkk1 was primarily colocalized with LRP6 in the nucleus.

In conjunction with our previous studies, we also set out to determine if Dkk1 is linked to HE4, a potential biomarker for PDAC that our lab has done extensive research on. In our previous work, we proved that HE4 is overexpressed in PDAC cells, serum, and tissue [123]. These results led us to check the effect of HE4’s overexpression on Dkk1 and we found that Dkk1’s proteins levels are increased when HE4 is overexpressed in Suit-2. Also, Dkk1 and HE4 are colocalized in the cytoplasm and nucleus in Suit-2 cells. Lastly our results also confirmed that if HE4 is present in Paclitaxel treated cells, Dkk1 is increased, decreasing the effect of Paclitaxel treatment. This result shows that Dkk1 has the potential to be used to monitor the effectiveness of Paclitaxel in vivo.

**Unanswered Questions & Future Direction**

By determining the pathway through which Dkk1 affects PDAC cells, we can in the future use this information in addressing its diagnosis. Based on the data obtained through our ELISA and IHC experiments, Dkk1 could be used as a method to diagnose and monitor PDAC progression.
While a previous study from Zhang et al. has shown administration of recombinant Dkk1 inhibits PanIN formation and stops PDAC initiation, our results show that for later stages of the disease, this treatment would be ineffective and could even cause it to significant increase in the rate of proliferation. Also, the use of Paclitaxel at the earlier stages of the disease could also be ineffective, as it would simply further downregulate Dkk1’s tumor suppressor activity. Paclitaxel would be more effective in the later stages of the disease. It would be important in future studies to determine what gives Dkk1 its dual functional role in PDAC and if there are other proteins that Dkk1 act on.
APPENDIX

FIGURES AND TABLES
FIGURES

Figure 1 - Dkk family of proteins: (A) Dkk1 has 266 amino acids with its first Cysteine domain from amino acid 85-139 and the second Cysteine domain from amino acid 189-263. (B) Dkk2 has 259 amino acids with its first Cysteine domain from amino acid 78-127 and the second Cysteine domain from amino acid 183-256. (C) Dkk3 has 350 amino acids with its first Cysteine domain from amino acid 147-195 and the second Cysteine domain from amino acid 208-284. (D) Dkk4 has 224 amino acids with its first Cysteine domain from amino acid 41-90 and the second Cysteine domain from amino acid 145-218. *Edited from Krupnik et al., 1999 [34].
Figure 2- Wnt canonical pathway: The binding of the Wnt ligand leads to disruption of the APC destruction complex, allowing β-catenin to remain active. Active β-catenin is translocated into the nucleus causing upregulation of proteins such as c-Myc, Cyclin D1 and Dkk1. *Edited from Fatima et al., 2011 [78]

Figure 3- Wnt canonical pathway: Dkk1 antagonizing and binding to the LRP5/6 receptors leads the destruction complex to phosphorylate and ubiquitinate β-catenin for degradation. *Edited from Fatima et al., 2011 [78]
Figure 4 - Dkk1 expression in PDAC cell tissues. (A) Medium/strong positive staining was observed normal pancreatic tissues, and (B) adjacent non-tumorous tissues. (C, D) Negative & weak positive stains was observed in pancreatic adenocarcinoma tissues with no metastasis (N0) and metastasis (N1). Magnification is 50X
**Figure 5** - Dkk1 serum concentration (pg/ml) is significantly decreased from normal to early stage PDAC (p=0.004) and from normal to late stage PDAC (p=0.02)

**Figure 6** - Dkk1 is Primarily Located in the Nucleus of Suit-2 Cells – A strong staining of Dkk1 is observed in the nucleus with a faint staining in the cytoplasm. NaATPase is cytoplasm membrane labeled with FITC (green) while Dkk1 is labeled with TRITC (red). The nucleus is stained with DAPI.
**Figure 7** - Dkk1 colocalization with CKAP4 in Suit-2 cells. Dkk1 (red) and CKAP4 (green) – Dkk1 & CKAP4 are mainly located in the nucleus with a faint staining in the cytoplasm – (A) Immunofluorescence staining for Dkk1 and CKAP4 (green) with DAPI. (B) Colocalization overlap of Dkk1 and CKAP4 alone and with DAPI.
Figure 8 - Dkk1 colocalization with CKAP4 in Suit-2 cells Dkk1 (red) and CKAP4 (green). (A) Immunofluorescence staining for Dkk1 and LRP6 (green) with DAPI. (B) Colocalization overlap of Dkk1 and LRP6 alone and with DAPI.
**Figure 9** - Endogenous Dkk1 expression in Pancreatic Cancer cell lines through Western blot analysis.

**Figure 10** – Immunoprecipitation using anti-IgG, anti-CKAP4, and anti-LRP6 antibodies in Suit-2 cells and Western blot analysis with anti-Dkk1 antibody.
**Figure 11** - Design of Dkk1 pCMV-Tag2B plasmid using DH5α E.coli cells as well as detection of Dkk1’s 800bp band on 2% agarose gel.

**Figure 12** - Dkk1 Upregulation leads to alteration of receptors CKAP4 and LRP6 – (A) mRNA levels of Dkk1 OE (B, C) Western blot analysis of Dkk1 overexpressed Suit-2 cells
**Figure 13** - Upregulation of p-Akt and Cyclin D1 by Dkk1 in Suit-2 Cells

**Figure 14** - Downregulation of Dkk1 by Paclitaxel in Suit-2 Cell Lines in the (A) mRNA, and (B) protein.
**Figure 15** - Dkk1 and HE4 Colocalization in both the cytoplasm and nucleus of Suit-2 cells

**Figure 16** - Dkk1 is a Downstream Target of HE4
**Figure 17** - HE4 Rescues Paclitaxel Knockdown of Dkk1

**Figure 18** - Dkk1’s Pathway in Late Stage PDAC
**Table 1.** Immunohistochemistry score in pancreatic tissue microarrays. Each tissue microarray (TMA) core of 10 normal, 10 adjacent, and 52 tumor tissues were scored as negative (-), 0-25% positive (+), 25-50% positive (++), 50-75% positive (+++), and 75-100% positive (++++). Analysis of the Dkk1 level of three groups showed a significant decrease of Dkk1 protein levels in tumor group compared to normal and adjacent group respectively. A negative correlation was observed between the normal and cancer cases (p = 0.001)

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<th>+++ (3)</th>
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Table 2. Categorizing of Dkk1 positive and negative cases in PDAC TMA. There is a higher number of cases that are positively stained with Dkk1 in normal tissues. There is a higher number of cases that are negatively/weakly stained with Dkk1 in cancer tissues.

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