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The Role of Dickkopf-3 Overexpression in Esophageal Adenocarcinoma

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Abstract

OBJECTIVES—Ninety percent of patients with esophageal adenocarcinoma (EAC) ultimately die of their disease highlighting the need for novel therapeutic targets. The goal of this study was to define the functional significance of overexpression of *Dickkopf-3 (DKK3)* in EAC.

METHODS—DKK3 expression was analyzed by real-time PCR in 95 chemonaive and 21 chemoresistant EACs. The EAC cell line OE33 was stably transfected with *DKK3* (OE33/DKK3) and evaluated using WST-1, matrigel, endothelial tube formation, and chemosensitivity assays. Tumorigenesis was evaluated by injecting 1×10^6 OE33/DKK3 and vector cells in NOD/SCIDγ mice.

RESULTS—*DKK3* was overexpressed (> 2-fold) in 75.8% (72/95) of EACs. DKK3 protein was present at moderate to high levels in 46.8% (29/62) of EACs on tissue microarray. Stable transfection of *DKK3* significantly increased proliferation ($p<0.05$) and matrigel invasion ($p<0.001$). Levels of SMAD4, a key mediator of the TGFß pathway, increased after activin treatment of OE33/DKK3, and *siSMAD4* significantly decreased matrigel invasion suggesting that DKK3 acts through the TGFβ pathway. OE33/DKK3 increased endothelial tube formation, were significantly more resistant to 5-FU and cisplatin, and *DKK3* expression was significantly higher in chemoresistant EACs ($p<0.005$). In NOD/SCIDγ mice, OE33/DKK3 cells resulted in tumors at all sites (8/8) while vector cells grew in only 1/8 sites. Nodal metastases were also significantly

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CENTRAL MESSAGE

DKK3 may mediate invasion in esophageal adenocarcinoma and could be a novel target in the treatment and prevention of metastases.

CENTRAL PICTURE

Figure 2A has been selected as the Central Picture.

CENTRAL PICTURE LEGEND

OE33/DKK3 had significantly increased matrigel invasion compared to controls ($p<0.001$).

increased in patients with EACs highly overexpressing *DKK3*, 28/32 (88%) versus 42/63 (68%) ($p<0.05$).

CONCLUSIONS—These findings suggest that DKK3 may be important in mediating invasion in EAC and could be a novel target in the treatment and prevention of metastatic disease.

INTRODUCTION

Dickkopf-3 (DKK3) is a divergent member of the Wnt inhibitor family,[1] and the significance of its interaction with the Wnt pathway is unclear. While DKK1, 2, and 4 inhibit the Wnt pathway by binding to LRP5/6, DKK3 does not bind to these proteins.[2] DKK3 binds to Kremen1/2;[3] however, DKK3 is a secreted protein and the significance of this intracellular interaction is uncertain. DKK3 was found to regulate FGF and Activin/Nodal through SMAD4, a central component of the TGFβ pathway, and stabilization of SMAD4 by DKK3 was found in the induction of mesoderm in *Xenopus* embryos.[4]

DKK3 has been proposed as a tumor suppressor, and overexpression of DKK3 suppresses cell growth and invasion of certain cancer cell lines.[5] However, *DKK3* is overexpressed in other cancers including hepatocellular carcinoma and hepatoblastoma.[6] DKK3 is a marker for neoangiogenesis in colon cancer,[7] and microvessels expressing DKK3 were increased in glioma, non-Hodgkin's lymphoma, and melanoma.[8] DKK3 has been associated with protection from apoptotic stress and with chemoresistance in Saos-2 osteosarcoma cells.[9] Using Oncomine (www.oncomine.org), a web-based application that allows evaluation of gene expression using cancer profiling data including 25 esophageal datasets with 751 samples, there was significant overexpression of *DKK3* in esophageal adenocarcinomas (EACs) relative to Barrett's metaplasia (BM) and normal esophagus (10.9 fold; $p<0.0001$).[10, 11] Conversely, *DKK3* expression was significantly decreased in lung adenocarcinoma. The expression and function of DKK3 appears to be tissue and tumor specific.

The incidence of EAC has increased greatly while the 5-year survival remains only 19%.[12] Metastatic disease accounts for the majority of deaths from EAC. While esophagectomy remains the primary treatment, there is an urgent need for novel therapies. In evaluating molecular changes in the progression from BM to EAC, overexpression of *DKK3* was identified in a significant subset of tumors. Interestingly, we found that a number of genes mediated by the TGFβ pathway were also overexpressed, suggesting that this pathway is important in EAC. This study was undertaken to delineate the expression and role of DKK3 in EAC. We hypothesized that DKK3 is a mediator of the TGFβ pathway in EAC and plays an important role in the proliferation and invasion of EAC. Inhibition of DKK3 and its downstream mediators could have a significant clinical impact on the treatment and prevention of micrometastatic disease, especially in patients with locally advanced or regional nodal disease.

MATERIALS AND METHODS

Patients and Tissues

This study was approved by the IRB, and after obtaining informed consent, tissues were obtained from patients undergoing esophagectomy at the University of Michigan. Specimens were transported in DMEM (Invitrogen) on ice and stored at −80°C. Samples with minimum 70% cellularity were identified using frozen sections including 95 chemonaive and 21 chemoresistant EACs.

Cell Lines

Flo, OE19, and OE33 (Sigma-Aldrich) were derived from EAC. Flo was grown in DMEM (Invitrogen) and OE33 and OE19 were grown in RPMI 1640 with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 1% Antibiotic-Antimycotic (Invitrogen) at 37°C in 5% CO_2/95% air. All cell lines and stable subclones underwent genotyping by the University of Michigan Sequencing Core to ensure cell line authenticity. To evaluate for chemosensitivity, cell lines were treated with cisplatin (5 ug/ml) and 5-FU (10 ug/ml) for 48 hours. Viability was assessed by WST-1 (Roche) and repeated in triplicate.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Real-time PCR was performed using 20 ng of total RNA and 0.2 μM of the forward and reverse primers. Cycling parameters included a 50°C hold for 2 minutes; 95°C hold for 10 minutes; 40 cycles at 95°C for 10 seconds; annealing for 15 seconds; and 72°C for 20 seconds. Significant differences in relative quantification were determined using the $2^{(-\Delta\Delta Ct)}$ method. Expression was normalized to GAPDH or β-actin. *DKK3* primers were forward 5′-TGAGGAACTGATGGAGGACA-3′ and reverse 5′-TTGCCAGGTTCACTTCTGAT-3′.

Western Blot

Western was performed using a 1:1000 dilution of DKK3 antibody (Santa Cruz) and a 1:5000 dilution of goat anti-rabbit secondary antibody (Vector). While the calculated molecular weight of DKK3 is 38 kDa, the size on Western has been reported as 50-55 kDa in reducing conditions due to glycosylation.[13] For SMAD4, a 1:2000 dilution of SMAD4 antibody (Abcam) was used with a 1:8000 dilution of anti-rabbit secondary antibody (Vector). ß-actin was used as a loading control with a 1:10000 dilution of ß-actin antibody (Abcam) and a 1:10000 dilution of anti-mouse secondary antibody (Vector).

Tissue Microarray

Tissue microarrays were constructed with formalin-fixed, paraffin-embedded tissues from 73 patients including 64 EAC, 8 dysplastic Barrett's mucosa, 11 BM, and 2 normal esophageal samples.[14] Immunohistochemical staining was done on the DAKO Autostainer using DAKO LSAB+ and 3,3′-diaminobenzidine as the chromogen. Dewaxed and rehydrated sections were labeled with DKK3 antibody (1:200 dilution; Santa Cruz). Microwave citric acid epitope retrieval was performed for 20 minutes. Slides were counterstained with hematoxylin. Samples were scored using a scale of 0 (no staining), 1+

(<10% staining), 2+ (10-50%) staining, or 3+ (≥50%).[15] The scoring was repeated to ensure reproducibility.

Construction of DKK3 Stable Cell Line

A *DKK3* mammalian expression construct (Plasmid 15496; Addgene) was PCR amplified using primers containing EcoRI and XbaI restriction sites for directional cloning into the pcDNA3(+) Vector (V79020; Invitrogen). The insert was sequenced to confirm that it contained *DKK3*. *DKK3* or empty vector constructs were transfected into OE33 or Flo using FuGENE 6 (Promega). Selected clones were maintained in medium containing 200 μg/mL geneticin.

Matrigel Invasion Assay

1×10^5 cells in serum-free RPMI 1640 medium were seeded in the upper chamber of the 24 well invasion chamber system (BD Biosciences), and 20% FBS was added to the lower chamber as a chemoattractant. After 48 hours, non-invading cells and Matrigel were removed with a cotton swab. Invasive cells on the lower side of the chamber were stained with crystal violet. Four 100x fields were counted, and assays were performed in duplicate.

SNP Array Analysis

Using the Genome-Wide Human Sty I 250K SNP Array (Affymetrix), 73 EAC DNAs were genotyped.[16] *DKK3* copy number analysis was performed using a log2 copy number ratio exceeding 0.848 for amplifications and −0.737 for deletions. Genomic positions were mapped in the hg18 genome build. SNP data was visualized using Integrative Genomics Viewer 1.3.1 (www.broadinstitute.org/igv).

Endothelial Tube Formation

HMVEC (Human Microvascular Endothelial Cells; Cascade Biologics) were stably transduced with lentiviral-expressed red fluorescent protein (RFP). OE33/DKK3 and OE33/Vector cells were stably transduced with lentiviral-expressed green fluorescent protein (GFP). RFP and GFP-positive cells were sorted by flow cytometry. Selected HMVECs were cultured as monolayers on Attachment Factor coated plates with Medium 131 mixed with Microvascular Growth Supplement (Cascade Biologics) and 1% FBS.[17] Cells were applied to collagen gel polymerized 6 well plates (2×10^5 cells/well) and incubated for 16 hours. A second collagen gel layer was overlaid with 2×10^5 OE33/DKK3 or vector cells/ml. VEGF (1 μg/ml; R&D Systems) was added to either OE33/Vector with endothelial cells or endothelial cells alone as positive controls. Cells were incubated at 37°C for 7 days, and media was changed every 3 days. Experiments were performed in triplicate.

Xenograft Models

NOD/SCIDγ (NSG) mice were received from the Unit for Laboratory Animal Medicine at 6-8 weeks of age. All procedures were approved by the University Committee on Use and Care of Animals, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog.php?record_id=12910). To evaluate whether stable transfection of *DKK3* increases the tumorigenicity of OE33, the

number of cells injected was titrated to the lowest number resulting in a palpable tumor after flank injection of OE33/DKK3 but not OE33/Vector. Cells were passed through a 40-μm filter. A single cell suspension was prepared with 100 μl of saline and 50% Matrigel (BD Biosciences). 2×10^6, 1×10^6, 1×10^5 and 5×10^4 cells were injected into the flank of NSG mice.[18] At 1×10^6 cells, OE33/DKK3 resulted in tumors at five weeks while OE33/Vector cells did not. Each experimental group consisted of 4 mice injected with 1×10^6 cells in each flank for a total of 8 sites per cell line. Mice were sacrificed after 5 weeks.

Oligonucleotide Microarray

Total RNA was isolated from stably transfected OE33/DKK3 and OE33/Vector cells using QIAzol (Qiagen) and purified with miRNeasy spin columns (Qiagen). RNA quality was confirmed by 1% agarose gel electrophoresis and A260:280 by NanoDrop 2000 spectrophotometer ratios. RNA quality was reassessed with the Agilent Bioanalyzer (Agilent Technologies) after double-stranded cDNA and cRNA synthesis. Hybridization and normalization of the Human Gene 2.1 ST Gene Chip data (Affymetrix) were performed by the University of Michigan Cancer Center Microarray Core. A summary statistic was calculated for the eleven probe pairs for each gene with the robust multichip average (RMA) method (14) using the Affymetrix library of Bioconductor (www.bioconductor.org). Expression values for OE33/DKK3 were compared with OE33/Vector. Pathway analysis was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov). Results were confirmed using real-time PCR.

Statistical Analysis

Statistical analysis was performed using SPSS version 22. Categorical variables were analyzed using the Fisher's exact or Chi-squared tests while continuous variables were analyzed using the Student's t-test. Survival was determined using the Kaplan-Meier method. For the analysis of the clinical characteristics, high *DKK3* expression was defined as expression relative to Barrett's mucosa with a threshold greater than 2 standard deviations above the mean.

RESULTS

DKK3 is overexpressed in esophageal adenocarcinoma

DKK3 was overexpressed greater than 2-fold in 75.8% (72/95) of EACs relative to BM using real-time PCR (Supplemental Figure 1A). qRT-PCR analysis of an additional 40 esophageal samples (6 BM, 7 BM/low-grade dysplasia, 6 low-grade dysplasia, 7 high-grade dysplasia, and 14 EACs) showed significant overexpression in EAC in the progression from BM ($p<0.05$) (Supplemental Figure 1B). The purpose was to identify molecular changes in the progression from BM to adenocarcinoma. Barrett's metaplasia was used as the control to avoid identifying genes with similar expression in BM and EAC but differential expression in normal esophagus. BM has been used as a reference control by other investigators.[19] Significant overexpression of *DKK3* in 95 primary EACs was also confirmed relative to normal esophagus using real-time PCR.

Staining of DKK3 on tissue microarray (Supplemental Table 1) showed moderate to high DKK3 expression (2-3+) in 46.8% (29/62) of EAC samples (Figure 1). 16.7% (2/12) of dysplastic samples and 20% (2/10) of BM samples had 2+ staining, but unlike EAC, none had 3+ staining. Normal esophageal samples showed mild 1+ DKK3 staining (2/2).

To evaluate the mechanism of overexpression, SNP array analysis of 73 EACs was performed, and *DKK3* was not amplified. While treatment of OE33 with 5-azacytidine did not increase DKK3 expression, a combination of 5-azacytidine and trichostatin A increased DKK3 expression on Western suggesting that histone acetylation is involved in the overexpression of *DKK3*.

OE33 transfection with *DKK3* increases cell proliferation and matrigel invasion

DKK3 overexpression was confirmed on qRT-PCR compared to native and vector controls and on Western, which also confirmed expression of a Flag tag. OE33, which does not natively express *DKK3,* had significantly increased proliferation on WST-1 assay after *DKK3* transfection compared to vector controls at 96 and 120 hours ($p<0.005$). OE33/DKK3 cells also had significantly greater matrigel invasion ($p<0.001$) (Figure 2). To ensure that these results were not cell line-specific, the EAC cell line Flo was transfected with *DKK3* and confirmed increased proliferation and matrigel invasion compared to vector controls.

Transfection of *DKK3* leads to stabilization of SMAD4 and activation of the TGFβ pathway

To determine the mechanism of action of DKK3 in EAC, the TOP-flash TCF-reporter assay was performed and showed no significant decrease in canonical Wnt pathway activation after transfection of *DKK3,* a divergent member of the Wnt inhibitor family, into OE33 compared to native and vector controls. However, treatment of OE33/DKK3 cells with the TGFβ ligand activin increased SMAD4 protein on Western (Figure 3A), suggesting its mechanism of action is through the TGFβ pathway. Activin treatment also significantly increased proliferation in OE33/DKK3 compared with vector controls. Inhibition with *siSMAD4* significantly decreased matrigel invasion of OE33/DKK3 ($p<0.001$) (Figure 3B and C). In addition, low and high SMAD4 staining correlated with DKK3 expression in the same tumors on immunohistochemistry (Figure 1). DAVID pathway analysis of Human Gene ST 2.1 array expression induced by the transfection of *DKK3* in OE33 showed that the TGFβ pathway was significantly increased compared with OE33 vector control ($p<0.05$).

DKK3 overexpression leads to increased neoangiogenesis

Endothelial tube formation assay demonstrated increased angiogenesis in the presence of OE33/DKK3 compared to OE33 native, vector, and endothelial cell controls (Figure 4). Endothelial tube formation was similar to that seen after adding VEGF to the OE33/Vector and endothelial only positive controls.

DKK3 overexpression increases chemoresistance in EAC cells

OE33/DKK3 cells were significantly more chemoresistant to 5-FU and cisplatin compared to vehicle control ($p<0.05$) (Figure 5A). *DKK3* expression was significantly higher on real-time PCR relative to BM in 21 chemoresistant EACs compared to 96 chemonaive EACs ($p<0.005$) (Figure 5B). To evaluate whether *DKK3* expression is induced by chemotherapy,

OE33 and Flo were treated with cisplatin and 5-FU with no significant increase in *DKK3* expression.

Injection of OE33/DKK3 cells in NOD/SCIDγ mice significantly increases the incidence of tumor growth

NSG mice were injected in the flank with 1×10^6 cells, the lowest number of cells to produce tumors with OE33/DKK3 but not OE33/Vector as described in the Methods. Five weeks after injection, only 1/8 OE33/Vector sites produced a palpable tumor (2 mm) while all OE33/DKK3 (8/8) tumor sites resulted in tumors (4-5 mm) (Supplemental Figure 2). Tumors were confirmed pathologically to be consistent with EAC. *DKK3* overexpression was also confirmed by RT-PCR and Western with no expression in the OE33/Vector tumor.

Nodal metastases were significantly increased in patients with EACs highly overexpressing *DKK3*

Clinical characteristics for 94 patients with chemonaive EACs, obtained before the routine use of preoperative chemotherapy, were analyzed for an association with *DKK3* overexpression (Supplemental Table 2). Nodal metastases were significantly associated with *DKK3* overexpression with 28/32 (88%) with nodal disease compared to 42/62 (68%) with lower *DKK3* expression (p=0.047). The pathological stage was also significantly higher with EACs highly overexpressing DKK3 with 26/32 (81%) EACs stage III+IVa versus 36/62 (58%) with lower *DKK3* expression (p=0.038). However, *DKK3* overexpression was not significantly associated with overall survival.

DISCUSSION

The TGFβ pathway is involved in proliferation, differentiation, and EMT, which has been associated with chemoresistance and tumor invasion. While TGFβ is a tumor suppressor at early stages of carcinogenesis, advanced cancers are resistant to its growth inhibition and TGFβ promotes metastasis and invasion.[20-22] TGFβ is overexpressed in EAC and is related to a poor prognosis.[23] DKK3 mediates the effects of activin, a TGFβ ligand, in Xenopus.[4] Our results suggest that DKK3 acts independently of the canonical Wnt pathway in EAC and that the mechanism of action is mediated through the TGFβ pathway with DKK3 stabilizing SMAD4 protein. Transfection of *DKK3* in OE33 resulted in increased activation of the TGFβ pathway. Various tumor biological processes were evaluated focusing on processes mediated by the TGFβ pathway, especially those previously associated with *DKK3* overexpression including invasion, angiogenesis, and chemoresistance. While DKK3 has been described as a tumor suppressor in some cancers, *DKK3* is overexpressed in hepatocellular carcinoma and hepatoblastoma.[6] The expression and function of DKK3, like the TGFβ pathway, depends on the tumor and tissue context. The overexpression and role of DKK3 have not been previously described in EAC.

In the current study, *DKK3* was overexpressed in a significant subset of EACs. Esophageal cancers are heterogeneous, and most molecular changes, including *DKK3* overexpression, will only be present in a subset of tumors. While *DKK3* is overexpressed in 75.8% of EACs on real-time PCR, 2-3+ DKK3 protein expression was found in 46.8% on

immunohistochemistry. Discordance in mRNA and protein expression in other genes has been reported in lung adenocarcinoma.[24] These differences may be the result of posttranscriptional regulation with changes in mRNA stability,[25] transcript localization,[26] or translational efficiency as well as differences in sensitivity between mRNA and antibody-based assays.

The mechanism behind *DKK3* overexpression in EAC was also evaluated. *DKK3* was not amplified on SNP array analysis, and treatment of OE33 cells with 5-azacitidine alone did not increase *DKK3* expression. However, a combination of 5-azacytidine and trichostatin A increased expression suggesting histone acetylation is involved in *DKK3* overexpression.

The majority of EAC patients die of metastatic disease.[12] *DKK3* overexpression may be important in tumor invasion, and matrigel invasion was increased following transfection of *DKK3* in Flo and OE33. Knockdown experiments could not be performed since none of the three available EAC cell lines overexpress *DKK3*. However, downstream inhibition of SMAD4 was able to decrease matrigel invasion of OE33/DKK3. Deckers, et al. found that inhibiting SMAD4 in MDA-MB-231 breast carcinoma cells inhibited bone metastases in nude mice.[27] While *SMAD4* inhibition may have effects independent of *DKK3* overexpression, it is notable for its ability to inhibit matrigel invasion of OE33/DKK3.

Neoangiogenesis is important in the progression from BM to adenocarcinoma and in supporting tumor invasion.[28] DKK3 supports capillary formation in gliomas and lymphoma,[8] and our results show an increase in endothelial tube formation in the presence of OE33/DKK3. While transfection of *DKK3* resulted in a more malignant phenotype with increased invasion and proliferation, there was no evidence that DKK3 is oncogenic in EAC. The three EAC cell lines available to us are cancer cell lines and therefore, were not able to be specifically tested for oncogenic transformation. However, this chromosomal region was not amplified on SNP array.

To determine if *DKK3* transfection was able to increase the tumorigenicity of OE33, the lowest number of OE33/DKK3, but not OE33/Vector, cells able to produce tumors was determined to be 1×10^6 cells. This is several fold less than previously described mice experiments using OE33 (2×10^6 to 1×10^7 cells).[29-32] In a tumorigenic assay, Zhao, et al. injected 1×10^5, 1×10^6, and 1×10^7 OE33 cells at 15 sites in BALB/c OlaHsd-Foxn1^{nu} mice.[18] OE33 cells produced tumors at 1/15 sites at 10 weeks and 2/15 at 29 weeks. In the current study, OE33/Vector cells produced tumors in 1/8 sites at 5 weeks while OE33/DKK3 cells resulted in tumors at all 8 sites supporting that *DKK3* expression is important in invasion and growth in EAC.

EACs highly overexpressing *DKK3* were significantly associated with nodal metastases in 88% ($p=0.047$) and higher stage III or IVa in 81% of EACs ($p=0.038$), consistent with the increased matrigel invasion seen with *DKK3* overexpression. While T and M stage were not significantly associated with *DKK3* expression, this is likely due to the fact that patients with invasion of surrounding structures or metastatic disease were not included in this series since they were not treated surgically.

Neoadjuvant chemoradiation followed by esophagectomy is standard in most large centers for EACs greater than T2 or with regional nodal disease to “downstage” the tumor and limit micrometastatic disease. However, only 21% of patients have a complete response.[34] *DKK3* overexpression is associated with chemoresistance in Saos-2 osteosarcoma,[9] and our results show that OE33/DKK3 cells were significantly more chemoresistant to cisplatin and 5-FU. Targeting DKK3 and other TGFβ pathway mediators may decrease this chemoresistance. If inhibiting DKK3 alone is not sufficient, combination with other therapies targeting the TGFβ pathway or downstream proteins may be a successful strategy.

DKK3 -/- knockout mice have significantly decreased natural killer cells, increased IgM and hemoglobin, and are hyperactive.[35] Dkk3 does not appear to function as a tumor suppressor in these knockout mice as the authors did not report any increase in tumors. In addition, these knockout mice show that DKK3 is a non-lethal target since the DKK3 -/- mice were viable.

There are some limitations to our study. None of the three EAC cell lines overexpress *DKK3* so we were unable to evaluate targeted inhibition of *DKK3*. However, we were able to evaluate the effect of transfection of *DKK3* in two EAC cell lines. Inhibition of *DKK3* using siRNA in an oral squamous cell carcinoma cell line natively overexpressing *DKK3* significantly decreased migration and invasion providing further support that DKK3 plays a role in these biological processes.[33]

While *DKK3* overexpression was not associated with decreased overall survival, the sample size was relatively small and only included operable patients. *DKK3* overexpression was increased in chemoresistant tumors, and transfection increased chemoresistance and invasion. *DKK3* overexpression may be associated with decreased survival in other populations including patients with metastatic disease or those treated with preoperative chemotherapy. We were not able evaluate these groups directly since our tumor bank consists mostly of chemonaive tumors obtained when patients were not treated routinely with preoperative chemotherapy. Katase, et al. reported *DKK3* negative oral squamous cell carcinoma patients had decreased nodal metastases and significantly longer disease-free survival.[33] These findings support that DKK3 overexpression is important clinically in cancers overexpressing DKK3.

The overall survival of patients with EAC remains poor with the majority of patients ultimately dying of metastatic disease. The results of the current study suggest that DKK3 may play an important role in tumor growth and invasion in EAC. *DKK3* is overexpressed in a significant subset of esophageal adenocarcinomas, and targeting DKK3 and its downstream mediators may be beneficial in the prevention and treatment of micrometastatic disease and potentially decreasing disease recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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PERSPECTIVE STATEMENT

Most esophageal adenocarcinoma patients die of metastases. *DKK3* was significantly overexpressed in esophageal adenocarcinoma and was associated with advanced tumors and nodal disease. Stable transfection of *DKK3* increases proliferation, invasion, and chemoresistance. DKK3 may be important in mediating invasion and could be a novel target in the treatment and prevention of metastatic disease.

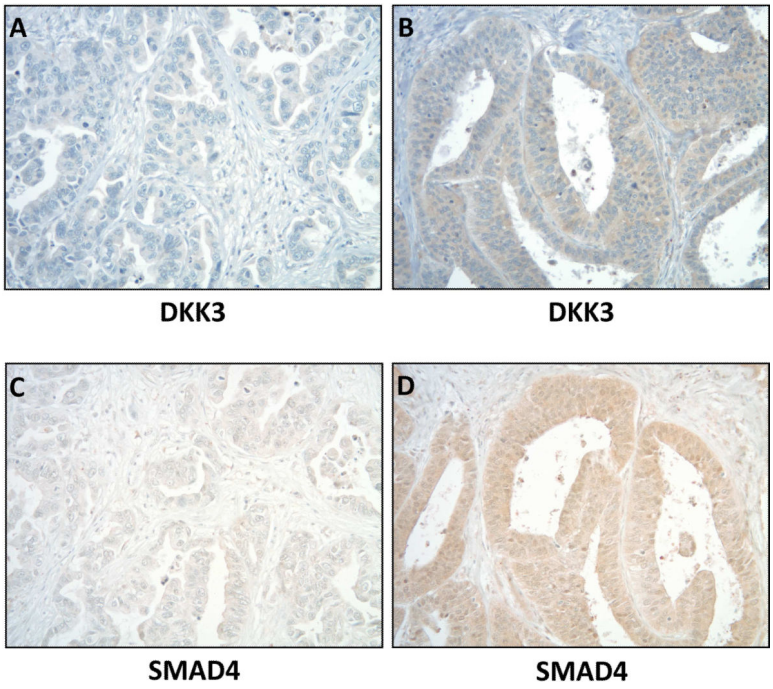

Figure 1.

Representative sections from a tissue microarray showing low (A) and high (B) cytoplasmic DKK3 staining in two esophageal adenocarcinomas. Moderate to high expression (2-3+) was found in 46.8% (29/62) of esophageal adenocarcinomas. Low (C) and high (D) SMAD4 staining correlated with DKK3 in the same tumors. Original magnifications are all 100x.

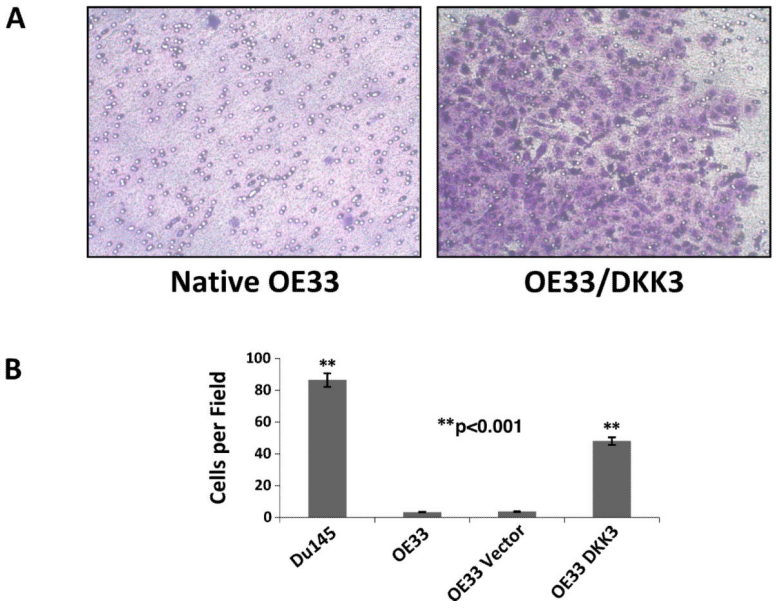

Figure 2.

A. OE33/DKK3 had significantly increased matrigel invasion compared to controls ($p<0.001$). Du145 prostate cancer cells served as a positive control. B. All assays were performed in duplicate.

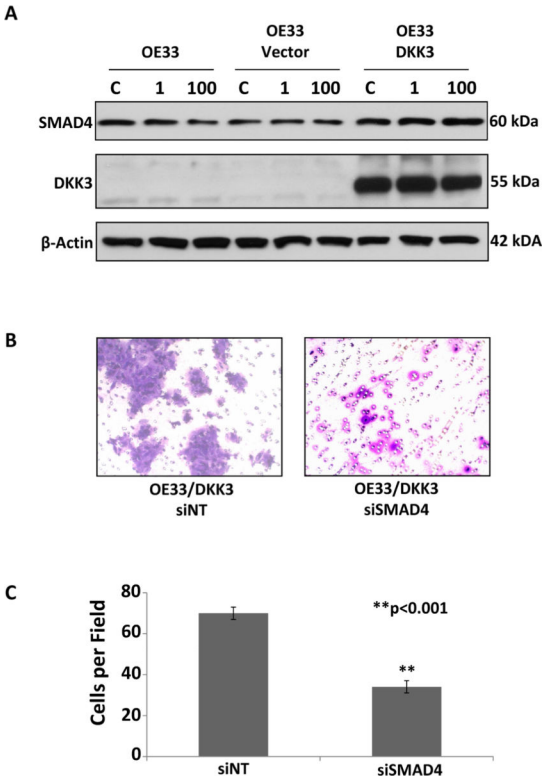

Figure 3.

A. SMAD4 was significantly increased on Western in OE33/DKK3 after treatment with 1 or 100 ng/ml of activin compared to native and vector controls (C, carrier control). B and C. Treatment of OE33/DKK3 cells with siSMAD4 resulted in significantly less matrigel invasion compared to a non-targeting control (**p<0.001).

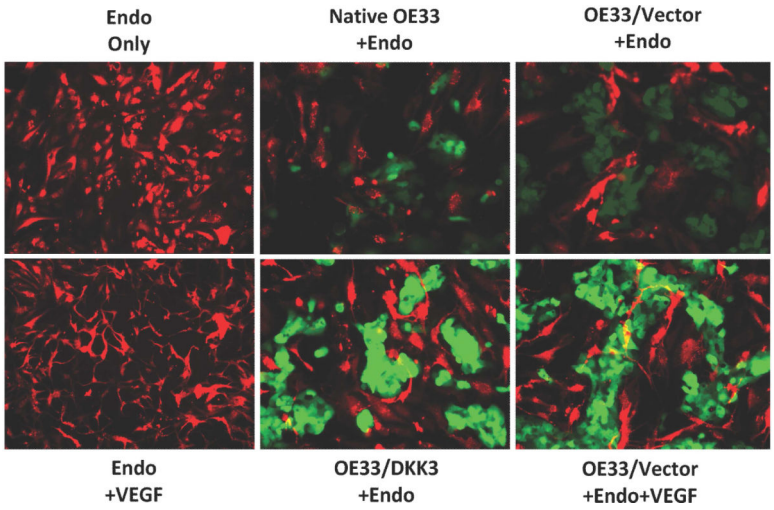

Figure 4.

The presence of stably-transfected OE33 (OE33/DKK3 +Endo) increased endothelial tube formation similar to the tube formation seen after adding VEGF to endothelial cells (Endo +VEGF) and to OE33/Vector (OE33/Vector +Endo +VEGF). Endothelial, native OE33, and vector controls showed minimal tube formation. OE33 and endothelial cells were labeled green and red respectively. Original magnifications were 200x.

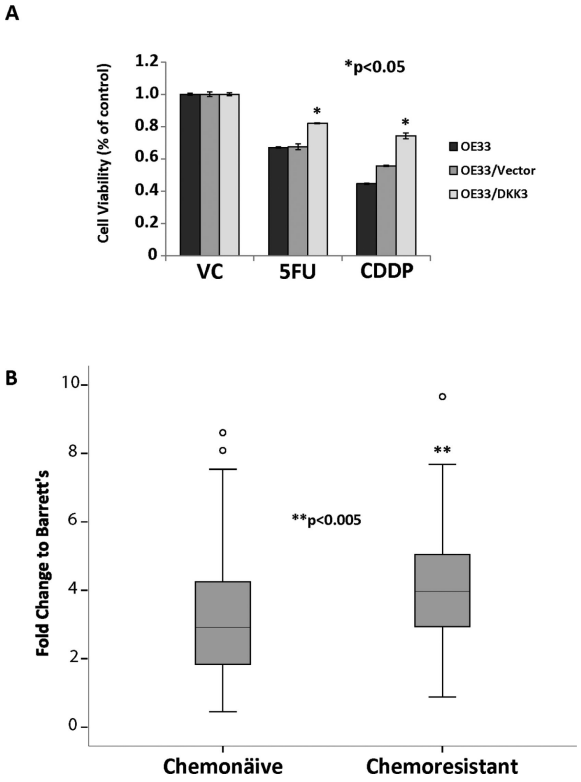

Figure 5.

A. OE33/DKK3 were significantly more resistant to 5-FU and cisplatin compared to treatment with vehicle control for 48 hours (*p<0.05). B. *DKK3* expression was significantly higher on real-time PCR in chemoresistant (n=21) compared to chemonaive esophageal adenocarcinomas (n=95) (**p<0.005).