The role of Dickkopf-3 overexpression in esophageal adenocarcinoma

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ABSTRACT

Objectives: Ninety percent of patients with esophageal adenocarcinoma 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 esophageal adenocarcinoma.

Methods: *DKK3* expression was analyzed by real-time polymerase chain reaction in 95 chemo-naïve and 21 chemoresistant esophageal adenocarcinomas. The esophageal adenocarcinoma cell line OE33 was stably transfected with *DKK3* (OE33/*DKK3*) and evaluated using WST-1 (Roche, Basel, Switzerland), Matrigel (BD Biosciences, San Jose, Calif), endothelial tube formation, and chemosensitivity assays. Tumorigenesis was evaluated by injecting 1×10^6 OE33/*DKK3* and vector cells in NOD/SCID γ mice.

Results: *DKK3* was overexpressed (>2-fold) in 75.8% (72/95) of esophageal adenocarcinomas. *DKK3* protein was present at moderate to high levels in 46.8% (29/62) of esophageal adenocarcinomas on tissue microarray. Stable transfection of *DKK3* significantly increased proliferation ($P < .05$) and Matrigel invasion ($P < .001$). Levels of SMAD4, a key mediator of the transforming growth factor- β pathway, increased after activin treatment of OE33/*DKK3*, and *siSMAD4* significantly decreased Matrigel invasion, suggesting that *DKK3* acts through the transforming growth factor- β pathway. OE33/*DKK3* cells increased endothelial tube formation and were significantly more resistant to 5-fluorouracil and cisplatin, and *DKK3* expression was significantly higher in chemoresistant esophageal adenocarcinomas ($P < .005$). In NOD/SCID γ mice, OE33/*DKK3* cells resulted in tumors at all sites (8/8), whereas vector cells grew in only 1 of 8 sites. Nodal metastases were also significantly increased in patients with esophageal adenocarcinomas highly overexpressing *DKK3*, 28 of 32 (88%) versus 42 of 63 (68%) ($P < .05$).

Conclusions: These findings suggest that *DKK3* may be important in mediating invasion in esophageal adenocarcinoma and could be a novel target in the treatment and prevention of metastatic disease. (J Thorac Cardiovasc Surg 2015;150:377-85)

OE33/*DKK3* had significantly increased Matrigel (BD Biosciences, San Jose, Calif) invasion compared with controls ($P < .001$).

Central Message

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

Perspective

Most patients with EAC die of metastases. *DKK3* was significantly overexpressed in EAC 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.

See Editorial page 288.

Supplemental material is available online.

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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. Although *DKK1*, 2, and 4 inhibit the Wnt pathway by binding to LRP5/6, *DKK3* does not bind to these proteins.² *DKK3* binds to Kremen1/2³; however, *DKK3* is a secreted protein, and the significance of this intracellular interaction is uncertain. *DKK3* was found to regulate fibroblast growth factor and Activin/Nodal through SMAD4, a central component of

Abbreviations and Acronyms

BM	= Barrett's metaplasia
DKK3	= Dickkopf-3
EAC	= esophageal adenocarcinoma
FBS	= fetal bovine serum
5-FU	= 5-fluorouracil
PCR	= polymerase chain reaction
RT-PCR	= real-time polymerase chain reaction
SNP	= single nucleotide polymorphism
TGF	= transforming growth factor
VEGF	= vascular endothelial growth factor

the transforming growth factor (TGF)- β 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 By 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; $P < .0001$).^{10,11} Conversely, *DKK3* expression was significantly decreased in lung adenocarcinoma. The expression and function of *DKK3* seem to be tissue and tumor specific.

The incidence of EAC has increased greatly, whereas the 5-year survival remains only 19%.¹² Metastatic disease accounts for the majority of deaths from EAC. Although 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. We found that a number of genes mediated by the TGF β pathway also were 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 TGF β 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 institutional review board, and after obtaining informed consent, tissues were obtained from patients undergoing esophagectomy at the University of Michigan. Specimens were transported in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, Calif) 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, St Louis, Mo) were derived from EAC. Flo was grown in Dulbecco's Modified Eagle Medium (Invitrogen), and OE33 and OE19 were grown in Roswell Park Memorial Institute 1640 with 10% fetal bovine serum (FBS) (Atlanta Biologicals Inc, Flowery Branch, Ga) and 1% Antibiotic-Antimycotic (Invitrogen) at 37°C in 5% $\text{CO}_2/95\%$ 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 $\mu\text{g/mL}$) and 5-fluorouracil (5-FU) (10 $\mu\text{g/mL}$) for 48 hours. Viability was assessed by WST-1 (Roche, Basel, Switzerland) and repeated in triplicate.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was performed using 20 ng of total RNA and 0.2 $\mu\text{mol/L}$ 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 $2^{(-\Delta\Delta\text{Ct})}$ method. Expression was normalized to GAPDH or β -actin. *DKK3* primers were forward 5'-TGAGGAAGTGTG GAGGACA-3' and reverse 5'-TTGCCAGTTCACTTCTGAT-3'.

Western Blot

Western blot was performed using a 1:1000 dilution of *DKK3* antibody (Santa Cruz Biotechnology Inc, Dallas, Tex) and a 1:5000 dilution of goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif). Although the calculated molecular weight of *DKK3* is 38 kDa, the size on Western blot has been reported as 50 to 55 kDa in reducing conditions due to glycosylation.¹³ For SMAD4, a 1:2000 dilution of SMAD4 antibody (Abcam, Cambridge, UK) was used with a 1:8000 dilution of anti-rabbit secondary antibody (Vector Laboratories). β -actin was used as a loading control with a 1:10000 dilution of β -actin antibody (Abcam) and a 1:10000 dilution of anti-mouse secondary antibody (Vector Laboratories).

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 (Carpinteria, Calif) LSAB+ and 3,3'-diaminobenzidine as the chromogen. Dewaxed and rehydrated sections were labeled with *DKK3* antibody (1:200 dilution; Santa Cruz Biotechnology Inc). 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+ ($\geq 50\%$).¹⁵ The scoring was repeated to ensure reproducibility.

Construction of *DKK3* Stable Cell Line

A *DKK3* mammalian expression construct (Plasmid 15496; Addgene, Cambridge, Mass) was polymerase chain reaction (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 Corp, Madison, Wis). Selected clones were maintained in medium containing 200 μ g/mL geneticin.

Matrigel Invasion Assay

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

Single Nucleotide Polymorphism Array Analysis

By using the Genome-Wide Human Sty I 250K single nucleotide polymorphism (SNP) Array (Affymetrix, Santa Clara, Calif), 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 were visualized using Integrative Genomics Viewer 1.3.1 (www.broadinstitute.org/igv).

Endothelial Tube Formation

Human microvascular endothelial cells (Cascade Biologics, Eugene, Ore) were stably transduced with lentiviral-expressed red fluorescent protein. OE33/*DKK3* and OE33/Vector cells were stably transduced with lentiviral-expressed green fluorescent protein. Red fluorescent protein and green fluorescent protein-positive cells were sorted by flow cytometry. Selected human microvascular endothelial cells 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. Vascular endothelial growth factor (VEGF) (1 μ g/mL; R&D Systems Inc, Minneapolis, Minn) was added to 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/SCID γ (NSG) mice were received from the Unit for Laboratory Animal Medicine at 6 to 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- μ m filter. A single cell suspension was prepared with 100 μ L 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 5 weeks, whereas 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 Inc, Valencia, Calif) and

purified with miRNeasy spin columns (Qiagen Inc). 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, Santa Clara, Calif) 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 11 probe pairs for each gene with the robust multichip average method¹⁴ 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 RT-PCR.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 (SPSS Inc, Chicago, Ill). Categorical variables were analyzed using the Fisher exact or chi-square tests, and continuous variables were analyzed using the Student *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 more than 2-fold in 75.8% (72/95) of EACs relative to BM using RT-PCR (Figure E1, A). Quantitative RT-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 ($P < .05$) (Figure E1, B). 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 RT-PCR.

Staining of *DKK3* on tissue microarray (Table E1) showed moderate to high *DKK3* expression (2-3+) in 46.8% (29/62) of EAC samples (Figure 1). Some 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. Although treatment of OE33 with 5-azacytidine did not increase *DKK3* expression, a combination of 5-azacytidine and trichostatin A increased *DKK3* expression on Western blot, suggesting that histone acetylation is involved in the overexpression of *DKK3*.

FIGURE 1. Representative sections from a tissue microarray showing low (A) and high (B) cytoplasmic DKK3 staining in 2 EACs. Moderate to high expression (2-3+) was found in 46.8% (29/62) of EACs. Low (C) and high (D) SMAD4 staining correlated with DKK3 in the same tumors. Original magnifications are all 100 $\times$. DKK3, Dickkopf-3.

OE33 Transfection With *DKK3* Increases Cell Proliferation and Matrigel Invasion

DKK3 overexpression was confirmed on quantitative RT-PCR compared with native and vector controls and on Western blot, 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 with vector controls at 96 and 120 hours ($P < .005$). OE33/*DKK3* cells also had significantly greater Matrigel invasion ($P < .001$) (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 with vector controls.

Transfection of *DKK3* Leads to Stabilization of SMAD4 and Activation of the Transforming Growth Factor- β Pathway

To determine the mechanism of action of *DKK3* in EAC, the TOP-flash TCF-reporter assay (Promega Corp., Madison, Wisc) 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 with native and vector controls. However, treatment of OE33/*DKK3* cells with the TGF β ligand activin increased SMAD4 protein on Western blot (Figure 3, A), suggesting its mechanism of action is through the TGF β pathway. Activin treatment also significantly increased proliferation in OE33/*DKK3* compared with vector controls. Inhibition with *siSMAD4* significantly decreased Matrigel invasion of OE33/*DKK3* ($P < .001$) (Figure 3, B 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 TGF β pathway was significantly increased compared with OE33 vector control ($P < .05$).

DKK3 Overexpression Leads to Increased Neoangiogenesis

Endothelial tube formation assay demonstrated increased angiogenesis in the presence of OE33/*DKK3* compared with OE33 native, vector, and endothelial cell controls (Figure 4). Endothelial tube formation was similar to that

FIGURE 2. A, OE33/DKK3 had significantly increased Matrigel (BD Biosciences, San Jose, Calif) invasion compared with controls ($**P < .001$). Du145 prostate cancer cells served as a positive control. B, All assays were performed in duplicate. *DKK3*, Dickkopf-3.

seen after adding VEGF to the OE33/Vector and endothelial only positive controls.

***DKK3* Overexpression Increases Chemoresistance in Esophageal Adenocarcinoma Cells**

OE33/*DKK3* cells were significantly more chemoresistant to 5-FU and cisplatin compared with vehicle control ($P < .05$) (Figure 5, A). *DKK3* expression was significantly higher on RT-PCR relative to BM in 21 chemoresistant EACs compared with 96 chemonaive EACs ($P < .005$) (Figure 5, B). 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/SCID γ Mice Significantly Increases 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 “Materials and Methods” section. Five weeks after injection, only 1 of 8 OE33/Vector sites produced a palpable tumor (2 mm), whereas all OE33/*DKK3* (8/8) tumor sites resulted in tumors (4-5 mm) (Figure E2). Tumors were confirmed

pathologically to be consistent with EAC. *DKK3* overexpression was also confirmed by RT-PCR and Western blot with no expression in the OE33/Vector tumor.

Nodal Metastases Were Significantly Increased in Patients With Esophageal Adenocarcinomas 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 (Table E2). Nodal metastases were significantly associated with *DKK3* overexpression, with 28 of 32 (88%) with nodal disease compared with 42 of 62 (68%) with lower *DKK3* expression ($P = .047$). The pathologic stage also was significantly higher with EACs highly overexpressing *DKK3*, with 26 of 32 (81%) EACs with stage III + IVa versus 36 of 62 (58%) with lower *DKK3* expression ($P = .038$). However, *DKK3* overexpression was not significantly associated with overall survival.

DISCUSSION

The TGF β pathway is involved in proliferation, differentiation, and epithelial-mesenchymal transition,

FIGURE 3. A, SMAD4 was significantly increased on Western blot in OE33/DKK3 after treatment with 1 or 100 ng/mL of activin compared with native and vector controls (C, carrier control). B and C, Treatment of OE33/DKK3 cells with siSMAD4 resulted in significantly less Matrigel invasion compared with a nontargeting control (** $P < .001$). DKK3, Dickkopf-3.

which has been associated with chemoresistance and tumor invasion. Although TGF β is a tumor suppressor at early stages of carcinogenesis, advanced cancers are resistant to its growth inhibition and TGF β promotes metastasis and invasion.²⁰⁻²² TGF β is overexpressed in EAC and related to a poor prognosis.²³ DKK3 mediates the effects of activin, a TGF β 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 TGF β pathway with DKK3 stabilizing SMAD4 protein. Transfection of *DKK3* in OE33 resulted in increased activation of the TGF β pathway. Various tumor biological processes were evaluated focusing on processes mediated by the TGF β pathway, especially those previously associated with *DKK3* overexpression, including invasion, angiogenesis, and chemoresistance. Although 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 TGF β pathway, depend 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 be present in only a subset of tumors. Although *DKK3* is overexpressed in 75.8% of EACs on RT-PCR, 2 to 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 post-transcriptional 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-azacytidine 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 patients with EAC die of metastatic disease.¹² *DKK3* overexpression may be important in tumor invasion, and Matrigel invasion was increased after transfection of *DKK3* in Flo and OE33. Knockdown experiments could not be performed because none of the 3 available EAC cell lines overexpress *DKK3*. However, downstream inhibition of *SMAD4* was able to decrease Matrigel invasion of OE33/DKK3. Deckers and colleagues²⁷ found that inhibiting *SMAD4* in MDA-MB-231 breast carcinoma cells inhibited bone metastases in nude mice. Although *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. Although 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 3 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 whether *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 and colleagues¹⁸ injected

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 200 $\times$. *VEGF*, Vascular endothelial growth factor.

1×10^5 , 1×10^6 , and 1×10^7 OE33 cells at 15 sites in BALB/c OlaHsd-Foxn1^{nu} mice. OE33 cells produced tumors at 1 of 15 sites at 10 weeks and 2 of 15 sites at 29 weeks. In the current study, OE33/Vector cells produced tumors in 1 of 8 sites at 5 weeks, whereas 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% ($P = .047$) and higher stage III or IVa in 81% of EACs ($P = .038$), consistent with the increased Matrigel invasion seen with *DKK3* overexpression. Although T and M stage were not significantly associated with *DKK3* expression, this is likely because patients with invasion of surrounding structures or metastatic disease were not included in this series because 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.³³ *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 TGF β pathway mediators may decrease this chemoresistance. If inhibiting *DKK3* alone is not sufficient, a combination with other therapies targeting the TGF β pathway or downstream proteins may be a successful strategy.

DKK3^{-/-} knockout mice have significantly decreased natural killer cells, have increased immunoglobulin-M and hemoglobin, and are hyperactive.³⁴ *Dkk3* does not appear to function as a tumor suppressor in these knockout mice because the authors did not report any increase in tumors. In addition, these knockout mice show that *DKK3* is a nonlethal target because the *DKK3*^{-/-} mice were viable.

Study Limitations

None of the 3 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 2 EAC cell lines. Inhibition of *DKK3* using small interfering RNA in an oral squamous cell carcinoma cell line natively overexpressing *DKK3* significantly decreased migration and invasion, providing

FIGURE 5. A, OE33/DKK3 were significantly more resistant to 5-FU and cisplatin compared with treatment with vehicle control for 48 hours (* $P < .05$). B, *DKK3* expression was significantly higher on RT-PCR in chemoresistant ($n = 21$) compared with chemonaive EACs ($n = 95$) (** $P < .005$). VC, Vector control; 5FU, 5-fluorouracil; CDDP, cis-diamminedichloroplatinum(II) (cisplatin); *DKK3*, Dickkopf-3.

further support that *DKK3* plays a role in these biological processes.³⁵

Although *DKK3* overexpression was not associated with decreased overall survival, the sample size was relatively small and included only 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 to evaluate these groups directly because our tumor bank consists mostly of chemonaive tumors obtained when patients were not treated routinely with preoperative chemotherapy. Katsue and colleagues³⁵ reported that patients with *DKK3* negative oral squamous cell carcinoma had decreased nodal metastases and significantly longer disease-free survival. These findings support that *DKK3* overexpression is important clinically in cancers overexpressing *DKK3*.

CONCLUSIONS

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 EACs, and targeting *DKK3* and its downstream mediators may be beneficial in the prevention and treatment of micrometastatic disease and potentially decreasing disease recurrence.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: Esophageal adenocarcinoma, Dickkopf-3, DKK3, tissue invasion, chemoresistance

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FIGURE E1. A, RT-PCR analysis of 95 EAC samples showed 2-fold overexpression of *DKK3* in 72/95 (75.8%) EACs relative to BM. B, A total of 40 esophageal samples including 14 EACs showed significant overexpression in EAC in the progression from BM (* $P < .05$). *BL*, Barrett's/low-grade dysplasia; *BM*, Barrett's metaplasia; *EAC*, esophageal adenocarcinoma; *LGD*, low-grade dysplasia; *HGD*, high-grade dysplasia.

FIGURE E2. NOD/SCID γ mice were injected in the flank with 1×10^6 cells. A, Only 1 of 8 OE33/Vector tumor sites produced a tumor (2 mm). B, All (8/8) tumor sites injected with OE33/DKK3 cells resulted in tumors (4-5 mm) after 5 weeks (3/4 mice are shown). C, Three OE33/DKK3 tumors are shown after explant. *DKK3* overexpression in the OE33/DKK3 tumors was confirmed on RT-PCR and Western blot.

TABLE E1. Immunohistochemical analysis of Dickkopf-3 expression in esophageal tissues on tissue microarray*

	0	1+	2+	3+
Normal esophagus	0/2 (0%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
Barrett’s metaplasia	3/10 (30%)	5/10 (50%)	2/10 (20%)	0 (0%)
Dysplasia	1/12 (8.3 %)	9/12 (75%)	2/12 (16.7%)	0 (0%)
Adenocarcinoma	11/62 (17.7%)	22/62 (35.5%)	24/62 (38.7%)	5/62 (8.1%)

*Significant staining includes moderate to high staining (scores of 2 to 3+).

TABLE E2. Clinical and pathologic characteristics of 94 chemonative esophageal adenocarcinomas and *DKK3* expression relative to Barrett’s metaplasia on quantitative real-time polymerase chain reaction

	<i>DKK3</i> Less than mean +2 SD n = 62	<i>DKK3</i> Greater than mean +2 SD n = 32	All n = 94	<i>P</i> value
Age, y, mean (SD)	69.3 (9.10)	64 (12.8)	68.5 (9.7)	.441
Gender				
M	55 (89%)	27 (84%)	82 (87%)	.535
F	7 (11%)	5 (16%)	12 (13%)	
Stage				
I + II	26 (42%)	6 (19%)	32 (34%)	.038
III + IVa	36 (58%)	26 (81%)	62 (66%)	
Barrett’s				
Y	34 (54%)	15 (47%)	49 (52%)	.524
N	29 (46%)	17 (51%)	46 (48%)	
GEJ				
Y	23 (37%)	13 (41%)	36 (38%)	.823
N	40 (63%)	19 (62%)	59 (62%)	
T stage				
1	9 (15%)	1 (3%)	10 (11%)	.132
2	10 (16%)	3 (9%)	13 (14%)	
3	41 (66%)	28 (88%)	69 (73%)	
4	2 (3%)	0 (0%)	2 (2%)	
N stage				
0	20 (32%)	4 (12%)	24 (25%)	.047
1	42 (68%)	28 (88%)	70 (75%)	
M stage*				
0	58 (94%)	26 (81%)	84 (89%)	.084
1a	4 (6%)	6 (19%)	10 (11%)	
1b	0 (0%)	0 (0%)	0 (0%)	

Boldface indicates *P* < .05. *DKK3*, Dickkopf-3; *GEJ*, gastroesophageal junction; *SD*, standard deviation. *M1a, positive celiac lymph nodes.TNM staging using *AJCC Cancer Staging Manual*. 6th ed. New York, NY: Springer; 2002.