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Silencing NKD2 by promoter region hypermethylation promotes esophageal cancer progression by activating Wnt signaling

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Abstract:	Introduction: Naked cuticle homolog 2 (NKD2) was found frequently methylated in human breast and gastric cancer. The epigenetic changes and mechanisms of NKD2 in human esophageal cancer remain unclear. Methods: Nine esophageal cancer cell lines and 154 cases of primary esophageal cancer samples were analyzed using methylation specific PCR, immunohistochemistry, western blot and a xenograft mouse model. Results: Loss of NKD2 expression and complete methylation were found in KYSE150 and TE1 cells. Reduced expression and partial methylation were observed in KYSE30, KYSE70, KYSE410, KYSE140 and COLO680 cells. High level expression and unmethylation were detected in KYSE450 and TE8 cells. Re-expression of NKD2 was induced by 5-aza-2'-deoxycytidine in NKD2 unexpressed or reduced cells. NKD2 was methylated in 53.2% (82/154) of human primary esophageal cancer samples, and promoter region hypermethylation was associated with reduced expression of NKD2 significantly ($p<0.01$). NKD2 methylation was associated with TNM stage and lymph node metastasis ($p<0.01$). The results suggest that NKD2 is regulated by promoter region methylation and methylation of NKD2 may serve as a prognostic marker in esophageal cancer. Our further studies demonstrate that NKD2 suppresses cell proliferation, colony formation, cell invasion and migration, as well as induces G1/S check point arrest in esophageal cancer cells. NKD2 suppressed xenograft tumor growth and inhibited Wnt signaling in human esophageal cancer cells. Conclusions: NKD2 is frequently methylated in human esophageal cancer, and the expression of NKD2 is regulated by promoter region methylation. NKD2 suppresses esophageal cancer progression by inhibiting Wnt signaling both in vitro and in vivo.
Keywords:	NKD2, DNA methylation, Wnt signaling, esophageal cancer

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Running title: NKD2 suppresses esophageal cancer growth by inhibiting Wnt signaling

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ABSTRACT

Introduction: Naked cuticle homolog 2 (NKD2) was found frequently methylated in human breast and gastric cancer. The epigenetic changes and mechanisms of NKD2 in human esophageal cancer remain unclear. **Methods:** Nine esophageal cancer cell lines and 154 cases of primary esophageal cancer samples were analyzed using methylation specific PCR, immunohistochemistry, western blot and a xenograft mouse model. **Results:** Loss of NKD2 expression and complete methylation were found in KYSE150 and TE1 cells. Reduced expression and partial methylation were observed in KYSE30, KYSE70, KYSE410, KYSE140 and COLO680 cells. High level expression and unmethylation were detected in KYSE450 and TE8 cells. Re-expression of NKD2 was induced by 5-aza-2'-deoxycytidine in NKD2 unexpressed or reduced cells. NKD2 was methylated in 53.2% (82/154) of human primary esophageal cancer samples, and promoter region hypermethylation was associated with reduced expression of NKD2 significantly ($p<0.01$). NKD2 methylation was associated with TNM stage and lymph node metastasis ($p<0.01$). The results suggest that NKD2 is regulated by

promoter region methylation and methylation of NKD2 may serve as a prognostic marker in esophageal cancer. Our further studies demonstrate that NKD2 suppresses cell proliferation, colony formation, cell invasion and migration, as well as induces G1/S check point arrest in esophageal cancer cells. NKD2 suppressed xenograft tumor growth and inhibited Wnt signaling in human esophageal cancer cells. **Conclusions:** NKD2 is frequently methylated in human esophageal cancer, and the expression of NKD2 is regulated by promoter region methylation. NKD2 suppresses esophageal cancer progression by inhibiting Wnt signaling both *in vitro* and *in vivo*.

Keywords: NKD2, DNA methylation, Wnt signaling, esophageal cancer

Introduction

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide [1]. The overall 5-year survival remains below 15% [2]. Poor outcomes in patients with esophageal cancer are related to diagnosis at advanced (metastatic) stages and the propensity for metastases [3]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal carcinoma worldwide [4]. Tobacco use and alcohol consumption are risk factors for ESCC, and the combination of tobacco and alcohol consumption further increases the risk of ESCC. Mutations in enzymes that metabolize alcohol have been associated with increased risk of ESCC [5-7]. Genetic and epigenetic

alterations are involved in esophageal carcinogenesis [8]. Aberrant expression of components in Wnt signaling pathway are found in many types of cancers, including esophageal cancer, and Wnt signaling pathway plays an important role in cancer progression [9, 10].

The naked cuticle (NKD) family includes Drosophila naked cuticle and its two vertebrate orthologs, NKD1 and NKD2. NKD1 is located in human chromosome 16q12.1, which has frequent loss of heterozygosity in human breast and hepatocellular carcinoma [11, 12]. NKD2 is located in chromosome 5p15.3. Loss of heterozygosity has been frequently found in these regions in multiple tumors [13-15]. In both zebrafish and mice, NKD inhibits canonical and non-canonical Wnt signaling [16-18]. The C-terminus of NKD2 is highly disordered, while the N-terminal region of NKD2 contains most of the functional domain, including myristoylation, an EF-hand motif, a Dishevelled binding region, and a vesicle recognition and membrane targeting motif [19-21]. NKD2 binds to multiple proteins and may function as a switch protein through its several functional motifs [22]. Both NKD1 and NKD2 have been proposed to interact with Dishevelled through their EF-hand-like motif. In addition, NKD2 has been reported to bind to Dishevelled through its TGFα binding region [21, 22]. NKD2 was reported to suppress tumor growth and metastasis in osteosarcoma through negative regulation of Wnt signaling [23]. Our previous study found that methylation of NKD2 promotes breast cancer growth by activating

Wnt signaling [24]. The methylation status and the function of NKD2 in esophageal cancer have yet to be elucidated. Therefore in this study we investigated the epigenetic changes and functions of NKD2 in human ESCC.

Materials and Methods

Human tissue samples and cell lines

Fifteen cases of human normal esophageal mucosa and 154 cases of human esophageal cancer samples were collected from the Chinese PLA General Hospital in Beijing. The median age of the cancer patients is 62.1 years old (range 46-87), and the ratio of males/females is 3.05:1. All cancer samples were classified according to TNM staging (AJCC 2010), including 5 cases of stage I, 99 cases of stage II and 50 cases of stage III. All samples were collected following the guidelines approved by the Institutional Review Board of the Chinese PLA General Hospital with written informed consent from patients (Reference No. 20090701-015).

Nine esophageal cancer cell lines (KYSE450, KYSE30, KYSE150, KESE70, TE8, KYSE410, TE1, KYSE140 and COLO680) were previously established from primary esophageal cancer and maintained in 90% RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

5-aza-2'-deoxycytidine treatment

Esophageal cancer cell lines were split to a low density (30%

confluence) 12 hours before treatment. Cells were treated with 5-aza-2'-deoxycytidine (5-AZA, Sigma, St. Louis, MO) at a concentration of 2μM. Growth medium conditioned with 5-AZA at a concentration of 2μM was exchanged every 24 hours for a total of 96 hours of treatment.

RNA isolation and semi-quantitative reverse transcription PCR

Total RNA was isolated by Trizol reagent (Life Technologies, Gaithersburg, MD). First strand cDNA was synthesized according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). PCR primers for NKD2 are 5'-ACAGGAGGTTGTCTGCACACG-3' (F) and 5'-GACTTGAGGAACTGCTTCTCC-3' (R). The primer sets for NKD2 were designed to span intronic sequences between adjacent exons in order to control for genomic DNA contamination. Semi-quantitative reverse transcription PCR (RT-PCR) was amplified for 33 cycles. GAPDH was used as an internal control.

Bisulfite modification, methylation-specific PCR and bisulfite sequencing

DNA was prepared by the proteinase K method. Bisulfite treatment was carried out as previously described [25, 26]. Methylation-specific PCR (MSP) primers were designed according to genomic sequences around transcriptional start sites (TSS) and synthesized to detect unmethylated (U) and methylated (M) alleles. Bisulfite sequencing (BSSQ) was

performed as previously described [9]. BSSQ products were amplified by primers flanking the targeted regions including MSP products. The MSP primers are as follows: NKD2-M-Forward 5'-GAGGTCGATGCGTTGCGGGTAGC-3' and NKD2-M-Reverse 5'-CGACGACCCGACTCCCTCTAAACG-3'; NKD2-U-Forward 5'-GTTGAGGTTGATGTGTTGTGGGTAGT-3' and NKD2-U-Reverse 5'-CAACAACAACCCAACTCCCTCTAAACA-3'. The bisulfite sequencing primers are 5'-GTTGGTGGGGTTTTAGGTTGG-3' (F) and 5'-AACTAAATTCTAAAACCRAAACC-3' (R).

Immunohistochemistry

Immunohistochemistry (IHC) was performed in human esophageal cancer samples and paired adjacent tissue samples. The NKD2 antibody was diluted 1:500 (Novus Biology, CO, USA). The staining intensity and extent of the staining area were scored using the German semi-quantitative scoring system as described previously [27, 28].

Plasmid construction

Human full-length NKD2 CDS (GenBank accession number NM_033120) was amplified and subcloned as described previously[24]. The primers used were 5′-GAGGATCCGCCACCATGGGGAAACTGCAGTCGAAG-3′ (F) and 5′-GATCTCGAGCTAGGACGGGTGGAAGTGGT-3′ (R). NKD2 expressing lentiviral or empty vectors were packaged using the

ViraPower™ lentiviral expression system (Invitrogen, San Diego, CA, USA). Lentivirus was added to the growing medium of KYSE150 and TE1 cells, and NKD2 stably expressed cells were selected by blasticidin (Invitrogen, San Diego, CA, USA) at a concentration of 2μg/ml.

Cell viability detection

Cells were plated into 96-well plates at 2×10^3 cells/well, and the cell viability was measured by MTT assay（KeyGEN Biotech, Nanjing, China) at 0, 24, 48 and 72h. Absorbance was measured on a microplate reader (Thermo Multiskan MK3, MA, USA) at a wavelength of 490 nm.

Colony formation assay

NKD2 unexpressed and stably expressed cells were seeded at 500 cells per well in 6-well culture plates in triplicate. The complete growth medium conditioned with blasticidin at 2ug/ml was exchanged every 72 hours. After 2 weeks, cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet (Beyotime, Nanjing, China) for visualization and counting.

Flow cytometry

NKD2 unexpressed and re-expressed KYSE150 and TE1 cells were starved 12 hours for synchronization, and the cells were re-stimulated with 10% FBS for 24 hours. Cells were fixed with 70% ethanol and treated using the Cell Cycle Detection Kit (KeyGen Biotech, Nanjing, China). The cells were then sorted by a FACS Caliber flow cytometer

(BD Biosciences, Mansfield, CA). The cell phase distribution was analyzed by the Modfit software (Verity Software House, ME, USA).

Transwell assay

NKD2 unexpressed and re-expressed KYSE150 and TE1 cells were suspended in serum-free medium. Cells (2×10^5) were placed into the upper chamber of an 8 μm pore size transwell apparatus (Corning, NY, USA) and incubated for 20 hours. Cells that migrated to the lower surface of the membrane were stained with crystal violet and counted in three independent high-power fields (×200). For invasion analysis, NKD2 unexpressed and re-expressed KYSE150 and TE1 cells (2×10^5) were seeded into the upper chamber of a transwell apparatus coated with extracellular matrix gel (ECM gel, BD Biosciences, San Jose, CA) and incubated for 36 hours. Cells that invaded into the lower membrane surface were stained with crystal violet and counted in three independent high-power fields (×200).

SiRNA knockdown technique

Selected siRNAs targeting NKD2 and the RNAi negative control duplex were used in this study. The sequences of the siRNAs targeting NKD2 and the RNAi negative control are as follows： NKD2-F: 5’-GGGAUUGAGAACUACACGUTT-3’ ， NKD2-R: 5’-ACGUGUAGUUCUCAAUCCCTT-3’ ， Negative Control-F: 5’-UUCUCCGAACGUGUCACGUTT-3’ and Negative Control-R:

5’-ACGUGACACGUUCGGAGAATT-3’. The RNAi oligonucleotide and RNAi negative control duplex were transfected into KYSE450 cells, which expressed high levels of NKD2.

In vivo tumorigenicity

NKD2 stably expressed and unexpressed KYSE150 cells (4×10^6 cells in 0.2 ml phosphate-buffered saline) were subcutaneously injected into the dorsal flank of 5-week-old female BALB/c nude mice. The tumor size was measured every 3 days for 24 days beginning 3 days after implantation. The tumor volumes were calculated according to the following formula: $V = L \times W^2/2$, where V, volume (mm^3); L, biggest diameter (mm); W, smallest diameter (mm). All procedures were approved by the Animal Ethics Committee of the Chinese PLA General Hospital.

Western blot

Protein samples from esophageal cancer cells were collected and western blot was performed as described previously [29]. Antibodies were diluted according to manufacturer’s instructions. The primary antibodies were as follows: NKD2 (Cell Signaling Technology, Danvers, MA), MMP2, MMP7, MMP9, cyclin D1, c-myc, p-β-catenin, β-catenin and β-actin (Bioworld Technology, MN, USA).

Statistical analysis

SPSS 17.0 software (IBM, NY, USA) was used for data analysis. All

data were presented as means ± standard deviation (SD) and analyzed using the Student's t test. The Chi-squared test and the Fisher's exact test were used to analyze the association of NKD2 methylation status with clinic-pathologic factors and the association of NKD2 expression with methylation status. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS

NKD2 expression is regulated by promoter region methylation in esophageal cancer cell lines

The expression of NKD2 was detected by semi-quantitative RT-PCR in human esophageal cancer cell lines. As shown in Figure 1A, loss of NKD2 expression was found in KYSE150 and TE1 cells. Reduced expression of NKD2 was observed in KYSE30, KYSE70, KYSE410, KYSE140 and COLO680 cells. High level expression of NKD2 was detected in KYSE450 and TE8 cells. The methylation status of the NKD2 promoter was examined by MSP. Complete methylation was found in KYSE150 and TE1 cells. Partial methylation was detected in KYSE30, KYSE70, KYSE410, KYSE140 and COLO680 cells. Unmethylation was observed in KYSE450 and TE8 cells (Figure 1B). These results demonstrate that loss of expression or reduced expression of NKD2 correlated with promoter region methylation in human esophageal cancer cells. To further reveal the methylation density and validate the MSP

results, BSSQ technique was used. As shown in Figure 1C, NKD2 was completely methylated in KYSE150 and TE1 cells, partially methylated in KYSE410 cells, and unmethylated in KYSE450 cells and normal esophageal mucosa. The results are consistent with MSP results (Figure 1C). To further analyze NKD2 expression is regulated by promoter region methylation, KYSE450, KYSE30, KYSE150, KYSE70, TE8, KYSE410, TE1, KYSE140 and COLO680 cells were treated with 5-AZA, a demethylating reagent. Restoration of NKD2 expression was induced by 5-AZA in KYSE150 and TE1 cells. Increased expression of NKD2 was observed in KYSE30, KYSE70, KYSE410, KYSE140 and COLO680 cells treated with 5-AZA, while, no expression changes were found in KYSE450 and TE8 cells before and after 5-AZA treatment (Figure 1A). These results suggest that the expression of NKD2 is regulated by promoter region methylation in human esophageal cancer cells.

NKD2 is frequently methylated in primary human esophageal cancer

To further explore the methylation status of NKD2 in primary human esophageal cancer, the methylation status was examined by MSP in 154 cases of esophageal cancer tissue samples and 15 cases of normal esophageal mucosa from non-cancerous patients. NKD2 was methylated in 53.2% (82/154) of primary esophageal cancer samples, and no methylation was detected in normal esophageal mucosa (Figure 2A and

B). As shown in table 1, NKD2 methylation was associated with TNM stage and lymph node metastasis significantly (both p <0.01), but no association was found between NKD2 methylation and age, gender, tumor size and differentiation (all p> 0.05). To further validate that NKD2 expression is regulated by promoter region methylation, 30 cases of available matched esophageal cancer and adjacent tissue paraffin samples were evaluated by IHC. NKD2 staining was observed mainly in the cytoplasm of the esophagus. NKD2 is highly expressed in adjacent tissue samples and reduced in primary cancer tissue samples (Figure 2C and D). Reduced expression of NKD2 is associated with the promoter region hypermethylation (p<0.01, Figure 2E). These results demonstrate that NKD2 is regulated by promoter region methylation in primary esophageal cancer.

Restoration of NKD2 expression suppresses cell proliferation and induces G1/S arrest in esophageal cancer cells

To evaluate the effects of NKD2 on cell proliferation, the cell viability was detected by MTT and colony formation assays. The OD value was 0.892 ± 0.027 vs. 0.763 ± 0.024 (p <0.05) in KYSE150 cells and 0.551 ± 0.024 vs 0.438 ± 0.011 (p <0.001) in TE1 cells before and after restoration of NKD2 expression (Figure 3A). The results demonstrated that NKD2 inhibited esophageal cancer cell viability. The effect of NKD2 on cell proliferation was evaluated by colony formation

assay. The clone numbers were 135.3 ± 6.8 vs. 57.7 ± 4.0 (p <0.001) in KYSE150 cells and 58.3 ± 4.7 vs. 29.7 ± 3.5 (p <0.001) in TE1 cells before and after restoration of NKD2 expression **(**Figure 3B**)**. These results suggest that NKD2 suppresses esophageal cancer cell growth.

To further understand the mechanism of NKD2 in esophageal cancer development, the role of NKD2 in cell cycle was analyzed by flow cytometry. In KYSE150 cells, the cell phase distribution before and after re-expression of NKD2 was as follows: G0/1 phase: 33.46 ± 0.58% vs. 41.82 ± 1.73%, S phase: 46.17 ± 2.21% vs. 37.15 ± 1.46%, and G2/M phase: 20.37 ± 2.21% vs. 21.03 ± 0.31%. The G0/1 phase is increased and the S phase is reduced significantly after re-expression of NKD2 (all p< 0.01).

In TE1 cells, the cell phase distribution before and after re-expression of NKD2 was as follows: G0/1 phase: 44.13 ± 2.60% vs. 61.73 ± 1.28%, S phase: 44.21 ± 3.88% vs. 22.93 ± 1.77%, and G2/M phase: 11.67 ± 1.38% vs. 15.34 ± 0.82% (Figure 3C). The G0/1 phase is increased and the S phase is reduced significantly after re-expression of NKD2 in TE1 cells (all p< 0.001). These results suggest that NKD2 induced G1/S check point arrest in esophageal cancer cells.

Restoration of NKD2 expression inhibits cell migration and invasion in human esophageal cancer cells

The transwell assay in the absence of ECM gel coating was employed to explore the effect of NKD2 on cell migration. The number of migrated cells for each high power field under the microscope was 105.7 ± 5.1 vs. 63.0 ± 4.0 in KYSE150 cells and 147 .0 ± 6.6 vs. 52.3 ± 5.7 in TE1 cells before and after restoration of NKD2 expression. The cell number was reduced significantly after re-expression of NKD2 in esophageal cancer cells (all p <0.001, Figure 4A). These results demonstrate that NKD2 inhibits esophageal cancer cell migration.

Next, the transwell assay with ECM coating was employed to evaluate the effect of NKD2 on cell invasion. The number of invasive cells for each high power field under the microscope was 114.7 ± 4.5 vs. 79.7 ± 4.5 in KYSE150 cells and 137.0 ± 4.0 vs. 65.0 ± 2.7 in TE1 cells before and after restoration of NKD2 expression. The cell number was reduced significantly after re-expression of NKD2 in KYSE150 and TE1 cells (all p<0.001, Figure 4B). These results suggest that NKD2 impedes esophageal cancer cell invasion.

To further understand the mechanism of NKD2 in esophageal cancer migration and invasion, the expression levels of MMP2, MMP7 and MMP9 were detected by western blot. As shown in Figure 4C, the expression levels of MMP2, MMP7 and MMP9 were reduced after re-expression of NKD2 in KYSE150 and TE1 cells. The inhibitory role of NKD2 on MMP2, MMP7 and MMP9 expression was further validated by

knocking down NKD2 in KYSE450 cells. Taken together, the above results suggest that NKD2 suppresses esophageal cancer cell migration and invasion.

NKD2 inhibits Wnt/β-catenin signaling in esophageal cancer

NKD2 has been reported to negatively regulate canonical Wnt signaling in multiple tumors [23, 24].To determine whether the canonical Wnt signaling pathway is regulated by NKD2 in human esophageal cancer, the key components in downstream of Wnt signaling pathway were detected by western blotting. The level of β-catenin was reduced and the level of phospho-β-catenin was increased after re-expression of NKD2 in KYSE150 and TE1 cells. The expression of Wnt signaling targeting genes, c-myc and cyclinD1, was reduced after re-expression of NKD2 in KYSE150 and TE1 cells (Figure 5A). These results demonstrate that NKD2 inhibits Wnt signaling in human esophageal cancer. To further validate the role of NKD2 on the Wnt signaling pathway, siRNA knockdown technique was employed. The expression of β-catenin, c-myc and cyclinD1 was increased, and the level of phospho-β-catenin was reduced after knockdown of NKD2 in KYSE450 cells (Figure 5B). These results suggest that NKD2 represses esophageal cancer cell proliferation by inhibiting Wnt signaling.

NKD2 suppresses tumor growth in esophageal cancer cell xenograft mice

To further validate the effects of NKD2 in esophageal cancer *in vivo*, NKD2 unexpressed and re-expressed KYSE150 cell xenograft mouse models were employed (Figure 6A). The volume of xenograft tumors was 345.12 ± 18.42 mm^3 in NKD2 unexpressed KYSE150 cells and 96.78 ± 17.29 mm^3 in NKD2 re-expressed KYSE150 cells. The tumor volume is smaller in NKD2 re-expressed KYSE150 cell xenografts compared to NKD2 unexpressed KESE150 cell xenografts ($p<0.001$, Figure 6A and B). The tumor weight was 220.62 ± 28.51 mg in NKD2 unexpressed KYSE150 cell xenografts and 22.35 ± 5.19 mg in NKD2 re-expressed KYSE150 cell xenografts. The tumor weight is lower in NKD2 expressed KYSE150 cell xenografts compared to NKD2 unexpressed KYSE150 cell xenografts ($p<0.001$, Figure 6C). To further validate NKD2 inhibits Wnt signaling *in vivo*, the expression of NKD2 and the levels of phospho-β-catenin was detected by IHC staining. The levels of phospho-β-catenin were increased in NKD2 expressing esophageal cancer cell xenografts (Figure 6D). These results suggest that NKD2 suppresses esophageal cancer cell growth by inhibiting Wnt signaling *in vivo*.

Discussion

The main risk factors for ESCC are cigarette smoking and alcohol consumption [30]. Genome-wide association analysis has demonstrated that gene-environment interaction promotes development of ESCC [31]. Other

studies have found that 2% of esophageal cancer and 11% of head and neck cancer patients develop a second cancer due to field cancerization [32, 33]. These studies support the idea that environment plays an important role in ESCC. Whole-genome and whole-exome sequencing in Chinese patients with ESCC revealed eight mutated genes, including six known tumor-associated genes (*TP53, RB1, CDKN2A, PIK3CA, NOTCH, and NFE2L2*) and two novel genes (*ADAM29 and FAM135B*) [34]. Additional genes were found frequently methylated in ESCC in previous studies [9, 27, 35-44]. Despite recent advances in treatment strategies, there has been no significant improvement in overall survival rate for advanced and metastatic disease [45]. New strategies are necessary for early detection and to improve treatment options in ESCC. Aberrant epigenetic changes can be induced by environmental factors, and epigenetic changes are reversible under certain circumstances [46, 47]. Therefore, more effective therapeutic strategies based on epigenetics are developing.

In this study, we demonstrated that NKD2 is frequently methylated in human ESCC and the expression of NKD2 is regulated by promoter region methylation. NKD2 methylation is associated with TNM stage and lymph node metastasis, suggesting that NKD2 methylation may serve as a poor prognostic marker in human ESCC. Further study found that NKD2 inhibits esophageal cancer cell proliferation, colony formation and induces G1/S check point arrest. In addition, NKD2 suppressed

esophageal cancer cell migration and invasion. These results suggest that NKD2 is involved in esophageal cancer progression and metastasis. The role of NKD2 in suppression of esophageal cancer growth was validated by an esophageal cancer cell xenograft model *in vivo*. We further explored the mechanism by which NKD2 suppresses esophageal cancer progression and metastasis. NKD2 impedes ESCC metastasis by down-regulating MMP2, MMP7 and MMP9 expression, and suppresses ESCC growth by inhibiting Wnt signaling.

At the time of diagnosis, more than 50% of esophageal cancer patients have been metastasized [48]. Though there are many approaches to treat metastatic disease, the overall survival time remains poor. Understanding the molecular events in ESCC may improve therapeutic strategies [49, 50]. Our finding provide more clues for epigenetic-based personalized medicine in esophageal cancer.

Conclusion

NKD2 is frequently methylated in human esophageal cancer and the expression of NKD2 is regulated by promoter region methylation. Methylation of NKD2 is associated with TNM stage and lymph node metastasis. NKD2 suppresses human esophageal cancer growth by inhibiting Wnt signaling.

List of abbreviations

5-AZA, 5-aza-2’-deoxycytidine; BSSQ, bisulfite sequencing;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ESCC，esophageal squamous cell carcinoma; IHC, immunohistochemistry; IVD, in vitro methylated DNA; ECM gel, extracellular matrix gel; MMP, matrix metalloproteinase; MSP, methylation specific polymerase chain reaction; NKD, naked cuticle; NL, normal lymphocyte DNA; RT-PCR, reverse-transcription polymerase chain reaction; TGFα, transforming growth factor α; TSS, transcription start sites.

Competing interests:

The authors declare no conflict of interest.

Authors' contributions

BC and WY performed experiments, analyzed data and wrote the manuscript. YJ, MZ, TH and QZ provided feedback and experimental advice. JGH and GZ provided experimental advice and manuscript editing. MG conceived the study design, supervised the experiments and edited the manuscript. All authors approved the final version of the submitted manuscript.

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Table 1. Clinical factors and NKD2 methylation in 154 cases of esophageal cancer

Clinical factor	No.	NKD2 methylation status		*p* * value
		Methylated n=82(53.2%)	Unmethylated n=72 (46.8%)	
Age (year)				
<50	10	5	5	*p*=0.9085
≥50	144	77	67	
Gender				
Male	116	65	51	*p*=0.3058
Female	38	17	21	
Tumor Size (cm)				
<5	99	50	49	*p*=0.4555
≥5	55	32	23	
Differentiation				
Well	9	6	3	*p*=0.3792
Moderate	97	54	43	
Poor	48	22	26	
TNM Stage				
Ⅰ+Ⅱ	104	47	57	*p*=0.0066<0.01
Ⅲ+Ⅳ	50	35	15	
Lymph node Metastasis				
N0	90	39	51	*p*=0.0058<0.01
N1	64	43	21	

* *p* values are obtained from chi-square test and the Fisher's exact test, significant difference, $p < 0.05$

Figure legends

Table 1. Clinical factors and NKD2 methylation in 154 cases of esophageal cancer

Figure 1. The expression and methylation status of NKD2 in esophageal cancer cells and normal esophageal mucosa

A. Semi-quantitative RT-PCR shows NKD2 expression levels in esophageal cancer cell lines. KYSE450, KYSE30, KYSE150, KESE70, TE8, KYSE410, TE1, KYSE140 and COLO680 are esophageal cancer cell lines. 5-AZA: 5-aza-2'-deoxycytidine; GAPDH: internal control of RT-PCR; H_2O: double distilled water. (-): absence of 5-AZA; (+): presence of 5-AZA.

B. MSP results of NKD2 in esophageal cancer cell lines. U: unmethylated alleles; M: methylated alleles; IVD: *in vitro* methylated DNA, serves as methylation control; NL: normal peripheral lymphocytes DNA, serves as unmethylation control; H_2O: double distilled water.

C. BSSQ results of NKD2. KYSE150, KYSE410, TE1, KYSE450: esophageal cancer cells, NE: normal esophageal mucosa. Double-headed arrow: MSP PCR product spanned 103 bp in NKD2. Bisulfite sequencing focused on a 287 bp region of the CpG island

(-287 bp to +38 bp) across the NKD2 transcription start site. Filled circles: methylated CpG sites, open circles: unmethylated CpG sites. TSS: transcription start site.

Figure 2. Methylation status and expression of NKD2 in primary esophageal cancer samples

A. MSP results of NKD2 in normal esophageal mucosa. NE: normal esophageal mucosa.

B. Representative results of MSP for NKD2 in primary esophageal cancer samples. EC: primary esophageal cancer samples.

C. Representative IHC results showing NKD2 expression in esophageal cancer and matched adjacent tissue samples (upper: ×100; lower: ×400).

D. NKD2 expression scores are shown as box plots, horizontal lines represent the median score; the bottom and top of the boxes represent the 25^{th} and 75^{th} percentiles, respectively; vertical bars represent the range of data. The expression level of NKD2 was significantly different between adjacent tissue and esophageal cancer samples. ***$p<0.001$.

E. The bar diagram shows the expression and DNA methylation status of NKD2 in different cancer samples. Reduced expression of NKD2 was significantly associated with promoter region methylation .

**p<0.01.

Figure 3. NKD2 inhibits esophageal cancer cell proliferation.

A. Growth curves represent the cell viability analyzed by the MTT assay in NKD2 re-expressed and unexpressed KYSE150 and TE1 cells. The experiment was performed in triplicate. *p<0.05, ***p<0.001.

B. Colony formation results show that colony number was reduced by re-expression of NKD2 in KYSE150 and TE1 cells. Each experiment was repeated three times. The average number of tumor clones is represented by bar diagram. *** p<0.001.

C. Cell phase distribution in NKD2 unexpressed and re-expressed KYSE150 and TE1 cells. The ratio is presented by bar diagram. Each experiment was repeated three times. **p<0.01, ***p<0.001.

Figure 4. Restoration of NKD2 expression inhibits cell migration and invasion

A. Cell migration in NKD2 unexpressed and re-expressed KYSE150 and TE1 cells. The ratio is presented by bar diagram. Each experiment was repeated three times. ***p<0.001.

B. Cell invasion in NKD2 unexpressed and re-expressed KYSE150 and TE1 cells. The ratio is presented by bar diagram. Each experiment was repeated three times. ***p<0.001.

C. The expression levels of NKD2, MMP-2, MMP-7 and MMP-9 were detected by western blot in NKD2 unexpressed and re-expressed KYSE150 and TE1 cells. Knockdown of NKD2 by siRNA was performed to validate the results in NKD2 highily expressed KYSE450 cells.

Figure 5. NKD2 inhibits canonical Wnt signaling in human esophageal cancer cells.

A. The expression levels of β-catenin, cyclin D1 and c-myc were reduced and the level of phosphorylated β-catenin (p-β-catenin) increased after re-expression of NKD2 in KYSE150 and TE1 cells.

B. The level of p-β-catenin was reduced and the expression of β-catenin, c-myc and cyclin D1 were increased after knockdown of NKD2 by siRNA in KYSE450 cells

Figure 6. NKD2 suppresses esophageal cancer cell growth in xenograft mice

A. Representative burdened nude mice in NKD2 re-expressed and unexpressed KYSE150 cells.

B. Subcutaneous tumor growth curves for xenograft mice in NKD2 unexpressed and re-expressed groups at different times. ***$p<0.001$.

C. Tumor weight in nude mice at the 24th day after inoculation of NKD2

unexpressed and re-expressed KYSE150 cells. Bars: mean of 6 mice. ***$p<0.001$.

D. Representative photographs of IHC analysis of NKD2 and p-β-catenin in xenografts. Staining of NKD2 and p-β-catenin was found in NKD2 re-expressed KYSE150 cell xenografts. Magnification: 400×.

Figure 1

Click here to download Figure Figure 1.tif

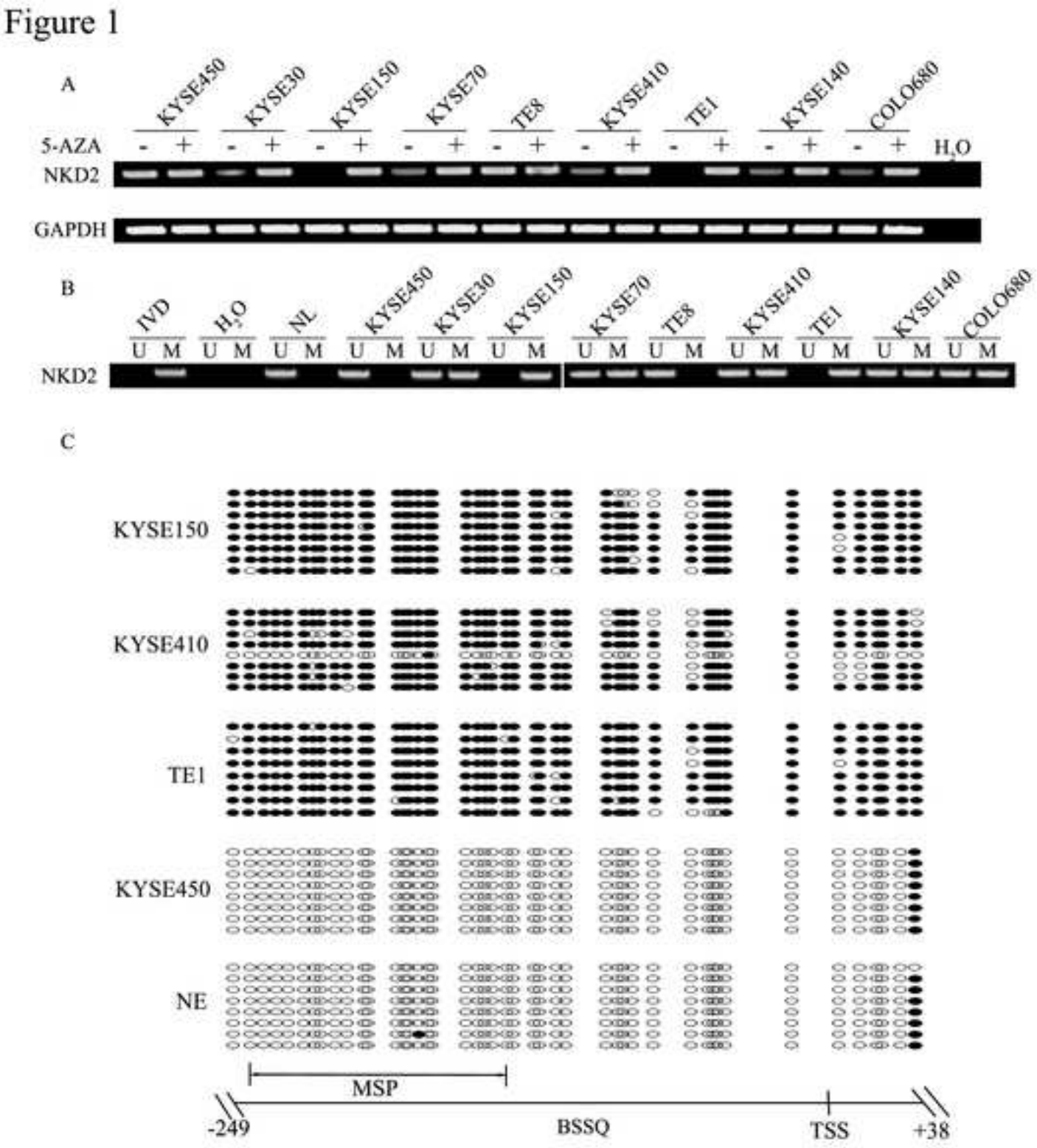
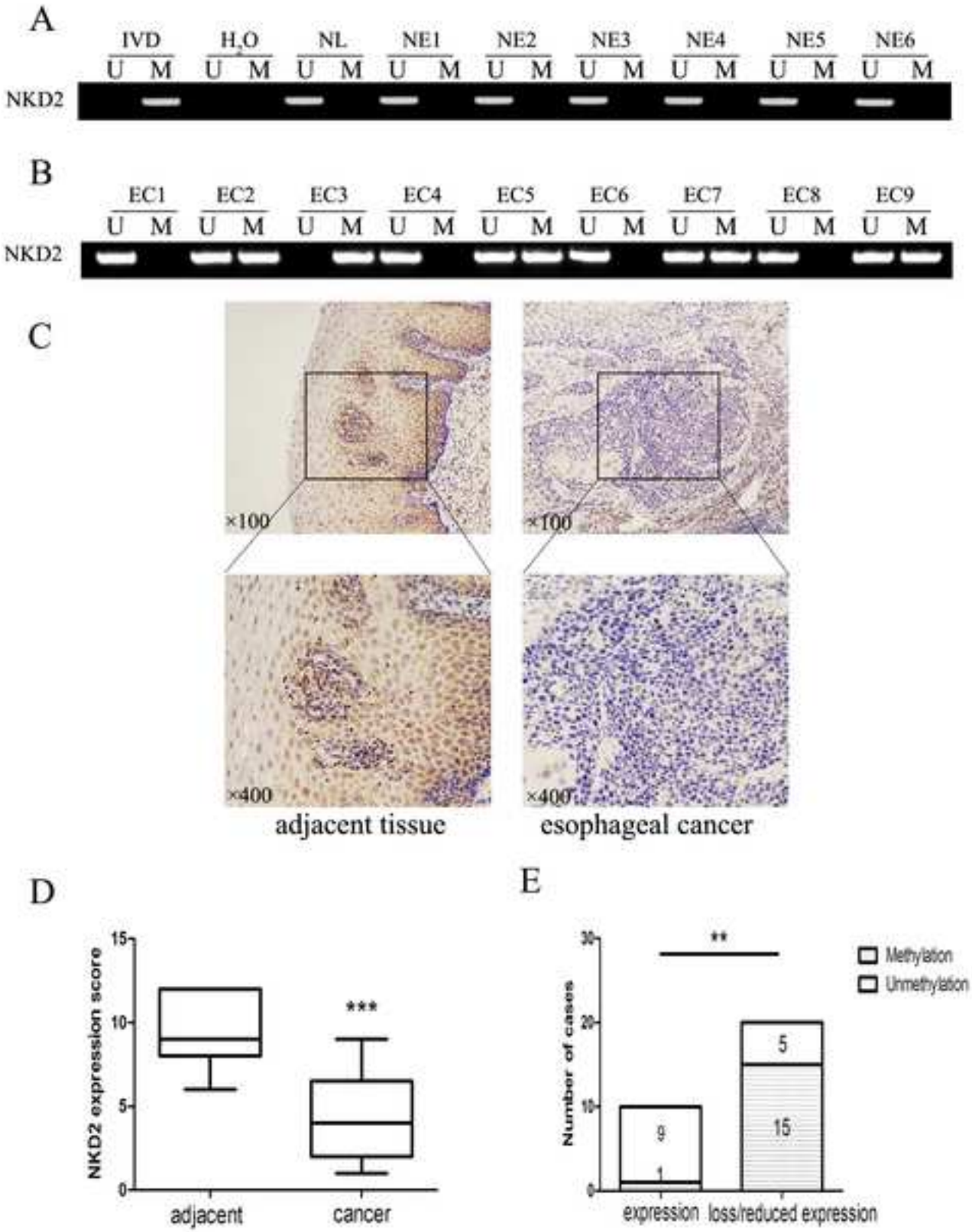


Figure 2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NL
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NE2
NE3
NE4
NE5
NE6
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NKD2
B
EC1
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EC4
EC5
EC6
EC7
EC8
EC9
U M
NKD2
C
×100
×100
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×400
adjacent tissue
esophageal cancer
D
NKD2 expression score
15
10
5
0
***
adjacent
cancer
E
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9
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5
15
Methylation
Unmethylation
expression
loss/reduced expression

Figure 3

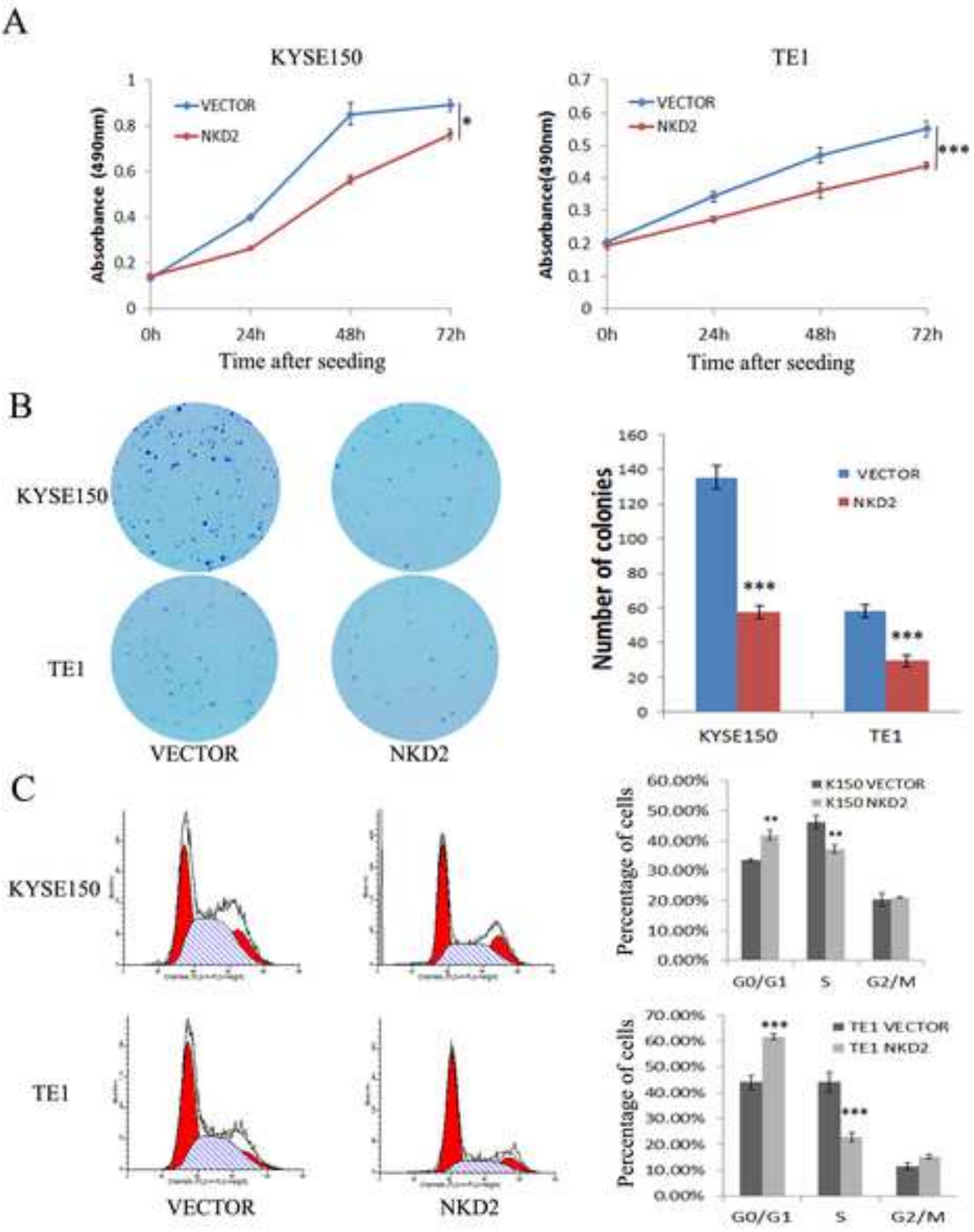
A
KYSE150
TE1
VECTOR
NKD2
Absorbance (490nm)
Absorbance(490nm)
0h
24h
48h
72h
Time after seeding
B
KYSE150
TE1
VECTOR
NKD2
Number of colonies
C
K150 VECTOR
K150 NKD2
TE1 VECTOR
TE1 NKD2
Percentage of cells
G0/G1
S
G2/M

Figure 4

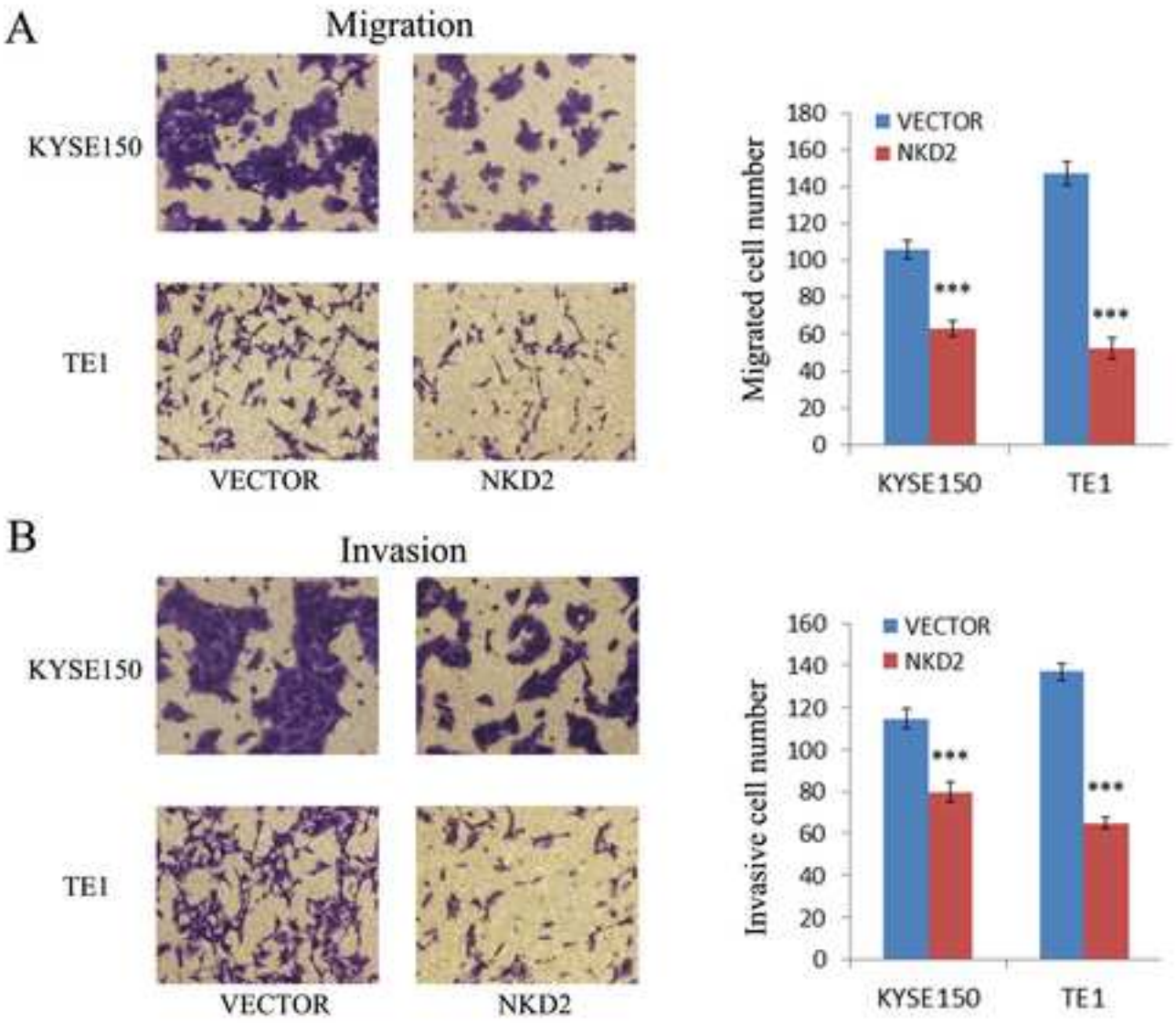

Figure 5

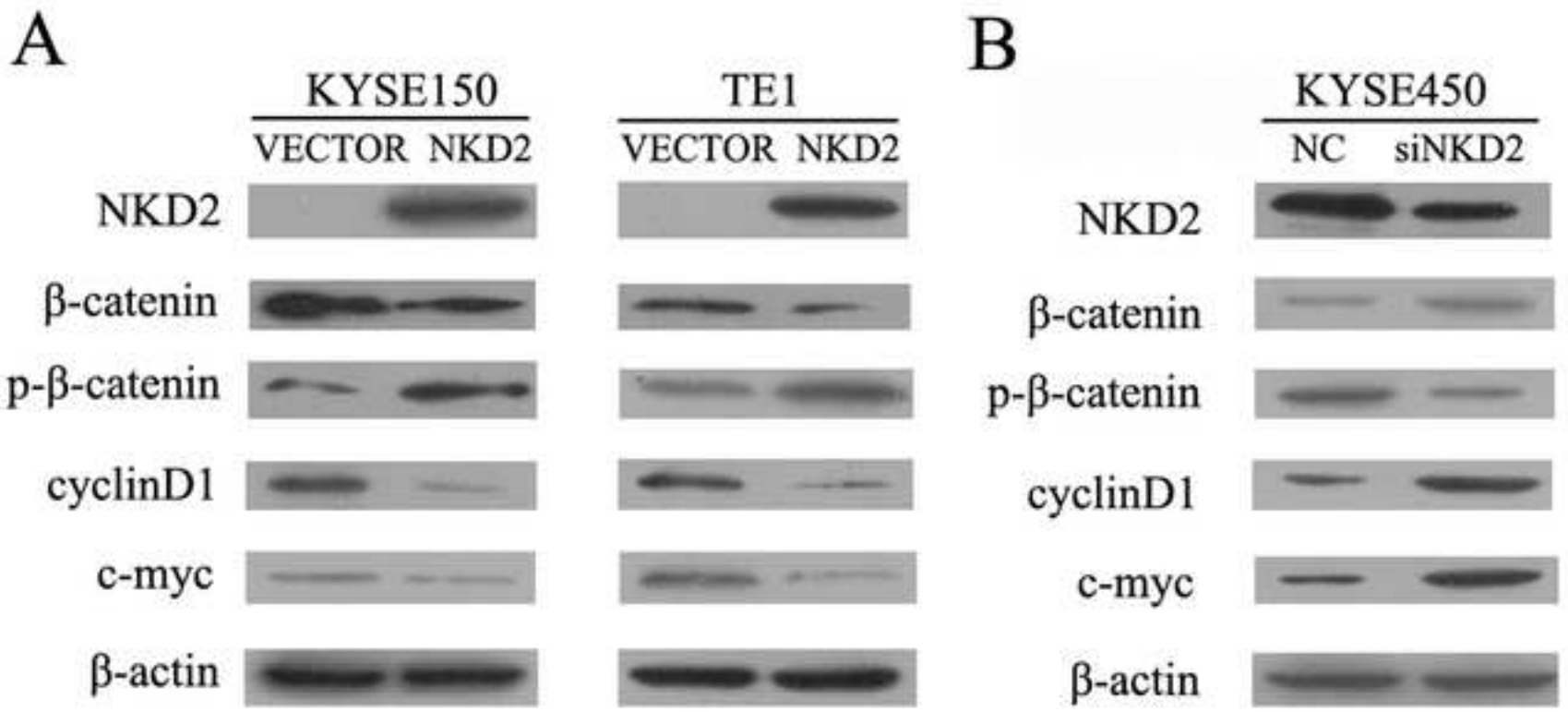

Figure 6