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Protein Kinase Casein Kinase 2–Mediated Upregulation of N-Cadherin Confers Anoikis Resistance on Esophageal Carcinoma Cells

Hyeonseok Ko¹, Seongrak Kim^{1,2}, Cheng-Hao Jin¹, Eunjung Lee^{1,2}, Sunyoung Ham^{1,2}, Jong In Yook^{3,4}, and Kunhong Kim^{1,2}

Abstract

Previously, we reported that high PKCK2 activity could protect cancer cells from death receptor–mediated apoptosis through phosphorylation of procaspase-2. Because anoikis is another form of apoptosis, we asked whether PKCK2 could similarly confer resistance to anoikis on cancer cells. Human esophageal squamous cancer cell lines with high PKCK2 activity (HCE4 and HCE7) were anoikis-resistant, whereas cell lines with low PKCK2 activity (TE2 and TE3) were anoikis-sensitive. Because the cells showed different sensitivity to anoikis, we compared the expression of cell adhesion molecules between anoikis-sensitive TE2 and anoikis-resistant HCE4 cells using cDNA microarray. We found that E-cadherin is expressed only in TE2 cells; whereas N-cadherin is expressed instead of E-cadherin in HCE4 cells. To examine whether PKCK2 activity could determine the type of cadherin expressed, we first increased intracellular PKCK2 activity in TE2 cells by overexpressing the PKCK2 α catalytic subunit using lentivirus and found that high PKCK2 activity could switch cadherin expression from type E to N and confer anoikis resistance. Conversely, a decrease in PKCK2 activity in HCE4 cells by knockdown of PKCK2 α catalytic subunit using shRNA induced N- to E-cadherin switching and the anoikis-resistant cells became sensitive. In addition, N-cadherin expression correlated with PKB/Akt activation and increased invasiveness. We conclude that high intracellular PKCK2 activity confers anoikis resistance on esophageal cancer cells by inducing E- to N-cadherin switching. *Mol Cancer Res*; 10(8); 1032–8. ©2012 AACR.

Introduction

Six essential alterations in cell physiology can result in malignant growth of cancer cells. These are described as "the hallmarks of cancer" (1) and include: (i) sustaining proliferative signaling, (ii) evading growth suppressors, (iii) resisting cell death, (iv) enabling replicative immortality, (v) inducing angiogenesis, and (vi) activating invasion and metastasis (1). Cancer cell invasion of surrounding tissues and metastasis to distant organs is the primary cause of death for most cancer patients. The sequential steps in the pathogenesis of cancer metastasis include vascularization, inva-

sion, detachment, embolization, survival in the circulation, arrest, extravasation, evasion of host defense, and progressive growth (2). Approximately one million circulating tumor cells are shed per gram of tumor tissue (3, 4) but fewer than 0.1% of cancer cells are viable within 24 hours after entry into the circulation and fewer than 0.01% survive to produce metastases (5). The low success rate of metastasis could result from the presence of physiologic barriers to metastasis such as anoikis and tumor surveillance.

Anoikis is a type of apoptosis that is induced by loss of cell adhesion to the extracellular matrix (ECM) and is an intrinsic characteristic of normal epithelial cells (6). In contrast to normal epithelial cells, cancer cells are resistant to anoikis and adhesion to the ECM is not required for their survival and proliferation. Accordingly, acquisition of anoikis resistance might be a molecular prerequisite for the successful metastatic spread of cancer cells. Cancer cells have developed strategies to become resistant to anoikis, which converge in the activation of survival signals (7). Such strategies include a switch in integrin expression (8, 9), reactive oxygen species–mediated Src oxidation/activation (10, 11), epithelial mesenchymal transition (EMT; refs. 12–14), constitutive activation of survival signaling components such as Src, PKB/Akt, or ERK (15–17), or overexpression of tropomyosin-related kinase B (TrkB; refs. 18 and 19).

PKCK2 is a constitutively active, growth factor–independent serine/threonine protein kinase with key roles in cell-

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cycle control, cellular differentiation, proliferation, and regulation of apoptosis (20–22). Changes in PKCK2 expression or activity have been reported in many cancer cells (21, 23–25) and overexpression of PKCK2 catalytic subunit can induce tumor formation (23). Inhibition of TNF-related apoptosis inducing ligand (TRAIL) death receptor–mediated apoptosis by PKCK2 might be important in the metastasis of cancer cells (21) because natural killer (NK) cells play a physiological role in tumor surveillance by presenting surface expressed TRAIL to incoming circulating cancer cells to induce apoptosis (26). Incoming cancer cells with high PKCK2 activity are resistant to TRAIL-mediated apoptosis and cannot be removed from the circulation, allowing their successful spread to distant sites (27). PKCK2 is also known as a positive regulator of the Wnt signaling pathway that is important for metastasis of cancer cells (28). Known PKCK2 substrates involved in Wnt signaling are dishevelled, β -catenin, and Snail. Snail is known to repress expression of the adhesion molecule E-cadherin (29, 30). Phosphorylation at Thr 393 on β -catenin protects it from proteosomal degradation, thereby increasing its nuclear translocation and cotranscriptional activity (31, 32). Increased expression of Axin2 by β -catenin results in transportation of GSK3 β out of the nucleus, thereby protecting Snail from GSK3 β -mediated degradation and inducing E-cadherin downregulation (33). In addition, phosphorylation of Snail at Ser 92 by PKCK2 results in its stabilization and thus downregulates E-cadherin expression (34).

Although PKCK2 acts as an antiapoptotic regulator in death receptor–mediated apoptosis, little is known about its association with anoikis of cancer cells. Accordingly, the purpose of this study was to examine whether PKCK2 confers anoikis resistance on esophageal cancer cells.

Materials and Methods

Cell culture and reagents

The human esophageal cancer cell lines TE2, TE3, HCE4, and HCE7 were grown as previously described (35). To induce anoikis, cells were plated on poly-2-hydroxyethylmethacrylate-coated culture plates (poly-HEMA; Sigma-Aldrich) as previously described (36).

cDNA microarray

The cDNA microarray assay was conducted using a GEArray™ series kit purchased from Superarray Bioscience Co. and was used according to the manufacturer's protocol.

Western blot analysis

Western blot analysis was carried out as previously described (37). Cells were lysed in RIPA buffer (1 M Tris [pH 8.0], 5 M NaCl, 100 mmol/L EGTA, 10% NP-40, and 0.25% sodium deoxycholic acid) with 1 \times complete protease inhibitor cocktail (Roche Diagnostics). Lysates were briefly vortexed and cleared by centrifugation at 12,000 r.p.m. for 10 minutes at 4°C. Supernatants were collected and protein concentration determined by Bradford assay (Bio-Rad).

SDS-PAGE (10–12%) was conducted followed by Western blotting. The blotted membranes were immunostained with antibodies specific for the following antigens: Akt, phospho-Akt (Ser-473), PARP, cleaved caspase-3 (Cell Signaling Technology Inc.); procaspase-3 (BD Pharmingen); E-cadherin, N-cadherin (Invitrogen Co.); keratin10, involucrin, vimentin, α -tubulin (Santa Cruz Biotechnology Inc.); PKCK2 α (Upstate), and β -actin (Sigma-Aldrich Co.). The signals were developed using a standard enhanced chemiluminescence method according to the manufacturer's protocol (Amersham Pharmacia Biotech Inc.).

Cell proliferation assay

Cells were plated on poly-HEMA-coated tissue culture plates and proliferation rates were measured using a sulforhodamine B colorimetric (SRB) assay. After collection of the cells and removal of the medium by aspiration, cells were fixed in 50 μ L ice-cold 10% trichloroacetic acid (TCA) and incubated at 4°C for 30 to 60 minutes. The cells were washed 5 times with water and allowed to air dry for 5 minutes. Fifty microliters of 0.4% sulforhodamine B solution in 1% acetic acid was added and the cells were incubated for 30 minutes at room temperature. The stained cells were washed 4 times with 1% acetic acid and allowed to air dry for 5 minutes, solubilized in 100 μ L of 10 mmol/L Tris (pH 10.5), and placed on an orbital rotator for 5 minutes. Supernatants were collected and absorbance at 570 nm was read using an UV-spectrophotometer.

RNA interference, lentiviral expression, and generation of stable cell lines

siRNA was purchased from Dharmacon, Inc. (Option A4). The target sequence for N-cadherin was CAUAUGUGAUGACCGUAAC (nucleotides 851–869). Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technology) according to the manufacturer's protocols. For overexpression of PKCK2 α , the lentivirus vector Lentipgk-GFP mCMV-HA-PKCK2 α IRESpuro was purchased from MacroGen and cotransfected into 293FT cells with packaging plasmids. The resulting supernatant containing PKCK2 α -expressing lentivirus was used to transduce cells according to the manufacturer's protocol (MacroGen). Transduced cells were selected using puromycin (1 μ g/mL). A human shRNA kit for silencing PKCK2 α expression was purchased from Origene Technologies Inc. To obtain stable transfectants, cells were transfected with the plasmid and then treated with puromycin (2 μ g/mL) for selection.

In vitro PKCK2 kinase activity assay

Intracellular PKCK2 activity was measured using an *in vitro* kinase assay as previously described with slight modification (25). Briefly, 3 μ g of bacterially expressed GST-CS (CK2 substrate) protein was incubated with glutathione Sepharose 4B beads for 60 minutes and washed twice with 1 \times kinase buffer (4 mmol/L MOPS, pH 7.2, 5 mmol/L β -glycerolphosphate, 1 mmol/L EGTA, 200 μ mol/L sodium orthovanadate, and 200 μ mol/L DTT). The beads were then incubated with 100 μ g cell lysate in a final volume of

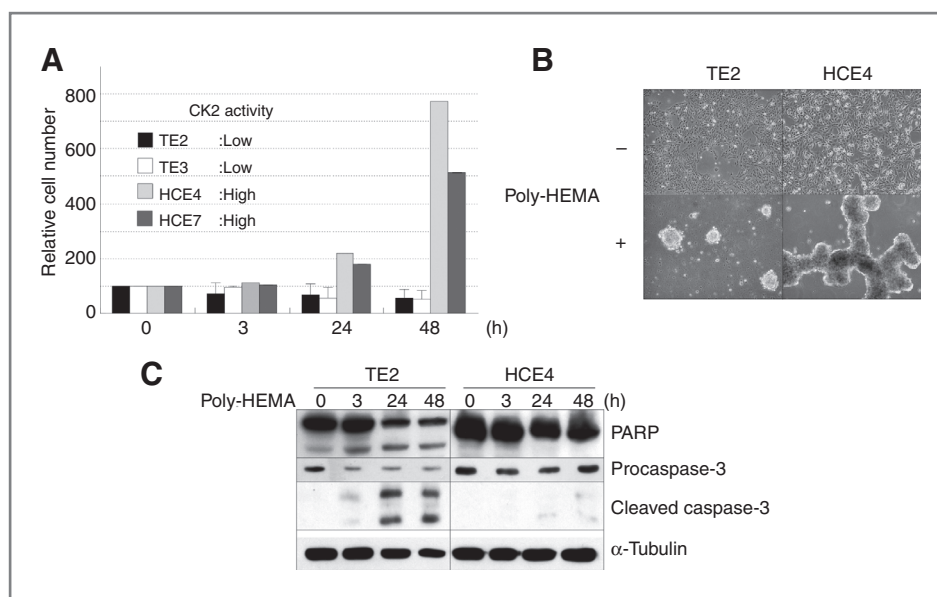


Figure 1. PKCK2 activity and anoikis. A, TE2, TE3, HCE4, or HCE7 cells were cultured on the poly-HEMA-coated tissue culture plates for the indicated times and tested for viability with the SRB assay. The data are expressed as mean \pm SD for triplicates. B, TE2 and HCE4 cells were plated on poly-HEMA-coated tissue culture plate (Poly-HEMA+) and photographed using phase contrast microscopy 16 hours after plating. C, Western blot analysis was conducted to confirm apoptosis using cell lysates for the indicated time points.

50 μ L kinase reaction buffer (10 μ L of 5 \times kinase buffer), 10 μ L magnesium/ATP cocktail solution [90 μ L of 75 mmol/L MgCl₂/500 mmol/L ATP plus 10 μ L (100 μ Ci) [γ -³²P]-ATP] for 20 minutes at 30°C. Reactions were stopped by washing twice with 1 \times kinase buffer. Samples were resuspended with 30 μ L of 2 \times SDS sample loading buffer, subjected to 12% SDS-PAGE followed by staining with Coomassie brilliant blue, and dried on Whatman papers. Incorporation of ³²P was detected by autoradiography.

Cell invasion assay

The cell invasion assay was conducted using a Cell Invasion Assay Kit purchased from CHEMICON International, Inc.

Results

Endogenous PKCK2 activity and anoikis

To examine whether intracellular PKCK2 activity is a determinant of anoikis resistance, esophageal cancer cell lines that show high (HCE4 and HCE7) or low (TE2 and TE3) PKCK2 activity (21) were cultured on poly-HEMA coated tissue culture plates. A cell viability assay showed that the number of HCE4 or HCE7 cells was increased 7.5- and 5-fold respectively, at 48 hours after suspension, whereas the number of TE2 or TE3 cells decreased 0.5-fold under the same conditions (Fig. 1A). Unlike anoikis-sensitive TE2 cells, anoikis-resistant HCE4 cells formed large, spheroid aggregates in suspension (Fig. 1B). Western blot analysis showed that TE2 cells began to undergo apoptosis after 24 hours in suspension but HCE4 cells did not, even after 48 hours of suspension (Fig. 1C). These results suggest that PKCK2 activity might be a determinant of anoikis resistance.

Cadherin expression and anoikis

Because the cancer cells responded differently to detachment, we compared the expression level of adhesion molecules between anoikis-sensitive TE2 and anoikis-resistant HCE4 cells using the GEArray™ Q series Human Extracellular Matrix & Adhesion Molecules Gene Array, which contains 96 genes encoding proteins important for the attachment of cells to their surroundings. E-cadherin expression was observed only in TE2 cells (data not shown) and this differential cadherin expression was confirmed by Western blot analysis (Fig. 2A and Supplementary Fig. S1). Although HCE4 cells express keratin 10 and involucrin, markers of terminal differentiation of epithelial cells, they also express vimentin, a marker for mesenchymal cells, and N-cadherin instead of E-cadherin, suggesting that the cells might be able to transit from epithelial to mesenchymal cells (Fig. 2A). To examine whether the expression of N-cadherin is required for survival of cancer cells in suspension, N-cadherin expression in HCE4 cells was silenced using siRNA. Although HCE4 cells that expressed N-cadherin were resistant to anoikis, N-cadherin-silenced HCE4 cells underwent anoikis (Fig. 2B). These data indicate that N-cadherin expression is necessary for cancer cells to become resistant to anoikis.

PKCK2 activity and cadherin switching

To examine whether there is a causal relationship between intracellular PKCK2 activity and the type of cadherin expressed in the cell, the PKCK2 α catalytic subunit was either overexpressed in TE2 cells using lentivirus or knocked-down in HCE4 cells using shRNA to increase or decrease intracellular PKCK2 activity, respectively. Overexpression of PKCK2 α catalytic subunit in TE2 cells

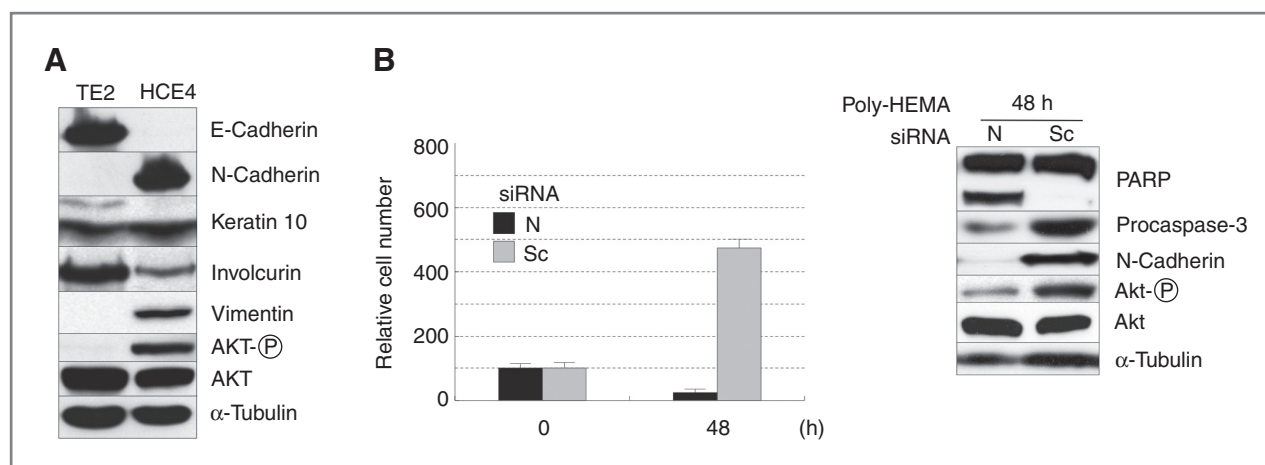


Figure 2. Cadherin expression and anoikis. A, Western blot analysis of epithelial or mesenchymal markers and PKB/Akt activation. B, N-cadherin downregulation, PKB/Akt inactivation, and anoikis. HCE4 cells were transfected with either a scrambled siRNA (Sc) or N-cadherin siRNA (N) for 48 hours. The cells were then cultured on poly-HEMA-coated plates for 48 hours. Cell viability assay (left) and Western blot analysis (right) were carried out. The data are expressed as mean \pm SD for triplicates.

resulted in a marked increase in PKCK2 activity, epithelial–mesenchymal transition as evidenced by E- to N-cadherin switching, and the induction of vimentin expression (Fig. 3A). As expected, TE2-GFP cells underwent anoikis whereas TE2-CK2 α cells were resistant to anoikis (Fig. 3B). When N-cadherin expression was knocked-down in TE2-CK2 α

cells using siRNA against N-cadherin, the cells reverted to anoikis sensitivity (Fig. 3C).

Conversely, knockdown of the PKCK2 α subunit in HCE4 cells (HCE4 CKD) resulted in a decrease in PKCK2 activity, mesenchymal–epithelial transition as evidenced by N- to E-cadherin switching, and reduction of vimentin

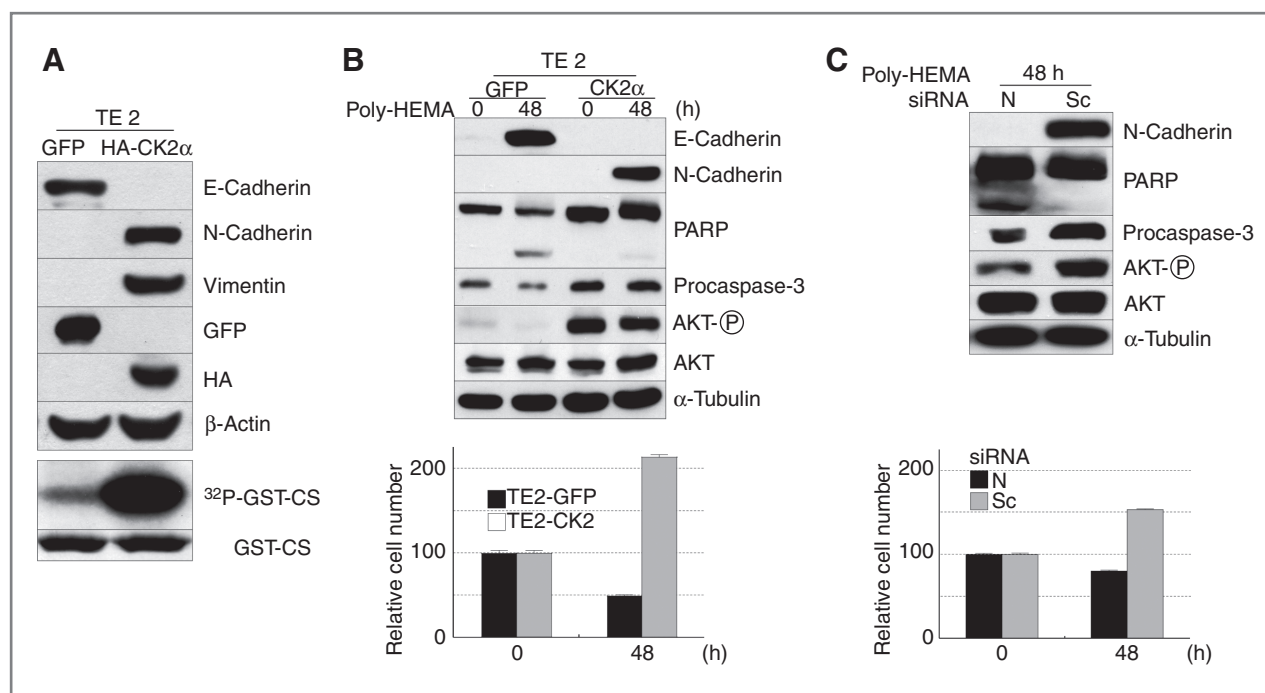


Figure 3. E- to N-cadherin switching and anoikis resistance. A, TE2 cells were transduced with lentivirus-expressing HA-tagged PKCK2 α catalytic subunit. Western blot analysis and *in vitro* kinase assay for intracellular PKCK2 activity were conducted. 32 P-GST-CS (GST-tagged recombinant CK2 Substrate) represents phosphorylated GST-CS and GST-CS represents Coomassie blue stained input GST-CS. B, TE2-GFP and TE2-CK2 α cells were cultured on poly-HEMA-coated plates for 48 hours. Western blot analysis (top) and cell viability assay (bottom) were conducted. The data are expressed as mean \pm SD for triplicates. C, TE2-CK2 α cells were transfected with either a scrambled siRNA (Sc) or N-cadherin siRNA (N) for 48 hours. The cells were then cultured on poly-HEMA-coated plates for 48 hours. Western blot analysis (top) and cell viability assay (bottom) were conducted. The data are expressed as mean \pm SD for triplicates.

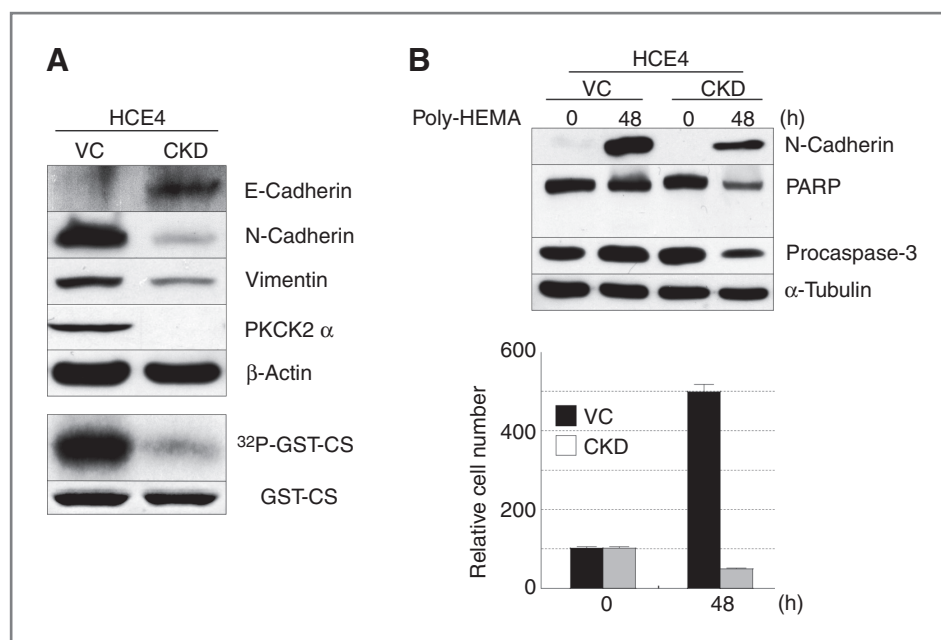


Figure 4. N-to E-cadherin switching and anoikis. A, HCE4 cells were stably transfected with shRNA against PKCK2 α catalytic subunit and treated with puromycin for selection. Western blot analysis and *in vitro* kinase assay for intracellular PKCK2 activity were conducted. B, HCE4-VC (Vector control) and HCE4-CKD (CK2 α knockdown) cells were cultured on poly-HEMA-coated plates for 48 hours. Western blot analysis (top) and cell viability assay (bottom) were conducted. The data are expressed as mean \pm SD for triplicates.

expression (Fig. 4A). The expression pattern of mRNA for each gene (E-cadherin, N-cadherin, vimentin, and PKCK2 α) was the same as that of protein (data not shown). Control HCE4 cells (HCE4 VC) were resistant to anoikis whereas HCE4 CKD cells became apoptotic after 48 hours of suspension (Fig. 4B). Taken together, these results indicate that PKCK2 activity determines the type of cadherin expressed on the cells.

N-cadherin-mediated PKB/Akt activation

Because PKB/Akt is a well-known survival signal, we measured PKB/Akt activity in these cells by Western blot analysis of Akt ser-473 phosphorylation (36, 37) and found that PKB/Akt was active in N-cadherin-expressing HCE4, HCE7 cells (Fig. 2A and Supplementary Fig. S1B) and TE2-CK2 α cells (Fig. 3B, top) that are resistant to anoikis. When N-cadherin expression was knocked-down using siRNA in HCE4 (Fig. 2B) or TE2-CK2 α cells (Fig. 3C), PKB/Akt activity was decreased and the cells became apoptotic in suspension. These results indicate that PKCK2-dependent upregulation of N-cadherin could activate PKB/Akt, thereby conferring anoikis resistance on cancer cells.

Increased invasiveness by N-cadherin

It has been known that N-cadherin-expressing cancer cells are more invasive and metastatic than E-cadherin-expressing cells (38–40). Thus, we examined invasiveness using a cell invasion assay kit and found that N-cadherin-expressing HCE4, HCE7, and TE2-CK2 α cells were more invasive than E-cadherin-expressing TE2, TE3, and TE2-GFP cells (Fig. 5A and B). In addition, N-cadherin-silenced HCE4 cells (N) were less invasive than N-cadherin-expressing HCE4 cells (Sc, Fig. 5C). These results indicate that cancer cells that have high intracellular PKCK2 activity become more invasive probably because of N-cadherin upregulation.

Discussion

This study shows that intracellular high PKCK2 activity confers anoikis resistance on esophageal cancer cells. We found that high intracellular PKCK2 activity could induce E- to N-cadherin switching (Fig. 3A). Upregulation of N-cadherin could activate PKB/Akt, thereby making cancer cells resistant to anoikis (Figs. 2 and 3B, Supplementary Fig. S1B) as previously reported in human melanoma and prostate carcinoma cells (41, 42).

To induce E- to N-cadherin switching, PKCK2 must play dual roles. The first is downregulation of E-cadherin expression. Snail is a well-known transcriptional repressor of E-cadherin gene expression (29, 30), and PKCK2 might stabilize Snail protein both directly and indirectly, thereby resulting in downregulation of E-cadherin. Direct phosphorylation on Snail at serine 92 could induce stabilization of Snail protein (34 and Supplementary Fig. S2). Phosphorylation on β -catenin at threonine 393 (31, 32) also could stabilize Snail protein indirectly through nuclear β -catenin-mediated upregulation of Axin2 expression, Axin2-mediated GSK3 β shuttling out from nucleus, and inhibition of GSK3 β -mediated Snail degradation (33 and Supplementary Fig. S3).

The second role of PKCK2 in E- to N-cadherin switching is upregulation of N-cadherin gene expression. Unlike E-cadherin gene regulation, little is known about PKCK2-dependent regulation of N-cadherin expression. PKCK2 phosphorylates many target proteins to regulate their function (43–45) and/or the expression of genes downstream (33, 45). Thus, it is possible that Myeloid Zinc Finger-1 (MZF1), a known transcriptional factor required for N-cadherin gene expression (46) could be a substrate of PKCK2 and may thus become stabilized and upregulate N-cadherin expression.

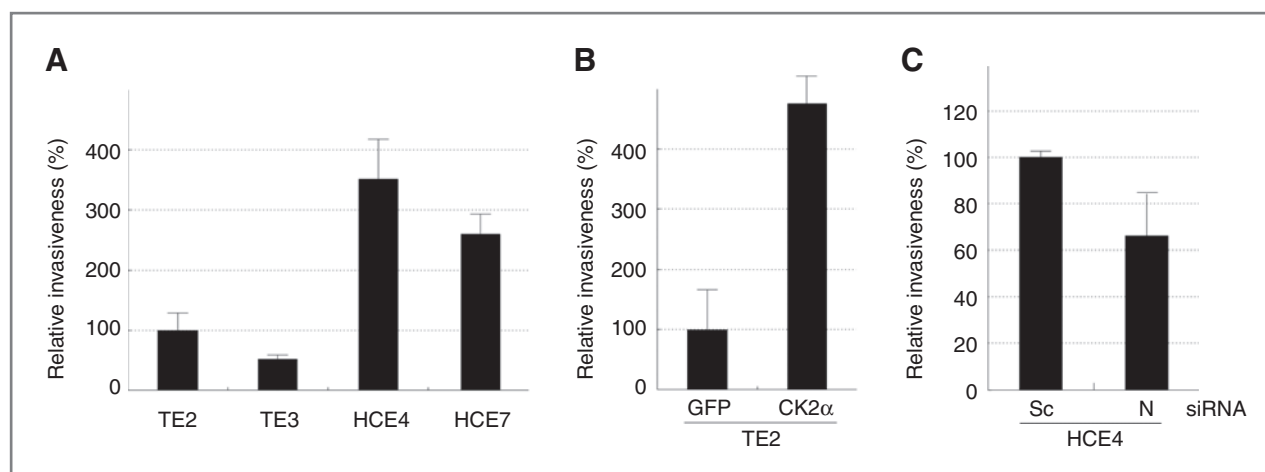


Figure 5. N-cadherin expression and invasiveness. **A**, *in vitro* invasiveness of TE2, TE3, HCE4, and HCE7 cells were measured using a Cell Invasion Assay Kit. **B**, *in vitro* invasiveness of TE2-GFP and TE2-CK2 α cells. **C**, *in vitro* invasiveness of HCE4 cells that were transfected with either a scrambled siRNA (Sc) or N-cadherin siRNA (N) for 48 hours. The data are expressed as mean \pm SD for triplicates.

In summary, we have shown that high intracellular PKCK2 activity confers anoikis resistance on cancer cells by inducing E- to N-cadherin switching. These findings suggest that PKCK2 may play important roles in cancer metastasis, because PKCK2 could induce EMT, confer anoikis resistance, and/or enhance NK cell tumor surveillance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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