

Frequent accumulation of nuclear E-cadherin and alterations in the Wnt signaling pathway in esophageal squamous cell carcinomas

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Esophageal squamous cell carcinoma is frequently associated with poor prognosis, as a result of high levels of lymph node metastasis. So far, very few genetic abnormalities have been associated with this disease, and its molecular etiology remains largely unknown. To assess whether the Wnt pathway contributes to esophageal squamous cell carcinoma, we characterized the expression and subcellular localization of the key Wnt signaling components in all 30 cases of esophageal squamous cell carcinomas analyzed. We found abnormal expression and/or localization in glycogen synthase kinase-3 α/β (34%), Axin2 (48%), α -catenin (31%), MYC (73%) and cyclin D1 in 46% of cases. Only 13% of tumors showed nuclear accumulation of β -catenin. By contrast, 60% showed nuclear expression of E-cadherin using an antibody that recognizes the cytoplasmic domain of E-cadherin. When the same tumors were stained with antibody raised against the extracellular domain of E-cadherin, the expression was lost. A direct correlation was found between nuclear E-cadherin and the increased nuclear cyclin D1, one of the AP-1 target genes in these tumors. By transfection experiments, the cytoplasmic portion of E-cadherin was found to activate the AP-1 transcription factor pathway and induced *cyclin D1* promoter activity, but β -catenin/Tcf transcription activity was unaffected. Nuclear expression of E-cadherin was also detected in tumors other than squamous cell carcinoma, including pancreatic and colon cancers, albeit at lower frequency. Nuclear accumulation of a portion of E-cadherin in esophageal squamous cell carcinoma and the other types of tumors indicates that, in addition to the previously implicated tumor suppressor activity of E-cadherin, modified forms of this glycoprotein might also play a role in growth promotion.

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Esophageal cancer is one of the deadliest but least studied forms of cancer and the sixth ranking cause of death from cancer in North America. It shows a varied geographical distribution and very poor survival rates.¹ Esophageal cancer accounts for 5% of all gastrointestinal cancers, and the overall number of new cases each year is steadily increasing.² Esophageal cancer can be divided into squamous cell carcinomas and adenocarcinomas. So far, very few genetic abnormalities have directly been linked to esophageal carcinogenesis. In this study, we sought to examine the expression and localiza-

tion of the key components of the Wnt signaling pathway that have previously been shown to play important roles in colorectal, breast, stomach and prostate cancer. Wnt signaling regulates cell growth, motility and differentiation. Upon binding to their receptor, specific Wnt ligands trigger phosphorylation of LRP5/6 (low density lipoprotein) by glycogen synthase kinase-3 (GSK3 α/β) and casein kinase-1 (CK1 γ), which distances Axin (Axin1 and Axin2) from the β -catenin destruction-complex.^{3–6} As a result, the β -catenin is stabilized and it accumulates in the cytosol which then, in association with TCF/Lef, translocates to the nucleus and induces the transcription of the Wnt target genes. In the absence of Wnt ligands, the serine/threonine kinases, CK1 and GSK3 α/β phosphorylate β -catenin bound to the APC–Axin complex and earmark it for ubiquitination and proteasomal degradation. In cancer cells, however, owing to either mutation or abnormal

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promoter methylation of genes encoding key components of the Wnt signaling system, such as *APC* (adenomatous polyposis coli), *Axin1*, *Axin2* or β -*catenin*, Wnt-induced transcription is abnormally turned on, leading to the overexpression of genes that are involved in tumorigenesis.

For our analysis, we focused on the expression of regulatory proteins of the pathway including *APC*, *Axin1*, *Axin2*, β -*catenin* (CTNNB1), γ -*catenin* (JUP), and α -*catenin* (CTNNA1) and of δ -*catenin* (P120CTN), *GSK3 α/β* , *E-cadherin* (CDH1), *MYC* and *cyclin D1*, in a series of esophageal squamous cell carcinomas. Although loss of heterozygosity of the *APC* gene has been reported in 60% of esophageal carcinoma, no mutations have been detected in this gene in esophagus tumors so far. In this study, we sought to examine the possible involvement of the key components of the Wnt signaling in a cohort of squamous cell carcinomas.

Materials and methods

Patient Samples and Cell Lines

Thirty esophageal squamous carcinoma cases were obtained from the Nelson R Mandela School of Medicine, Durban, South Africa. A summary of the clinical data for the esophageal carcinomas has been provided in Table 1. Primary colorectal cancers (82 cases), their liver metastases (36 cases), intraductal papillary mucinous neoplasm of the pancreas (16 cases), endocrine pancreatic tumors (51 cases) and corresponding normal tissues were obtained from the UHN (University Health Network) tissue bank, Toronto, Canada. In addition, HEK293 embryonic kidney cell line was obtained from ATTC (American Type Tissue Culture).

Tissue Array Construction and Immunohistochemical Analysis

Tissue arrays for esophageal squamous cell carcinoma, primary colorectal cancers and liver metastases, intraductal papillary mucinous neoplasm and endocrine pancreatic tumors and corresponding normal tissues were generated as described previously.⁷ Briefly, formalin-fixed, paraffin-embedded tissue blocks containing normal or tumor tissue were cored and transferred to a recipient master block,

using a Tissue Microarrayer (Beecher). The blocks were then processed and cut for staining. For immunohistochemistry, the slides were dried at 60°C for a few hours, deparaffinized with xylene and transferred through changes of ethanol. Antigen unmasking was performed by boiling the slides in a 10 mM sodium citrate buffer. Endogenous hydrogen peroxide activity was blocked by a 10 min incubation in 3% hydrogen peroxide buffer. The slides were then washed in TBS-Tween20 buffer and blocked with avidin and biotin for 15 min each (Vector Laboratories). Primary antibodies diluted in antibody dilution buffer (DakoCytomation) were applied to the slides overnight at 4°C. After rinsing in TBS-Tween20 buffer, the slides were incubated with biotin-conjugated anti-rabbit/anti-mouse secondary antibodies for 30 min at room temperature and washed in buffer and incubated with enzyme-labeled-Ultra Streptavidin (ID Lab) for another 30 min. The slides were rinsed and visualized with 3,3'-diaminobenzidine solution, counterstained with hematoxyline (DakoCytomation), dehydrated and mounted with Permount mounting solution (Sigma). The slides were scanned by ScanScope CS (Aperio Technologies), a bright field scanner at $\times 20$ or $\times 40$ magnification. Cores were examined with an ImageScope image viewer program (Aperio Technologies) and microscope (Leica). Each tissue microarray core was then analyzed for localization and expression levels of different markers according to nuclear, membrane, cytoplasmic or lost. Primary antibodies used in this study were as follows: *Axin1* (1:50, in-house) and *Axin2* (1:500, in-house), *GSK3 β* (1:25, Cell Signaling Technologies), *GSK3 α/β* (1:100, BioSource), *APC* (1:2000, Abcam), β -*catenin* (1:2000, Signal Transduction Labs), *cyclin D1* (1:500, Calbiochem), γ -*catenin* (1:100, NeoMarkers), α -*catenin* (1:100, NeoMarkers), *MYC* (1:50, Santa Cruz), *E-cadherin* (1:2000, BD Signal Transduction Laboratory) cytoplasmic domain, *E-cadherin* (1:5000, Vector Labs) extracellular domain, δ -*catenin* (1:1000, Epitomics), *Histone H3* (1:1000, Signal Transduction Labs) and *GAPDH* (1:10 000, Abcam) and anti-Flag-M2 (1:2000, Sigma) antibodies.

Fractionation and Western Blot Analysis

Cell lysates were prepared from transiently transfected cells, stable cell lines or tissues as noted. Nuclear and cytoplasmic extracts were prepared using NE-PER™ nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions (Pierce). For total cell lysates, cells or tissue were lysed in boiling SDS buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM Tris pH 7.4, protease inhibitor), microwaved for 10 s and centrifuged at 15°C for 10 min. Protein concentrations was measured by either BCA protein assay (Pierce) or DC protein assay (Bio-Rad). A 20 μ g portion of total, nuclear or cytoplasmic protein from each

Table 1 Summary of clinical data for squamous cell carcinomas

Age	24–92 (~ 55 years)
Survival (weeks)	0.3–284
Gender	60% female 40% male
Lymph node	65% (17/26)
Grade	77% moderately differentiated 13% well differentiated 10% poorly differentiated

preparation was separated on 8, 10 or 12% SDS-PAGE gels by electrophoresis and transferred onto Hybond-P polyvinylidene difluoride membranes. Membranes were probed with primary antibodies diluted in TBS containing 0.2% Tween-20 and 5% non-fat dry milk, followed by incubation with goat anti-rabbit/mouse IgG (H + L)-HRP-conjugated antibodies (1:10 000, Bio-Rad) for detection by enhanced chemiluminescence (Pierce) on BioMAX MR Film.

DNA Constructs and Cell Transfection

The C-terminal domain of *E-cadherin* was amplified from cDNA generated from normal colon epithelial cells by forward primers: Ecad-1904F 5'-AAAAAGC TTATACCAGAACCTCGAACTATATTC-3', Ecad-2301F 5'-AAAAAGCTTCTGCTCTTGCTGTTTCTTCGG-3' and reverse primer Ecad-2762R 5'-AAATCTAGATC GAGTCCCCTAGTCGCTCTC-3' (Figure 1). The PCR fragments were cloned into TOPO vector (Invitrogen). After antibiotic selection and DNA preparation, fragments were digested by *Hind*III and *Xba*I restriction enzymes and were re-ligated to p3 × FLAG-CMV10 vector (Sigma). The sequence of each construct was verified by DNA sequencing. The Ecad-2301 construct expresses the E-cadherin cytoplasmic domain from amino acids (aa) 725–882, while Ecad-1904 expresses the cytoplasmic portion of E-cadherin and one extracellular tandem repeat

(aa 594–882). Each construct was transfected into cells by Lipofectamine 2000 (Invitrogen). After 48 h, the cells were processed for western blot analysis. For the generation of stable lines, the day after transfection with DNA, HEK293 cells were grown in DMEM media containing 1 µg/ml G418. Stable lines were isolated after 3 weeks of antibiotic selection.

Luciferase Gene Reporter Assays

To determine whether the induction of E-cadherin cytoplasmic domain activates the β-catenin/Tcf response element, we performed Top/Fop luciferase assays. All transfections were performed in six-well plates using the Lipofectamine 2000 reagent. Cells were cotransfected for 43 h with 0.5 µg of the Wnt/β-catenin pathway luciferase TOPFLASH or FOPFLASH constructs (gift from Dr RD Moon), effector plasmids (Ecad-2301 or Ecad-1904) and pcDNA3-β-galactosidase plasmid as internal control. Luciferase assays were performed using Luciferase Assay System Kit according to the manufacturer's protocol (Applied Biosystem Tropix). Reporters from Luciferase System 3 (designed for studies of cell growth and proliferation) were used to monitor the activity of multiple signal transduction pathways upon E-cadherin cytoplasmic domain induction. HEK293 cells were cotransfected with different luciferase reporter vectors (System 3 reporter from Clontech, Palo Alto, USA) including c-Jun/c-fos, ATF2/CREB, E2F/DP1, STAT1/STAT2, Elk-1/SRF) and either Ecad-1904 or Ecad-2301 constructs. Cells were harvested after 43 h and assayed for luciferase activity. Similarly, the human *cyclin D1* promoter fragments linked to the luciferase gene (gift from Dr RG Pestell) were used to monitor *cyclin D1* promoter activity upon E-cadherin induction.

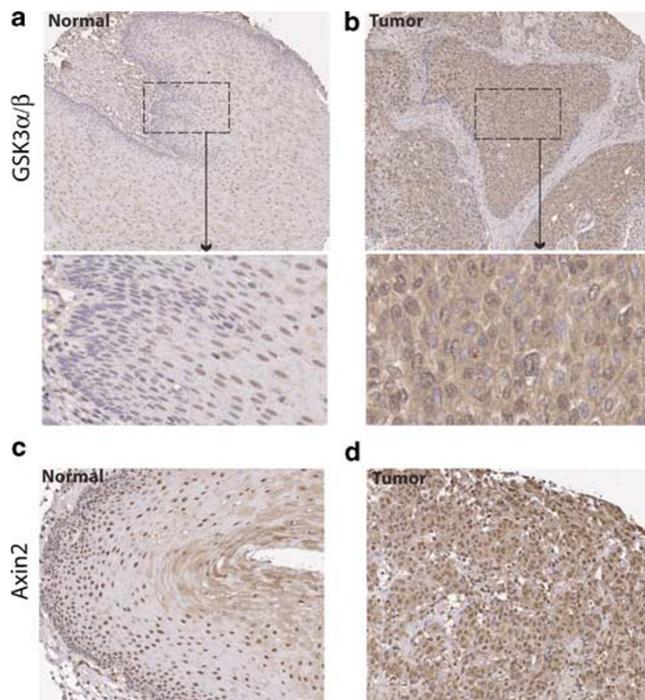


Figure 1 Increased cytoplasmic GSK3α/β and Axin2 in esophageal squamous carcinomas. Increased cytoplasmic levels of GSK3 (a and b) and Axin2 (c and d) detected in many esophageal tumors indicates that the Wnt signaling pathway is deregulated in some squamous cell carcinomas.

Results

Deregulation of the Key Components of the Wnt Signaling Pathway in Esophageal Squamous Cell Carcinoma

Tissue arrays containing cored samples from esophageal tumors and corresponding normal tissues were immunostained with antibodies against different components of the Wnt signaling pathway. Cytoplasmic expression of APC was detected in all but one sample. There was, however, marked variation in the intensity of expression in different tumors. We found increased cytoplasmic levels of GSK3α/β in 34% (10/29) of tumors. Increased levels of Axin2 and α-catenin were also detected in 48% (14/29) and 31% (9/29) of the cases, respectively (Figure 1). Abnormal nuclear expression of α-catenin, cyclin D1 and MYC was detected in 17, 46 and 73% of the tumors, respectively (Table 2). Nuclear β-catenin accumulation was detected in 13% (4/30) of the cases, whereas 50% (14/30) of the cases showed increases in cytoplasmic β-catenin.

Table 2 Abnormalities detected in esophagus tumors in the key components of the Wnt signaling pathway

<i>Abnormal nuclear localization and expression</i>	
β -catenin	13% (4/30)
α -catenin	17% (5/29)
E-cadherin	60% (18/30)
Cyclin D1	46% (14/30)
MYC	73% (21/30)
<i>Increased cytoplasmic level</i>	
Axin2	48% (14/29)
β -catenin	50% (14/30)
E-cadherin	33% (10/30)
α -catenin	31% (9/29)
GSK3 α/β	34% (10/29)

Unexpectedly, we found high nuclear expression of E-cadherin in 60% (18/30) of esophageal squamous cell carcinomas when we used an antibody that specifically recognizes the cytoplasmic domain of E-cadherin (Figure 2a). Interestingly, in three cases with high-grade dysplasia in mucosa overlying the tumor, E-cadherin was observed within the nucleus. E-cadherin expression in these tumors was also examined with a distinct E-cadherin antibody that recognizes the extracellular domain of the protein. These results showed a reduction in or loss of membrane staining and the presence of cytoplasmic expression of E-cadherin in these tumors (Figure 2a). These results indicate that the nuclear-localized E-cadherin in these samples is unlikely to be full-length E-cadherin.

Translocation of E-cadherin C-Terminal Domain

To examine whether the cytoplasmic tail of E-cadherin might translocate to the nucleus, we generated two truncated *E-cadherin* expression constructs: one expressed only the cytoplasmic tail of E-cadherin (Ecad-2301), whereas the second expressed the cytoplasmic domain, the transmembrane domain and one of the extracellular tandem repeats of *E-cadherin* (Ecad-1904) (Figure 2a). Each *E-cadherin*-cDNA expression construct was then transfected into HEK293 cells, and at 2 days post-transfection, cells were isolated, lysed and the cytoplasmic and nuclear fractions were separated. Subcellular localization of endogenous and exogenous E-cadherin was examined by SDS-PAGE western blot analysis. Interestingly, Ecad-2301 protein, comprising only the cytoplasmic domain, was significantly cleaved and fragments were predominantly found in the cytosol. The longer Ecad-1904 protein was detected in both the nuclear and cytoplasmic fractions of the cell lysate (Figure 2b). These data indicate that the intact cytoplasmic domain can be transported into the nucleus, whereas the smaller, proteolysed fragments are likely not.

Cytoplasmic Domain of E-Cadherin Stabilizes β -Catenin

To examine the effect of E-cadherin on β -catenin expression, we generated stable HEK293 cell lines

expressing either Ecad-2301 or Ecad-1904 recombinant proteins. Expression of either E-cadherin construct led to β -catenin stabilization (Figure 3a). However, cells expressing Ecad-2301 recombinant protein that does not contain the transmembrane or any extracellular domain showed higher expression of β -catenin (Figure 3a). One possibility is that the free and unbound E-cadherin (Ecad-2301) interacts and competes for β -catenin-binding sites with components of β -catenin-destruction complex, such as Axin or APC, and thereby inhibits efficient β -catenin ubiquitination and degradation. Of note, the increased β -catenin in these cells did not affect the endogenous level of E-cadherin (Figure 3a). There was a correlation between membrane expression or increased cytoplasmic levels of E-cadherin and β -catenin, where most tumors expressing nuclear E-cadherin lacked nuclear β -catenin expression (Figure 3b).

Correlation of Nuclear E-Cadherin Accumulation with Nuclear Cyclin D1

Cells expressing nuclear E-cadherin also displayed increased cyclin D1 and MYC expression. Nuclear accumulation of E-cadherin correlated with nuclear expression of cyclin D1 in esophageal tumors, but not directly with β -catenin (Figures 3b and 4a). These results indicate that nuclear E-cadherin or β -catenin expression in this type of tumor might occur through independent pathways. In all cases where E-cadherin cytoplasmic levels were increased, we found increased cytoplasmic β -catenin expression (Figure 3b). This is likely because E-cadherin competes for binding sites on β -catenin with other β -catenin binding partners, in particular, the β -catenin destruction complex. Some studies have suggested that loss of E-cadherin expression does not induce Wnt signaling, whereas others have suggested a role of E-cadherin in modulation of Wnt-dependent gene expression.⁸⁻¹⁰ However, previous studies have reported that none of the studied germline *E-cadherin* mutations is responsible for an increase in the β -catenin/Lef transcriptional activity.^{11,12}

E-Cadherin Cytoplasmic Domain Induces Cyclin D1, AP-1 and STAT1/STAT2 Promoter Activities

To address whether overexpression of cytoplasmic E-cadherin could modulate Wnt signaling, we performed TOP/FOP luciferase assays to examine the effect of the E-cadherin cytoplasmic tail on β -catenin/TCF promoter activity. Expression of neither Ecad-2301 nor Ecad-1904 induced the β -catenin/TCF pathway. As a control, overexpression of a mutant form of β -catenin induced the β -catenin/TCF promoter activity by 1000-fold, as expected (Figure 4b). We then used different reporter vectors containing specific *cis*-acting DNA sequences

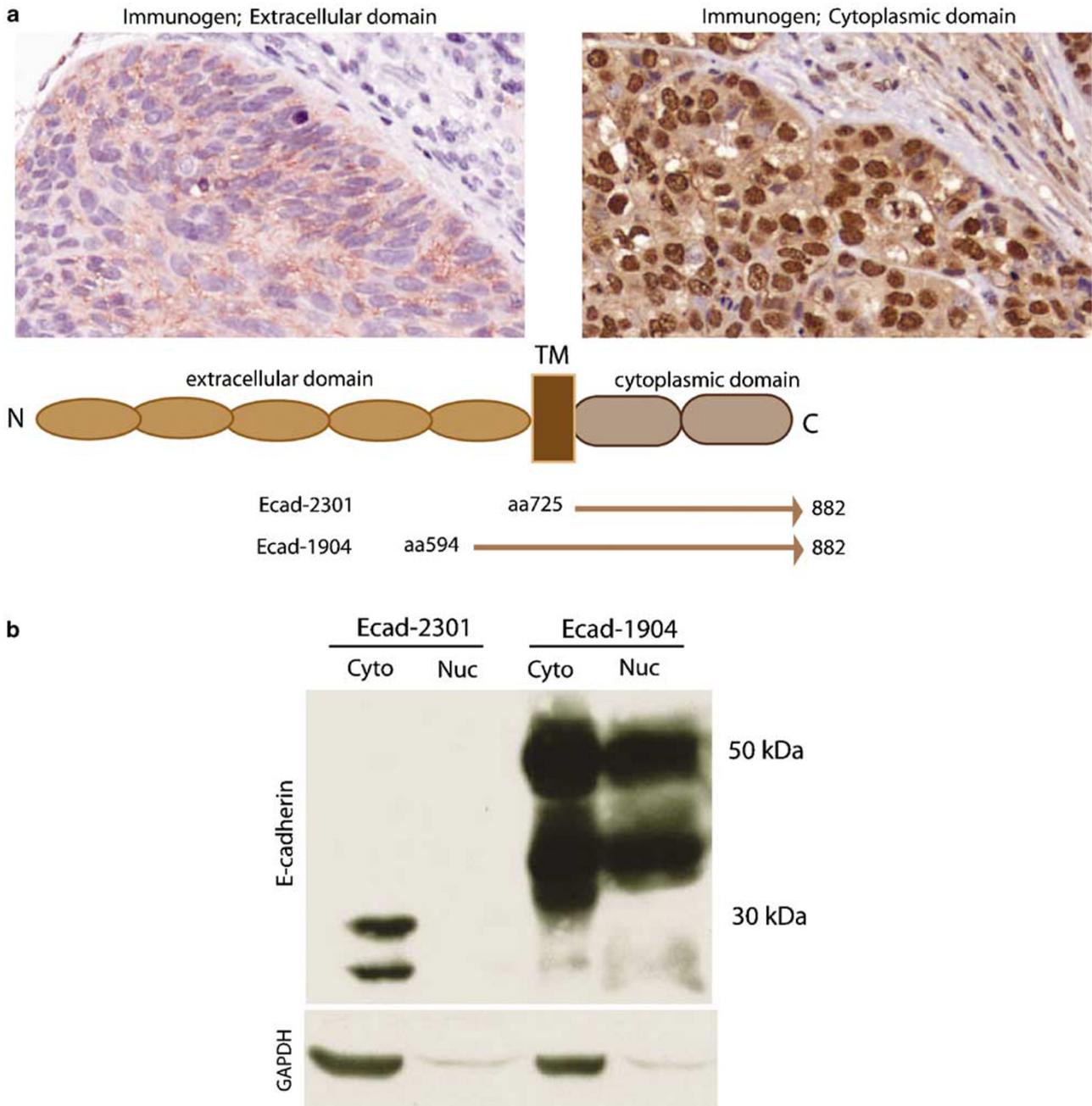


Figure 2 Subcellular localization and protein structure of E-cadherin. Two different E-cadherin antibodies were used to localize E-cadherin in tumors. An E-cadherin extracellular domain-based immunogen showed loss or reduced E-cadherin expression, whereas the same tumors showed increased nuclear accumulation of E-cadherin when an E-cadherin antibody raised against the intracellular domain was used (a). These results indicate that extracellular part of E-cadherin is lost in tumors, whereas the C-terminal domain is released into the cytoplasm and accumulates in the nucleus. The two constructs used in this study expressed aa 725–882 (Ecad-2301) and aa 594–882 (Ecad-1904), the latter of which, in addition to intracellular domains of E-cadherin, also contains one extracellular domain and the transmembrane domain (a). To examine whether the cytoplasmic domains of E-cadherin can translocate to the nucleus, HEK293 cells were transfected with either Ecad-2301 or Ecad-1904 constructs (b). Cell extracts were fractionated and cytoplasmic and nuclear lysates were resolved by western blot analysis using an E-cadherin antibody raised against the cytoplasmic tail of the protein. Ecad-1904 clearly translocated to the nucleus, whereas Ecad-2301 was efficiently cleaved and was found predominantly in the cytosol. Both E-cadherin recombinant proteins (Ecad-2301 and Ecad-1904) undergo cleavage (b).

coupled to a reporter gene to examine whether overexpression of cytoplasmic domain of E-cadherin could activate other signaling pathways (c-Jun/c-fos, ATF2/CREB, E2F/DP1, STAT1/STAT2, Elk-1/SRF reporter elements). The luciferase assay and

pathway profiling systems revealed 3.2- and 6.5-fold induction of AP-1 promoter activity and 3.8- and 2.4-fold induction of STAT1/STA2 promoter activity by Ecad-2301 or Ecad-1904 constructs, respectively (Figure 4c). *Cyclin D1* promoter activity was also

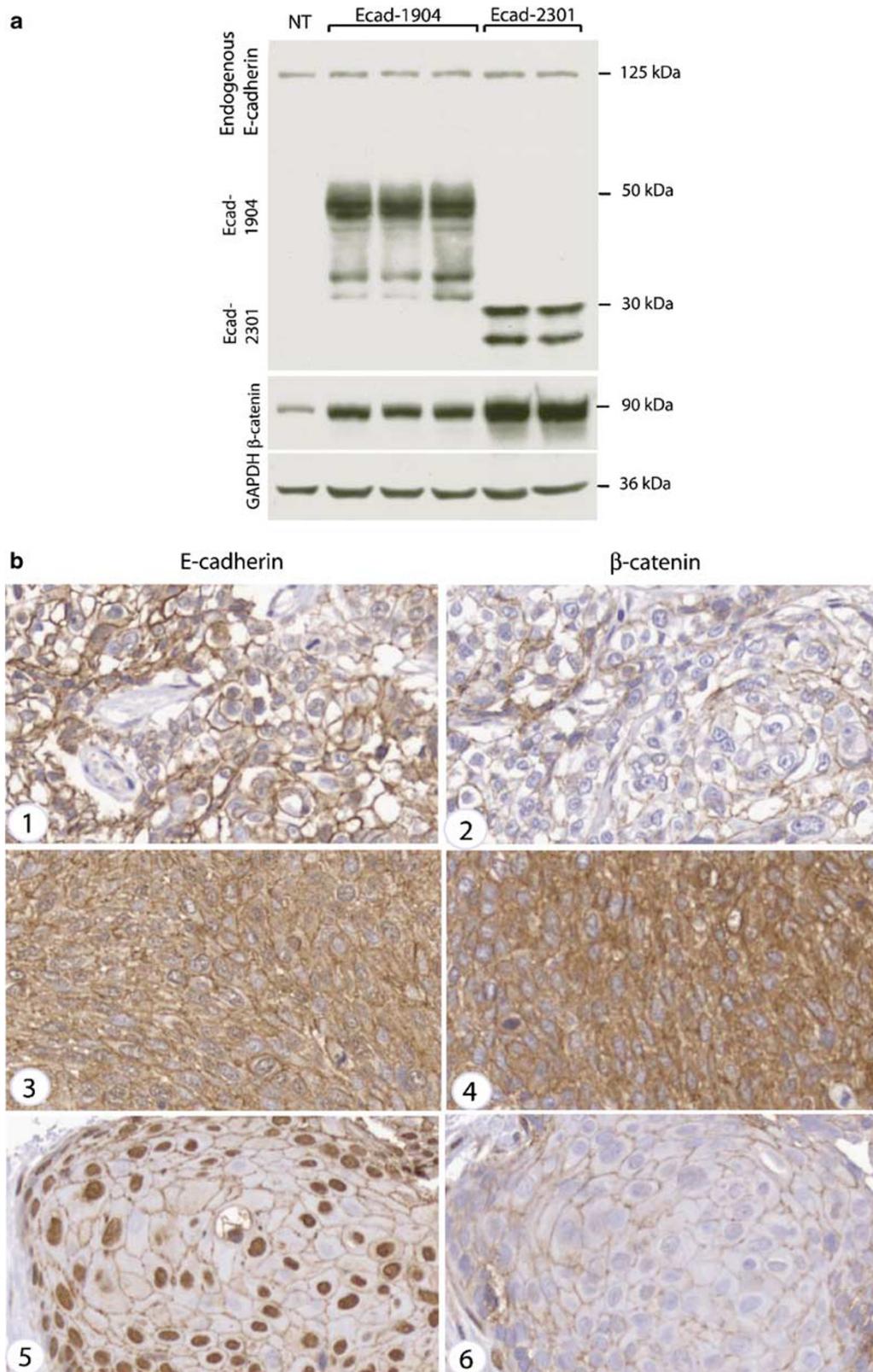


Figure 3 Increased E-cadherin stabilizes β -catenin in cells. E-cadherin and β -catenin levels were examined in HEK293 cells that stably express Ecad-2301 and Ecad-1904. Both E-cadherin constructs stabilized β -catenin levels in the cells (a). The constructed tissue microarray from esophageal tumors was stained with antibodies against E-cadherin and β -catenin. Within the same slide, we found variation in expression and subcellular localization of both markers (b). Tumors with high cytoplasmic levels of E-cadherin (panel 3) also showed increased β -catenin (panel 4). However, cells expressing nuclear E-cadherin (panel 5) mostly showed normal β -catenin distribution in esophagus tumors (panel 6) while cells with normal E-cadherin distribution showed normal β -catenin expression in these esophagus tumors (panels 1 and 2).

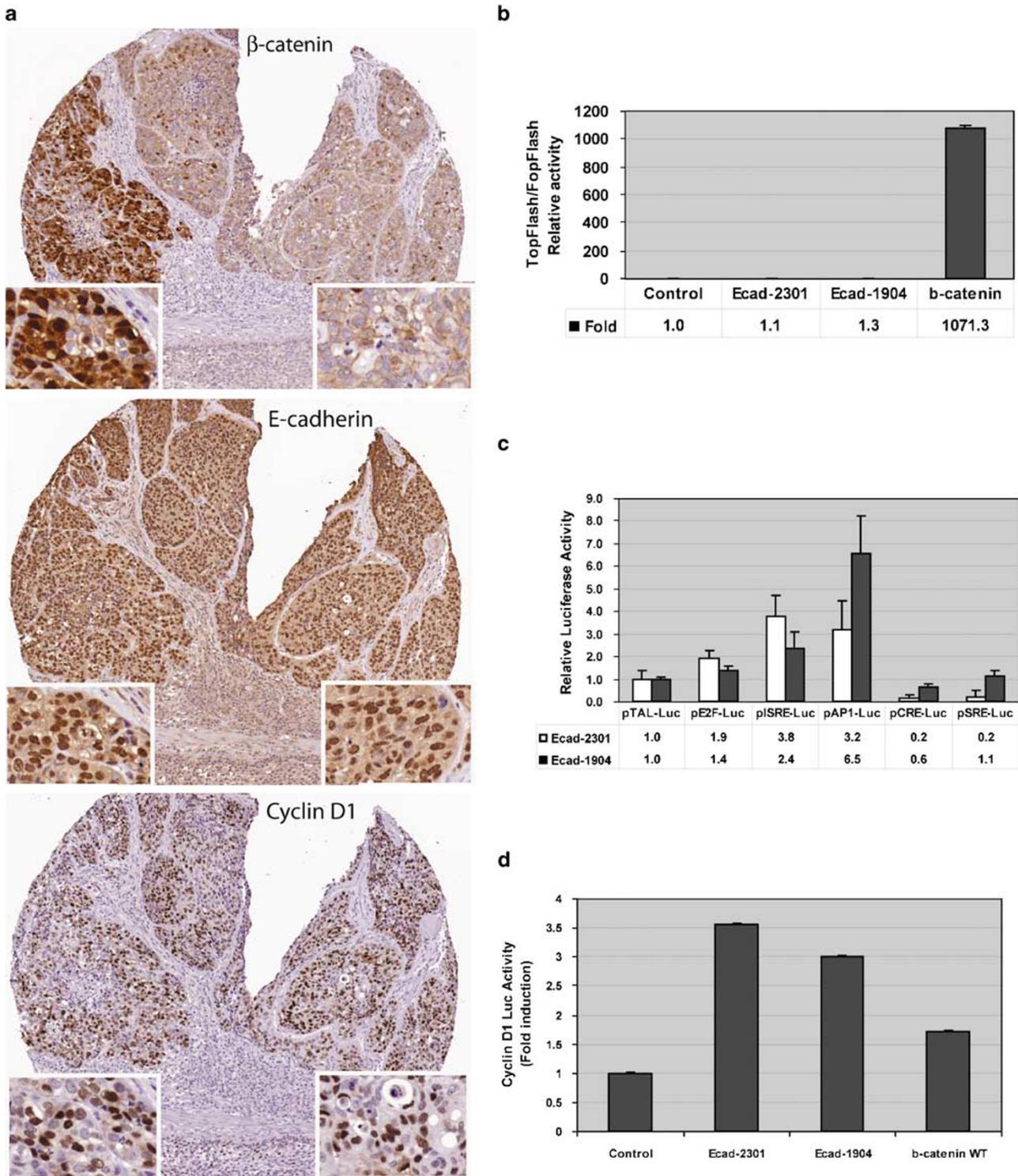


Figure 4 E-cadherin-induced *cyclin D1* promoter activation does not require β -catenin/Lef activity. Immunohistochemical analysis of the same tumor section showed that, in most cases, nuclear E-cadherin correlates with nuclear expression of cyclin D1, but not with nuclear expression of β -catenin (a). To determine whether induction of E-cadherin cytoplasmic domain activates β -catenin/Lef response elements, we performed luciferase assays. Neither Ecad-2301 nor Ecad-1904 induced Tcf-responsive (TOP-FLASH) or mutant (FOP-FLASH) reporter constructs. Expression of either Ecad-2301 or Ecad-1904 resulted in a 3.2- and 6.5-fold induction of pAP1-Luc (AP-1 promoter activation) and 3.8- and 2.4-fold pISRE-Luc induction (activation of STAT1/STAT2 transcription factors), respectively. Activation of each reporter was compared with the basal promoter activity of pTAL-Luc, which does not contain any enhancer elements (c). Ecad-2301 and Ecad-1904 overexpression induced *cyclin D1* promoter activity (3.6- and 3-fold, respectively) (d).

assessed in cells transfected with E-cadherin C-terminal domain. Expression of the E-cadherin constructs also induced cyclin D1 promoter activity by 3.6- and 3-fold, respectively (Figure 4d). Together, these results indicate that overexpression of the E-cadherin-cytoplasmic domain induces signaling and invasion likely through activation of the non-canonical Wnt signaling coupled to *AP-1* promoter and STAT1/STAT2 transcription activities.

Prevalence of E-Cadherin Nuclear Accumulation in Other Tumors

We next examined whether nuclear accumulation of the cytoplasmic tail of E-cadherin could be detected in other types of tumors. A series of sporadic colorectal tumors and liver metastases was labeled immunohistochemically and examined for E-cadherin expression. Only 5 colorectal tumors of 82 (6%) showed nuclear E-cadherin expression. Interestingly, one colorectal cancer case with high nuclear E-cadherin also showed a focal increase of E-cadherin in the corresponding liver metastasis (Figure 5a). Protein fractionation of this tumor showed an increased level of E-cadherin in the nuclear fraction compared with corresponding normal tissue (Figure 5b). In total, we found nuclear accumulation of E-cadherin in 6% (2/36) of liver metastases. Nuclear E-cadherin was also found in 24% (12/51) and 13% (2/16) of endocrine pancreatic tumors and intraductal papillary mucinous neoplasm of the pancreas, respectively (Table 3). These results indicate that nuclear translocation of E-cadherin can also be found in other tumor types, albeit at lower frequencies, except for solid-pseudo-papillary tumors, which perhaps is a unique tumor, because the vast majority of these also harbor mutations of the β -catenin gene.¹³

Discussion

The results presented here provide strong evidence that Wnt signaling and, in particular, E-cadherin expression or subcellular localization, are deregulated in esophageal squamous cell carcinoma. E-cadherin, a transmembrane protein, is primarily expressed at the membrane. E-cadherin is required for formation and maintenance of epithelia, mediates cell-cell adhesion and is an essential component of intercellular adhesion complexes. The extracellular domain of *E-cadherin* contains five tandem repeat sequences, which are involved in Ca^{2+} -dependent, homophilic interactions. The juxtamembrane domain of E-cadherin interacts with δ -catenin, β -catenin and γ -catenin, linking E-cadherin to the cytoskeleton. E-cadherin is an invasion suppressor and its expression is suppressed in advanced invasive carcinomas, whereas normal E-cadherin levels suppress invasion, metastasis and proliferation. Recent studies of early onset hereditary diffuse

gastric cancer and colorectal cancer indicate that *E-cadherin* acts as a tumor suppressor and its deregulation is an initiating event in tumorigenesis.^{14–17} In this study, we show that abnormal nuclear E-cadherin expression, in addition to esophageal squamous cell carcinoma, is found in other tumors and metastases as well. Inactivation of *E-cadherin* through gene mutation, transcriptional inactivation or promoter methylation has been detected in many types of cancer.^{18–21} Abnormal E-cadherin in breast carcinomas has been associated mainly with *E-cadherin* promoter methylation and very few mutations in the gene have been reported.^{22,23} Previous studies showed that decreased immunoreactivity of E-cadherin statistically correlated with the presence of lymph node metastases in esophageal squamous cell carcinoma, and loss of intercellular adhesion activates a transition from low- to high-grade squamous cell carcinoma, thus indicating an important role for E-cadherin as a prognostic marker in the progression of tumors.^{24,25} Increased expression of the 80 kDa fragment of E-cadherin in metastatic prostate cancer in association with increased levels of several metalloproteinases, which are believed to be responsible for cleavage of short E-cadherin fragment from the full-length E-cadherin, are suggested to induce tumor progression in this tumor.²⁶ Metalloproteinases differentially expressed by tumor and stromal cells are finely regulated by interaction with distinct cellular and extracellular components of the tumor environment. Presenilin 1 controls a γ -secretase-like cleavage of E-cadherin. The cleavage occurs between E-cadherin residues 731 and 732 and is stimulated by calcium influx or apoptosis. The proteolytic cleavage of E-cadherin dissociates cadherins, β -catenin and α -catenin from the cytoskeleton, thus promoting disassembly of the E-cadherin-catenin adhesion complex and leads to loss of cell-cell adhesion. Furthermore, this cleavage releases the cytoplasmic E-cadherin to the cytosol and increases the levels of soluble β - and α -catenins. Thus, the presenilin 1/ γ -secretase system stimulates disassembly of the E-cadherin-catenin complex and increases the cytosolic pool of β -catenin.^{27,28} Recent studies suggest that E-cadherin modulates Wnt-dependent gene expression by regulating the availability of β -catenin.⁹ We also found increased cytoplasmic levels of β -catenin in tumors with high levels of cytoplasmic E-cadherin. In addition, we show increased β -catenin levels in cells which stably express the E-cadherin cytoplasmic domain. One possibility is that E-cadherin competes for binding for β -catenin with the Axin and APC destruction complex, thereby indirectly controlling β -catenin-regulated gene expression. Mutational analysis of some of the Wnt components including Axin, APC and β -catenin so far have identified very few genetic abnormalities in esophageal cancer, indicating that mechanisms other than mutations are responsible for activation of this pathway in esophageal cancer.^{29–31}

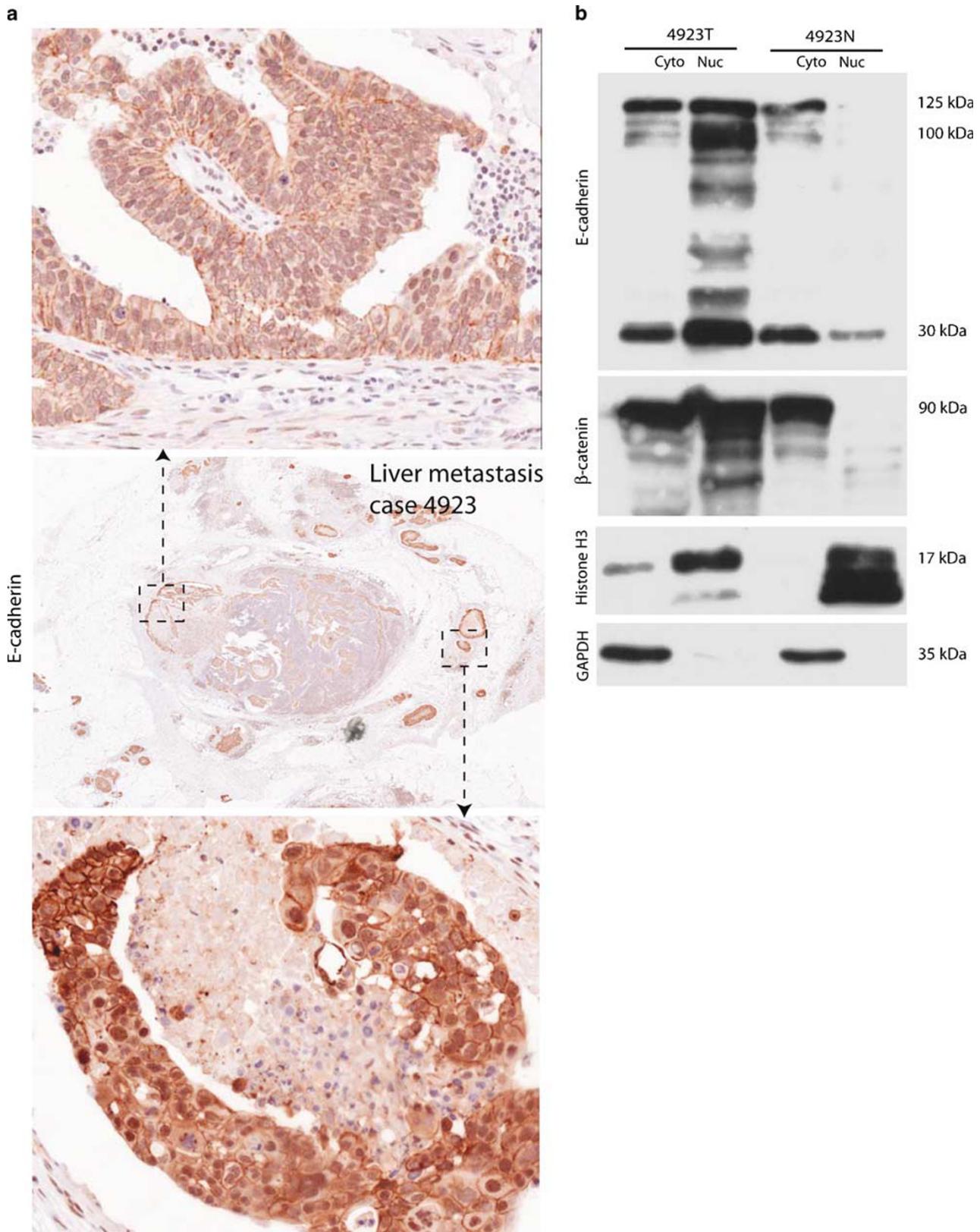


Figure 5 E-cadherin nuclear translocation was also found in primary colorectal cancer and metastasis. E-cadherin nuclear translocation was detected in primary colorectal cancers and in some areas of the corresponding liver metastasis of a colorectal tumor with nuclear E-cadherin (a). Cytoplasmic and nuclear fractionation of tissue extracts verified nuclear translocation of E-cadherin as initially detected by immunohistochemical analysis (b). Histone H3 and GAPDH were used as nuclear and cytoplasmic markers, respectively.

Table 3 The prevalence of nuclear localization of the cytoplasmic tail of E-cadherin in different tumors

Esophagus squamous carcinoma	60% (18/30)
Endocrine pancreatic tumor	24% (12/51)
<i>Sporadic colorectal cancer</i>	
Primary tumor	6% (5/82)
Liver metastasis	6% (2/36)
Intraductal papillary mucinous neoplasm	13% (2/16)

Recent studies have suggested a role for E-cadherin in the nuclear export of δ -catenin (p120 catenin).³² The Armadillo sequence-rich protein δ -catenin, associates with the cytoplasmic domain of E-cadherin and together with β -catenin and α -catenin accumulates at cell–cell junctions. An increased level of cytoplasmic δ -catenin has been found in breast carcinomas.³³ Although we found variation in δ -catenin expression in different esophageal tumors, there was no correlation between E-cadherin and δ -catenin expression or subcellular localization in these cases. One of the main mechanisms by which E-cadherin is regulated appears to be via proteasomal degradation. In the absence of calcium ions, E-cadherin loses its homotypic interactions and it is targeted for proteasome degradation. Downregulation of E-cadherin is found in many types of cancer and is correlated with transition to metastasis. In normal cells, after E-cadherin is synthesized at the endoplasmic reticulum, it binds to catenin and then transports via the secretory pathway to the cell surface. When cadherins from adjacent cells bind to each other (binding is calcium-dependent), then an adherens junction is established. During apoptosis, the trafficking of E-cadherin to the cell surface is lost, and as E-cadherin is turned over, cells detach from one another. This process is believed to be deregulated in cancer cells, where cell:cell architecture is disorganized.

The mechanism by which E-cadherin is over-expressed, stabilized or translocated to the nucleus is not clear. One possibility is that the cleaved E-cadherin, which normally is degraded by the 26S proteasome, accumulates and subsequently translocates to the nucleus where it might participate in regulation of gene expression. In malignant melanoma, loss of E-cadherin leads to upregulation of NF- κ B activity.³⁴ Similarly, E-cadherin-dependent cell–cell adhesion has been shown to control the nuclear abundance of the nuclear transcription factors such as apolipoprotein A-IV.³⁵ Cell–matrix and cell–cell adhesion play a central role in the control of cell proliferation and differentiation, and E-cadherin expression appears to influence these processes in epithelial cells. Some studies have suggested that loss of E-cadherin expression does not induce Wnt signaling, while others have suggested a role of E-cadherin in modulation of Wnt-dependent gene expression.^{8,9} Recently,

abnormal promoter methylation of a number of Wnt signaling components including APC have been reported in Barrett's esophagus.³⁶ These results suggest that Wnt signaling plays an important role in esophageal carcinoma.

In summary, in this study, we have shown that Wnt signaling is deregulated in esophageal squamous cell carcinoma. Aberrant expression of E-cadherin, cyclin D1 and MYC was found in the majority of esophageal squamous lesions examined. We also showed that the cytoplasmic domain of E-cadherin accumulates in the nucleus. In addition to nuclear localization of E-cadherin in esophageal squamous carcinoma, E-cadherin abnormalities were found in other tumor types, generally at lower frequencies (except the solid-pseudopapillary tumors). This is the first study to report the abnormal nuclear E-cadherin expression in these types of tumors, and our data indicate that E-cadherin may, in addition to its proposed role as tumor suppressor, act as an oncogene in some types of tumors.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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