

## ORIGINAL ARTICLE

**The human cadherin 11 is a pro-apoptotic tumor suppressor modulating cell stemness through Wnt/ $\beta$ -catenin signaling and silenced in common carcinomas**L Li<sup>1,7</sup>, J Ying<sup>1,2,7</sup>, H Li<sup>1</sup>, Y Zhang<sup>1</sup>, X Shu<sup>1</sup>, Y Fan<sup>1</sup>, J Tan<sup>3</sup>, Y Cao<sup>4</sup>, SW Tsao<sup>5</sup>, G Srivastava<sup>6</sup>, ATC Chan<sup>1</sup> and Q Tao<sup>1</sup>

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Genetic alterations of 16q21-q22, the locus of a 6-cadherin cluster, are frequently involved in multiple tumors, suggesting the presence of critical tumor suppressor genes (TSGs). Using 1 Mb array comparative genomic hybridization (aCGH), we refined a small hemizygous deletion (~1Mb) at 16q21-22.1, which contains a single gene *Cadherin-11* (*CDH11*, *OB-cadherin*). *CDH11* was broadly expressed in human normal adult and fetal tissues, while its silencing and promoter CpG methylation were frequently detected in tumor cell lines, but not in immortalized normal epithelial cells. Aberrant methylation was also frequently detected in multiple primary tumors. *CDH11* silencing could be reversed by pharmacologic or genetic demethylation, indicating an epigenetic mechanism. Ectopic expression of *CDH11* strongly suppressed tumorigenicity and induced tumor cell apoptosis. Moreover, *CDH11* was found to inhibit Wnt/ $\beta$ -catenin and AKT/Rho A signaling, as well as actin stress fiber formation, thus further inhibiting tumor cell migration and invasion. *CDH11* also inhibited epithelial-to-mesenchymal transition and downregulated stem cell markers. Thus, our work identifies *CDH11* as a functional tumor suppressor and an important antagonist of Wnt/ $\beta$ -catenin and AKT/Rho A signaling, with frequent epigenetic inactivation in common carcinomas.

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**Introduction**

Various genetic analyses using microsatellite markers have demonstrated the frequent loss of heterozygosity at 16q in multiple tumors, including nasopharyngeal carcinoma (NPC; ~40%) (Hui *et al.*, 1999), hepatocellular carcinoma (HCC; ~50%) (Yeh *et al.*, 1996), breast cancer (~77%) (Callen *et al.*, 2002), lung cancer (~60%) (Stanton *et al.*, 2000), prostate cancer (~53%) (Suzuki *et al.*, 1996) and gastric cancer (~45%) (Nishioka *et al.*, 2001). One critical deletion region has been mapped to 16q22.1-16q24.3, suggesting the presence of candidate tumor suppressor gene(s) (TSG). Several candidate TSGs have already been identified in this region, including *WWOX* (Aqeilan *et al.*, 2007), *CBFA2T3* (Kochetkova *et al.*, 2002), *ATBFI* (Sun *et al.*, 2005), *CMTM3* (Wang *et al.*, 2009) and *E-cadherin* (*CDH1*) (Margulis *et al.*, 2005).

Cadherins comprise an important group of cell–cell adhesion molecules that mediate intercellular adhesion by Ca<sup>2+</sup>-dependent homophilic interactions (Yagi and Takeichi, 2000). By forming homodimers, cadherins can cluster through a zipper-like mechanism, while their intracellular domain is anchored to the actin cytoskeleton through  $\alpha$ -catenin and  $\beta$ -catenin (Angst *et al.*, 2001). These interactions have crucial roles in maintaining tissue architecture and cell polarity, as well as limiting cell movement and proliferation, thus resulting in tumor inhibition (Berx and van Roy, 2009). Six classical cadherin family members, including *CDH1*, *CDH3* (*P-cadherin*), *CDH5* (*VE-Cadherin*), *CDH8*, *CDH11* (*OB-cadherin*) and *CDH13* (*H-cadherin*), are located on 16q22.1-16q24.3, as a so-called six-cadherin cluster (Kremmidiotis *et al.*, 1998). Some cadherins have been identified as functional tumor suppressors, such as *CDH1* (Margulis *et al.*, 2005) and *CDH13/H-cadherin* (Andreeva and Kutuzov, 2010), involved in inhibiting cell proliferation and invasiveness, and promoting apoptosis.

Epigenetic alterations of TSGs, including promoter CpG methylation and histone modifications, are

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frequently involved in tumor development and progression (Bird, 2002). Remarkably, epigenetic silencing of *CDH1* (Hiraguri *et al.*, 1998; Eads *et al.*, 2001; Wheeler *et al.*, 2001; Kroeger *et al.*, 2008) and *CDH13* (Toyooka *et al.*, 2001, 2002; Roman-Gomez *et al.*, 2003; Kroeger *et al.*, 2008) in tumors has been reported in multiple epithelial tumors and hematopoietic malignancies, indicating that promoter CpG methylation-mediated silencing is an important regulatory mechanism for disrupting cadherin members in tumorigenesis.

We have previously performed 1-Mb array comparative genomic hybridization (aCGH) analysis of carcinoma cell lines, and identified *CDH11* as the only gene located at a 1-Mb hemizygous deletion detected at 16q22.1. We thus hypothesized that *CDH11* could be a critical tumor suppressor gene implicated in tumorigenesis. Our present epigenetic and functional studies demonstrated that *CDH11* was frequently inactivated by promoter methylation in multiple carcinomas and functioned as a tumor suppressor, by inducing tumor cell apoptosis and inhibiting cell motility and invasion, as well as cell stemness through Wnt/ $\beta$ -catenin and AKT/Rho A signaling.

## Results

### Identification of *CDH11* as a candidate TSG at 16q21-22.1

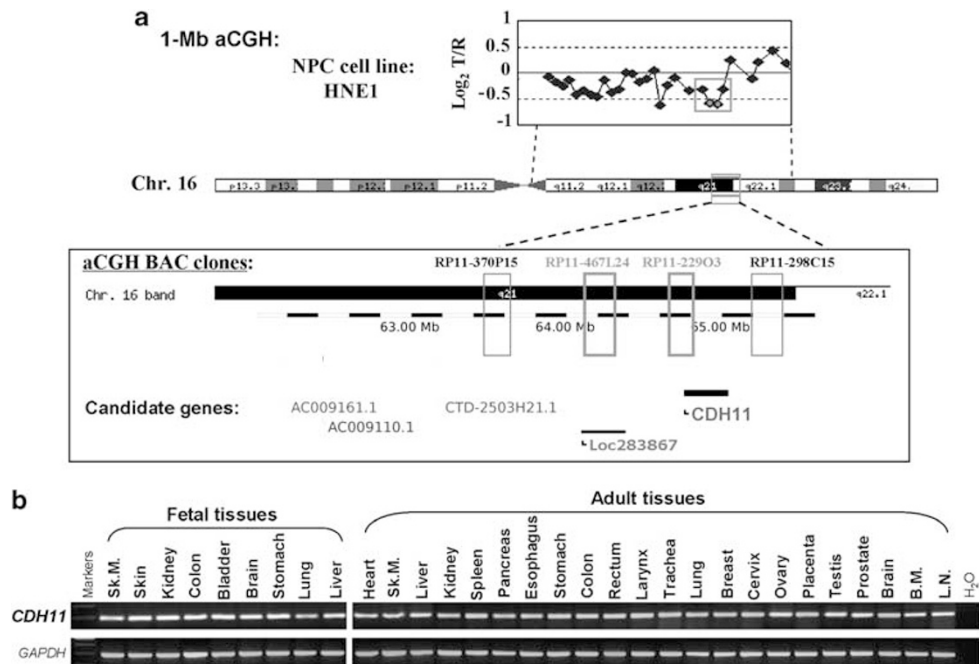
Genome-wide identification of gene deletions using aCGH identifies candidate TSG loci in tumors. Pre-

viously, we performed 1 Mb aCGH to identify DNA copy number aberrations in tumor cell lines including NPC (Ying *et al.*, 2006), and identified an  $\sim$ 1-Mb hemizygous deletion at 16q21-22.1 in three NPC cell lines (Figure 1a). Only one known gene—OB-cadherin (*CDH11*) is located at this deletion, indicating that *CDH11* could be a candidate TSG for 16q21-22.1 deletion. We further assessed its expression in a series of human normal adult and fetal tissues using semi-quantitative RT-PCR and detected its broad expression in normal tissues, though with variable expression levels (Figure 1b).

We then examined the expression levels of *CDH11* in a series of tumor as well as immortalized but non-transformed normal epithelial cell lines. As shown in Figure 2b, significant reduction or silencing of *CDH11* expression was frequently observed in multiple tumor cell lines of nasopharyngeal, esophageal, gastric, hepatocellular, colon, breast and cervix, infrequently in lung carcinoma cell lines (Supplementary Figure S1), but not in any of the normal cell lines. These results indicate that frequent downregulation of *CDH11* is involved in multiple tumorigenesis.

### Frequent silencing of *CDH11* by promoter CpG methylation in common carcinomas

Gene downregulation could result from either genetic or epigenetic mechanism. We checked whether *CDH11* reduction could be due to genetic deletion. No homozygous deletion was detected in any silenced or down-regulated cell line, as well as expressing normal epithelial



**Figure 1** (a) Representative 1 Mb aCGH result showing a small hemizygous deletion including the *CDH11* locus in NPC cell lines. Cytoband of 16q is shown. Normalized log<sub>2</sub> signal intensity ratios from  $-1$  to  $1$  are plotted. Each dark blue-colored dot represents a single BAC clone. Two BAC clones closest to the *CDH11* locus (RP11-467L24 and RP11-229O3) are labeled with red dots and red rectangle frames. The *CDH11* locus is shown in lower panel as in Ensemble Human Contig view (<http://www.ensembl.org/>). (b) *CDH11* is broadly expressed in human normal adult tissues and fetal tissues, with *GAPDH* as a control. Sk.M., skeleton muscle; B.M., bone marrow; L.N., lymph node.

cells (Supplementary Figure S2), suggesting that the downregulation/silencing of *CDH11* is not due to genetic deletion, but rather epigenetic silencing.

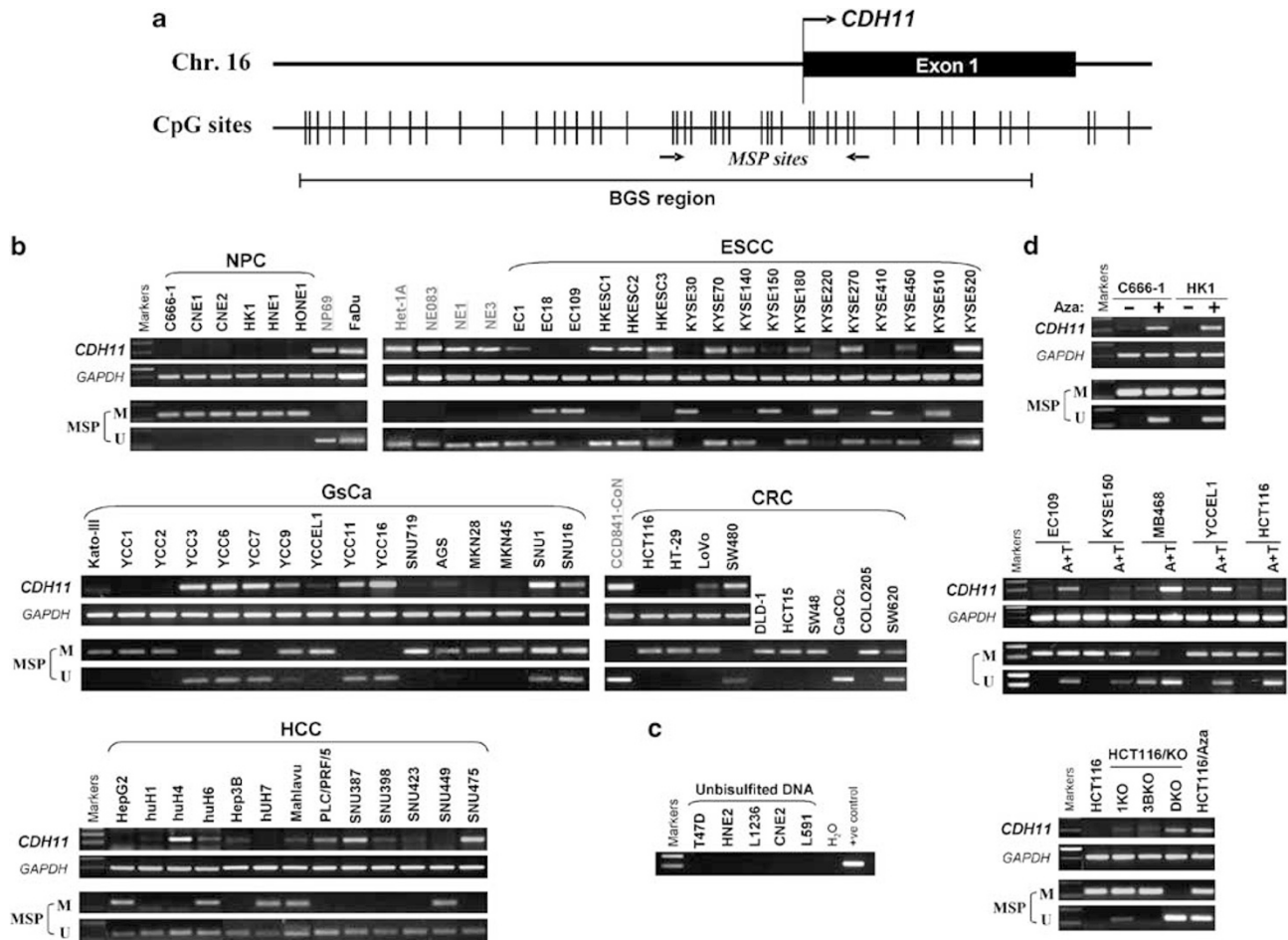
Further analysis of *CDH11* promoter showed that it contains a typical CpG island (<http://ccnt.hsc.usc.edu/cpgislands2>; Figure 2a), suggesting that *CDH11* is likely subject to methylation-mediated silencing. We then analyzed *CDH11* promoter methylation using a methylation-specific PCR (MSP) system, which was shown to be specific using DNA not bisulfite treated (Figure 2c). MSP analysis showed that *CDH11* was frequently methylated in cell lines of nasopharyngeal, esophageal, gastric, hepatocellular, colorectal, breast and cervix carcinomas, well correlated with the expression levels (Figure 2b; Supplementary Figure S1). In contrast, no *CDH11* methylation was found in any of the eight normal epithelial cell lines, suggesting that *CDH11* methylation is tumor specific.

To examine the methylation status of *CDH11* promoter in more detail, bisulfite genomic sequencing (BGS) analysis was performed with 44 CpG sites

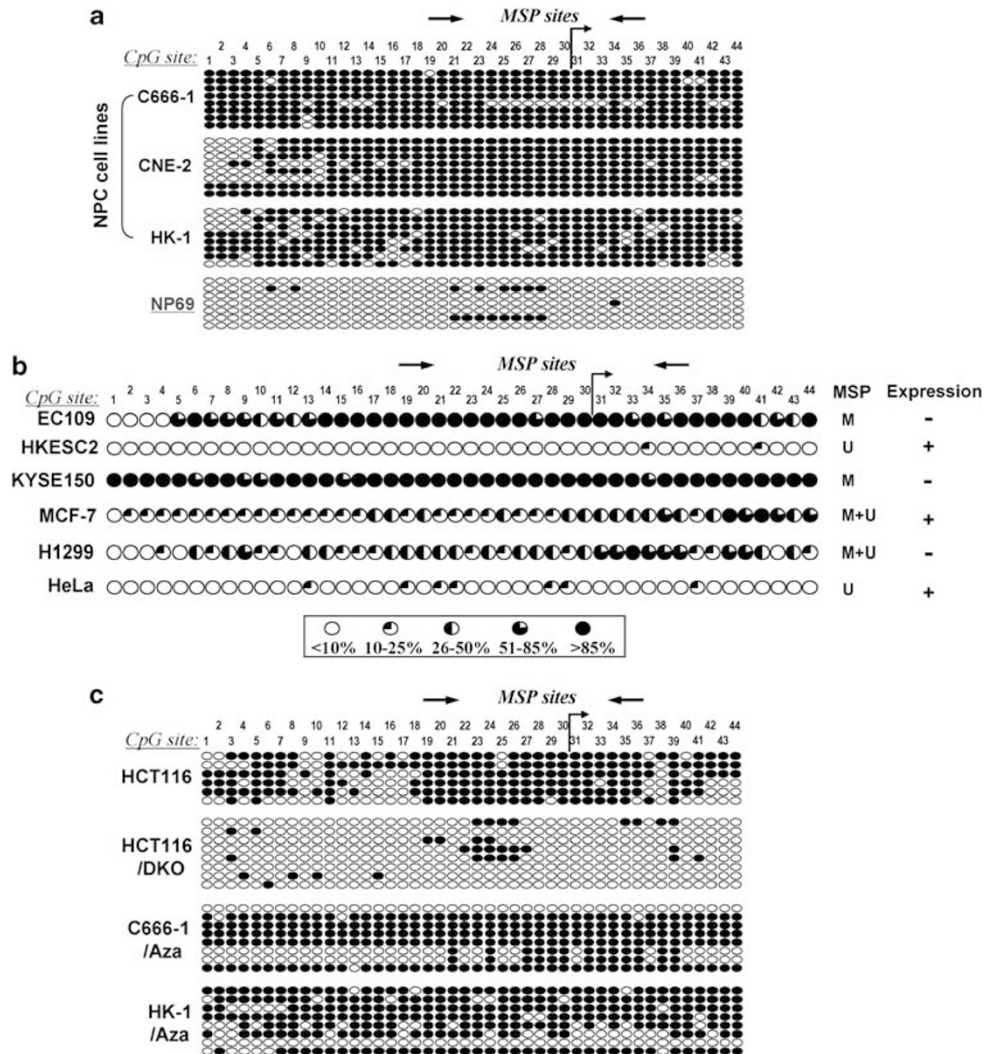
spanning the *CDH11* core promoter and exon 1 in a 416-bp region analyzed. The BGS results confirmed the MSP data (Figures 3a and b). Thus, *CDH11* silencing by DNA methylation is a widespread event in multiple tumors and might be one of the critical events involved in tumorigenesis.

*Demethylation restored CDH11 expression and frequent methylation of CDH11 in multiple primary tumors*

To test whether methylation directly mediates silencing of *CDH11*, several carcinoma cell lines with methylated and silenced *CDH11* were treated with DNA methyltransferase inhibitor Aza, alone or combined with the HDAC inhibitor trichostatin A. Aza or/and trichostatin A restored the expression of *CDH11* in these cell lines, accompanied by a decrease of methylated promoter alleles and an increase of unmethylated alleles, as analyzed by both MSP and BGS (Figures 2d and 3c). *CDH11* could also be activated in colon cell line HCT116 by genetic disruption of both *DNMT1* and *DNMT3B*, accompanied by *CDH11* promoter



**Figure 2** (a) Schematic structure of the *CDH11* CGI. Exon 1 (indicated with a black rectangle), CpG sites (short vertical lines), MSP sites, and BGS region analyzed are indicated. (b) *CDH11* is frequently silenced and methylated in multiple carcinoma cell lines but expressed and unmethylated in immortalized but non-transformed epithelial cell lines (underlined). ESCC, esophageal carcinoma; GsCa, gastric carcinoma; CRC, colorectal cancer; M, methylated; U, unmethylated. (c) Validation of the specificity of MSP system for *CDH11*. No product was obtained for unisulfited DNA. (d) Pharmacologic demethylation with Aza alone or combined with trichostatin A (A + T), or genetic demethylation in DKO cell line restored *CDH11* expression in methylated/silenced tumor cell lines.



**Figure 3** Detailed BGS analyses of *CDH11* promoter methylation. (a) NPC cell lines and immortalized normal cell line NP69. (b) Summarized percentage methylation of *CDH11* in multiple carcinoma cell lines. (c) Pharmacologic and genetic demethylation of *CDH11*. The *CDH11* transcription start site is shown as bent arrows. Circles, CpG sites analyzed; row of circles, an individual promoter allele that was cloned, randomly selected, and sequenced; filled circle, methylated CpG site; open circle, unmethylated CpG site.

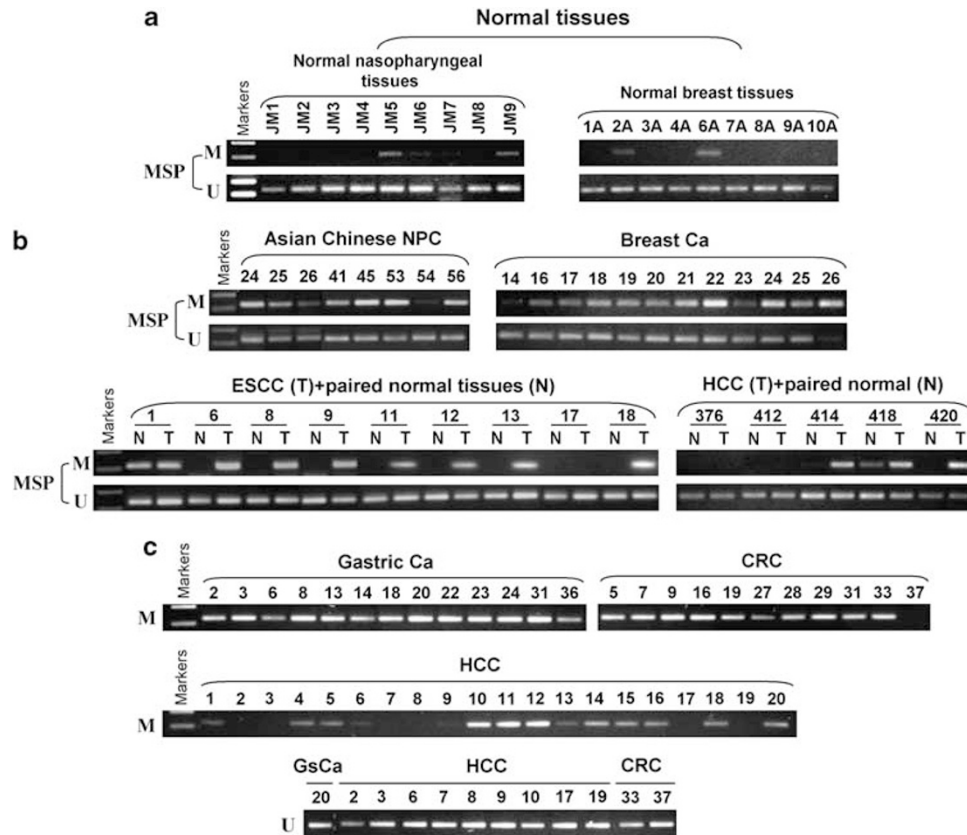
demethylation, but not in single knockout (KO) of either DNA methyltransferase 1 (DNMT1) or DNMT3B (Figure 2d), similarly to other known and novel TSGs we previously studied, indicating that the maintenance of *CDH11* promoter methylation is mediated by DNMT1 and DNMT3B.

*CDH11* methylation was further detected in multiple primary carcinomas with variable frequencies, as summarized in Supplementary Table S2. *CDH11* methylation was frequently detected in 94% (17 of 18) of NPC, 93% (13 of 14) of esophageal, 100% (13 of 13) of gastric, 66% (28 of 42) of HCC, 90% (10 of 11) of colonic and 91% (11 of 12) of breast carcinomas (Figure 4; Supplementary Table S2), while only seldom detected in normal tissues (nine normal nasopharynx, nine normal breast tissues, paired normal tissues of esophageal carcinoma and HCC; Figure 4). These

results suggest that hypermethylation of the *CDH11* promoter is a common event in multiple tumorigenesis.

#### *CDH11* inhibited tumor cell clonogenicity and induced apoptosis

Immunostaining showed that CDH11 was primarily expressed in the cell membrane in *CDH11*-expressing HONE1 and KYSE150 cells, while no detectable expression of CDH11 was found in control cells (data not shown) (Supplementary Figure S3A). Confocal microscopy assay further demonstrated clear cell membrane localization of CDH11, with E-cadherin as a comparison, in *CDH11*-transfected HONE1 cells (Figure 5a). We also examined the expression of the two alternatively spliced forms of CDH11 protein. Western blot showed that the wild-type CDH11



**Figure 4** (a–c) Representative MSP analysis of *CDH11* methylation in primary carcinomas and normal tissues. Ca, carcinoma; ESCC, esophageal carcinoma; CRC, colorectal cancer; GsCa, gastric carcinoma; N, paired tumor-adjacent normal tissues; T, tumor; M, methylated; U, unmethylated.

( $M_r \sim 110$  kDa) was abundantly expressed in normal tissues including brain, testis, lung and trachea, as well as in *CDH11*-transfected tumor cells (Figures 5b and e), while no E-cadherin was detected in human testis as expected (Andersson *et al.*, 1994; Conrad *et al.*, 2008). In addition, a very weak band at  $M_r \sim 85$  kDa was also detected using *CDH11* monoclonal antibody, but not with anti-Flag antibody, indicating that the wild-type *CDH11* is the predominant form expressed in cells, thus serves as the major executor for its functions.

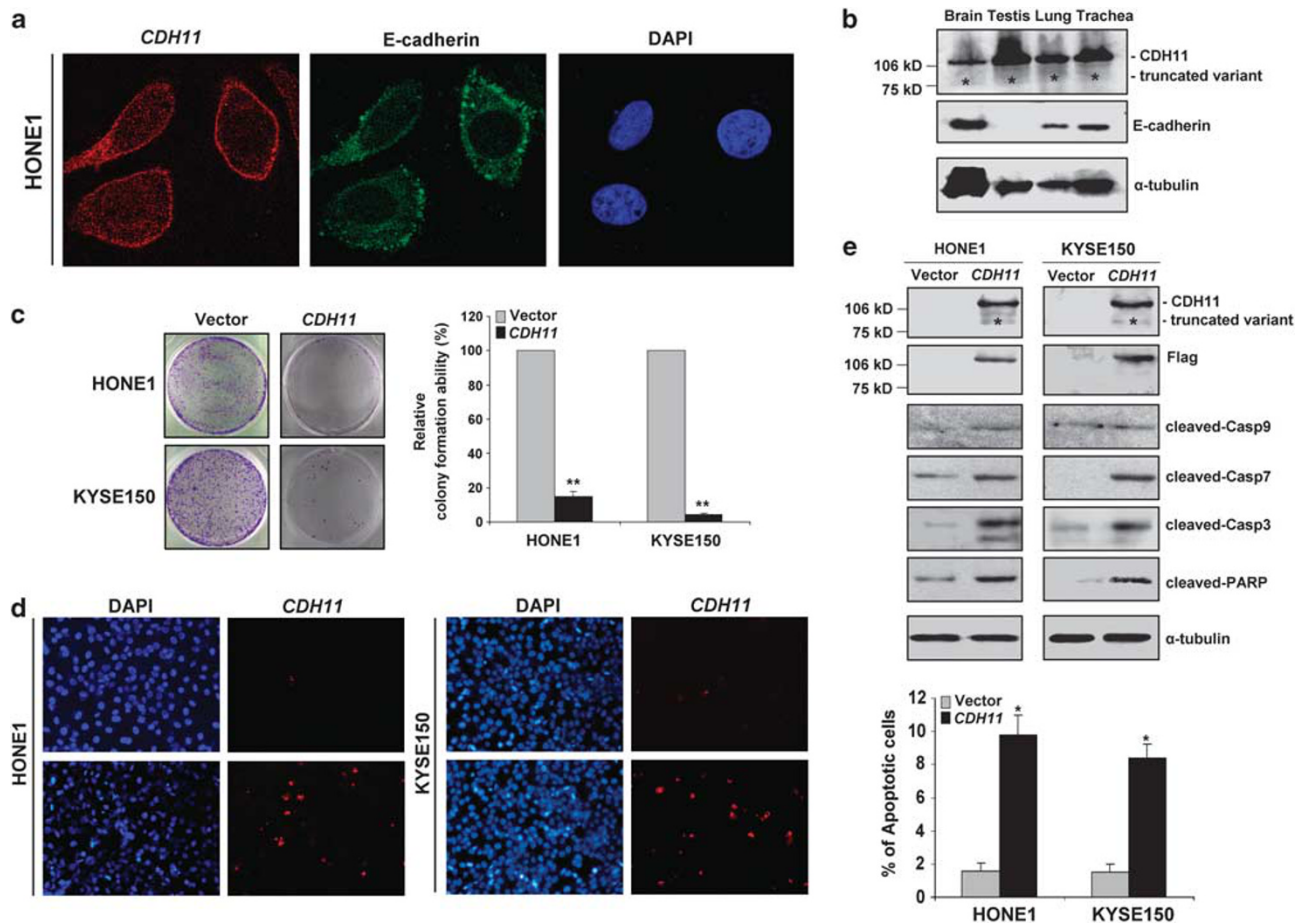
The frequent downregulation and methylation of *CDH11* in common tumors indicated that it is likely a tumor suppressor. Thus, we further explored the functional impact of *CDH11* expression in tumor cells. Four cell lines (HONE1, KYSE150, CNE-2 and EC109) with complete methylation and silencing were selected. Results of colony formation assays showed that ectopic expression of *CDH11* significantly suppressed the numbers of tumor cell colonies to <50% of the control cells (Figures 5c; Supplementary Figure S3B), indicating that *CDH11* indeed suppresses the tumorigenicity of tumor cells.

We then checked whether *CDH11* expression could induce tumor cell apoptosis. HONE1 and KYSE150 cells stably expressing *CDH11* were tested using TUNEL (terminal nucleotidyltransferase-mediated nick end labeling) assay. Results showed that *CDH11*

expression significantly increased the number of apoptotic HONE1 and KYSE150 cells when compared with controls (from  $\sim 1.5$  to  $\sim 10\%$ ;  $*P < 0.05$ ; Figure 5d). The data were confirmed by western blot for caspase-mediated cleavage of caspase 7, caspase 9, caspase 3 and poly (ADP-ribose) polymerase as apoptosis markers (Figure 5e), suggesting that *CDH11* indeed induces apoptosis through the intrinsic caspase-dependent pathway.

#### *CDH11* antagonized Wnt/ $\beta$ -catenin signaling pathway

To gain more insight of the molecular mechanisms underlying the tumor suppression by *CDH11*, we first examined whether *CDH11* could counteract Wnt/ $\beta$ -catenin signaling. By TOPflash reporter assay measuring endogenous  $\beta$ -catenin/TcF activity, it was found that *CDH11*-expressing tumor cells showed 2–3-fold reduced TOPflash activities when compared with controls (Figure 6a), suggesting that *CDH11* led to decreased  $\beta$ -catenin/TcF activities. Three genes as typical transcriptional targets of the Wnt/ $\beta$ -catenin/TcF signaling pathway, *CCND1* (cyclin D1), c-Myc and *MMP7* were thus evaluated. Results showed that *CDH11* markedly repressed the promoter activities and expression of *CCND1*, c-Myc and *MMP7* (Figures 6a and b).



**Figure 5** Subcellular localization and functional analyses of CDH11. (a) Confocal microscopy assay showed that CDH11 (red) is clearly expressed on the cell membrane in *CDH11*-transfected HONE1 cells using mouse anti-CDH11 monoclonal antibody, with double immunostaining for E-cadherin (green) as a control. DAPI counterstaining (blue) was used to visualize DNA. (b) CDH11 and E-cadherin protein expression detected in human normal tissues of brain, testis, lung and trachea by western blot. \*Indicates truncated variant band. (c) CDH11 inhibits tumor cell growth. Representative colony formation assays. Quantitative analyses of colony numbers are shown in the right as values of mean  $\pm$  s.d., \* $P < 0.05$ , \*\* $P < 0.01$ . (d) CDH11 induces tumor cell apoptosis. TUNEL assays of *CDH11* and vector-expressing tumor cells. \* $P < 0.05$ . (e) Western blot showing upregulation of cleaved caspase 9, 7, 3 and cleaved poly (ADP-ribose) polymerase (PARP) in *CDH11*-expressing tumor cells.

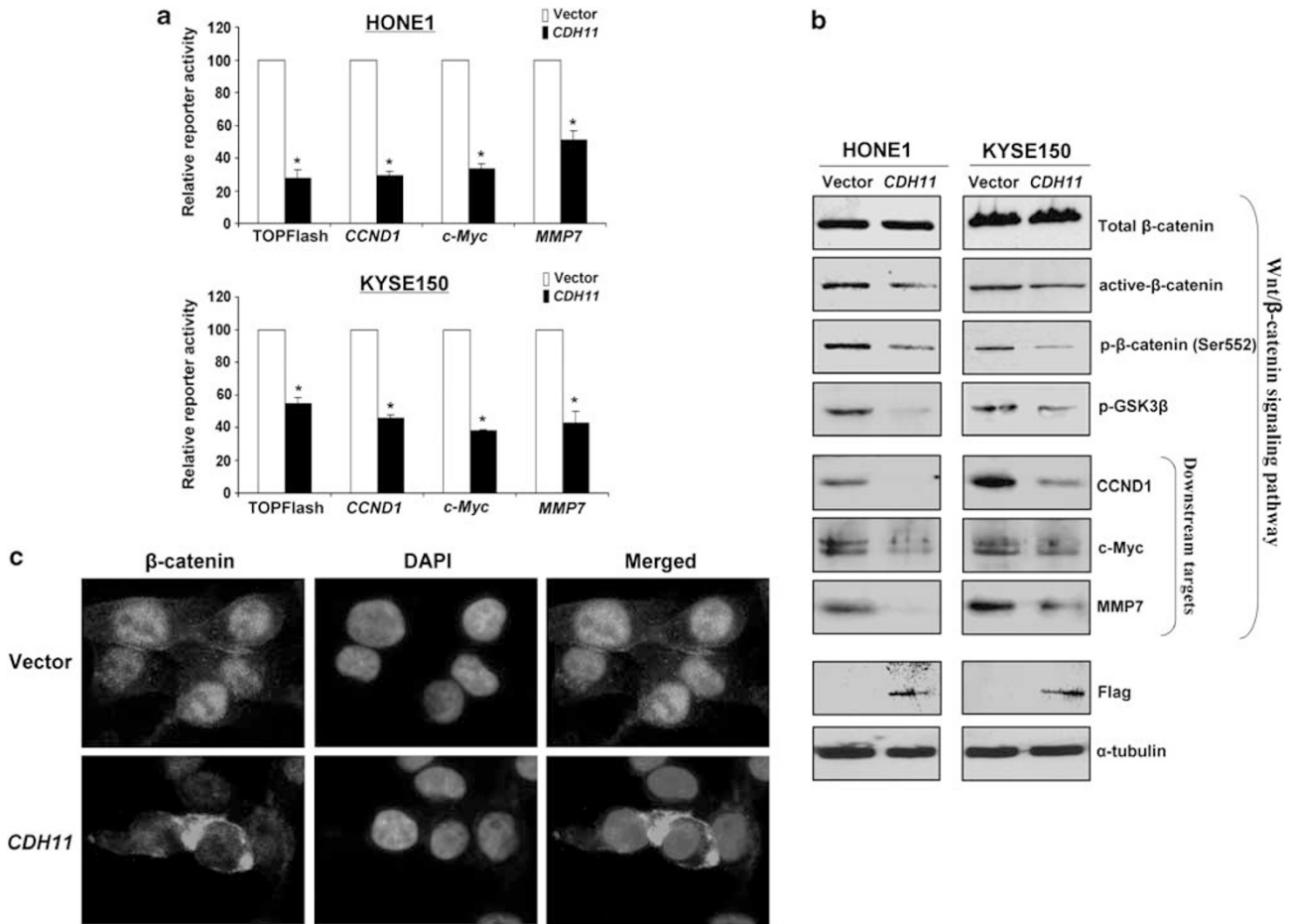
$\beta$ -Catenin, when enters the nucleus, has an important role as an activator of the transcription factor Tcf/LEF. The expression and localization of the  $\beta$ -catenin were examined by western blot and immunostaining. No obvious change of total  $\beta$ -catenin protein levels was noted, however, levels of active- and phospho- $\beta$ -catenin (ser552) that are the active forms dramatically decreased in *CDH11*-expressing tumor cells when compared with control cells, accompanied by downregulated GSK-3 $\beta$  phosphorylation (Figure 6b). In *CDH11*-transfected tumor cells,  $\beta$ -catenin translocated from nucleus to cytoplasm (Figure 6c), indicating that the extranuclear sequestration and inactivation of  $\beta$ -catenin mediated by *CDH11* are responsible for reduced  $\beta$ -catenin/Tcf-mediated transcriptional activity.

#### *CDH11* regulated the organization of actin cytoskeleton, cell migration and invasion

Classical cadherins are directly or indirectly linked to actin cytoskeleton organization. The impact of CDH11

expression on actin filament integrity was investigated by immunofluorescence. As shown in Figure 7a, the formation of actin stress fibers was disassembled in *CDH11*-expressing cells but not in control cells. Furthermore, downregulation of phospho-Rho A and AKT, as well as upregulation of phospho-JNK was found in *CDH11*-expressing cells by western blot (Figure 7b), consistent with their roles in impairing stress fibers.

As remodeling of actin cytoskeleton is critical for changes in cell shape, migration and invasion, the effect of CDH11 on tumor cell migration and invasion was further explored. Scratch wound-healing assays showed that *CDH11*-expressing cells were less proficient in closing an artificial wound than the vector-transfected cells on a confluent monolayer (Figure 7c, \* $P < 0.05$ ). Moreover, *CDH11*-expressing cells displayed significantly suppressed invasiveness compared with controls in matrigel invasion assays (Figure 7d, \* $P < 0.05$ ). Collectively, these results indicate that CDH11 expres-



**Figure 6** (a) Inhibition of TcF activity and *CCND1*, *c-Myc* and *MMP7* promoter reporter activities in *CDH11*-expressing tumor cells. \* $P < 0.05$ . (b) Ectopic expression of *CDH11* disrupted Wnt/ $\beta$ -catenin signaling pathway. Western blot was performed using antibodies against total  $\beta$ -catenin, active  $\beta$ -catenin, phospho- $\beta$ -catenin (Ser552), phospho-GSK3 $\beta$ , *CCND1*, *c-Myc* and *MMP7*.  $\alpha$ -Tubulin was used as a control. (c) *CDH11* sequestered nuclear  $\beta$ -catenin to the cytoplasm. Endogenous  $\beta$ -catenin localization was visualized in *CDH11*- and vector-transfected HONE1 cells by indirect immunofluorescence. Original magnification,  $\times 400$ .

sion could inhibit tumor cell metastasis and migration via disrupting stress filament integrity mediated by multiple signaling pathways.

#### *CDH11* regulated epithelial–mesenchymal transition and stemness

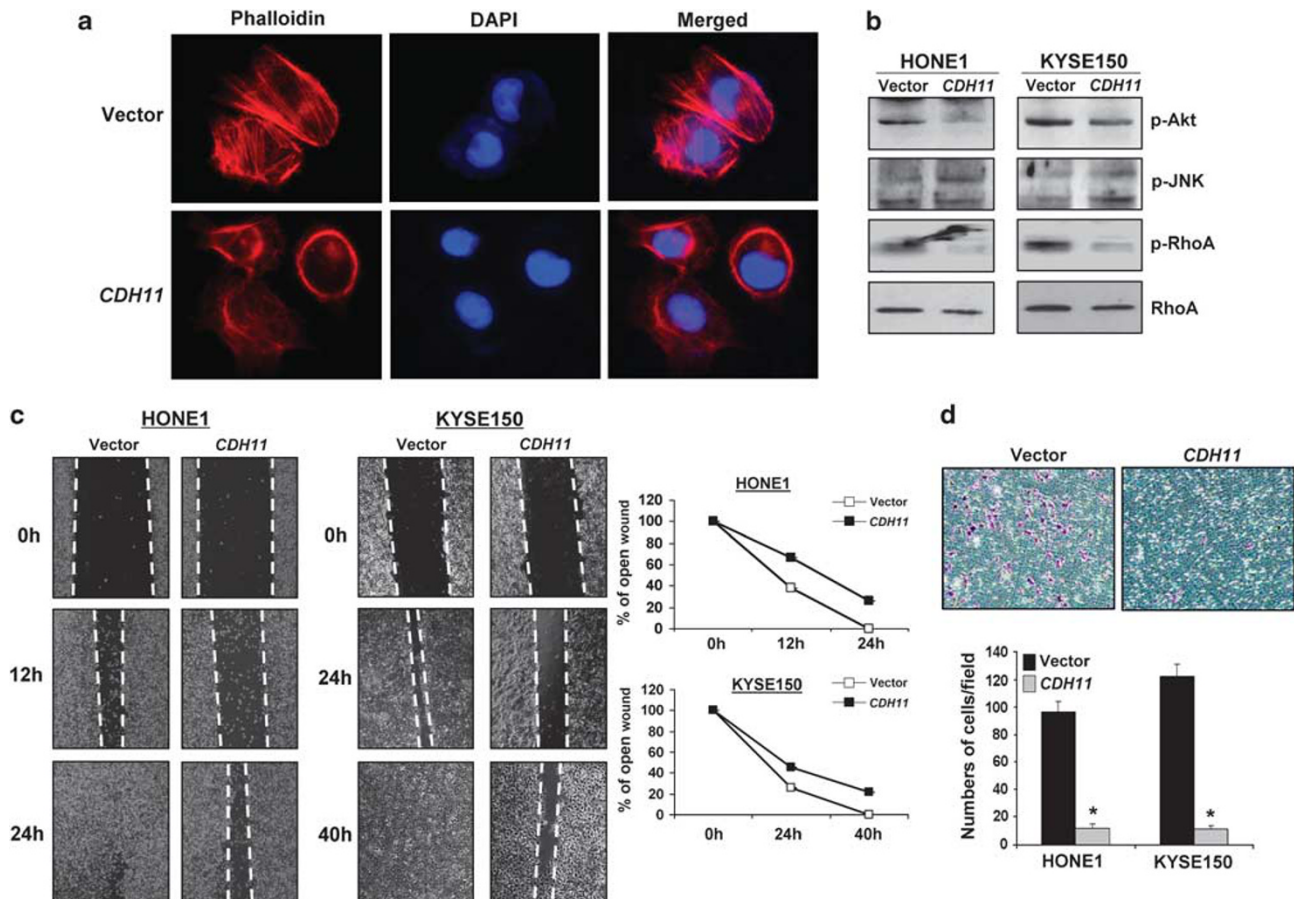
Epithelial–mesenchymal transition (EMT) has a critical role in tumor cell metastasis by reducing cell–cell contact and increased motility. The influence of *CDH11* expression on EMT regulation in tumor cells was further assessed. Dramatic morphological changes were observed in *CDH11*-expressing tumor cells, in which spindle-like, fibroblastic morphology was replaced by the typical cobblestone-like appearance of normal epithelial cells (Figure 8a), indicating that *CDH11* most likely reverses tumor cell EMT. Epithelial and mesenchymal markers were examined to determine whether *CDH11* negatively regulates EMT. *CDH11*-expressing cells exhibited a reversed EMT phenotype, including upregulated epithelial marker E-cadherin and downregulated mesenchymal marker Vimentin

(Figure 8c), which was further confirmed by immunofluorescence (Figure 8b).

As EMT is also linked to epithelial stem cell properties, we further examined whether *CDH11* could inhibit the tumor cell stemness. Representative stem cell markers were analyzed by RT–PCR. *CDH11* expression downregulated 6 out of 7 stem cell markers: *NANOG*, *DPPA5*, *ABCG2*, *c-Myc*, *MAD2* and *OCT4*, at the transcriptional level, except for *KLF4* (Figure 8d). Taken together, these results suggest that *CDH11* inhibits both EMT and stemness of carcinoma cells.

#### Discussion

In this study, we found that although *CDH11* is ubiquitously expressed in human normal adult and fetal tissues, it is frequently methylated and silenced in multiple common carcinoma cell lines and primary tumors but seldom in normal tissues. Pharmacologic or genetic demethylation resulted in the demethylation and restoration of *CDH11* expression. We also found that



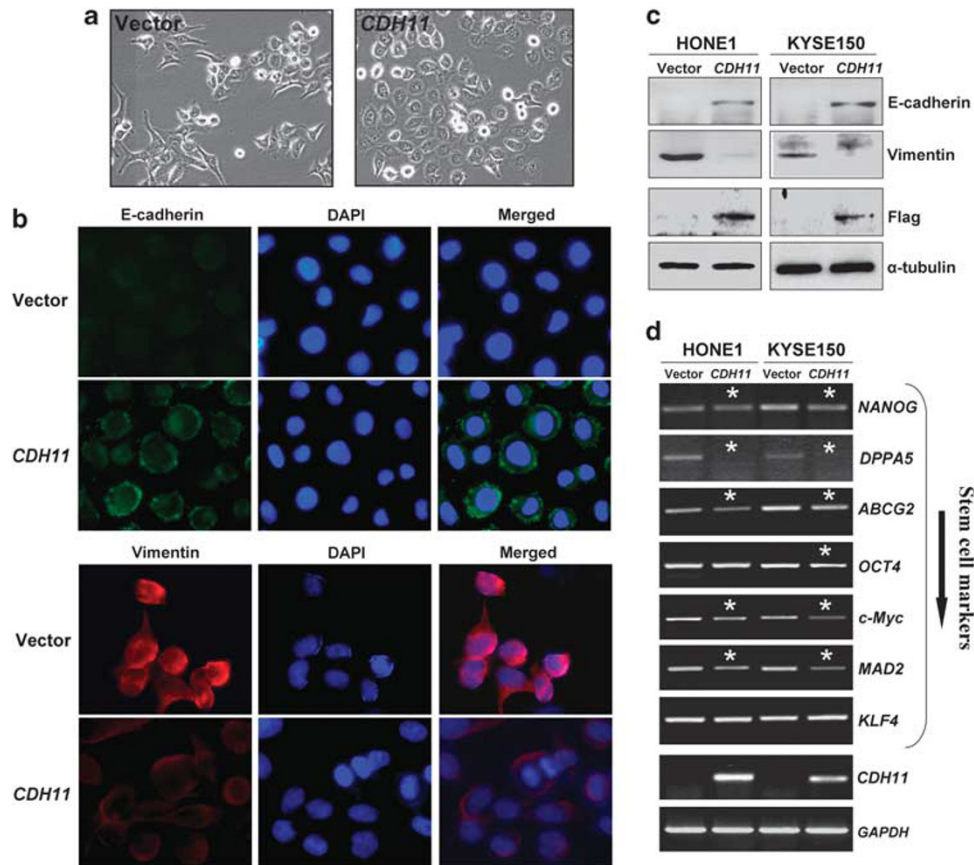
*CDH11* functions as a pro-apoptotic tumor suppressor, through antagonizing Wnt/ $\beta$ -catenin and AKT/Rho A signaling and disrupting stress fiber formation, and further inhibits the EMT and stemness of tumor cells and their migration and invasion. Our study appears to be the first study demonstrating the functional mechanisms of *CDH11*, and strongly supports the notion that *CDH11* is a tumor suppressor for multiple carcinomas.

*CDH11*, one of the type II classical cadherins, was originally identified in osteoblasts (Okazaki *et al.*, 1994), subsequently found overexpressed in certain invasive tumors (Pishvaian *et al.*, 1999; Chu *et al.*, 2008; Huang *et al.*, 2010). Earlier studies suggested that *CDH11* was a candidate oncogene for some tumor types like prostate cancer (Chu *et al.*, 2008; Huang *et al.*, 2010), breast cancer (Pishvaian *et al.*, 1999) and oral cancer (Choi *et al.*, 2008). Recently, increasing evidence indicate that *CDH11* might be a candidate tumor suppressor for locus 16q, a frequent loss of heterozygosity region in multiple cancers. For example, genetic loss of *CDH11* has been identified in more than half of retinoblastoma, implicating *CDH11* as a potential TSG in retinoblastoma (Marchong *et al.*, 2004). Using 16q-specific aCGH,

*CDH11* was identified to be a candidate tumor suppressor in breast tumorigenesis (Roylance *et al.*, 2006). Integrative epigenomic and genomic analysis of malignant pheochromocytoma also confirmed the potential of *CDH11* as a candidate tumor suppressor (Sandgren *et al.*, 2010). *CDH11* overexpression suppressed pulmonary metastasis of osteosarcoma *in vivo* (Nakajima *et al.*, 2008). These results are in line with ours and support the view that *CDH11* is a potent tumor suppressor for certain tumors.

TSGs are more frequently inactivated through epigenetic mechanisms such as promoter CpG methylation, or a combination of genetic and epigenetic inactivation, than biallelic genetic inactivation alone (Jones and Baylin, 2007). Three cadherin members (*CDH1* and *CDH13* at 16q, *CDH4*; Miotto *et al.*, 2004) as TSGs being inactivated genetically and/or epigenetically in various tumors and involved in tumor cell invasion and metastasis have been previously reported. We reported here that *CDH11* is the fourth cadherin member functioning as a TSG in multiple tumors, but silenced by promoter CpG methylation in multiple carcinomas. We also found that no methylation was detected in few carcinoma cell lines





**Figure 8** (a) Morphology changes of HONE1 cells transfected with CDH11 or empty vector by phase-contrast microscopy. Original magnification,  $\times 400$ . (b) Indirect immunofluorescence detecting the expression of E-cadherin and Vimentin in *CDH11*- or vector-transfected HONE1 cells. (c) Western blot showing the expression of E-cadherin and Vimentin in *CDH11*- or vector-transfected cells.  $\alpha$ -Tubulin was used as a loading control. (d) Downregulation of representative stem cell markers in *CDH11*-transfected tumor cells. \*Indicates significantly downregulated bands.

with reduced *CDH11* expression, suggesting that histone modification may be an alternative mechanism for *CDH11* downregulation in tumorigenesis.

*Cdh11* was first identified to act as a candidate tumor suppressor in a murine retinoblastoma model by facilitating tumor cell death (Marchong *et al.*, 2010), but how it functions in human tumorigenesis is not well understood. *CDH11* is unique among cadherins as two alternatively spliced forms are expressed simultaneously in cells: the intact form and a COOH terminus-truncated variant lacking homophilic cell–cell adhesion ability thus as a dominant-negative product which is also the secreted form (Kashima *et al.*, 1999; Pishvaian *et al.*, 1999). We found that when the full-length *CDH11* was introduced into tumor cells, its intact form ( $\sim 110$  kDa) is the predominant isoform expressed, although a tiny amount of its truncated variant was also detected, in line with previous studies (Kashima *et al.*, 1999; Pishvaian *et al.*, 1999; Feltes *et al.*, 2002). Subcellular localization of *CDH11* in *CDH11*-expressing tumor cells exhibited the clear membrane-localizing feature of cadherin family members. Furthermore, we identified that the intact form *CDH11* significantly suppressed the malignant properties of transfected cells including proliferation, migration/invasion and promoted tumor cell apoptosis,

acting as a functional tumor suppressor, in line with the previous finding that intact *CDH11* inhibits the invasion of breast cancer cells (Pishvaian *et al.*, 1999; Feltes *et al.*, 2002). While an invasion-promoting effect of its truncated variant has been reported (Feltes *et al.*, 2002), more studies are needed to delineate the different role of the truncated variant of *CDH11* in tumorigenesis.

Cadherin-associated  $\beta$ -catenin pool having a distinct role from its nuclear transcription role (Berx and van Roy, 2009). As a functional adherens junction molecule, *CDH11* mediates calcium-dependent cell–cell adhesion via recruiting  $\alpha$ -catenin,  $\beta$ -catenin and p120<sup>cas</sup> to the membrane, involved in the regulation of Wnt/ $\beta$ -catenin signaling pathway (Feltes *et al.*, 2002; Huang *et al.*, 2010). In our study, *CDH11* was found to facilitate extranuclear sequestration of  $\beta$ -catenin, and reduce  $\beta$ -catenin/Tcf-mediated proliferation and transcriptional activity, thus disrupting Wnt/ $\beta$ -catenin signaling, as well as cross-talking with Rho A, AKT and JNK signaling. Consistent with this mechanism, we observed disrupted actin polymerization and reversed EMT phenotypes after *CDH11* expression. EMT promotes the production of cells with property of self-renewing stem cells, thus facilitating the execution of the invasion-metastasis

cascade (Mani *et al.*, 2008; Giannoni *et al.*, 2010). The role of cadherin family members on cell stemness is complicated, depending on coordinately regulated cadherins (Hosokawa *et al.*, 2010). E-cadherin has been identified to be a critical molecule for embryonic stem cell pluripotency (Karpowicz *et al.*, 2009; Chen *et al.*, 2010; Redmer *et al.*, 2011). In our study, the down-regulation of several stem cell markers by CDH11 suggests the potential of CDH11 in modulating tumor cell stemness, which may be due to increased E-cadherin and further link to its tumor suppressive role.

In summary, our study identifies CDH11 as a functional tumor suppressor and an important regulator of Wnt/ $\beta$ -catenin and AKT/Rho A signaling, with frequent epigenetic inactivation in common carcinomas. Our study further extends the current understanding on the role of cadherin family in tumorigenesis.

## Materials and methods

### Cell lines and tissue samples

A series of tumor cell lines were used (Wang *et al.*, 2009; Cheng *et al.*, 2010). Immortalized, non-transformed normal epithelial cell lines (NP69, Het-1A, NE083, NE1, NE3, CCD841-CoN, HMEC and HMEpC) were used as normal controls. HCT116 cell lines with genetic KO of DNMTs: HCT116 DNMT1<sup>-/-</sup> (1KO), HCT116 DNMT3B<sup>-/-</sup> (3BKO) and HCT116 DNMT1<sup>-/-</sup>DNMT3B<sup>-/-</sup> (DKO) cells (gifts of Bert Vogelstein, Johns Hopkins University, Baltimore) were also used. Cell lines were obtained either from American Type Culture Collection or from collaborators. Cell lines were treated with 10  $\mu$ mol/L 5-aza-2'-deoxycytidine (Aza, Sigma-Aldrich, St Louis, MO, USA) for 3 days or further treated with 100 nmol/L trichostatin A (Cayman Chemical Co., Ann Arbor, MI, USA) for additional ~16 h as described previously (Qiu *et al.*, 2004; Ying *et al.*, 2006). Normal adult and fetal tissue RNA and protein samples were purchased commercially (Stratagene, La Jolla, CA, USA or Millipore-Chemicon, Billerica, MA, USA). DNA samples of normal nasopharyngeal and breast tissues, Asian Chinese NPC, paired Hong Kong Chinese esophageal carcinomas (T) and the matching surgical marginal normal tissues (N), and other carcinomas have been described previously (Cui *et al.*, 2008; Cheng *et al.*, 2010).

### Antibodies

Antibodies used were cleaved caspase-7 (9491), cleaved caspase-9 (9505), cleaved caspase-3 (9661), cleaved poly (ADP-ribose) polymerase (9541), phospho- $\beta$ -Catenin (Ser552), phospho-GSK-3 $\beta$  (Ser9), phospho-AKT (Ser473), phospho-SAPK/JNK (Thr183/Tyr185), RhoA (2117) and E-cadherin (4065) (Cell Signaling, Beverly, MA, USA); Flag M2 (F3165), Vimentin (V6630) (Sigma-Aldrich) and active  $\beta$ -catenin (anti-ABC, 05-665, Upstate, Lake Placid, NY, USA); total  $\beta$ -catenin (M3539), anti-mouse Ig G-HRP (P0161), anti-rabbit Ig G-HRP (P0448) (Dako, Glostrup, Denmark); CCND1 (sc-20044), c-myc (sc-764), phospho-RhoA (Ser188) (sc-32954) (Santa Cruz, CA, USA); MMP7 (MS-813-P0, Thermo Scientific, Fremont, CA, USA); monoclonal CDH 11 antibody (32-1700) (Invitrogen, Carlsbad, CA, USA);  $\alpha$ -tubulin (Lab Vision Corporation, Fremont, CA, USA).

### Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed as described before (Jin *et al.*, 2007). *GAPDH* was amplified as a control. Primers used in this study are listed in Supplementary Table S1.

### Deletion analysis of CDH11 by multiplex PCR

Homozygous deletion of *CDH11* was examined using multiplex genomic DNA PCR as previously described (Qiu *et al.*, 2004; Cui *et al.*, 2008). Primer sequences are shown in Supplementary Table S1.

### Bisulfite treatment and promoter methylation analysis

Bisulfite modification of DNA, MSP and BGS were carried out as described previously (Tao *et al.*, 1999, 2002). MSP and BGS primers are listed in Supplementary Table S1.

### Construction of CDH11-expressing vector and generation of stable cell lines

Full-length cDNA of *CDH11* was PCR cloned from human trachea RNA (BD Clontech, Palo Alto, CA, USA) with the primers (Supplementary Table 1) and sequence verified. pcDNA3.1(+)-Flag-CDH11 plasmid was then constructed as previously (Cheng *et al.*, 2010; Li *et al.*, 2010). This expression construct was transfected into HONE1 and KYSE 150 cells using Lipofectamine 2000 (Invitrogen). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and selected in 400  $\mu$ g/ml of G418 for 20–30 days to establish stable cell pools.

### Colony formation assay

Colony formation assays were carried out as previously described (Jin *et al.*, 2007; Ying *et al.*, 2008). Briefly, cells were cultured overnight in a 12-well plate (1.0  $\times$  10<sup>5</sup> per well) and transfected with empty vector or CDH11-expressing plasmid using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, the transfectants were replated in triplicate and cultured for 10–15 days in complete medium containing G418 (400  $\mu$ g/ml). Surviving colonies were stained with gentian violet after methanol fixation and visible colonies ( $\geq$  50 cells) were counted.

### TUNEL assay

Cells cultured on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 2 min on ice. TUNEL staining was done using the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction.

### Western blot

Cell lysates were prepared by incubating the cell pellets in lysis buffer (50 mmol/l Tris-HCl, pH 8.0; 150 mmol/l NaCl, 0.5% NP-40) for 30 min on ice, followed by centrifugation at 14 000  $\times$  g for 15 min at 4  $^{\circ}$ C. For western blot, membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4  $^{\circ}$ C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using Western blot Luminol reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

### Dual-luciferase reporter assay

TcF transcriptional activity and its target genes promoter activities were determined by luciferase reporter assays. The TcF-responsive luciferase construct pTopFlash was kindly provided by Prof. Christof Niehrs (German Cancer Research Center, DKFZ). *CCND1*-, *c-Myc*- and *MMP7*-promoter reporter constructs containing TcF-responsive element were cloned in our laboratory. Cells were transiently transfected in

triplicate with one of the luciferase reporters and pRL-TK (Promega, Madison, WI, USA). After 48 h of transfection, luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). Relative luciferase activities were determined and normalized using Renilla reniformis luciferase activity as an internal control.

#### Indirect immunofluorescence

Cells grown on coverslips were stained by indirect immunofluorescence as described previously (Hu *et al.*, 2009). Briefly, cells were incubated with primary antibodies against CDH11,  $\beta$ -catenin, E-cadherin, or Vimentin and then incubated with Alexa Fluor 594- (Invitrogen Molecular Probes, Carlsbad, CA, USA) or FITC-conjugated (Dako) secondary antibody against mouse or rabbit IgG. To analyze the effects of CDH11 on actin stress fiber formation, cells were serum starved for 24 h before incubation in serum-containing 10% fetal bovine serum medium. After 1 h, cells were fixed and stained by Rhodamine-labeled phalloidin (Invitrogen Molecular Probes). Cells were then counterstained with DAPI and imaged with an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) and Leica TCS SP5 confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). Confocal images of 2048  $\times$  2048 pixels were acquired and assembled.

#### Wound-healing assay and matrigel invasion assays

Cell motility was assessed using a scratch wound-healing assay (Ying *et al.*, 2008). Stably transfected cells were cultured in 6-well plates until confluent. The cell layers were carefully

wounded using sterile tips and washed twice with fresh medium. After incubation for 12, 24 and 40 h, the cells were photographed under a phase-contrast microscope. The experiments were performed in triplicate.

*In-vitro* invasion assays were carried out in BD BioCoat Matrigel chambers (Transwell, BD Biosciences, Heidelberg, Germany) as described previously (Hu *et al.*, 2009).

#### Statistical analysis

Results are shown as values of mean  $\pm$  s.d. Statistical analyses were performed using the Student's *t*-test to determine *P*-values.

#### Conflict of interest

The authors declare no conflict of interest.

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