N-cadherin expression is correlated with metastasis of spindle cell carcinoma of head and neck region

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Running title: N-cadherin expression in SpCC

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Summary

Spindle cell carcinoma (SpCC) is a biphasic tumor composed of conventional squamous cell carcinoma and a malignant spindle cell component. SpCC expresses both epithelial and mesenchymal markers by immunohistochemical analysis. There is mounting evidence for sarcomatoid transformation from the epithelial component, supporting the theory that SpCC is a monoclonal neoplasm originating from a stem cell giving rise to both components. The loss of E-cadherin and the gain of N-cadherin expression are known as the “cadherin switching”. Cadherin switching is a major hallmark of epithelial-mesenchymal transition (EMT). EMT is a crucial process in cancer progression providing cancer cells with the ability to escape from the primary focus, to invade stromal tissues and to migrate to distant regions. Although E-cadherin down-regulation is well known in various cancers, there are a few studies on N-cadherin expression in cancer. Here, therefore, we investigated N-cadherin expression in the progression of head and neck SpCC. First we examined cadherin swithching in our established SpCC cell lines, SpCC-1 and SpCC-2. SpCC-1 and SpCC-2 cells were spindle in shape and showed cadherin switching. Moreover, we examined N-cadherin expression in 15 SpCC cases by immunohistochemistry. Although N-cadherin expression was not observed in non-neoplastic squamous epithelium, high expression of N-cadherin was observed in 10 of 15 SpCC cases. Interestingly, 6 of 7 SpCC cases with metastasis showed high expression of N-cadherin. In conclusion, our findings suggest that N-cadherin may play an important role in metastasis of SpCC in addition to the pathogenesis of SpCC of the head and neck.

Introduction

Spindle cell carcinoma (SpCC) is a rare and peculiar biphasic malignant neoplasm that occurs mainly in the upper aerodigestive tract. SpCC has been referred to by a variety of names, including pseudosarcoma (1), carcinosarcoma (2) or pleomorphic carcinoma (3-5), which reflect the divergent interpretation of the sarcomatoid component as reactive or neoplastic, mesenchymal or epithelial. SpCC is thought of as a variant of squamous cell carcinoma, which shows biphastic proliferation of conventional SCC component and malignant spindle shape cells with sarcomatous appearance. It is generally accepted that the sarcomatoid cells are derived from squamous cells. We previously reported that the epithelial nature of the sarcomatoid component of SpCC was clearly revealed by a combination of immunohistochemical staining for keratins and electron microscopic demonstration of tonofilament-like filaments and/or desmosome-like structures (6). Moreover, we found co-expression of cytokeratins, vimentin and Wnt-5a in SpCC cell line (7). These our previous findings support the theory of epithelial origin and mesenchymal transition of SpCC.

It has recently been suggested that epithelial-mesenchymal transition (EMT) might play an important role in the pathogenesis of SpCC (8-12). EMT is a crucial process in cancer progression, providing cancer cells with the ability to escape from the primary focus, to invade stromal tissues and to migrate to distant regions (13, 14). Cadherin switching, which is characterized by the loss of E-cadherin and the gain N-cadherin expression, is a major hallmark of EMT (13). Cadherins are a family of transmembrane proteins that mediate Ca^{2+}-dependent cell-cell adhesion. Of the members of the cadherin family, E-cadherin is distributed widely and is the most important intercellular adhesion molecule in epithelial cells. Although E-cadherin is well documented as an invasive suppressor for cancer cells, the role of anomalously expressed N-cadherin in epithelial cells has only recently been identified more clearly (15). Unlike E-cadherin, N-cadherin is normally expressed in neuroectodermal and mesodermal-derived tissue and is involved in a lot of processes, such as cell-cell

adhesion, differentiation, embryogenesis, migration, invasion and signal transduction (15, 16). Aberrant expression of N-cadherin is considered to be associated with increased tumor progression in certain cancers (15). In particular, increased expression of N-cadherin and concomitant down-regulation of E-cadherin are suggested to be a feature of EMT in cancer (17). Previous report has shown that cadherin switching was observed in 19 of 30 SpCC cases. However, little is known about the role of aberrant expression of N-cadherin in SpCC.

This study attempted to investigate the hypothesis that spindle cell phenotype might be related to the cadherins which form adherens junctions between cells, whether it is due to EMT representing invasive potential or simply a metaplastic/bidirectional differentiation process of the cancer cells. To find out the involvement, we examined the expression of N-cadherin and compared it with E-cadherin expression and clinico-pathological parameters in SpCC.

Materials and methods

Cell lines and cell culture

SpCC cell line, SpCC-1 was previously established in our laboratory (Kudo et al., 2006). SpCC-2 cell line was recently established from SpCC tissue sample. Tissue sample was obtained from a SpCC of a 32-year-old-Japanese female tumor in gingiva. The tissue sample was cut into small pieces and placed on 90-mm Petri dishes (3003, Falcon, Becton Dickinson, Franklin Lakes, NJ) with DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Invitrogen) and 100 U/ml penicillin-streptomycin (Gibco) under conditions of 5% CO_2 in air at 37 ˚C. When these outgrowth cultures formed confluent monolayer, the cells were subcultured after enzymatic removal with 0.05 % trypsin-EDTA to passage 1 (P1). Then, we subcultured the cells with the same medium until over P100 and used for the following analyses.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated and purified from cells by using RNeasy Mini Kit (Qiagen, Hilden, Germany). Preparations were quantified and their purity was determined by standard spectrophometric methods. cDNA was synthesized from 1μg total RNA according to the ReverTra Dash (Toyobo Biochemicals, Tokyo, Japan). Primer sequences included the following;

human N-cadherin: 5'-GACAATGCCCCTCAAGTGTT-3' (forward)
5'-CCATTAAGCCGAGTGATGGT-3' (reverse)

human E-cadherin: 5'-TGCCCAGAAAATGAAAAAGG-3' (forward)
5'-GGATGACAGCGTGAGAGA-3' (reverse)

human Vimentin: 5'-CCCTCACCTGTGAAGTGGAT-3' (forward)
5'- TCCAGCAGCTTCCTGTAGGT-3' (reverse)

human GAPDH: 5'-ACAGTCAGCCGCATCTTCTT-3 (forward)
5'-TTGATTTTGGAGGGATCTCG-3' (reverse)

Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen), and amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 25-35 cycles after an initial 30 sec denaturation at 94 ˚C, annealed for 30 sec at 60 ˚C, and extended for 1 min at 72 ˚C in all primers. The amplification reaction products were resolved on 1.2 % agarose/TAE gels, electrophoresed at 135 mV, and visualized by ethidium-bromide staining.

Immunofluoresence

SpCC-1 cells grown on coverslips were fixed in 4 % paraformaldehyde for 10 min at room temperature, rinsed three times with ice-cold PBS, and then permeabilized in 0.1 % Triton X-100 in PBS for 15 min at room temperature. After rinsing three times with PBS, cover slips were incubated with the primary antibody in 10 % DMEM. We used N-cadherin monoclonal antibody (1:100; BD Transduction Laboratories) for

primary antibody and anti-mouse Alexa594 (1:1000; Molecular Probes, Eugene, OR) for secondary antibody. DNA was visualized by DAPI staining. Immunostaining of cell preparations was recorded using an epifluorescence Zeiss Axioplan 2 (Zeiss Inc., Thorwood, NY) microscope attached to a CCD camera.

Clinical data and paraffin-embedded tissue samples

15 paraffin-embedded SpCC cases were selected from the Surgical Pathology Registry of Hiroshima University Hospital. Clinical details and lymph node metastasis were gathered from surgical records of the patients. Clinical data of the patients are summarized in Table 1. Among the 15 SpCC patients, 4 were males and 11 were females. At the time of diagnosis their age ranged from 32 to 98 years. Lymph node metastasis was observed in 7 cases. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Informed consent was obtained from all patients for this study.

Immunohistochemistry

Unstained 4.5 μm sections were cut from each paraffin block and de-paraffinized, rehydrated by routine techniques. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol in 30 minutes before doing retrieval antigen by microwave treatment in Tris-EDTA buffer pH 9.0 and in citrate phosphate buffer for N-cadherin and E-cadherin, respectively in 20 minutes. The sections were then incubated with protein block serum-free solution in 10 minutes. N-cadherin and E-cadherin monoclonal antibodies (BD Transduction Laboratories) were diluted in PBS (1:30 and 1:150, respectively) and incubated for overnight at 4 ˚C. After washing three times by PBS, the sections were incubated with appropriate peroxidase-labeled secondary antibodies for 1 hour at room temperature. The sections were then labeled by diaminobenzidine and were counterstained with Mayer's haematoxyline, dehydrated and mounted. Evaluation of immunostaining was based on proportion of stained

cancer cells and location of staining. For N-cadherin, the samples were then divided into two groups; low (less than 20% of the cancer cells were stained) and positive (over 20% of the cancer cells were stained). E-cadherin was defined as preserved (over 80% of cancer cells exhibited positive staining similar to that in normal epithelial cells) and reduced (less than 80% of the cancer cells exhibited positive staining similar to that in normal epithelial cells).

Statistical analysis

Correlations between the expression pattern of N-cadherin and E-cadherin and the clinico-pathological features of SpCC were examined using Chi-square test. Statistical analysis was performed using SPSS 13.0 and statistical significance was defined as $P<0.05$.

Results

Establishment of SpCC cell line

We established a SpCC cell line from a SpCC arisen in gingival of 32-year-old female. The patient did not have a history of pre-existing squamous cell carcinoma and was treated by radical surgical excision without irradiation and chemotherapy. Histologically, the tumor widely infiltrated into connective tissues, muscles and mandible with ulcer formation and metastasized to cervical lymph nodes. The tumor was mainly composed of pleomorphic spindle cells accompaning small areas of squamous cell carcinoma (Fig. 1A and 1D). The surface epithelium blended imperceptibly with the spindle cell component (Fig. 1B). SpCC cells metastasized to cervical lymph nodes (Fig. 1C). Immunohistochemically, the SpCC cells were positive for cytokeratins, detected by AE1/AE3 and CAM5.2 (Fig. 1E and 1F). SpCC cells were also positive for vimentin (Fig. 1G). α-smooth muscle actin (αSMA), S100 and HMB45 were negative in SpCC cells (Fig. 1H-J). The diagnosis of SpCC was made on the basis of these histological and immunohistochemical findings. We could subculture

this tumor cells up to 100 passages. We previously established another SpCC cell line, SpCC-1 (7). In similar to SpCC-1 cells, established SpCC cells (SpCC-2) showed spindle shape (Fig. 2A). SpCC-1 and SpCC-2 cells showed vimentin expression and loss of E-cadherin expression demonstrated by RT-PCR (Fig. 2B). Oral squamous cell carcinoma cell lines, Ca9-22 and Ho-1-U-1 cells showed E-cadherin expression. Then, we examined N-cadherin expression in SpCC cells. Both SpCC-1 and SpCC-2 cells showed high expression of N-cadherin (Fig. 2B) and N-cadherin expression was observed in the cytoplasm of SpCC cells (Fig. 2C). Thus, as we expected, cadherin switching was observed in SpCC cell lines.

N-cadherin expression and its correlation with metastasis

Next, we examined the expression of N-cadherin in 15 SpCC cases by immunohistochemistry. Although non-neoplastic mucous squamous epithelium was completely negative, high expression of N-cadherin was observed in SpCC cells (Fig. 3A). High expression of N-cadherin was observed in 10 of 15 SpCC cases (Fig. 3B and Table 1). All cases showed reduced expression of E-cadherin in comparison with non-cancerous epithelial cells (~~data not shown~~Table 1). Interestingly, 6 of 7 SpCC cases with metastasis showed high expression of N-cadherin (Fig. 3C and Table 1).

Discussion

It is known that cellular changes resulting in a more mesenchymal-like state driven by EMT in cancer are thought to play a major role in tumor progression. The loss of E-cadherin and gain of N-cadherin expression are known as “cadherin switching” (13). Cadherin switching is thought to reflect an EMT, whereby tumor cells are released from E-cadherin-dependent cell-cell interations and acquire a motile phenotype through the induction of N-cadherin. In this study, we found the varying patterns of cadherin expression, associated with morphological transition from epithelial to spindle cell phenotype, loss of epithelial markers and expression of mesenchymal

markers. Cadherin switching was observed in all 2 SpCC cell lines and 10 of 15 SpCC cases. These features are reminiscent of EMT. Importantly, 6 of 7 SpCC cases with metastasis showed high expression of N-cadherin. As SpCC is rare to be found in head and neck, we could collect only a small group of patients. So far, there are a few studies on N-cadherin expression in SpCC (11, 12), and clinical benefit of N-cadherin expression is not clarified. Here we have shown that N-cadherin expression was well correlated with lymph node metastasis in SpCC. We suggest that N-cadherin may be a marker for prediction of metastasis. To prove this, further experiments using a large number of SpCC cases are required.

It is well known that loss of E-cadherin expression is frequently found during tumor progression in most epithelial cancers (18). We also previously demonstrated that methylation of E-cadherin is involved in invasion and metastasis in head and neck squamous cell carcinoma (19). On the other hand, aberrant de novo expression of N-cadherin has been noted in breast, prostate, and bladder cancers, where it parallels a down-regulation of E-cadherin and a decrease in tumor differentiation (20-22). As the result shown, N-cadherin expression was found in membrane and cytoplasm of SpCC cells by immunohistochemistry and immunofluorescence analysis. It has been shown that aberrant expression of N-cadherin in cancer cells can contribute to invasiveness and metastasis by making the cells more motile (23-25). It is interesting to examine the molecular mechanism of N-cadherin in metastasis of SpCC. It has been shown that cadherin switching is necessary for increased motility but is not required for the morphological changes that accompany EMT (26). In addition to N-cadherin and E-cadherin, other type of cadherins including P-cadherin and VE-cadherin are involved in EMT induction of cancer. P-cadherin was shown as one major component in reconfiguring mesenchymal cells with epithelial features by triggering GSK-3ß-mediated inactivation and cytoplasmatic translocation of Snail in oral squamous cell carcinoma (27). VE-cadherin was induced during EMT in mammary tumor cells and was aberrantly expressed in invasive human breast

carcinomas (28). However, mechanism of EMT induction in cancer is still unknown (29, 30). We suggest that SpCC cells can be a useful tool for understanding EMT induction in cancer.

In conclusion, our study indicates N-cadherin may play an important role in metastasis of SpCC and EMT might play an important role in the pathogenesis of SpCC of the head and neck.

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FIGURE LEGENDS

Figuer 1. Histologic and immunohistochemical findings of primary tumor of SpCC. (A) H&E staining; low magnification of SpCC (x40). Scale bar; 50 μm. (B) H&E staining; The surface epithelium of SpCC (x40). (C) Metastasis of SpCC to cervical lymph node (x40). (D) H&E staining; high magnification of SpCC (x200). Scale bar; 250 μm. (E) Immunohistochemical staining; Expression of cytokeratins (AE1/AE3) in SpCC. (x200). (F) Immunohistochemical staining; Expression of cytokeratins (CAM5.2) in SpCC. (x200). (G) Immunohistochemical staining; Expression of vimentin in SpCC. (x200). (H) Immunohistochemical staining; Expression of αSMA in SpCC. (x200). (I) Immunohistochemical staining; Expression of S100 in SpCC. (x200). (J) Immunohistochemical staining; Expression of HMB45 in SpCC. (x200).

Figure 2. Cadhein switching in SpCC. (A) Morphology of SpCC cells. H & E staining of SpCC-1 and SpCC-2 cells was shown. (B) N-cadherin, E-cadherin and Vimentin mRNA expression was examined by RT-PCR in SpCC cells. GAPDH expression was used as a positive control. (C) N-cadherin expression was examined by immunofluoresence analysis in SpCC-1 cells. After fixing with paraformaldehyde, SpCC-1 cells were stained with anti-N-cadherin antibody (green). Nuclei of the cells were stained by DAPI (blue).

Figure 3. Immunohistochemical expression of N-cadherin in SpCC cases. (A) Left panel shows lower magnification (x40) and right panel shows higher magnification (x100). Scale Bar; 50 μm and 250 μm. (B) N-cadherin expression in 15 SpCC cases. Graph shows the number of SPCC cases with low or high expression of N-cadherin. (C) Correlation between N-cadherin expression and lymph node metastasis (negative or positive). Graph shows the number of SpCC cases with or without lymph node metastasis.

Table 1. Clinical data of SpCC patients and immunohistochemical results

Case No.	Sex	Age	Site	Metastasis	E-cadherin	N-cadherin
1	Male	50	Mandible	Positive	Reduced	High
2	Male	83	Tongue	Negative	Reduced	Low
3	Female	76	Tongue	Positive	Reduced	High
4	Female	82	Buccal mucosa	Negative	Reduced	Low
5	Female	75	Palate	Negative	Reduced	Low
6	Female	84	Mandible	Positive	Reduced	High
7	Female	60	Tongue	Positive	Reduced	High
8	Male	86	Tongue	Negative	Reduced	High
9	Female	69	Mandible	Positive	Reduced	Low
10	Female	98	Buccal mucosa	Negative	Reduced	Low
11	Female	85	Buccal mucosa	Negative	Reduced	High
12	Male	73	Mandible	Negative	Reduced	High
13	Female	77	Tongue	Positive	Reduced	High
14	Female	85	Mandible	Positive	Reduced	High
15	Female	32	Maxilla	Negative	Reduced	High

Figure 1

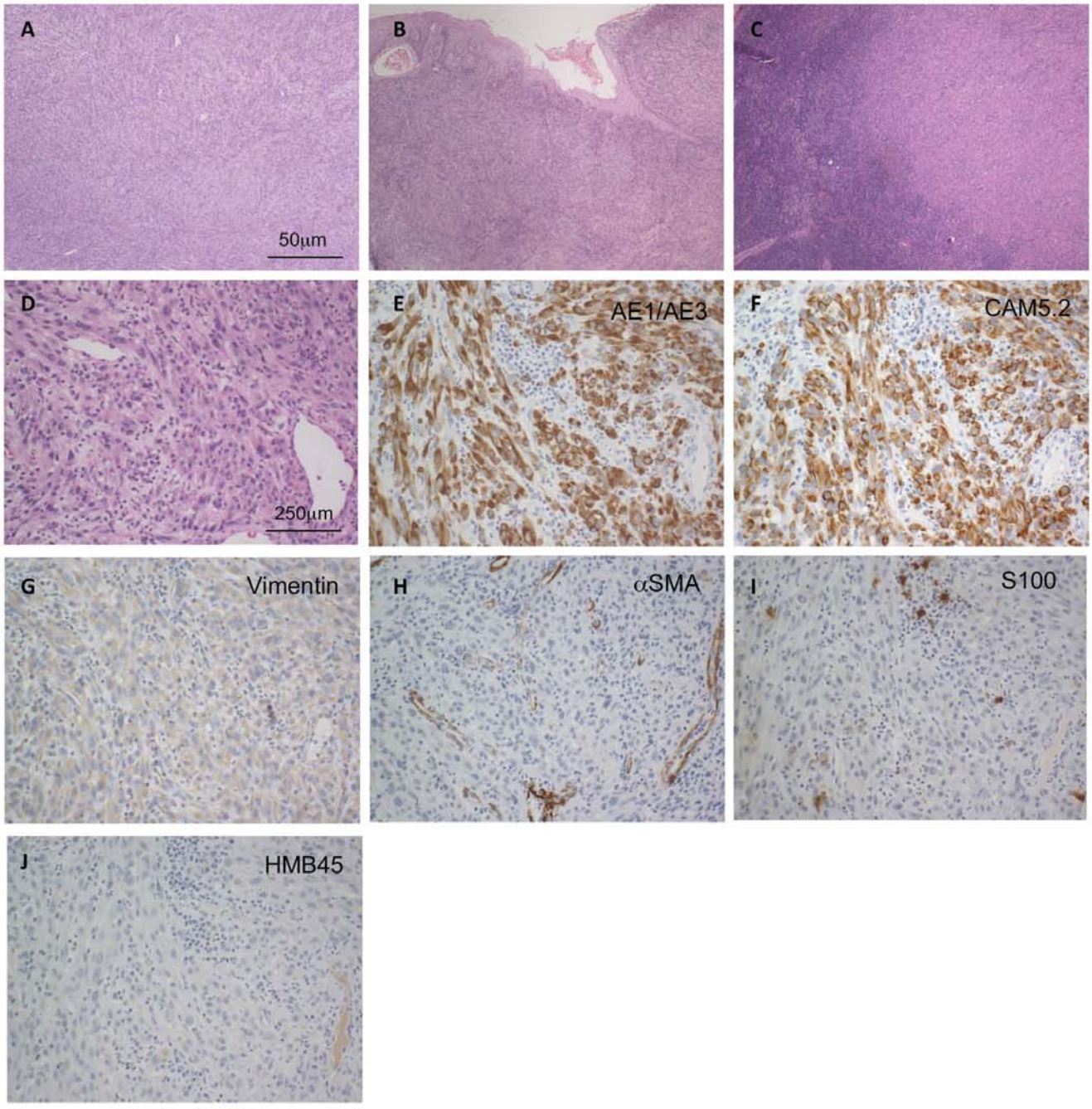

A
50μm
B
C
D
250μm
E
AE1/AE3
F
CAM5.2
G
Vimentin
H
αSMA
I
S100
J
HMB45

Figure 2

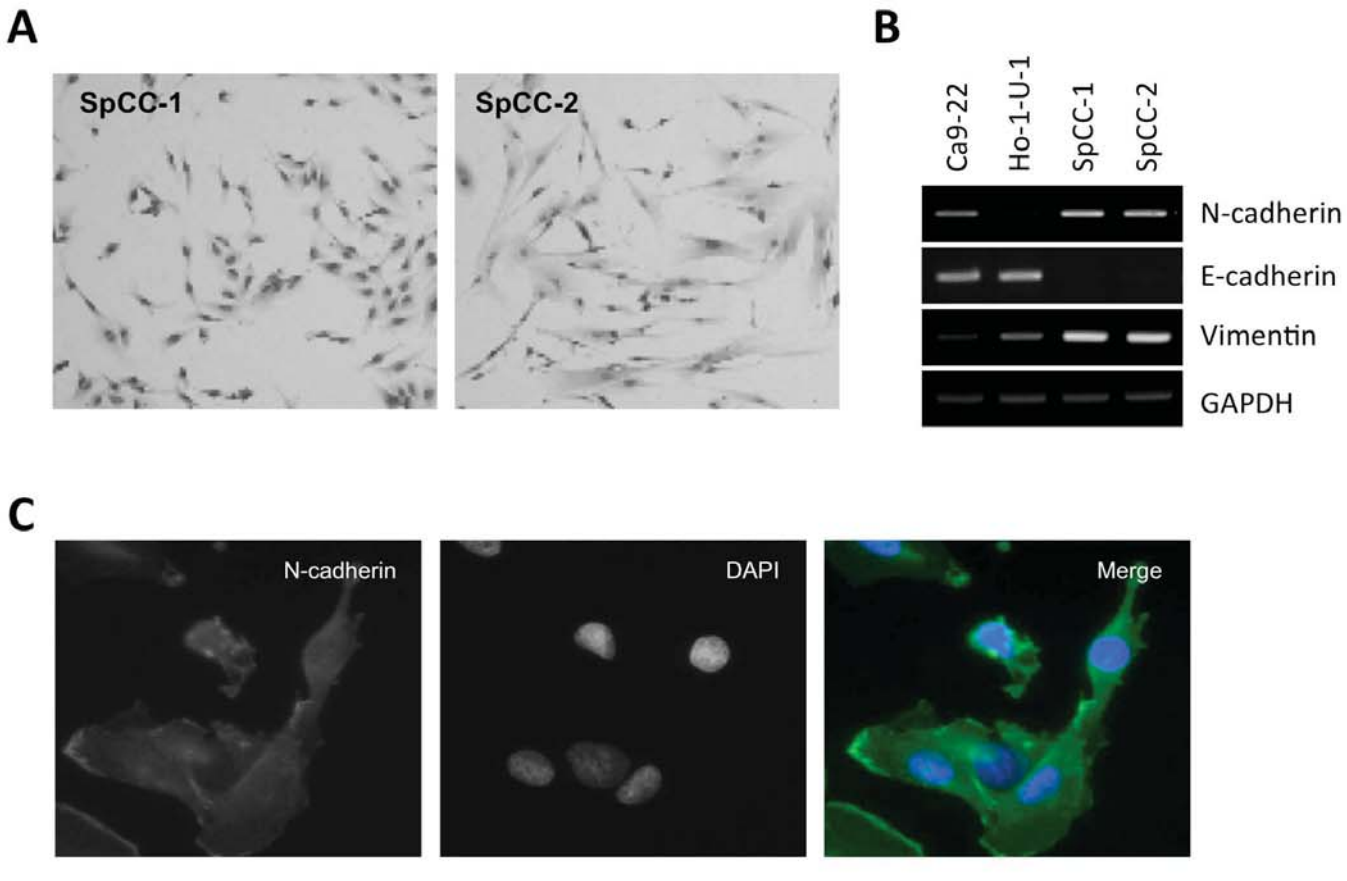
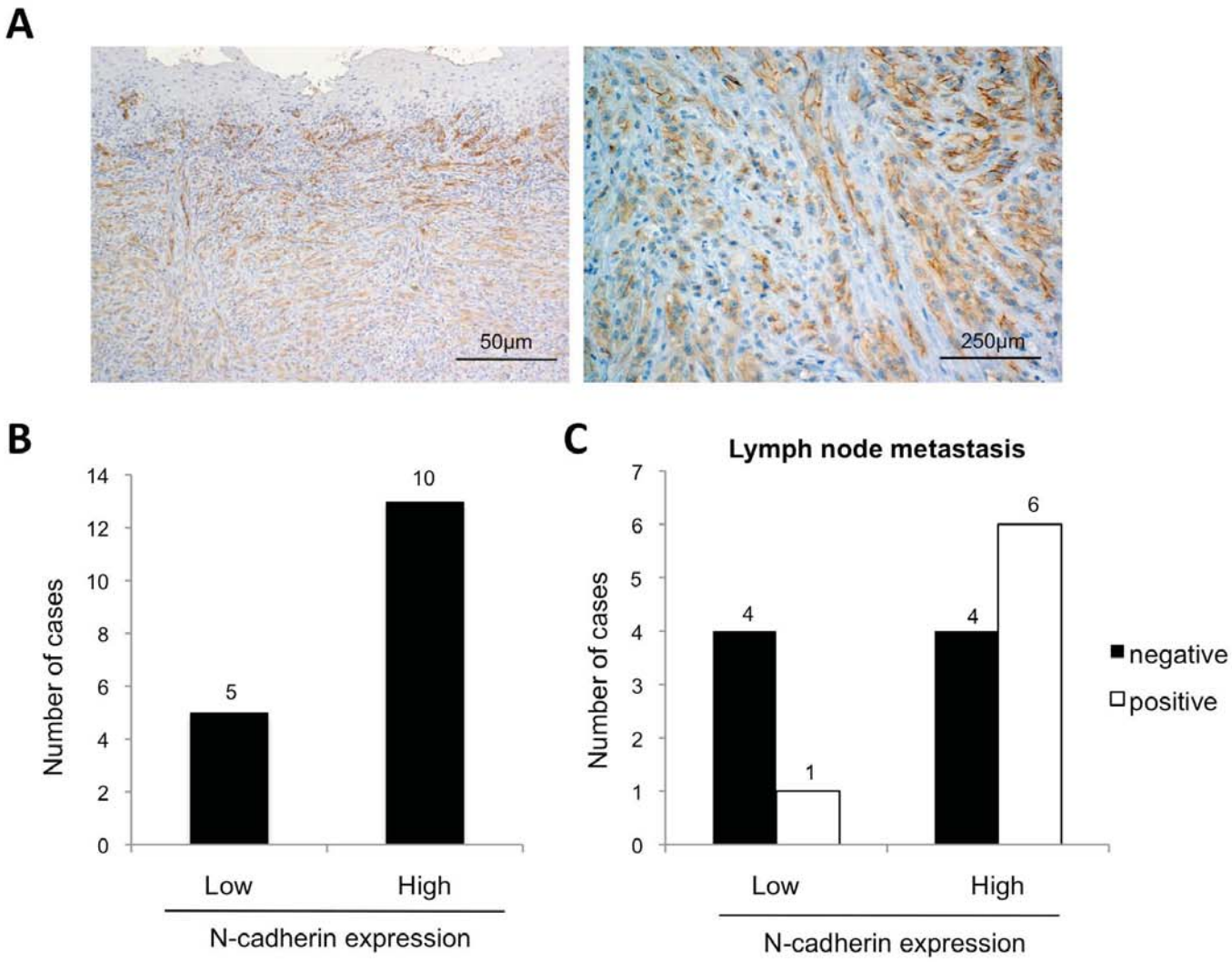

A
SpCC-1
SpCC-2
B
Ca9-22
Ho-1-U-1
SpCC-1
SpCC-2
N-cadherin
E-cadherin
Vimentin
GAPDH
C
N-cadherin
DAPI
Merge

Figure 3


A
50μm
250μm
B
Number of cases
14
12
10
8
6
4
2
0
5
10
Low
High
N-cadherin expression
C
Lymph node metastasis
Number of cases
7
6
5
4
3
2
1
0
4
1
4
6
Low
High
N-cadherin expression
negative
positive