

Characteristics of bladder transitional cell carcinoma with E-cadherin and N-cadherin double-negative expression

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Abstract. The aim of the present study was to examine the characteristics of bladder transitional cell carcinoma with E-cadherin and N-cadherin double-negative expression. An immunofluorescence assay was used to detect E-cadherin and N-cadherin expression in infiltrative bladder cancer tissues, and immunofluorescence and western blot analysis were used to detect E-cadherin and N-cadherin expression in human urinary bladder grade II carcinoma 5637, transitional cell carcinoma UMUC-3 and invasive bladder carcinoma EJ cells. Cell proliferation, migration, invasion and plate colony formation assays were used to detect the proliferative, migratory and invasive abilities and the efficiency of plate colony formation of 5637, UMUC3 and EJ cells. A tumor xenograft formation assay was used to evaluate the tumorigenic abilities of 5637, UMUC-3 and EJ cells *in vivo*. E-cadherin and N-cadherin double-negative expression was identified in various pathological grades of infiltrative bladder cancers. E-cadherin positive and N-cadherin negative expression was exhibited by 5637 cells. By contrast, E-cadherin negative and N-cadherin positive expression was exhibited by EJ cells, and E-cadherin and N-cadherin double-negative expression was exhibited by UMUC-3 cells. The ability of cells to proliferate, migrate, invade, and the efficiency of plate colony formation and tumorigenic abilities of the cells were significantly different among 5637, UMUC-3 and EJ cells. These cell characteristics were significantly increased in UMUC-3 cells compared with 5637 cells; however, the characteristics were significantly decreased compared with EJ cells. The biological characteristics of bladder cancer cells with E-cadherin and N-cadherin

double-negative expression was between bladder cancer cells that exhibited a E-cadherin positive and N-cadherin negative expression, and bladder cancer cells that exhibited E-cadherin negative and N-cadherin positive expression. The present study deduces that the status of E-cadherin and N-cadherin double-negative expression may participate in the process of epithelial-mesenchymal transition in the pathogenesis of bladder urothelial carcinoma.

Introduction

In the USA and other regions of the world, bladder cancer is one of the most common forms of urologic cancers (1). Furthermore, bladder cancer is the primary cause of death among urinary tumors in China (2). An individual afflicted with non-muscle-invasive bladder cancer is susceptible to a high recurrence rate, due to the aggressive nature of this type of cancer, and may rapidly progress to muscle-invasive disease (3). The prognosis of individuals with muscle-invasive bladder cancer is extremely poor, due to the high rate of metastasis (4).

It is well known that the most common type of bladder cancer is bladder urothelial carcinoma. There is a great deal of evidence that supports the theory that epithelial-mesenchymal transition (EMT) is pivotal in tumor invasion and metastasis, since it provides cells with a more motile and invasive phenotype (5-7). The primary characteristics of EMT is the disappearance of epithelial cell polarity, acquisition of mesenchymal cell properties (8) and abnormal E-cadherin and N-cadherin expression (9). The EMT process is extremely important for progression of the tumor. E-cadherin and N-cadherin are considered biomarkers of EMT (10). In tumor development, E-cadherin and N-cadherin functions are varied. However, the exact process of EMT remains unclear, and the function of E-cadherin and N-cadherin double-negative expression is rarely studied. In order to gather additional knowledge regarding the process of EMT, the present study investigated E-cadherin and N-cadherin double-negative expression in various pathological grades of infiltrative bladder urothelial carcinoma tissues, and examined the biological characteristics of these cells and deduced the association of E-cadherin and N-cadherin expression with EMT.

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Materials and methods

Tissue samples and cell culture. Human bladder cancer tissues were obtained from patients at Nanfang Hospital, which is affiliated to the Southern Medical University (Guangzhou, China). The Bioethics Committee of Nanfang Hospital approved the present study. All participants were informed about the purpose of the study and provided their written consent. Three specimens were collected (male; aged 65, 83 and 88 years), which were pathologically diagnosed biopsy specimens of low-, mid- and high-level infiltrative bladder urothelial carcinoma. Human urinary bladder grade II carcinoma 5637, transitional cell carcinoma UMUC-3 and invasive bladder carcinoma EJ cells were obtained from Guangzhou Jennio Biological Technology Co., Ltd., (Guangzhou, China), and the cells were preserved in the laboratory. The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) that contained 10% fetal bovine serum (FBS; HyClone™, GE Healthcare, Logan, UT, USA) at 37°C in a 5% CO₂ humidified incubator.

Immunofluorescence of tissue samples. Tissue samples were cut into 10 μm thick sections by freeze-sectioning. The sections of tissue samples were placed onto 12x12 mm glass slides. The 10 μm-thick sections were fixed with fixing liquid (70% acetone and 30% anhydrous methanol; 4°C precooling) for 10 min, washed 3 times with phosphate-buffered saline (PBS; 1 wash/5 min), blocked in PBS with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min, washed again with PBS, then incubated at 4°C in a refrigerator overnight with rabbit polyclonal anti-E-cadherin (Cell Signaling Technology, Inc., Danvers, MA, USA; dilution, 1:100; catalog no. 3195p) and mouse monoclonal anti-N-cadherin antibodies (Abcam, Cambridge, MA, USA; dilution, 1:100; catalog no. ab98952). Following an additional wash with PBS, the sections were incubated with species-specific secondary antibodies (goat anti-mouse Dylight 488, catalog no. ZF-0512; and goat anti-rabbit Dylight 594, catalog no. ZF-0516; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China; dilution, 1:100) at 37°C for 90 min and washed with PBS. Subsequently, the sections were incubated with fluorescein isothiocyanate (FITC)-phalloidin (catalog no. P5282; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min in the dark and washed with PBS again, and the slides were then stained with 4',6-diamidino-2-phenylindole (DAPI; Zhongshan Golden Bridge Biotechnology Co., Ltd.) for nuclear staining. Images were captured and analyzed with a microscope (Olympus Corporation, Tokyo, Japan).

Immunofluorescence of bladder cancer cells. Human bladder cancer cells (1x10⁵) were cultured in a confocal dish and subjected to immunofluorescence analysis at a 80-90% confluence of cells. The cells were washed 3 times with PBS (1 wash/5 min). Following fixing with 4% paraformaldehyde, permeabilization with 0.2% Triton X-100 and washing with PBS, the cells were blocked using 5% BSA in PBS for 30 min. Subsequently, the cells were washed with PBS, then incubated at 4°C overnight with anti-E-cadherin and anti-N-cadherin antibodies (dilution, 1:100). Following washing with PBS,

the cells were incubated with species-specific secondary antibodies (goat anti-mouse Dylight 488; goat anti-rabbit Dylight 594; dilution 1:100) at 37°C for 90 min. Following another wash with PBS, the cells were incubated with FITC-phalloidin at 37°C for 2 min in the dark. Subsequent to a final wash with PBS, the nuclei of the cells were stained with DAPI, and images were photographed and analyzed with an Olympus microscope.

Western blotting. Protein samples were extracted from bladder cancer cells with M-PER™ (Mammalian Protein Extraction Reagent, catalog no. 78501; Thermo Fisher Scientific Inc.), Equivalent quantities of proteins (50 μg) were separated with 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk in PBS with Tween 20 and incubated at 4°C overnight with the primary antibodies, rabbit polyclonal anti-E-cadherin and mouse monoclonal anti-N-cadherin antibody, and a mouse monoclonal anti-Tubulin antibody (Cell Signaling Technology, Inc.; catalog no. T6199; dilution, 1:1,000). The cells were then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (anti-rabbit or anti-mouse; catalog no. 7074 and 7076, respectively; dilution, 1:1,000; Cell Signaling Technology, Inc. Danvers, MA, USA) for 1 h at room temperature. Detection of the protein bands was performed by a FluorChem® FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA).

Cell proliferation assay. The bladder cancer 5637, UMUC-3, and EJ cells were seeded in a 96-well plate at a density of 600 cells/well, and incubated for 7 days. Following the incubation period, a cell proliferation assay was performed by adding 10 μl cell counting kit-8 solution (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at a set time each day over the 7 day period. Following incubation for 2 h with CCK-8, the absorbance values were detected by EnSpire® 2300 multi-label reader (PerkinElmer, Inc., Waltham, MA, USA) at 570 nm.

Migration abilities in vitro. In total, 200 μl serum-free RPMI-1640 media containing tumor cells (5x10⁴ cells/well) was added to the upper chamber of a Transwell chamber (Corning Incorporated, Corning, NY, USA), and 500 μl RPMI-1640 containing 10% FBS was added to the lower chamber as a chemo-attractant. Following incubation for 12 h (37°C; 5% CO₂), the non-migratory tumor cells were removed with cotton swabs. Subsequently, the migratory tumor cells that were located on the lower surface of membrane were fixed for 25 min using 4% paraformaldehyde. The cells were then stained with hematoxylin for 20 min. Following rinsing with PBS, the Transwell chambers were inspected via inverted microscopy in 5 random visual fields.

Invasion abilities in vitro. In total, 50 μl Matrigel (dilution, 1:5 with RPMI-1640) was added to a Transwell chamber. A total of 200 μl serum-free RPMI-1640 containing tumor cells (1x10⁵ cells/well) was added to the upper chamber, and 500 μl RPMI-1640 containing 10% FBS was added to the lower chamber as a chemotactic factor. Following incubation

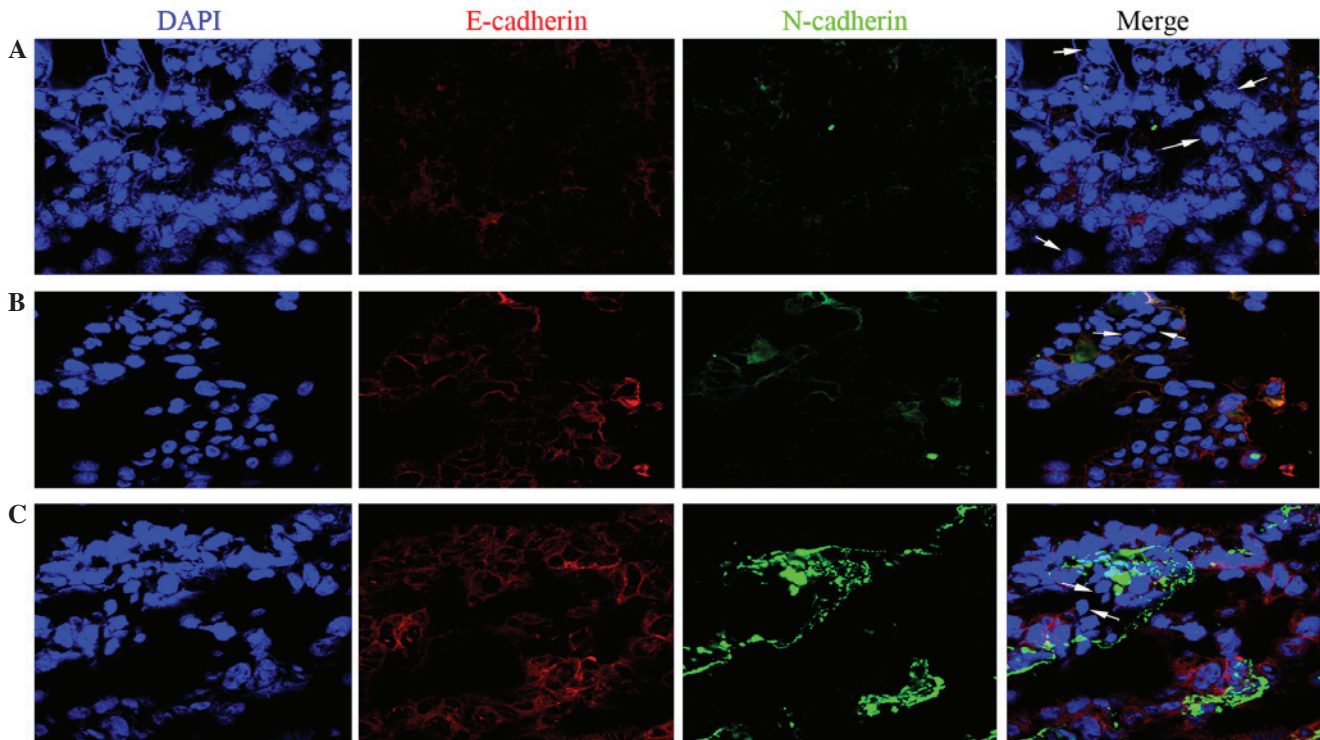


Figure 1. E-cadherin and N-cadherin expression in infiltrative bladder urothelial carcinoma tissues using immunofluorescence assay. (A) Low-, (B) mid- and (C) high-level infiltrative bladder urothelial carcinoma. E-cadherin is marked with red fluorescence; N-cadherin is marked with green fluorescence. E-cadherin and N-cadherin double-negative expression in infiltrative bladder urothelial carcinoma tissues is indicated by the arrows. Original magnification, x90. DAPI, 4',6'-diamidino-2-phenylindole.

for 36 h (37°C; 5% CO₂), the non-invading tumor cells were removed with cotton swabs. Subsequently, 4% paraformaldehyde was used to fix the invading tumor cells that were located in the lower surface of membrane for 25 min. The cells were then stained with hematoxylin for 20 min. Following rinsing with PBS, the Transwell chambers were inspected via inverted microscopy in 5 random visual fields.

Plate colony formation test. In total, ~2x10² cells/well were added to 6-well culture plates. Following incubation for 8 days (37°C; 5% CO₂), 4% paraformaldehyde was used to fix the tumor cells for 25 min. The cells were washed 3 times with PBS and stained with hematoxylin for 25 min, and rinsed again with PBS. The number of colonies (≥50 cells) were counted under a microscope, and the following equation was used: Colony formation efficiency = (number of colonies / number of inoculating cells) x 100%.

Tumorigenicity in vivo assay. The Ethics Committee of the Southern Medical University approved experimental procedures with animals in the present study (contract no., 2011016). Female, 5-week-old, immune deficient mice (n=9) were maintained at the Center of Experimental Animals, Southern Medical University. All mice were maintained at room temperature under specific pathogen-free conditions and exposed to 12 h light/dark cycles. A total of 3 animals (per group) were kept in each cage with *ad libitum* access to water and food.

The left and right flanks of the immune deficient mice received subcutaneous injections with tumor cells (100 μl; 2x10⁶ cells; n=3 per group). The tumor size was measured with

a ruler every three days between day 6 and day 24 following the injection. Four weeks later, the subcutaneous tumors were resected and the mice were sacrificed. The subcutaneous tumor was cut into 10-μm-thick sections using freeze-sectioning and observed by hematoxylin and eosin (H&E) staining. The tumor volume was calculated with the following formula: Tumor volume = d² x D / 2, where d is the shortest diameter and D is the longest diameter (11).

Statistical analysis. SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data was expressed as the mean ± standard deviation. Statistical analysis was performed with Student's t-test between two groups, or one-way analysis of variance for more than three groups. P<0.05 was considered to indicate a statistically significant difference.

Results

E-cadherin and N-cadherin double-negative expression in bladder urothelial carcinoma tissues. Immunofluorescence analysis of tissue samples revealed that E-cadherin and N-cadherin double-negative expression was detected in low-, mid- and high-level infiltrative bladder urothelial carcinoma (Fig. 1). Therefore, the assay demonstrated that E-cadherin and N-cadherin double-negative expression was present in infiltrative bladder urothelial carcinoma.

E-cadherin and N-cadherin expression in human bladder cancer 5637, UMUC-3 and EJ cells. Western blotting revealed that E-cadherin and N-cadherin double-negative expression

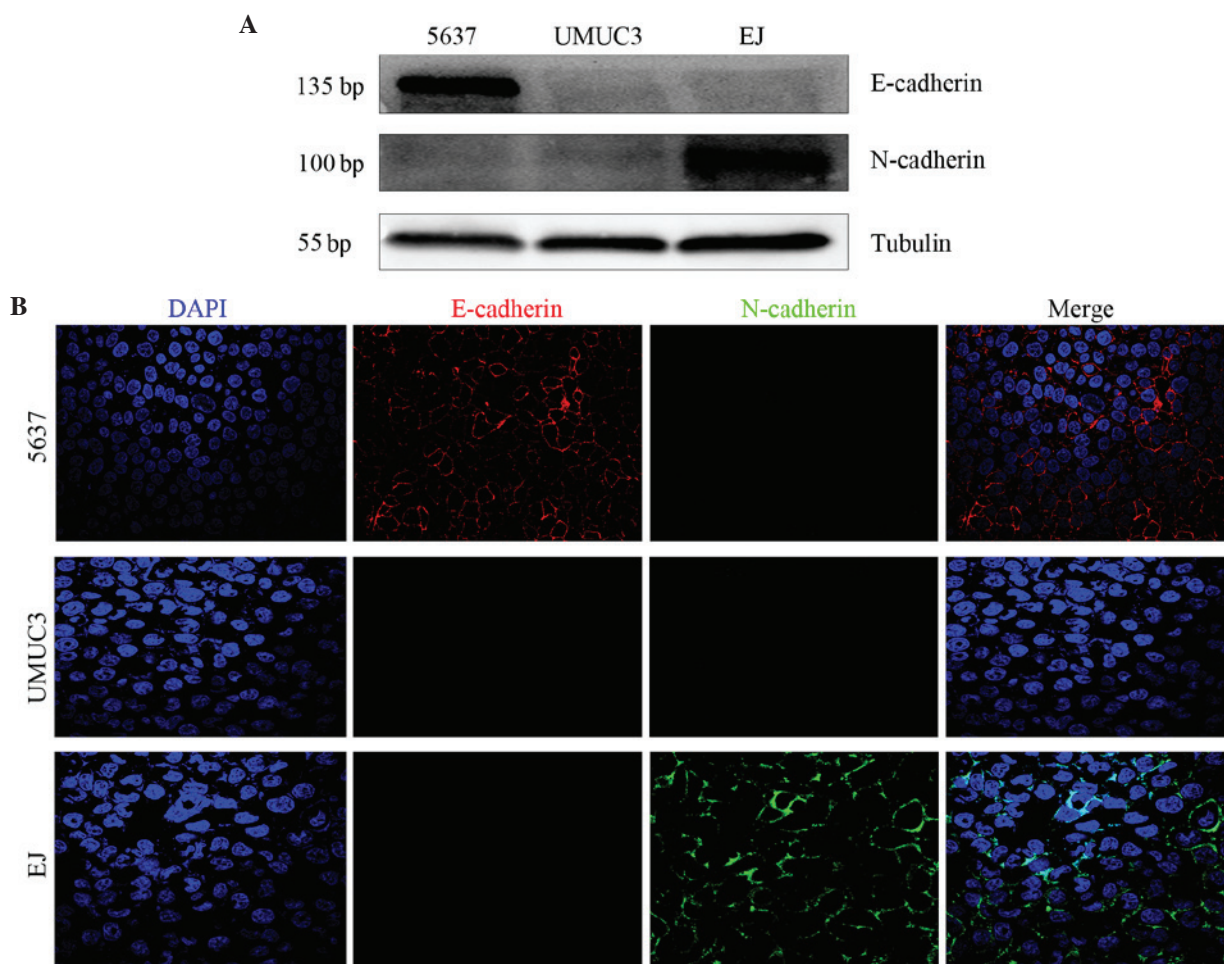


Figure 2. E-cadherin and N-cadherin expression was detected and confirmed by (A) western blotting and (B) immunofluorescence analysis in human bladder cancer 5637, UMUC-3 and EJ cells. E-cadherin is marked with red fluorescence; N-cadherin is marked with green fluorescence. The results revealed that there is E-cadherin and N-cadherin double-negative expression in UMUC-3 cells, E-cadherin positive and N-cadherin negative expression in 5637 cells, and E-cadherin negative and N-cadherin positive expression in EJ cells (original magnification, x60).

was present in UMUC-3 cells. However, E-cadherin positive and N-cadherin negative expression was identified in 5637 cells, while E-cadherin negative and N-cadherin positive expression was identified in EJ cells (Fig. 2A). Immunofluorescence analysis of the bladder cancer cells demonstrated the same result as the western blot analysis (Fig. 2B). Therefore, the two assays revealed that E-cadherin and N-cadherin double-negative expression was detected only in UMUC-3 cells.

Functional comparison of bladder cancer 5637, UMUC-3 and EJ cells. A cell proliferation assay revealed that the ability of UMUC-3 cells to proliferate was significantly increased compared with 5637 cells on day 3, 4, 5, 6 and 7 using a CCK-8 assay ($P<0.001$); however, the cell proliferative abilities of the UMUC-3 cells was significantly weaker compared with the EJ cells ($P=0.004$; Fig. 3A). The plate colony formation assay revealed that UMUC-3 cells formed larger and more numerous colonies compared with the 5637 cells ($P<0.001$). However, in comparison to EJ cells, there was a significant decrease in the quantity of colonies of UMUC-3 cells ($P<0.001$; Fig. 3B). The results of the two assays revealed that the proliferative abilities of UMUC-3 cells was decreased compared with EJ cells and increased compared with 5637 cells.

Using the same number of cells and the same incubation conditions, UMUC-3 cells exhibited a significant increase in motility and invasion abilities compared with 5637 cells (both $P<0.001$). However, in comparison to EJ cells, the motility and invasion abilities of the UMUC-3 cells exhibited a significant decrease (both $P<0.001$; Fig. 3C and D). Therefore, the migration and Matrigel invasion assays revealed that the transmembrane activity of UMUC-3 cells was decreased compared with EJ cells and increased compared with 5637 cells.

Under the same conditions, an injection of UMUC-3 cells led to the development of increased tumor volumes compared with 5637 cells in immune deficient mice ($P<0.001$). Compared with an injection of EJ cells in immune deficient mice, the subcutaneous tumor volume of UMUC-3 cells was significantly smaller ($P=0.017$; Fig. 3E). The change in tumor volume of the 5637 cells group change was not significant with time elapsed ($P=0.138$), however, the tumor volumes of the UMUC-3 and EJ cell groups significantly increased with time elapsed (both $P<0.001$; Fig. 3E). The morphology of subcutaneous tumor sections were stained with H&E and observed under a microscope; the tumor cells showed diffuse patchy distribution, with a clearly abnormal shape, deeply stained nuclei and common mitotic figures (Fig. 3E). Consequently,

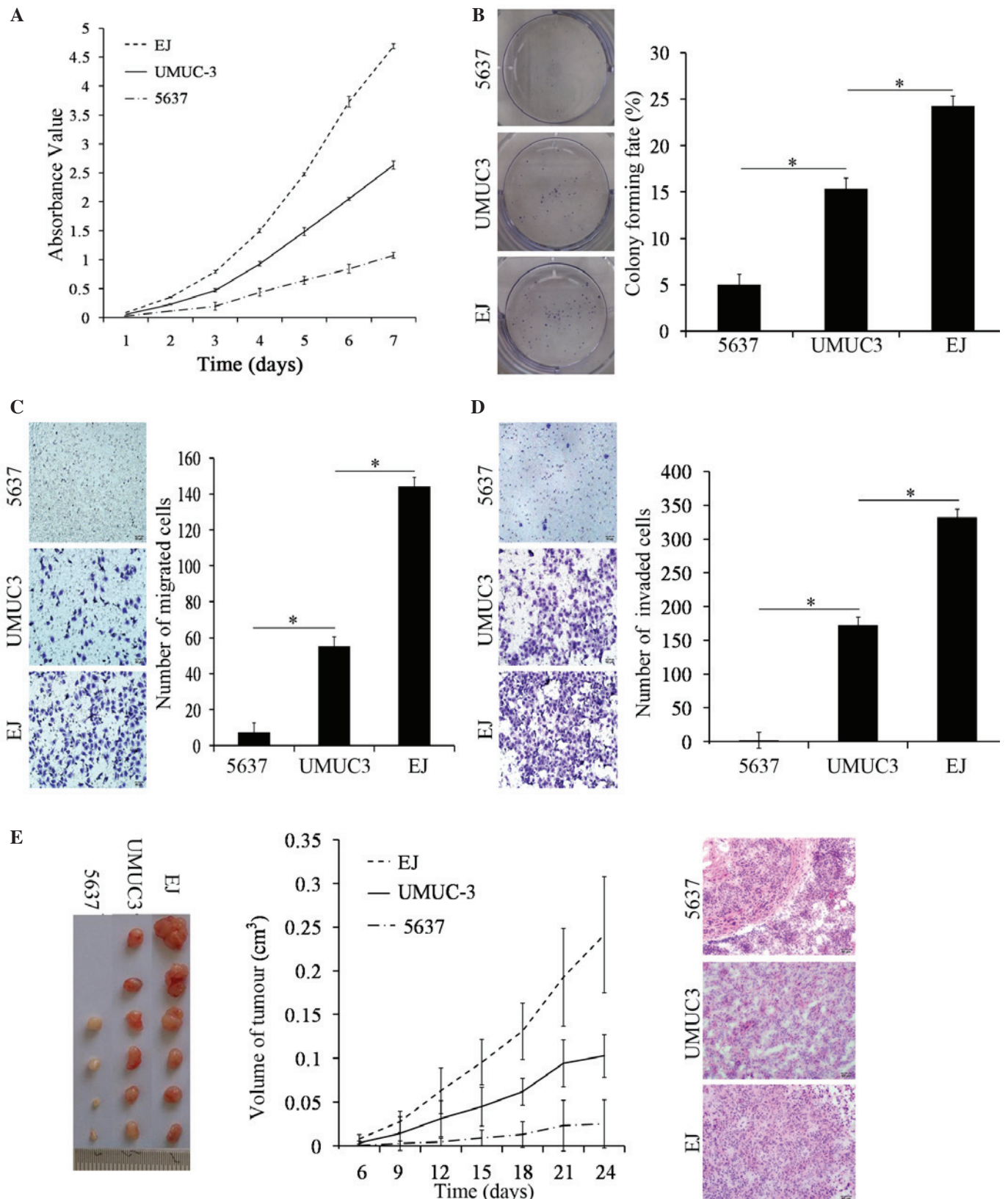


Figure 3. Functional characteristic comparison among human bladder carcinoma 5637, UMUC-3 and EJ cells. (A and B) Comparison of proliferative abilities. (A) The cell proliferation growth curve using cell counting kit-8 revealed that the UMUC-3 cells exhibited a higher absorbance value compared with 5637 cells, but a lower absorbance value compared with EJ cells. (B) Light microscopy images of the colony formation assay demonstrated that UMUC-3 cells formed larger and more numerous colonies compared with 5637 cells, but smaller colonies compared with EJ cells. (C) Comparison of migration abilities. Light microscopy images of the Transwell migration assay revealed that the number of migrated UMUC-3 cells was significantly increased compared with 5637 cells and decreased compared with EJ cells. (D) Comparison of invasion abilities. Light images of the Transwell invasion assay revealed that the number of invading UMUC-3 cells was significantly increased compared with 5637 cells and decreased compared with EJ cells. (E) Comparison of tumorigenic abilities. Light images of subcutaneous tumor xenograft formed in immune deficient mice. The comparison of tumor growth volume revealed that mice injected with UMUC-3 cells developed larger tumors compared with mice injected with 5637 cells, but smaller tumors than mice injected with EJ cells. Microphotographs of hematoxylin and eosin stained tumor tissue sections. * $P < 0.001$.

the animal assays revealed that the *in vivo* tumorigenic abilities of the UMUC-3 cells were decreased compared with the EJ cells and increased compared with the 5637 cells.

Discussion

Greenberg and Hay (12) proposed the theory of EMT in 1982; it was revealed that cultured lens epithelial cells morphologically converted into mesenchyme-like cells with pseudopodia in the gel. EMT is a multistep process. The primary characteristics of EMT are the disappearance of epithelial cell polarity and acquisition of mesenchymal cell properties (8). The important barrier functions of epithelial cells are promoted by their tight cell-cell junctions (13), and the disappearance of cell-cell junctions may cause morphological alterations and increase the invasion and metastatic abilities of epithelial cells. The primary mediator of cell-cell junction is cadherin, a transmembrane glycoprotein, that is observed in epithelial tissue. Cadherin is a calcium-dependent adhesion protein, which promotes tight cell-cell junction molecules that promote the formation and growth of malignant tumors (14). The primary cadherins identified are E-cadherin, P-cadherin and N-cadherin (15). E-cadherin not only adjusts the connections of nearby epithelial cells, but also maintains cell phenotype and cell polarity. Therefore, E-cadherin is an extremely key role in epithelial cell-cell junctions (16). E-cadherin is also crucial for tumor suppression as it prevents tumor cell invasion (17). Several studies have revealed that the loss of E-cadherin function may induce EMT and increase tumor invasion and metastasis (18-21).

N-cadherin was first identified in muscle and neural cells; however, a recent study revealed that N-cadherin was also identified in mesenchymal cells (22). N-cadherin is located at the adherens junction, where it promotes dynamic contact between the matrix and cells, and between the cells themselves (23). The cytoplasmic expression of N-cadherin participates in multiple intracellular signaling pathways (24). Much study has indicated that N-cadherin may increase tumor cell motility and promote tumor cell metastasis and invasion in numerous experimental models (25). In addition, N-cadherin has been used as a biomarker of mesenchymal differentiation in the study of EMT (26).

N-cadherin and E-cadherin are typical cadherins, and are biomarkers of EMT (10). The prominent characteristic of EMT is E-cadherin decrease and N-cadherin increase, also referred to as cadherin switching. In the majority of tumor cells, cadherin switching plays an extremely crucial role (27). Cadherin switching has been observed to be an extremely crucial process in bladder cancer development (28), and cadherin switching promotes tumor invasion and metastasis in bladder cancer development (29,30). Much study has demonstrated the potential effect of EMT in bladder cancer development (5,6,31). Although there are numerous studies concerning EMT, the study of E-cadherin and N-cadherin double-negative expression has rarely been examined, and the exact process of EMT remains unclear.

The present study demonstrated that E-cadherin and N-cadherin double-negative expression is observed in non-muscle invasive bladder cancers using immunohistochemical staining (32). In addition, the present study detected

E-cadherin and N-cadherin double-negative expression in low-, mid- and high-level infiltrative bladder urothelial carcinoma using immunofluorescence assays. These results suggest that E-cadherin and N-cadherin double-negative expression exists in bladder urothelial carcinoma. It is known that the prominent characteristic of EMT is E-cadherin decrease and N-cadherin increase. Therefore, in order to study the characteristics of bladder cancer cells with E-cadherin and N-cadherin double-negative expression and deduce its association with EMT, bladder cancer cells of E-cadherin positive and N-cadherin negative expression, and E-cadherin negative and N-cadherin positive expression were selected as controls. Western blotting and immunofluorescence assays revealed that E-cadherin and N-cadherin double-negative expression were identified in the bladder cancer UMUC-3 cell line. E-cadherin positive and N-cadherin negative expression were identified in the bladder cancer 5637 cell line, and E-cadherin negative and N-cadherin positive expression were identified in the bladder cancer EJ cell line. By comparing the functions among these three cell lines, the present study demonstrated that the biological characteristics of UMUC-3 cells were significantly stronger compared with 5637 cells, and significantly weaker compared with EJ cells.

In conclusion, the present study revealed that E-cadherin and N-cadherin double-negative expression exists in bladder urothelial carcinoma. Analysis of the biological characteristics of bladder cancer cells with E-cadherin and N-cadherin double-negative expression were performed by comparing these type of cells with bladder cancer cells of E-cadherin positive and N-cadherin negative expression, and E-cadherin negative and N-cadherin positive expression. The present results suggest that the biological characteristics of bladder cancer cells with E-cadherin and N-cadherin double-negative expression were significantly stronger compared with bladder cancer cells of E-cadherin positive and N-cadherin negative expression. However, the biological characteristics of double-negative cells were significantly weaker compared with bladder cancer cells that exhibited E-cadherin negative and N-cadherin positive expression. Therefore, the biological characteristics of bladder cancer cells with E-cadherin and N-cadherin double-negative expression existed between bladder cancer cells of E-cadherin positive and N-cadherin negative expression and bladder cancer cells of E-cadherin negative and N-cadherin positive expression. Overall, the present study deduces that the status of E-cadherin and N-cadherin double-negative expression may participate in the process of EMT in the pathogenesis of bladder urothelial carcinoma. The present study may aid in the understanding of the process and effect of EMT in the pathogenesis of bladder cancer.

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