

CAB39 mediates epithelial-mesenchymal transition via activation of NF- κ B signaling to facilitate bladder cancer invasion and metastasis

Haichao Chao

The second Affiliated hospital of Nanchang University

Lifen Peng

Jiangxi Provincial People's Hospital Affiliated to Nanchang University

Leihong Deng

The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Zhaojun Yu

Nanchang University - Qianhu Campus: Nanchang University

Huanhuan Deng

Nanchang University

Fanghua Xu

Jiangxi Pingxiang People's Hospital

Xiangda Xu

The second affiliated hospital of Nanchang University

Jianbiao Huang

Nanchang University

Tao Zeng (✉ 491641369@qq.com)

The Second Affiliated Hospital of NanChang University

Research

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Abstract

Background Bladder cancer (BC) is the most common urinary cancer among men with a high mortality rate despite of constant advancement in medical and therapeutic treatment. Recent evidence demonstrated that CAB39 plays a critical role in BC pathogenesis by exhibiting various biological activities, but the underlying molecular mechanisms remain unclear. The aim of this research was to define the expression patterns of CAB39 in normal and tumor tissues and explore its biological function in epithelia-mesenchymal transition (EMT) in human BC. Methods Immunohistochemistry and Quantitative RT-PCR analyses were used respectively to examine the expression of CAB39 in BC tissues and cell lines with different metastatic potentials. In addition, the clinical significance of CAB39 expression was also evaluated. Wound-healing assay, cell invasion assay, and CCK8 proliferation assay in cell lines in which CAB39 was knocked down by shRNA, as well as xenograft tumor models in nude mice, were performed to assess the effect of CAB39 reduction on invasion, migration, and proliferation of BC cells. The GSEA database was used to analyze panel of genes enriched as a result of elevated CAB39 expression in BC cells, and the results were validated by western blot analysis. Results The level of CAB39 protein was up-regulated in invasive but not in noninvasive bladder cancer tissues. Elevated expression of CAB39 was inversely correlated with prognosis of the malignant disease. Additionally, CAB39 was differentially expressed in T24, 5637, and J82 bladder cancer cell lines with highest expression in T24, the most invasive cell line among the three. However, shRNA-mediated attenuation of endogenous CAB39 in T24 and 5637 cell lines reversed such invasive and metastatic effects as demonstrated by the inhibition of tumorigenesis in nude mice xenografts. Furthermore, we demonstrated that CAB39 could mediate EMT through upregulation of N-cadherin and downregulation of E-cadherin in BC via NF- κ B signaling pathway. Conclusions Our study reveals a previously unknown mechanism of CAB39-mediated EMT in promoting invasion and metastasis of BC and provides a rationale for future investigation of CAB39 as a potential target for the development of novel therapeutic agents to fight the malignancy.

Background

Bladder cancer (BC) is the 4th most frequently occurring malignant cancers and the 9th most common cause of death worldwide[1]. About 80,000 BC patients were diagnosed in the United States with an incidence rate in men ranked second behind only prostate cancer in 2017[2]. The invasive and metastatic form of the cancer is the main cause of death or unfavorable prognosis for BC patients, but its exact underpinning molecular mechanism remains largely unknown. Hence the overall 5-year survival rate did not significantly improve in the past thirty years even though numerous therapeutic approaches have been developed for its clinical treatment[3]. Therefore, it is imperative to continuously seek novel therapeutic targets for more effective interventions for BC.

Epithelial-mesenchymal transition (EMT) is a cellular program that plays crucial roles in various physiological processes such as embryogenesis, tissue morphogenesis, and wound healing in addition to pathological processes such as tumor migration, invasion, and metastasis[4]. Neoplastic progression including invasion and metastasis relies on EMT activation to confer malignant traits associated with

cancer stem cells[5, 6]. Several protein markers, such as cell surface epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), vimentin, and fibronectin, can be used to distinguish between epithelial and mesenchymal states[7]. As epithelial cells undertake morphological changes from polygonal shape to spindle-like structure associated with mesenchymal state, they exhibit decreased expression of E-cadherin and increased expression of N-cadherin and vimentin. EMT is regulated by a unique set of transcription factors including snail homolog 1(SNAIL1), twist basic helix-loop-helix transcription factor 1(TWIST1), and zinc-finger-E-box-binding homeobox 1(ZEB1)[6]. In BC, E-cadherin is negatively correlated with tumor grade and stage in invasive bladder carcinomas[8]. Recently, it has been reported that SNAIL-induced EMT promotes metastasis in a xenograft model of BC while transcriptional silencing of TWIST1 inhibits EMT and invasiveness of the tumor cells[9, 10]. Goulet et al observed that paracrine IL-6 from fibroblasts associated with non-invasive cancer could induce EMT and promote aggressive BC[11]. In addition, aberrant signaling in NF- κ B pathway has been implicated in promoting EMT in BC[12]. Despite a burgeoning body of evidence to imply important roles of EMT in BC invasion and metastasis, the mechanism underlying EMT process in this particular malignant disease still remains poorly defined.

Calcium binding protein 39 (CAB39) was originally thought to be a highly conserved protein with calcium binding motif that expressed during the early stage of mouse embryogenesis[13]. It is a scaffold protein partnering with STE20-related adaptor (STRAD) and LKB1, a tumor suppressor protein kinase, to stabilize the interaction between STRAD and LKB1 and to establish an active heterotrimeric complex [14, 15]. LKB1 complex is a major actor of the AMPK/mTOR pathway connecting cellular metabolism, cell growth and tumorigenesis[16]. LKB1 contributes to cell cycle regulation and directly involves in cell divisions[17-19]. In addition, the complex contributes to apico-basal cell polarity, and LKB1 activity ensures that the epithelial state maintained[20]. With more and more research advance in recent years, CAB39 has been identified as an activator of various STE20 kinases, affecting the evolution of a plethora of diseases including cancers. For instance, Jiang et al. identified CAB39 as an oncogene by promoting the growth and metastasis of hepatocellular carcinoma through ERK pathways[21]. Godlewski et al. reported that miR-451 can modulate the adaptation to metabolic stress in glioma through regulating CAB39[22]. In addition, miR-451 has also been found to target and regulate CAB39 both at mRNA and protein levels in colorectal cancer, pancreatic cancer, and lung cancer[23-25]. Although emerging evidence regarding the role of CAB39 has been demonstrated in some cancers, its role in the development and progression of BC remains elusive.

In this study, we investigated the expression of CAB39 in BC cell lines and tissues and its correlation with common clinicopathological features. We explored the effect of shRNA-mediated reduction of CAB39 protein in BC cell lines on migration, invasion, proliferation, as well as tumorigenesis in a mouse xenograft model. We further analyzed expression levels of typical EMT markers including E-cadherin and N-cadherin in addition to activated NF- κ B signaling as a potential mechanism underlying the functional roles of CAB39 in BC. We propose that CAB39 is a novel potential therapeutic target for highly aggressive form of human BC.

Materials And Methods

Specimens and cell lines

In total, 89 BC tissue specimens and 10 adjacent normal paired tissues were obtained from individuals who had undergone a radical cystectomy at the Second Affiliated Hospital of Nanchang University from January 2014 to December 2019. No patients had received chemotherapy or radiotherapy before surgery. Written informed consent was obtained from all patients according to ethical standards, and the study was approved by the Ethics Committee and Institutional Review Board of the Second Affiliated Hospital of Nanchang University. The histopathology of these tissue samples was determined and confirmed by two pathologists according to the criteria of the World Health Organization and the Nevin staging system.

The bladder cancer lines T24 and 5637 cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS; Gibco). J82 cells were maintained in minimum essential medium (MEM) (Gibco) with 10% FCS. All cells were maintained at 37 °C in a 5% CO₂ incubator.

Immunohistochemistry (IHC)

The expression of CAB39 was examined by IHC assay on paraffin-embedded tissue sections. Anti-CAB39(ab51132, 1:100 dilution), anti-E-cadherin(ab1614, 5µg/ml dilution), and anti-N-cadherin(ab207608, 1:500 dilution) were purchased from Abcam (Cambridge, MA, USA). Tissue sections were prepared and stained as previously described [26]. Then, images were taken with a tissue chip scanner and analyzed using paired software. Histochemistry score (H-score) based on the percentage of positive cells and degree of staining was computed to quantify CAB39 expression as previously described by Yeo et al.[27]. Patients were classified into two groups (low and high expression) based on the overall scores.

RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA samples from cultured cells and tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) The forward and reverse primer sequences for CAB39 were 5'GCATTTGCCACATTCAAGGATT3' and 5'GCTGCGTCTTGTTAGGATTGG3', respectively. Quantitative real-time PCR (qRT-PCR) was performed on a BioRad CFX96 real-time PCR machine (BioRad, Hercules, CA, USA) as described previously[26]. Glyceraldehyde-3 phosphate dehydrogenase(GAPDH) was used as the housekeeping gene for internal quantitative control. The concrete setups were performed as we described previously.

Western blot analysis

Western blot was performed as previously described[26] e using Anti-CAB39(ab51132, Abcam, Cambridge, MA, USA, 1:2000 dilution), Anti-E-cadherin(ab1614, Abcam, Cambridge, MA, USA, 1:1000 dilution), Anti-N-cadherin(ab207608, Abcam, Cambridge, MA, USA, 1:1000 dilution) and Anti- NF-κB (ab86299, Abcam, Cambridge, MA, USA, 1:4000 dilution). Anti-β-actin (Servicebio, Wuhan, China, Cat.

GB12001) was hired as an endogenous control. The density value of each sample was normalized to its β -actin density value to get its relative quantity value.

Lentivirus-mediated RNA interference (RNAi)

Target cells were cultured with a good growth status, and experimental conditions were designed according to experimental results of lentiviral infection. T24 and 5637 cells were infected with a lentivirus carrying a CAB39-knockdown (KD) plasmid (CAB39-KD-1 or CAB39-KD-2) or a control plasmid (GV248, Shanghai Genechem, Shanghai, China) at the good growth status. To silence CAB39 two target sequences :5'TCACACAATTGGTGTGAA'3 which named CAB39-KD-1 and 5'GGAGCTCTTATGGTCTATG'3 which named CAB39-KD-2 were cloned. The target sequence for the control plasmid was 5'TTCTCCGAACGTGTCACGT'3. At 48 h after infection, the lentivirus carrying a copy of the green fluorescent protein (GFP) and the infection efficiency were assessed by fluorescence microscopy based on the numbers of GFP-expressing cells. CAB39 gene and protein expressions in infected cells were tested by the qRT-PCR and Western blotting.

Functional experiments

Wound healing assay, matrigel invasion assay and cell counting kit-8 (CCK-8) assay, which were used to analyze the invasion, migration, and proliferation of BC cells, were performed as previously reported [26].

Gene Set Enrichment Analysis (GSEA)

The datasets of *CAB39* mRNA expression in BC samples analyzed by microarray were obtained from TCGA-BC for Cancer Genomics. Additional ethical approval was not required for downloading these data, for TCGA was publicly available database. Subsequently, GSEA was performed using these downloaded datasets to find target genes up- or down-regulated by *CAB39* in BC[28]. It was considered statistically significant when statistical *P* value < 0.01 and false discovery rate (FDR) $q < 0.05$.

Animal experiments

Animal work was performed in accordance with a protocol approved by the Second Affiliated Hospital of Nanchang University Animal Care and Use Committee (Jiangxi, China). BALB/c female nude mouse at 6~7 weeks old were purchased from LingChang Bio-Technique (Shanghai, China) and subcutaneously injected with 2.5×10^6 of T24 cells with control or CAB39 shRNA (CAB39-KD-1) in a 50% MatrigelTM matrix (BD Co.). The tumor size was measured weekly in a blinded manner with calipers, and the tumor volume was calculated using the following formula: tumor volume = $(4/3) \times (L/2) (W/2)^2$, where L is the length and W the width. Results are presented as the mean \pm standard error (SE) for each experimental group.

Statistic analysis

Data were expressed as mean \pm standard deviation (SD), and all statistical analyses were performed using SPSS software version 20.0 (IBM SPSS Inc, Chicago, IL, USA). Differences of CAB39 expression between different groups were statistically analyzed using the t-test, while the chi-square test and Fisher's exact test were used to analyze the relationship between CAB39 expression and the clinicopathological characteristics, and Kaplan-Meier was used to plot the survival curves. Comparisons were performed by two-sided independent Student's test. Statistical significance was accepted when a *P* value is less than 0.05.

Results

Elevated expression of CAB39 in BC is correlated with clinicopathological characteristics and patients survival

Tian et al reported that CAB39 expression was elevated in glioma and seemed to correlate with staging of the disease[29]. Therefore we first examined CAB39 expression in a BC cohort of 89 Chinese Han ethnicity from our hospital by IHC method. As shown in Fig. 1A-B, CAB39 staining was much stronger and its expression level was significantly higher in BC cases than that of normal tissues ($p=0.0124$). Then we performed a multivariate analysis of CAB39 expression in cancer tissues against multiple clinicopathological features. The CAB39 expression is not associated with age and gender (Table 1). However, there was a positive correlation between elevated expression of CAB39 and severity of the malignancy including status of lymph node metastasis ($p=0.002$), disease staging ($p=0.006$), and tumor differentiation ($p=0.007$)(Table 1). When we further divided the BC patients into two groups, one of invasive form and the other noninvasive, as shown in Fig. 1C, there was also a statistically significant difference between the two groups. Such differential expression also existed in BC cell lines, in which T24 with the greatest metastatic potential had the highest expression level of CAB39 compared with 5637 and J82 (Fig. 1E, $p<0.01$ and $p<0.001$). Subsequently, we explored the possible relationship between CAB39 expression and survival of BC patients after tumor surgeries. As shown in Fig. 1D, BC patients with high expression of CAB39($n=45$) exhibited poorer survival outcome than those with low expression ($n=44$) ($p=0.0025$).

Reduced expression of CAB39 facilitates migration, invasion, proliferation of BC cells

5637 and T24 cells stably infected with lentiviral vector carrying CAB39 shRNA were used as the cell models and efficiency of infection was tested by western blot and q-PCR (Fig. 3A-3B). Both CAB39 mRNA and protein were significantly reduced in the presence of two different shRNAs, KD1 and KD2, relative to NC and mock transfected cells. As a result, T24 and 5637 with reduced CAB39 expression proliferated at a much slower rate than their parental and mock counterparts (Fig. 3C-3E, $p < 0.001$). Furthermore, knockdown of CAB39 attenuated their ability to migrate and invade in wound healing experiments (Fig. 2A-2B, $p < 0.10$ for 5637 and $p < 0.001$ for T24) and transwell assays (Fig. 2C, $p < 0.001$). To further examine the knockdown effect of CAB39 on BC growth in vivo, 5637 modified cells were injected subcutaneously into the immunodeficient nude mice. Tumors formed by injected 5637 cells depleted of

CAB39 grew less rapidly than those from normal control ones. At the terminating point of experiment, there was significant reduction in tumor size (Fig. 3E-3F, $p < 0.01$) and weights (Fig. 3G, $p < 0.01$) for CAB39-deficient 5637 tumors. These data strongly imply that CAB39 functions to facilitate proliferation, migration, and invasion of BC cells.

CAB39 mediates EMT in BC cells via NF- κ B signaling pathway

To delineate the mechanism through which CAB39 promotes invasion and metastasis of BC, we performed GSEA bioinformatics analysis (<https://www.gsea-msigdb.org/gsea/index.jsp>) of CAB39-related signaling pathways among BC tissue samples from TCGA database ($n = 414$). As shown in Fig. 4A, CAB39 expression was positively correlated with a total of 126 EMT signaling pathway genes—such as *LAMA3*, *SNAI2* (snail homolog 2), *TGFBI*, as shown in Supplemental Table S1 and Figure S1, which were enriched in BC tissues with elevated expression of CAB39 (NES =1.559, $P=0.061$, FDR $q=0.109$, FWER $p=0.502$). In addition, CAB39 may mediate EMT through NF- κ B signaling pathway (Fig. 4B). CAB39 expression was positively correlated with a total of 123 NF- κ B signaling pathway genes, such as *F3*, *IL7R*, *BIRC3*, as shown in Supplementary Table S2 and Figure S2 (NES=1.907, $P=0.0$, FDR $q=0.202$, FWER $p=0.076$). This was further confirmed by IHC staining of BC tissue specimens, showing that the expression level of CAB39 was inversely correlated with that of E-cadherin, a tumor suppressor in EMT, in normal, noninvasive and invasive BC tissues (Fig. 5A, $p < 0.01$ and $p < 0.001$). In contrast, endogenous CAB39 expression is positively correlated with expression of (Fig. 5b, $p < 0.01$ and $p < 0.001$), key molecular markers of EMT. To further elucidate the role of CAB39 in EMT, we examined expression of the above-mentioned critical regulators of EMT in CAB39-deficient 5637 cells. Consistently, knockdown of CAB39 induced up-regulation of E-cadherin but down-regulation of N-cadherin (Fig. 4C, $p < 0.001$). Furthermore, reduction of CAB39 was associated with decreased phosphorylated form of NF- κ B, but the amount of total protein remained unchanged (Fig. 4D, $p < 0.001$). Immunofluorescence experiment further confirmed that the modified 5637 cells possessed less mesenchymal characteristics than control and mock cells with weakened staining of vimentin (Fig. 4E), indicating an important role of CAB39 in EMT and metastasis of BC as a critical regulator of downstream effectors in the NF- κ B signaling pathway.

Discussion

In the current study, we found that the levels of both CAB39 mRNA and protein were up-regulated in invasive BC tissues compared with non-invasive ones and in T24 cells which had a higher metastatic potential than those of 5637 and J82. The endogenous expression of CAB39 was inversely correlated with prognosis of the malignant disease and patients' survival. However, shRNA-targeted attenuation of endogenous CAB39 in T24 and 5637 cell lines reduced such tumorigenic and metastatic effects as demonstrated by suppression of cell proliferation, reversal of EMT transition, impediment of invasion, reduction of metastatic potential, as well as the growth retardation of xenografts in nude mice. Furthermore, we demonstrated that elevated CAB39 could facilitate EMT transition in BC as indicated by down-regulation of E-cadherin and up-regulation of N-cadherin via activation of NF- κ B signaling pathway.

The correlation of CAB39 expression with the degree of invasiveness and metastatic potential of BC, as well as unfavorable prognosis and poor survival, is in line with similar findings from several other types of malignancies. Jiang and colleagues reported frequent upregulation of CAB39 in hepatocellular carcinoma, which was associated with tumor metastasis, poor prognosis, and low survival rate[21]. From analysis of TCGA database, Ruhl et al. noted increased CAB39 expression at the protein level in a significant portion of colon cancer patients associated with poorer overall survival[23]. Additionally, studies on microRNAs targeting CAB39 in gastric cancer, lung cancer, colorectal cancer, and glioma also suggest that CAB39 upregulation has stimulatory effect on cell proliferation, invasion, and oncogenic autophagy [25, 29-31]. Since CAB39 is evolutionarily conserved and ubiquitously expressed in a variety of human tissues, its oncogenic function is likely to be fundamental in most cell types and the association with tumor invasion and metastasis observed in BC can also occur in other types of cancers[32].

The oncogenic role of CAB39 in bladder carcinogenesis as a measure of aggressiveness cannot simply be explained by LKB1 activity as a tumor suppressor[16]. If higher metastatic potential is interpreted as suppressed LKB1 complex activity, it is unlikely to be mediated by CAB39, a stabilizing scaffold protein to maintain tumor-suppressing activity of LKB1[33]. In fact, CAB39 can bind to several other STE20 family kinases including SPAK, OSR1, MST3, MST4, and YSK1 to play roles beyond stabilizing LKB1 complex[34]. A recent finding indicated that CAB39 could activate an extracellular signal-regulated kinase (ERK) pathway, promoted b-catenin nuclear accumulation, and upregulate MMP9[21]. In addition, Xu et al. reported in a study of gastric cancer, that suppression of cancer progression and oncogenic autophagy was achieved through reduction of CAB39, an upstream regulator of the AMPK/mTOR signaling pathway[31]. Furthermore, miRNA-451 targeting CAB39 suppressed proliferation and invasion of glioblastoma and lung cancer cells via mTOR/HIF-1a/VEGF and PIK3/AKT signaling pathways, respectively[25, 32]. Finally, a link between NF- κ B activation and transcriptional regulation of EMT-TFs has been indicated in certain human cancers[35-38]. In our current investigation, we have identified a novel downstream effector of CAB39, the phosphorylated form of NF- κ B protein. Further investigation of the effect of activated NF- κ B signaling pathway on possible upregulation of EMT genes in BC as a result of direct binding of target promoters is warranted.

Since previous studies have revealed enhanced CAB39 expression in many types of cancers including colorectal cancer, gastric cancer, and hepatocellular cancer promoting cell proliferation and metastasis, the oncogene has become a favorable target for the treatment of these types of cancers. [23, 25, 29-32]. Ruhl et al. Found that in response to g-radiation, a specific miRNA-451a could be rapidly upregulated to target and degrade CAB39[23]. Consistently, Kelley and colleagues measured the expression of CAB39 in rectal cancer patients with or without response to radiotherapy and found a significantly higher CAB39 level in patients without response than those with complete or partial response[39]. However, Liang et al. have found that CAB39 was upregulated as a result of treatment of dichloroacetate (DHA) and associated with increased chemosensitivity to oxaliplatin[30]. In addition, a recent report has suggested that CAB39 act as a tumor suppressor in pancreatic cancer, which inhibits cell proliferation, reduces cell invasion, promotes cell apoptosis, and induces cell cycle arrest in pancreatic cancer cell lines PANC-1 and AsPC-1[40]. The seemingly contradicting roles of CAB39 in different types of cancers maybe attributed to

particular signaling pathways activated as a result of specific tissue type and environmental cue. Hence it is paramount to clearly define the role of CAB39 in BC and understand the molecular mechanism underpinning its response to various therapeutic interventions in order to derive more effective treatment. Nevertheless, the current study suggests that CAB39 could serve as a potential therapeutic target to treat invasive BC for better overall survival and favorable prognosis.

Conclusions

In conclusion, we identified CAB39 as a tumor promoter in BC and further uncovered a molecular mechanism in which CAB39 modulates EMT through upregulation of N-cadherin and downregulation of E-cadherin via NF- κ B signaling pathway, leading to the invasion and metastasis of BC cells.

List Of Abbreviations

BC bladder cancer

CAB39 calcium binding protein 39

EMT epithelial-mesenchymal transition

RT-qPCR real-time quantitative reverse-transcription polymerase chain reaction

CCK-8 Cell Counting Kit-8

TCGA the Cancer Genome Atlas

SNAIL1 snail homolog 1

TWIST1 twist basic helix-loop-helix transcription factor 1

ZEB1 zinc-finger-E-box-binding homeobox 1

STRAD STE20-related adaptor

MEM minimum essential medium

IHC immunohistochemical

GAPDH Glyceraldehyde-3 phosphate dehydrogenase

RNAi Lentivirus-mediated RNA interference

GFP green fluorescent protein

FDR false discovery rate

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee and Institutional Review Board of the Second Affiliated Hospital of Nanchang University, and the Written informed consent was obtained from all patients according to ethical standards. All the research was carried out in accordance with the provisions of the declaration of Helsinki of 1975. All Animal experiments complied with the national guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflict of interest to disclose.

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Authors' contributions

H.C. Chao, L.F. Peng, L. H. Deng, Z.J.Yu, and H.H. Deng performed the experiments and analyzed the data. H.C. Chao drafted the manuscript. Z. Tao revised the

manuscript critically. H.C.Chao, L.F. Peng, F.H.Xu, X.D.Xu, and J.B.Huang designed or/and supervised this project and revised the manuscript. Final approval of manuscript: All authors.

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Table

Table 1: Correlation between CAB39 and clinicopathological characteristics in BC patients

characteristics	Number of patients	CAB39 low expression	CAB39 high expression	χ^2 -value	P-value
Gender					
Male	80	37[46.25%]	43[53.75%]	0.032	0.859
female	9	5[55.56%]	4[44.44%]		
Age					
<60	51	25[49.02%]	26[50.98%]	0.114	0.736
>60	38	20[52.63%]	18[47.37%]		
Lymph node metastasis					
M ₀	59	34[57.63%]	25[42.37%]	9.414	0.002
M ₁	30	7[23.33%]	23[76.67%]		
Tumor staging					
T ₁	30	22[73.33%]	8[26.67%]	12.309	0.006
T ₂	29	12[34.48%]	17[58.62%]		
T ₃	20	8[40.00%]	12[60.00%]		
T ₄	10	2[20.00%]	8[80.00%]		
Tumor differentiation					
High	42	29[69.05%]	13[30.95%]	9.836	0.007
Moderate	31	12[38.71%]	19[61.29%]		
low	16	5[31.25%]	11[68.75%]		

Figures

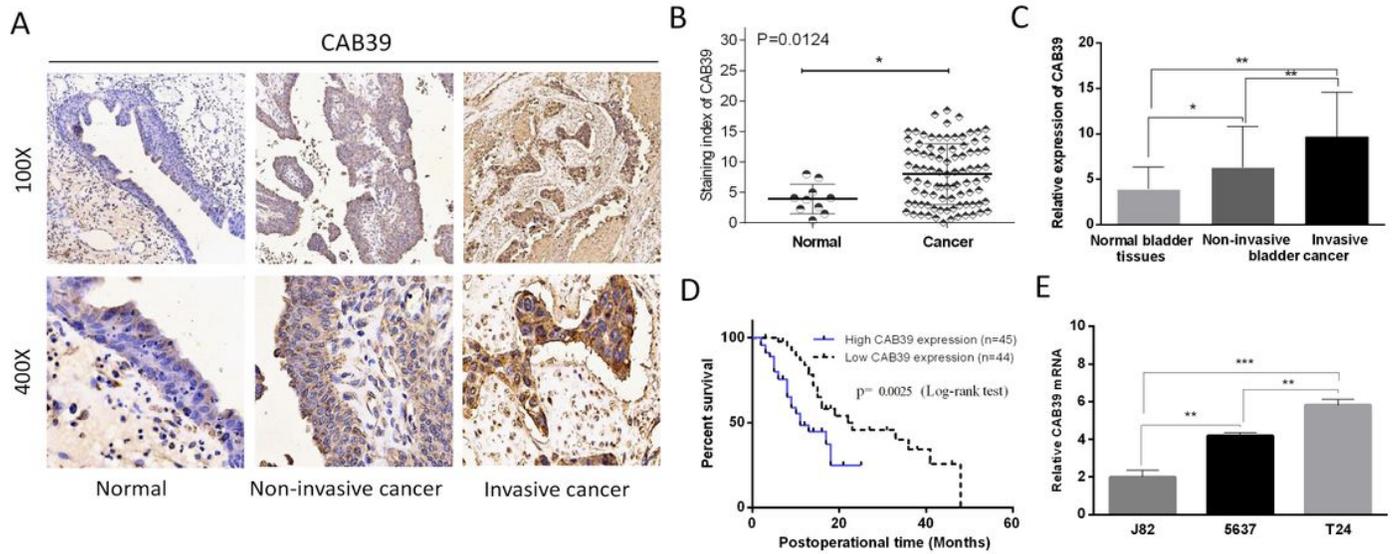


Figure 1

CAB39 expression in primary BC tissues. A. Increasing cellular expression of CAB39 in normal, non-invasive, and invasive cancerous tissues of the bladder was shown by IHC (100X, 400X); B. Expression levels of CAB39 were compared between cancer tissues and normal ones by histogram. C. Relative expression levels of CAB39 were compared between normal, non-invasive, and invasive tissues; D. Kaplan-Meier curves showed that protein levels of CAB39 were inversely correlated with survival of the BC patients; E. Transcriptional activation of CAB39 mRNA was quantified in bladder cancer cell lines T24, 5637 and J82 by quantitative RT-PCR.

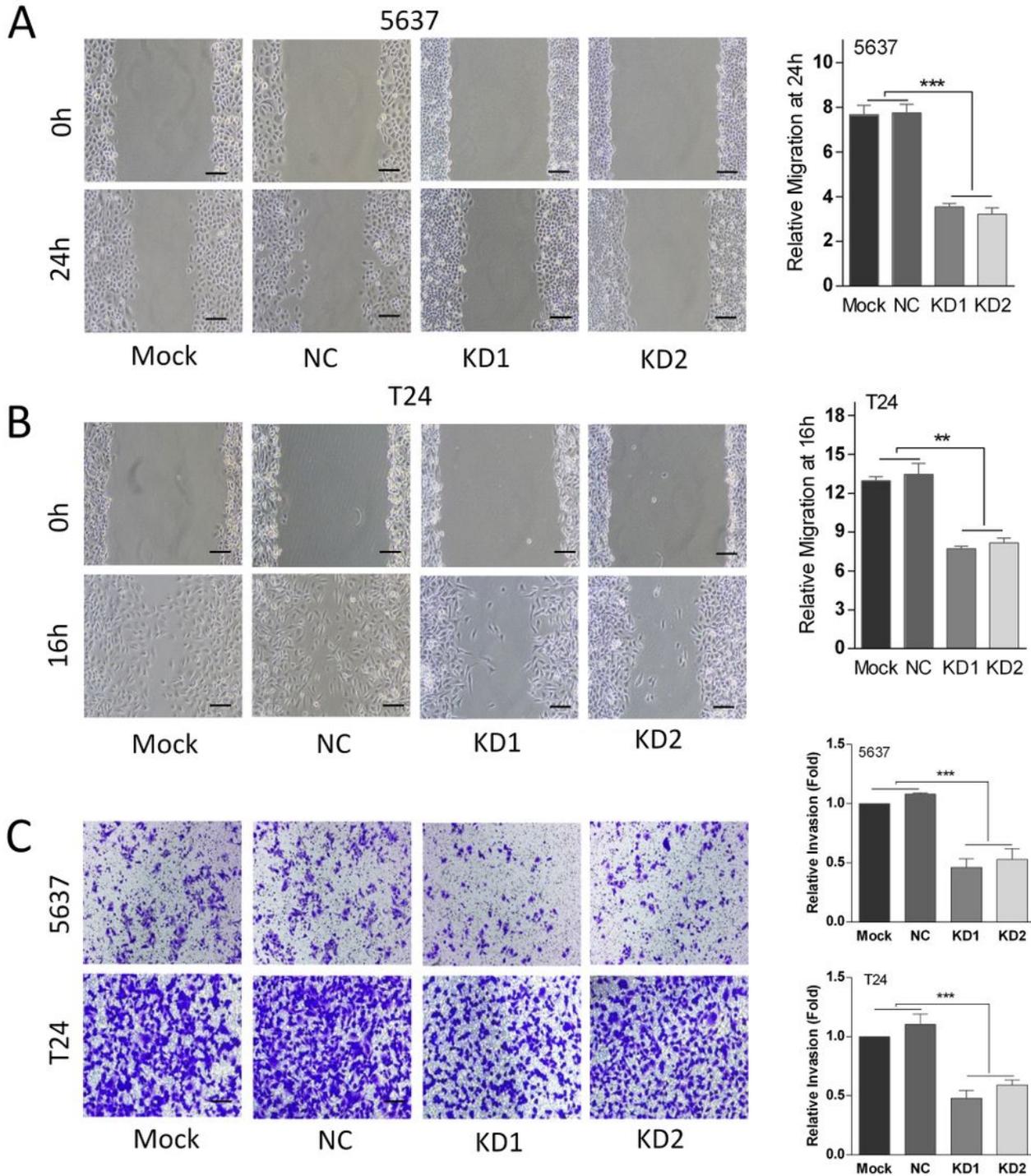


Figure 2

Functional effects of knockdown of CAB39 expression in BC cell lines on migration and invasion. A, B. Wound healing assays were performed on 5637 and T24 cells, and cells were photographed every 24 h and 16 h after scratching. The wound healing percentage represents mean \pm SD of at least three experiments at each time point. Statistical results were shown by bar or linear graph. ** $p < 0.01$, *** $p <$

0.001 ; C. Transwell invasion (DAPI staining) assays of 5637 and T24 cells were performed after transduction by CAB39 knockdown lentivirus. *** $p < 0.001$.

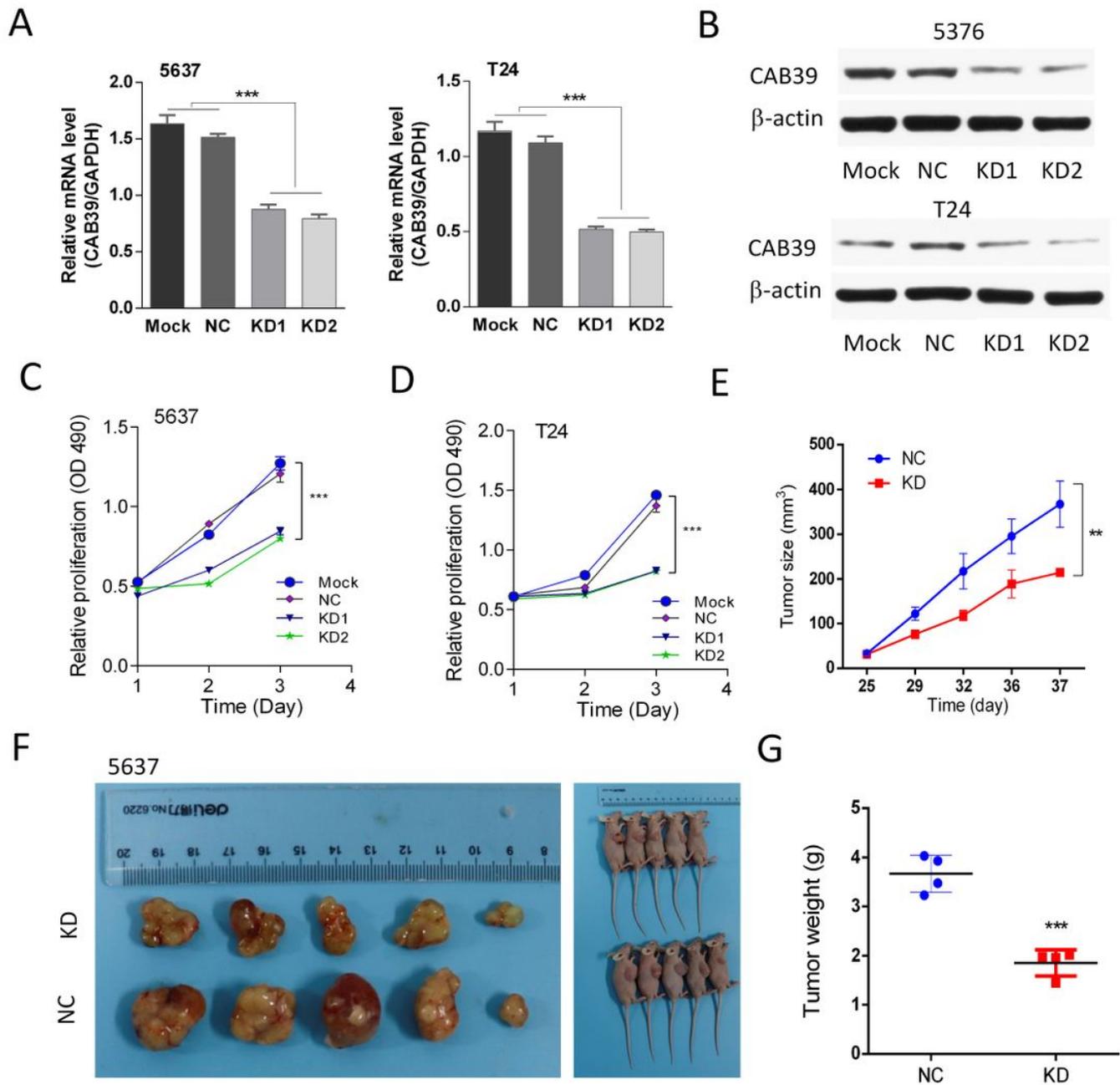


Figure 3

In vivo examination of knockdown effects of CAB39 expression in nude mice. A. Transcriptional inhibition of CAB39 mRNA was quantified by real-time quantitative fluorescent PCR; B. Reduction of protein expression of CAB39 was shown by western blot analysis; C, D. Proliferation of 5637 and T24 cells infected with lentiviral vectors with CAB39-shRNA was assessed by CCK-8 assays compared to normal control and mock. *** $p < 0.001$; E. Tumor growth for subcutaneously seeded parental and modified 5637 cells in nude mice was monitored for 37 days. ** $p < 0.01$; F. At the endpoint of experiment, tumor

xenografts from 5637 cells in nude mice were excised and shown side-by-side; G. Excised tumors were weighted and shown in dotted histogram. $**p < 0.01$.

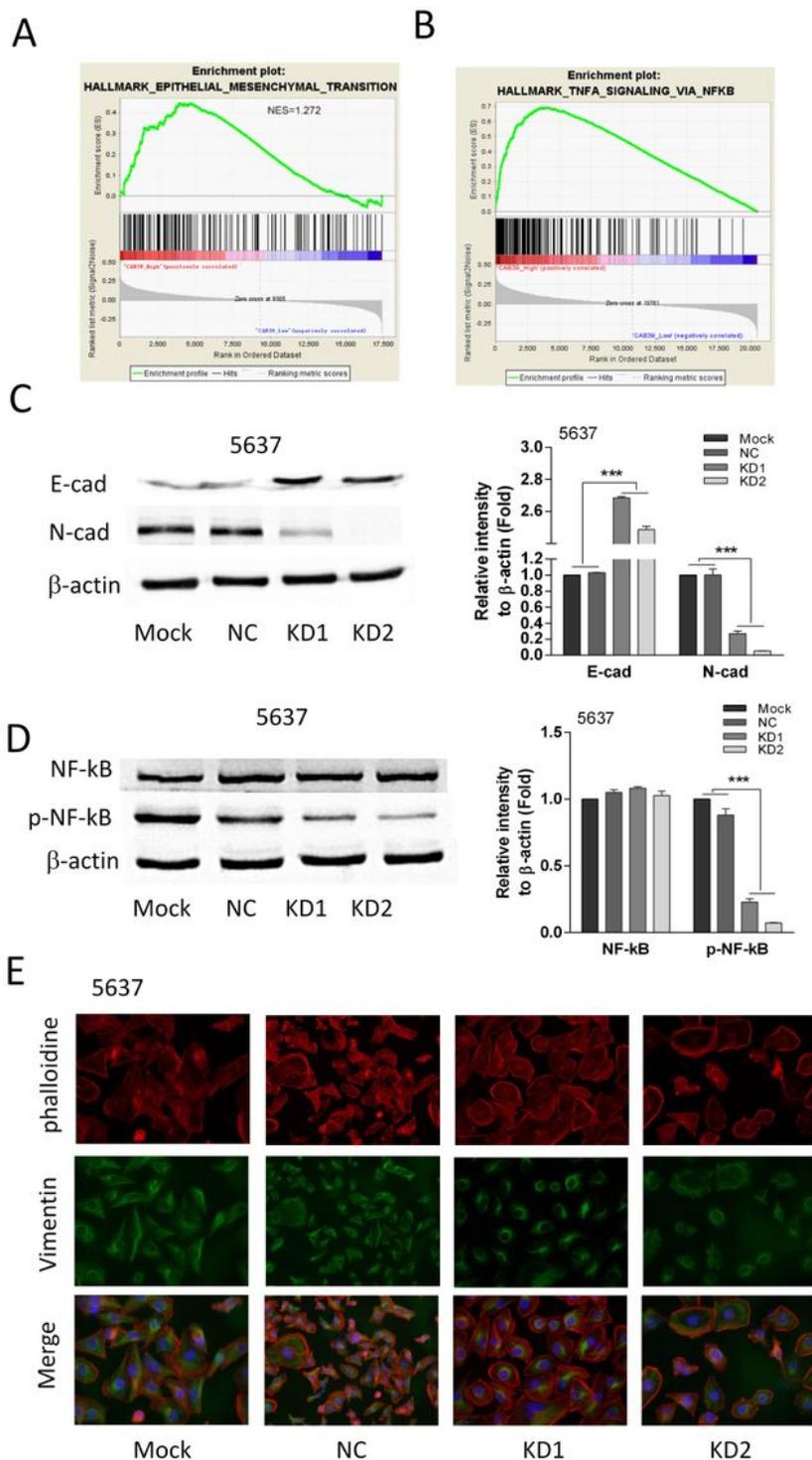


Figure 4

Activation of NF- κ B to facilitate EMT in BC cells. A. Correlation of CAB39 expression with genes enriched in EMT was shown in BC cells by GSEA bioinformatics analysis; B. Activation of NF- κ B signaling pathway coincided with CAB39 expression was shown by GSEA bioinformatics analysis; C. Protein levels of E-

cadherin and N-cadherin in modified 5637 cells were shown by western blot analysis. $***p < 0.001$; D. Quantification of NF- κ B and p-NF- κ B, key components of the NF- κ B signaling pathway, was performed by western blot analysis. $***p < 0.001$; E. Cellular topography of modified 5637 cells were observed by immunofluorescence (IF) staining of actin filaments by phalloidin (red), vimentin (green), nucleus (dapi).

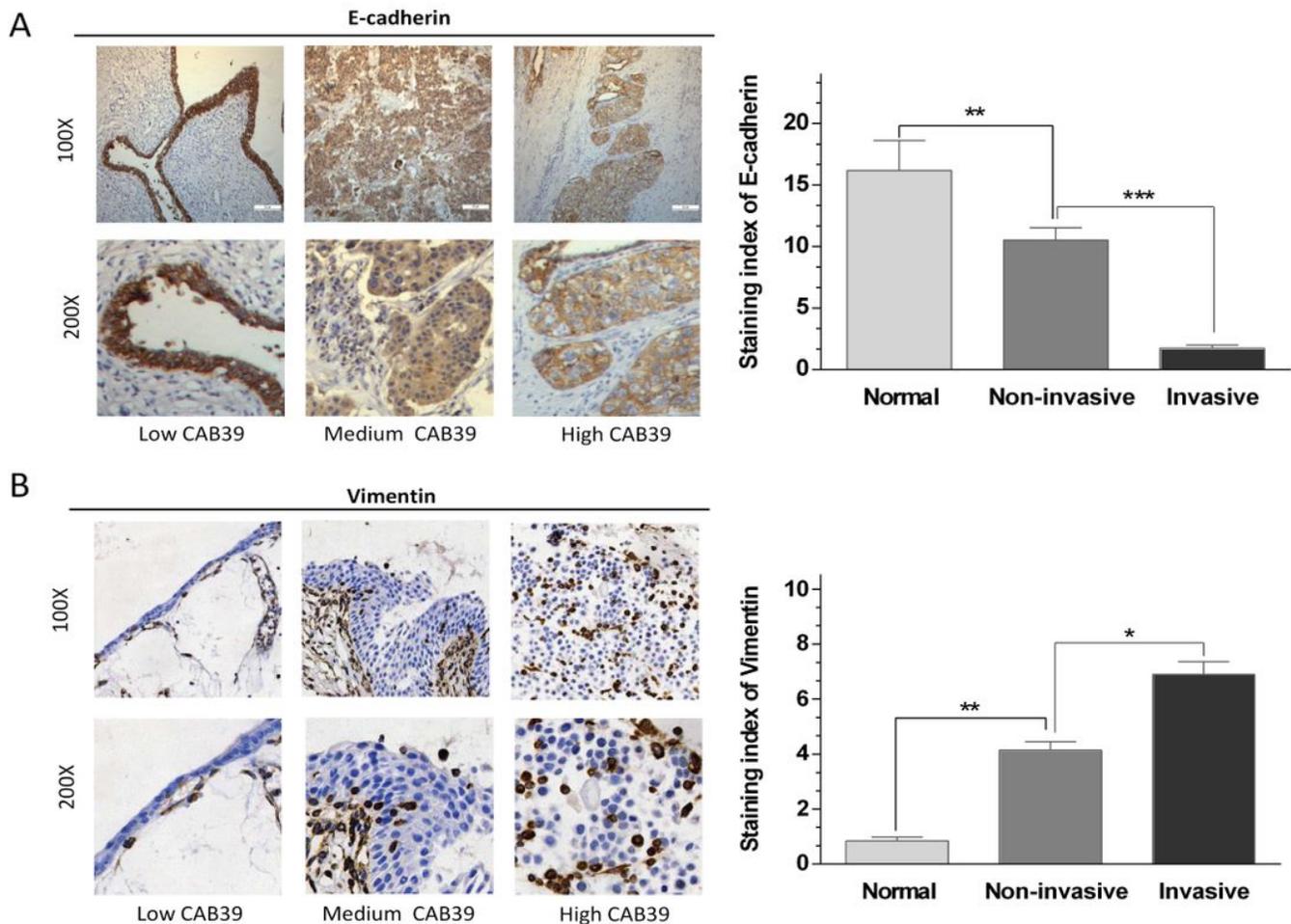


Figure 5

Quantitative analysis of expression of E-cadherin and vimentin in relation to CAB39 by IHC. A. Inverse correlation between expression of E-cadherin and CAB39 in normal and cancerous bladder tissues. $**p < 0.01$, $***p < 0.001$; B. Positive correlation between expression of vimentin and CAB39 in normal and cancerous bladder tissues. $**p < 0.01$, $***p < 0.001$.

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