

L1CAM promotes epithelial to mesenchymal transition and formation of cancer initiating cells in human endometrial cancer

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Abstract. Identification of novel factors critical for epithelial to mesenchymal transition (EMT) and cancer initiating cell (CIC) formation may aid in the identification of novel therapeutics for the treatment of endometrial cancer. The present study demonstrated that L1 cell adhesion molecule (CAM) is critical for EMT and formation of CICs in endometrial cancer. Overexpression of L1CAM may promote EMT with increased formation of CICs in HEC-1A endometrial cancer cells. CICs and mesenchymal status resist chemotherapeutic drugs and may regenerate the various cell types in tumors, thereby resulting in relapse of the disease. The present study demonstrated that overexpressing L1CAM promoted paclitaxel resistance and regulated paclitaxel resistance-associated microRNA expression in HEC-1A cells. Furthermore, it was demonstrated that overexpressing L1CAM promoted anoikis resistance in HEC-1A cells. This link between L1CAM and EMT/CICs may provide a novel target for advancing anticancer therapy.

Introduction

Endometrial carcinoma (EC) is the most common gynecologic malignancy and is associated with a poor prognosis when diagnosed at an advanced stage (1). Endometrial cancer is traditionally classified into type I and type II subtypes (2). Type I cancers account for 80-85% of EC cases, are of endometrioid histology, more often well differentiated and associate with favorable prognosis (2). In contrast the type II

cancers are non-endometrioid carcinomas, poorly differentiated and associate with poorer survival (2). However, patients with deep myometrial invasion, poor differentiation, serous or clear cell histology or extension of disease to other organs or lymph nodes within the pelvic region are at higher risk for disease recurrence (3,4). Therefore, it is imperative to find new therapeutic targets to elaborate the molecular mechanisms underlying progression of endometrial carcinogenesis.

L1 cell adhesion molecule (L1CAM, CD171) is a 200-220-kDa transmembrane glycoprotein composed of 6 immunoglobulin-like domains, 5 fibronectin-type III domains, a transmembrane stretch, and a short cytoplasmic tail (5). L1CAM was originally identified as a neural cell adhesion molecule in the central nervous system that plays an important role in initiating cerebellar cell migration and neurite outgrowth (6). L1CAM expression is also found in other cell types such as lymphoid and myelomonocytic cells, kidney tubule epithelial cells, and intestinal crypt cells (7-10). In addition, L1CAM expression has been identified in a variety of tumor types and correlates with poor prognosis and metastasis (11). L1CAM functions mostly in proliferation, migration, invasion, and survival through L1CAM homophilic interaction or heterophilic interactions with other cell adhesion molecules, integrins, or growth factor receptor, while the cellular properties are not homogeneous among different types of cancers (12). Recently, it has been reported that L1CAM was involved in progression of endometrial cancer (13).

Cyclophilin A (CypA) is a highly abundant protein, accounting for up to ~0.6% of the total cytosolic protein content (14). CypA is involved in a growing number of biological processes, including protein folding, signal transduction, viral infection, trafficking, receptor assembly, immune response, and transcription regulation (15). Although several proteins have been identified to interact with CypA (16-19), the underlying mechanism of the CypA action and the physiological implications of the interactions remain in most cases unknown. CypA exhibits peptidyl-prolyl *cis-trans* isomerase (PPIase) activity by catalyzing *cis-trans* isomerization of peptide bonds preceding proline residues (20). CypA can in principle act as an enzyme or a binding partner (21) in mediating the biological processes.

In this study, we showed that L1CAM promotes EMT with increased characteristics of CICs and paclitaxel resistance in human endometrial cancer.

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Abbreviations: CICs, cancer initiating cells; EMT, epithelial to mesenchymal transition; EC, endometrial cancer

Key words: endometrial cancer, cancer initiating cells (CICs), epithelial to mesenchymal transition (EMT), L1CAM, paclitaxel resistance

Materials and methods

HEC-1A cells line. HEC-1A cells were obtained from Peking Union Medical College (Beijing, China). Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Shanghai, China) containing 10% fetal bovine serum (FBS; Shanghai ExCell Biology, China) and 100 mg/ml penicillin and streptomycin (Gibco, Shanghai, China) at 37°C in a humidified atmosphere with 5% CO₂.

LICAM expressing plasmids/empty vectors and transfection experiments. LICAM expressing plasmids and empty vectors (pcDNA3.1) were obtained from Tiangen (Beijing, China). Transfections were performed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA) following the manufacturers' protocols.

Western blot analysis. It was performed as described previously (22,23). Total protein was prepared using extraction buffer comprising NaCl/P_i containing 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and complete protease inhibitors (Roche). The concentration of each protein lysate was determined using a BCATM protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of total protein were subjected to 12% SDS/PAGE. Then samples were transferred to nitrocellulose membranes and blocked for 60 min at room temperature in 5% skim milk powder (w/v) in NaCl/P_i. The membranes were immunoblotted using primary anti-body anti-LICAM (1:500; Abcam, Cambridge, MA, USA), anti-E-cadherin (1:500; Abcam, Cambridge, MA, USA), anti-Vimentin (1:500; Abcam, Cambridge, MA, USA), anti-Musashi-1 (1:500; Abcam, Cambridge, MA, USA), anti-CD133 (1:500; Abcam, Cambridge, MA, USA), anti-Cyclophilin A (1:500; Abcam, Cambridge, MA, USA) and anti-β-actin (1:500; Abcam, Cambridge, MA, USA) overnight at 4°C, anti-rabbit secondary antibodies (1:10,000; Abcam, Cambridge, MA, USA) were used for 30 min at room temperature. The specific proteins were visualized by OdysseyTM Infrared Imaging System (Gene Company, Lincoln, NE, USA). β-actin expression was used as an internal control to show equal loading of the protein samples.

Immunofluorescence staining. It was performed as described previously (24,25). Cells were plated on glass coverslips in six-well plates and transfected as indicated. At 48 h after transfection, the cells were fixed in 4% paraformaldehyde for 15 min, and then blocked with goat serum blocking solution for 20 min at room temperature. Coverslips were stained with the mentioned antibody mentioned anti-LICAM antibodies (1:500; Abcam, Cambridge, MA, USA), After washing three times with NaCl/P_i, cells were incubated with appropriate secondary antibodies (Abcam, Cambridge, MA, USA) for 30 min at 37°C. 4',6'-diamidino-2-phenylindole (DAPI) staining (blue) was used to indicate nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Fluorescence intensities were calculated from a few viewing areas for 300 cells per coverslip and analyzed by ImageJ 1.37v software (<http://rsb.info.nih.gov/ij/index.html>).

Quantitative real-time RT-PCR (qRT-PCR). Quantitative real-time RT-PCR were described before (21). The specific

primer sets for PCR were as follows: GAPDH, forward primer: 5'-GAAGGTGAAGGTCGGAGTCA-3', and reverse primer: 5'-GAAGATGGTGATGGGATTTC-3'; E-Cadherin, forward primer 5'-TCAACGATCCTGACCAGCAGTTTCG-3' and reverse primer 5'-GGTGAACCATCATCTGTGGCGATG-3'; N-cadherin, forward primer 5'-CATCCCTCCAATCAACTTGC-3' and reverse primer 5'-ATGTGCCCTCAAATGAAAAC-3'; Vimentin, forward primer 5'-GACAATGCGTCTCTGGCAGTCTT-3' and reverse primer 5'-TCCTCCGCCTCC TGCAGGTTCTT-3'; ZEB1, forward primer 5'-TTAGTTGCTCCCTGTGCAGTT-3' and reverse primer 5'-TAGGAGCCA GAATGGGAAAAG-3'. GAPDH was a loading control.

MTT assay. The proliferation of cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma, St Louis, MO, USA). The MTT analysis was performed as described previously (26-29). In brief, the cells were plated in 96-well plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at a density of 8x10³ cells per well at 37°C in a 5% CO₂ incubator for 12 h. Cells were transfected with LICAM expressing plasmid or empty vectors for 24 h and then were treated with different doses of paclitaxel (10⁻⁴-10²). After 24 h, MTT (5 mg·ml⁻¹) was added to the wells (20 μl per well). The plates were incubated in a cell incubator for 4 h, then the supernatant was removed and 150 μl of dimethyl sulfoxide was added to each well. After incubation for 10 min, the absorbance of each well was measured using a SynergyTM 4 (BioTek Instruments, Winooski, VT, USA) with a wavelength of 570 nm, with the reference wavelength set at 630 nm. Absorbance was directly proportional to the number of survival cells.

Sphere formation assay. It was performed as described previously (30). Cells (10³/ml) in serum-free RPMI1640/1 mM Na-pyruvate were seeded on 0.5% agar precoated 6-well plates. After 1 week, half the medium was exchanged every third day. Single spheres were picked and counted.

Anoikis assays. It was performed as described previously (31). Anoikis resistance was evaluated by seeding 7.5x10⁴ cells in ultralow attachment plates (Corning). After 24 h of anchorage-independent culture, cells were transfected as indicated and resuspended in 0.4% trypan blue (Sigma, St. Louis, MO, USA) and cell viability was assessed.

miRNA microarray. It was performed as described previously (32). Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). cRNA for each sample was synthesized by using 3' IVT EXPRESS KIT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocols. The purified cRNA was fragmented by incubation in fragmentation buffer (provided in the 3'IVT express kit) at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to MicroRNA2.0 Array (Affymetrix, Santa Clara, CA, USA) and hybridized in Genechip hybridization oven 640 (Affymetrix, Santa Clara, CA, USA) at 45°C for 18 h. After washing and staining in Genechip fluidics station 450 (Affymetrix, Santa Clara, CA, USA), the arrays were scanned by using Genechip scanner

3000 (Affymetrix, Santa Clara, CA, USA). The gene expressions levels of samples were normalized and compared by using Partek GS 6.5 (Partek, Inc, St. Louis, MO, USA). Average-linkage hierarchical clustering of the data was applied by using the Cluster [Eisen *et al* (33), Stanford, Stanford University, CA, USA; <http://rana.lbl.gov>] and the results were displayed by using TreeView [Eisen *et al* (33), Stanford, Stanford University, CA, USA; <http://rana.lbl.gov>].

Statistical analysis. Results were analyzed using SAS software (9.4). Data were presented as mean \pm standard error of the mean (SEM) of separate experiments (n=3). P-values less than 0.05 were considered to be significant.

Results

LICAM promotes EMT in endometrial cancer HEC-1A cells. To investigate whether LICAM can affect epithelial or mesenchymal status of HEC-1A cells, we performed western blot to test whether LICAM expressing plasmids could express LICAM protein in HEC-1A cells. The results of western blot showed that LICAM expressing plasmids can significantly up-regulate LICAM protein expression in the cells (Fig. 1A). To determine whether LICAM can promote EMT, we transfected HEC-1A cells with LICAM expressing plasmids and then observed that its overexpression promoted evident changes in the cells morphology (EMT, epithelial to mesenchymal transition) (Fig. 1B). To confirm that the changes of morphology are induced by EMT, we performed immunofluorescence analysis to detect epithelial and mesenchymal markers of HEC-1A cells transfected with LICAM expressing plasmids and empty vectors. We found that the E-Cadherin protein (epithelial marker) was inhibited and Vimentin protein (mesenchymal marker) were induced by LICAM in HEC-1A cells (Fig. 1C). To further analyze whether LICAM could affect E-Cadherin and Vimentin protein, we used western blotting to detect their expression in the cells transfected with LICAM expressing plasmids and empty vectors. The results demonstrated that E-Cadherin was downregulated and Vimentin was upregulated by LICAM (Fig. 1D and E). We also performed real-time PCR to detect epithelial and mesenchymal markers. As anticipated, we found that epithelial marker (E-cadherin) was downregulated and mesenchymal markers (such as N-Cadherin, Vimentin, and ZEB1) was upregulated by LICAM in HEC-1A cells (Fig. 1F).

LICAM promotes formation of CICs in HEC-1A cells. EMT can contribute to increased formation of CICs in cancer cells (34-37). To determine whether LICAM could affect characteristics of CICs, we performed sphere forming assay to evaluate the formation of CICs in HEC-1A cells. The results of sphere forming assay showed that formation of spheres were increased by LICAM in HEC-1A cells (Fig. 2A). Moreover, we performed western blot to detect whether LICAM could regulate CICs markers-Musashi-1 and CD133 expression in the cells. We found that Musashi-1 and CD133 protein were evidently upregulated by LICAM in HEC-1A cells (Fig. 2B and C).

LICAM promotes paclitaxel resistance in human endometrial cancer HEC-1A cells. To further identify whether LICAM can affect paclitaxel efficacy in HEC-1A cells, we performed MTT

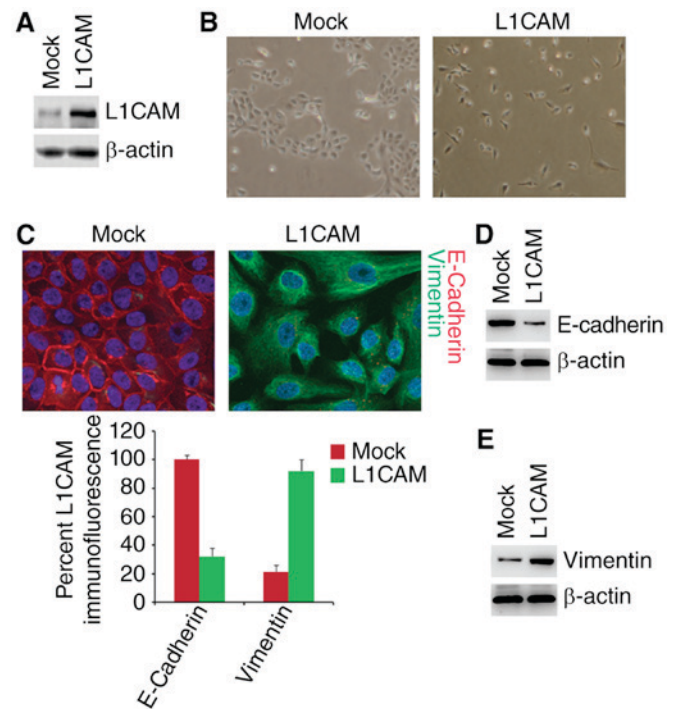


Figure 1. LICAM promotes EMT in endometrial cancer HEC-1A cells. (A) Western blot of LICAM in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3. (B) HEC-1A cells transfected as indicated were photographed after 72 h of transfection. n=3. (C) Immunofluorescence assay of E-cadherin and vimentin in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3. (D) Western blot of E-cadherin in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3. (E) Western blot of vimentin in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3.

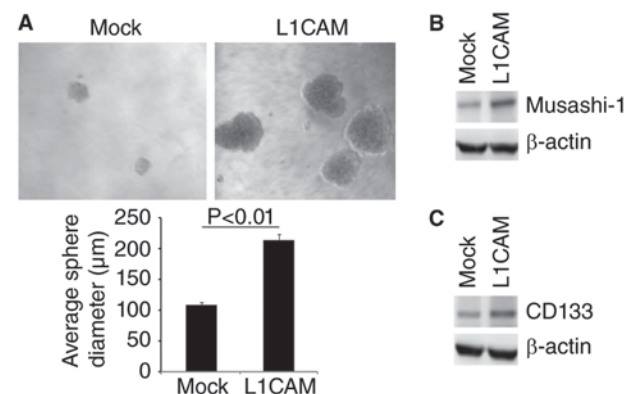


Figure 2. LICAM promotes formation of CICs in endometrial cancer HEC-1A cells. (A) Sphere growth of HEC-1A cells transfected with LICAM expressing plasmids and empty vectors (mock). (B) Western blot of Musashi-1 in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3. (C) Western blot of CD133 in LICAM expressing plasmids and empty vectors (mock) transfected HEC-1A cells. n=3.

assay in HEC-1A cells treated as indicated (Fig. 3A). The results showed that overexpressing LICAM could promote paclitaxel resistance (Fig. 3A). In addition, we performed western blot to analyze cyclophilin A protein expression in LICAM expressing plasmids and empty vectors transfected HEC-1A cells. We found that cyclophilin A protein can be increased by LICAM (Fig. 3B).

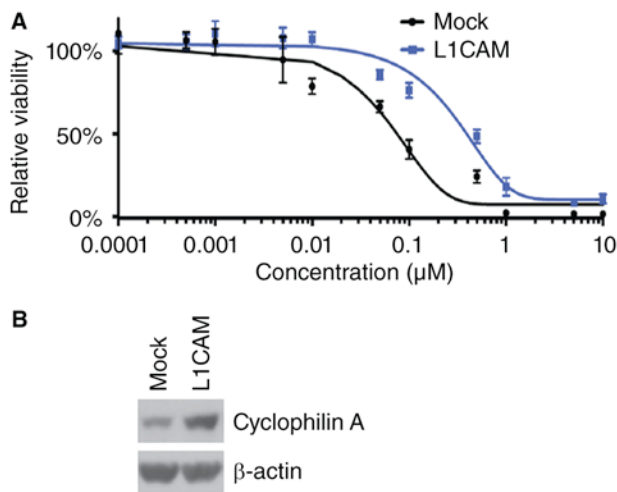


Figure 3. LICAM promotes paclitaxel resistance in endometrial cancer HEC-1A cells. (A) MTT of HEC-1A cells transfected with LICAM expressing plasmids and empty vectors (mock) were treated with different concentration of paclitaxel. $n=3$. (B) Western blot of cyclophilin A in LICAM and empty vectors (mock) transfected HEC-1A cells. $n=3$.

LICAM regulates paclitaxel resistance-associated microRNAs expression in HEC-1A cells. Oncogenes can promote endometrial cancer progression by regulating microRNA expression and microRNA involved in endometrial cancer pathogenesis can function as oncogene or tumor suppressor gene (38-40). Thus, we reasoned that LICAM could function as an oncogene by regulating miRNAs expression. We performed microarrays to detect miRNA expression. RNAs isolated from LICAM expressing plasmids or empty vectors transfected HEC-1A cells were hybridized to a custom miRNA microarray platform. We found that miR-135a, miR-375, miR-200c, miR-182, let-7e, miR-31, miR-21, miR-200b, miR-143 and miR-145 were changed more than 10 folds in the cells (Fig. 4A).

LICAM promotes anoikis resistance in human endometrial cancer HEC-1A cells. To study the roles of LICAM on metastasis, we used anoikis assays to detect its role regulating anoikis resistance. Cells transfected with LICAM expressing plasmids showed about 200% increased resistance to anoikis-mediated cell death (Fig. 4B).

Discussion

The expression of LICAM is a strong predictor of poor outcome in endometrial cancer (41). EMT plays an important role in invasion and metastasis of endometrial cancer and enables cancer cells to obtain malignant characters and traits of CICs (42). Critical molecular features of this process are the deregulation of E-cadherin and vimentin expression (43). Consistent with previous report that LICAM was inversely associated with E-cadherin expression (44), we found that overexpressing LICAM induced EMT and inhibited E-cadherin expression in endometrial cancer HEC-1A cells.

CICs have been proposed as the major power of EMT and responsible for poor survival (45). Musashi-1 and CD133 have been proposed as markers of CICs for endometrial cancer (46). In line with previous report that LICAM is required for

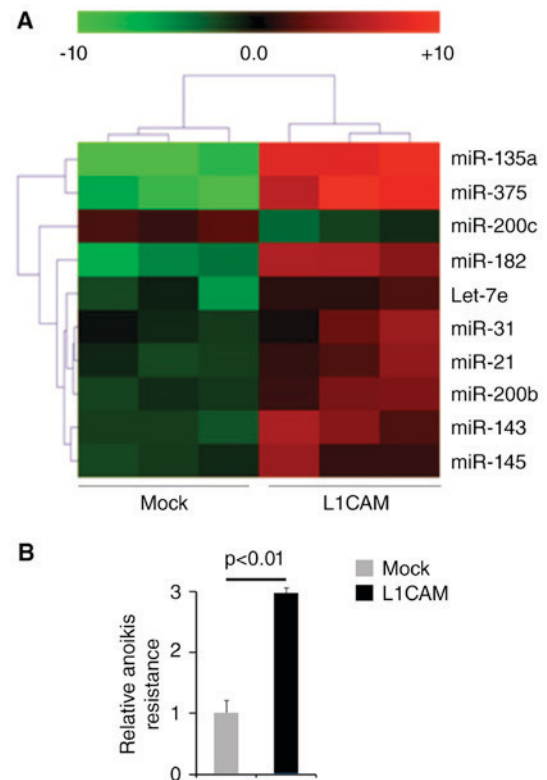


Figure 4. LICAM regulates paclitaxel resistance-associated microRNAs expression and promotes anoikis resistance in endometrial cancer HEC-1A cells. (A) miRNA microarray analysis for LICAM expressing plasmids or empty vectors (mock) transfected HEC-1A cells. $n=3$. (B) Anoikis assays for LICAM expressing plasmids or empty vectors (mock) transfected HEC-1A cells. $n=3$.

maintaining CICs and targeting LICAM may represent a novel therapeutic strategy (47), we showed that its overexpression evidently promoted formation of CICs traits and upregulated Musashi-1 and CD133 protein. All the results indicated that LICAM might be a therapeutic target for eradicating CICs in endometrial cancer.

Chemotherapy is a common therapeutic strategy for cancer, but it fails to eradicate cancer cells, because of primary resistance or acquired drug resistance. Elucidating the mechanisms of drug resistance for cancer will yield vital information about how to improve cancer chemotherapy and circumvent the resistance. In line with previous report that LICAM can confer chemoresistance in malignant tumor (48,49), we showed that over-expressing LICAM could promote paclitaxel resistance in endometrial cancer HEC-1A cells. Cyclophilin A expression was increased in paclitaxel-resistant endometrial cancer cells, as well as silencing Cyclophilin A reversed paclitaxel resistance (50). We showed that Cyclophilin A was upregulated by LICAM. microRNAs have recently been identified as key genes implicated in mechanisms of chemoresistance. Upregulation of miR-135a and miR-375 can contribute to paclitaxel resistance. Up-regulating miR-200c in ovarian cancer reduced tumor burden and improved paclitaxel sensitivity. We showed that LICAM significantly upregulated miR-135a and miR-375 expression and downregulated miR-200c expression. The results indicated that LICAM may induce paclitaxel resistance by regulating microRNAs.

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