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Function of CSE1L/CAS in the secretion of HT-29 human colorectal cells and its expression in human colon

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Abstract The secretion of colorectal epithelium is important for maintaining the physiological function of colorectal organ. Herein, we report that cellular apoptosis susceptibility (CAS) (or CSE1L) protein regulates the secretion of HT-29 human colorectal cells. Polarity is essential for directed secretion of substances produced by epithelial cells to the external (luminal) compartment; CAS overexpression induced polarization of HT-29 cells. CAS was punctate stained in the cytoplasm of HT-29 cells, and CAS overexpression increased the translocation of CAS-stained vesicles to the cytoplasm near cell membrane and cell protrusions. CAS overexpression increased the secretion of carcinoembryonic antigen (CEA) and cathepsin D. Immunohistochemistry showed CAS was positively stained in the goblet cells of colon mucosa and cells in the crypts of Lieberkühn of human colon as well as the glands in metastatic colorectal cancer tissue. Our results suggest that CAS regulates the secretion of colorectal cells and may regulate the metastasis of colorectal cancer.

Keywords CAS · Colon · Colorectal cancer · Polarity · Secretion

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Introduction

In addition to absorption, the colorectal epithelium also has secretory function, and the secretions of colorectal epithelium cells are essential for maintaining the normal physiological functions of colorectal tissues [1]. Abnormality in the secretion of colorectal epithelium cells has a close relationship with the development of various colorectal diseases including the invasion and metastasis of colorectal cancer [2, 3]. The formation of cell polarity is essential for establishing the selective permeability barriers between biological compartments (lumen and serosa) and the specialized vectorial functions in absorption and secretion [4, 5]. The membrane of a polarized cell is specialized and can be characterized by apical, lateral, and basal domains. The apical membrane domains of polarized cells face the lumen, and vectorial absorption and secretion occur between the apical membranes and the lumen of polarized cells. In colorectal tissues, the colorectal epithelium cells are also well polarized and this is essential for the normal absorption and secretion of colorectal organs.

The cellular apoptosis susceptibility (CAS) protein is the human homologue of the yeast chromosome segregation gene product, CSE1 [6]. CAS can regulate apoptosis induced by cypermethrin [7], interferon-γ [8], chemotherapeutic

drugs [9, 10], *Pseudomonas* exotoxin, diphtheria toxin, and tumor necrosis factors [11]. Pathological studies showed that CAS was highly expressed in various cancers, and the expression of CAS was positively correlated with high cancer stage, high cancer grade, and worse outcome in cancer patients (reviewed in [12]). Therefore, CAS may play an important role in regulating the development and progression of cancer. Our recent study showed that CAS regulates invasion and metastasis but not proliferation of cancer cells [13].

HT-29 cells, a colorectal epithelial cell line derived from human colorectal carcinoma, are frequently used in the study of colorectal epithelium secretion [14–17]. The effect of CAS overexpression on the secretion of HT-29 cells and the distribution of CAS in human colon and colorectal cancer were examined in this study. Our results suggest that CAS may play an important role in regulating the secretion of colorectal cells.

Materials and methods

Antibodies

Antibodies used in the experiment were anti-CAS (clone 24) and anti-cathepsin D (clone 49) (BD Pharmingen, San Diego, CA, USA), anti-CAS (clone 3D8) (Abnova, Taipei, Taiwan), anti-CEA (clone COL-1) (Thermo Fisher Scientific, Fremont, CA, USA), anti-α-tubulin (clone TU-01) (Zymed, South San Francisco, CA, USA), and goat anti-mouse IgG secondary antibodies coupled to Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA).

Vectors

We isolated total cellular RNA from HT-29 cells with the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction was carried out using the 1st-strand cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA). The reverse transcription reaction mixture (20 μl) containing 1 μg of DNase-treated total RNA, 20 pmol oligo $(dT)_{18}$ primer, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each of dNTP, 1 unit RNase inhibitor, and 200 units/μg RNA of MMLV reverse transcriptase was incubated at 42°C for 1 h. The PCR reaction was done in a 50 μl reaction mixture containing 5 μl of the reverse transcription reaction mixture, 100 ng each of primer, 0.3 mM Tris–HCl pH 8.0, 1.5 mM KCl, 1 μM EDTA, 1% glycerol, 0.2 mM each of dNTP, and 1 μl of 50× Advantage 2 polymerase mix (Clontech Laboratories). Primers used to amplify *CAS* cDNA were 5′-TATAGC AATGGAACTCAGCGATGC (sense) and 5′-AGTTTAA AGCAGTGTCACACTGGC (antisense). The DNA was amplified in a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA) for 35 cycles using the following parameters: 94°C for 30 s, 65°C for 30 s, and 72°C for 200 s with a final extension step at 72°C for 10 min. The amplified products were resolved in 1% agarose gel with ethidium bromide. The DNA was eluted and cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA), and was then subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen) to obtain pcDNA-CAS vector. The identity of the DNA sequences was determined by DNA sequencing.

Cells culture and vector transfection

Human 293 kidney cells and HT-29 colorectal cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cultures of cells were as previously described [18]. HT-29 cells grown in a non-polarized manner in medium containing high concentration (25 mM) of glucose, whereas in the absence of glucose the cells will grow in a polarized manner [19]. Therefore, we cultured the HT-29 cells in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate at 37°C under a humidified 5% CO_2 atmosphere.

Cells were transfected with vectors using the Lipofectamine plus reagent (Invitrogen). Transfected cells were selected with a high concentration of G418 for 3 weeks. Multiple drug-resistant colonies (>100) were pooled together and amplified in mass culture. The transfected cells were maintained in media containing 200 μg/ml G418. For the experiments, cells were cultured in media without G418.

Immunoblotting

Cells were washed with PBS and harvested by scraping. The harvested cells were washed with PBS and lysed in RIPA buffer (25 mM Tris–HC1 [pH 7.2], 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Fifty micrograms of each protein sample was resolved with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Buckinghamshire, UK). The membrane was blocked at 4°C for overnight with blocking buffer (1% BSA, 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20). The blots were incubated for 1 h at room temperature with primary antibodies followed by incubating

with secondary antibodies conjugated to horseradish peroxidase for 1 h. The levels of proteins were detected by enhanced chemiluminescence with an ECL Western blotting detection system (Amersham Pharmacia).

Immunofluorescence

Cells grown on coverslips (12 × 12 mm) were cytospun at 1000 rpm for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 4% paraformaldehyde, and blocked with PBS containing 0.1% bovine serum albumin (BSA) and 0.5% Tween-20. Samples were incubated with primary antibodies, washed with PBS, incubated with goat anti-mouse IgG secondary antibodies coupled to Alexa Fluor 568. DAPI (4′, 6-diamidino-2-phenylindole) was used to stain the nuclei. Samples were then examined with a Zeiss Axiovert 200 M inverted fluorescence microscope (Carl Zeiss, Jena, Germany).

Cell secretion assay

Equal numbers of cells (3×10^5 cells/dish) were seeded on 100-mm culture dishes. Cells were grown to sub-confluence, washed with PBS, and changed to media without FBS. After incubation for another 36 h, the conditioned media were harvested and the cell numbers were determined. The cell number standardized conditioned media were resolved in 10% SDS-PAGE and stained with Coomassie Blue-250.

CEA and cathepsin D secretion analysis

Equal numbers of cells (3×10^5 cells/dish) were seeded on 100-mm culture dishes. Cells were grown to sub-confluence. The conditioned media were harvested and the cell numbers were determined. The cell number standardized conditioned media were resolved in 10% SDS-PAGE and subjected to immunoblotting with the anti-CEA and anti-cathepsin D antibodies.

Immunohistochemistry

Immunohistochemistry was performed on 6-μm formalin-fixed/paraffin-embedded tissue sections. After deparaffinization in xylene and rehydration in graded ethanol, the sections were immersed in citrate buffer (pH 6.0) at 95°C for 10 min for antigen retrieval. Immunohistochemical detection was performed with the use of a labeled streptavidin-biotin method with a Histostain kit (Zymed, San Francisco, CA, USA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water, and nonspecific staining was blocked by incubation with 5% BSA for 1 h at room temperature. Sections were incubated with a 50-fold dilution of the clone 3D8 anti-CAS antibodies for 1 h at room temperature. Sections were then reacted with biotinylated secondary antibodies and peroxidase-labeled streptavidin. The sections were finally developed with diaminobenzidine and counterstained with Mayer's hematoxylin.

Results

CAS overexpression induces polarization of HT-29 cells

We transfected the HT-29 colorectal cells with a eukaryotic expression vector (pcDNA3.1) carrying the human *CAS* cDNA (i.e., the pcDNA-CAS vector). HT-29 cells overexpressing CAS (i.e., the HT-CAS cells) were found to organize themselves in a well-polarized manner (Fig. 1). The cells were in close contact and those at the edges of colonies regularly arrange themselves; furthermore, their lateral membrane domains were cohesively associated. Many large intercellular lumens were observed in colonies, and many polarized cells were organized around the lumens (Fig. 1). Polarity formation was hardly observed in the parent HT-29 cells and the HT-EV cells, an HT-29 cell line transfected with the empty pcDNA3.1 vector (Fig. 1). Thus, CAS regulates the polarization of HT-29 cells.

Vesicle-like staining of CAS in the cytoplasm near cell membrane and cell protrusions

The effect of CAS overexpression on cellular distribution of CAS was studied. CAS can associate with microtubules [6] and importin-α, a nuclear-transport receptor [20]. Therefore, CAS should show granule-like staining in the perinuclear areas of cells due to its association with importin-α, or show microtubule-like staining due to its association with microtubules in immunofluorescence microscopy. However, in addition to granule-like staining at the cytoplasm surrounding perinuclear areas, CAS also showed vesicle-like staining in the protrusions of HT-29 cells (Fig. 2a). Human 293 kidney cells have larger cytoplasm areas and longer cell protrusions, and vesicle-like staining of CAS in cell protrusions was more evident in human 293 kidney cells (Fig. 2b).

CAS overexpression induced polarization of HT-29 cells and thus the cells showed increased cell–cell contact (Fig. 2a). The results of immunofluorescence showed that enhanced CAS expression in HT-29 cells resulted in an increase in the distribution of the CAS-stained vesicles in the cytoplasm near cell membrane and cell protrusions (Fig. 2a, arrowheads); whereas in the HT-EV control cells,

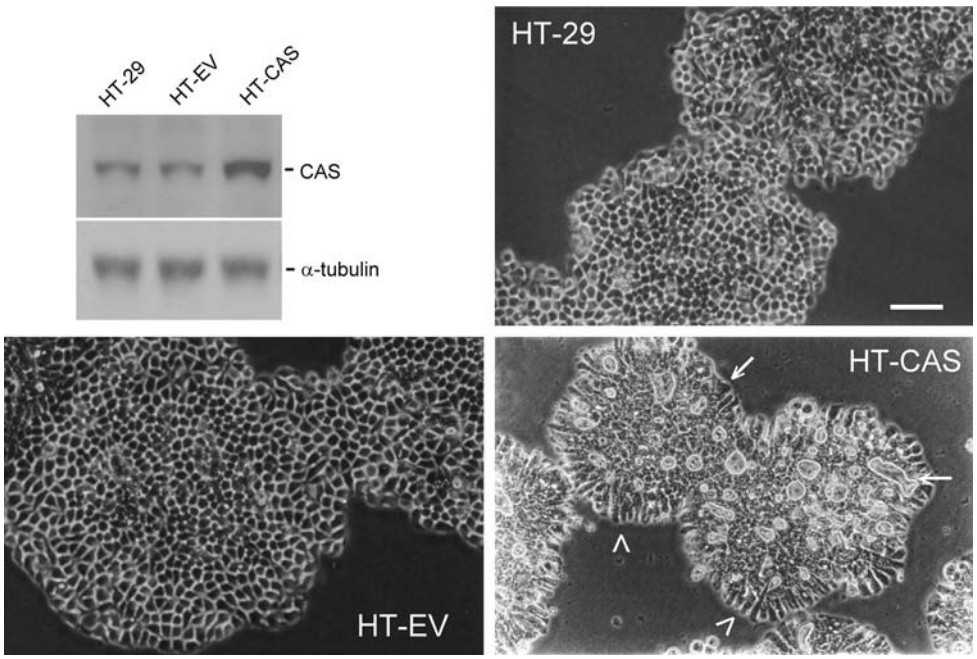

Fig. 1 CAS induces polarization of the HT-29 colorectal cells. The expression levels of CAS protein in HT-29, HT-EV, and HT-CAS cells were analyzed by immunoblotting with the clone 24 anti-CAS antibodies. HT-29, HT-EV, and HT-CAS cells were cultured in media for 6 days as described under Materials and methods and were photographed using a microscope. CAS overexpression stimulated polarization of HT-29 cells as shown by the regulatory cell arrangement at the edges of colonies (arrowheads) and the enhanced intercellular lumen (arrows) formation of HT-CAS cells. The scale bar represents 60 μm

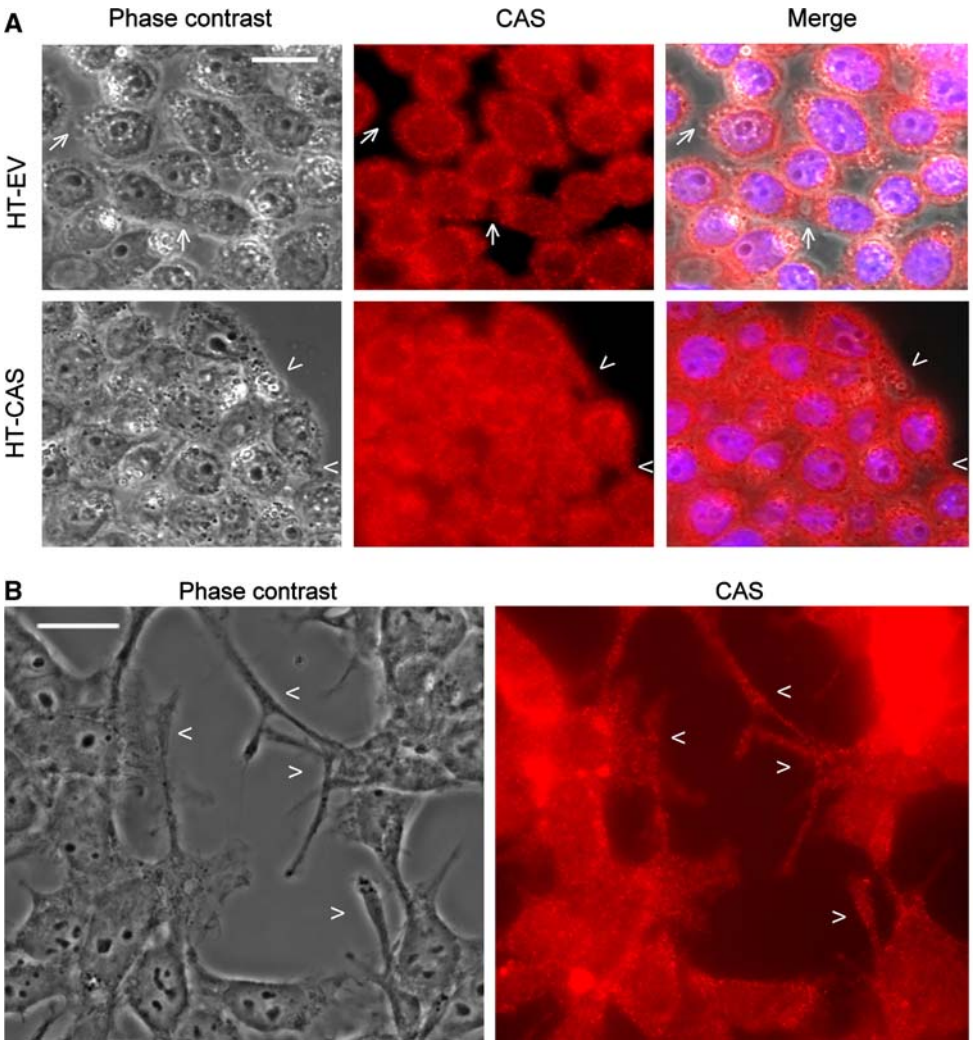

Fig. 2 Vesicle-like staining of CAS in the cytoplasm near cell membrane and cell protrusions. **a** HT-EV and HT-CAS cells were subjected to immunofluorescence with the clone 24 anti-CAS antibodies. DAPI was used to stain the nuclei. The fluorescence was examined with a fluorescence microscope. Note the increased distribution of CAS-stained vesicles in the cytoplasm near the membrane and protrusions of HT-CAS cells (arrowheads), and rare distribution of CAS-stained vesicles in the cytoplasm near the membrane and protrusions of HT-EV cells (arrows). The scale bar represents 10 μm. **b** Human 293 kidney cells were subjected to immunofluorescence with the anti-CAS antibodies. Note the vesicle-like staining of CAS in the cytoplasm near the membrane and protrusions of human 293 kidney cells (arrowheads). The scale bar represents 20 μm

the CAS-stained vesicle was mainly located in the cytoplasm surrounding perinuclear areas and rarely distributed in the cytoplasm near cell membrane and cell protrusions (Fig. 2a, arrows). Cytoplasm vesicles play an important role in regulating the secretion of cells [21]. Thus, these results indicate that CAS may play a role in regulating cell secretion.

CAS overexpression increases the secretion of HT-29 cells

The effect of CAS overexpression on the secretion of HT-29 cells was studied. In cell culture, we observed that the color of the conditioned media of HT-CAS cells were darker yellow when compared to that of HT-EV cells (Fig. 3a). The difference in the color of the conditioned media of HT-EV and HT-CAS cells indicates that the secretory abilities of the two cell lines might be different. We cultured HT-EV and HT-CAS cells to sub-confluence and changed the culture media to media without FBS. After incubation for 36 h, the conditioned media were harvested and the proteins were resolved with SDS-PAGE and stained with Coomassie Blue-250. The results showed that the intensity of protein bands of the conditioned media harvested from HT-CAS cells was obviously stronger than that of HT-EV cells (Fig. 3b). CAS overexpression induced polarization of HT-29 cells and reduced the growth of HT-CAS cells, thus the cell number of HT-CAS cells was lower than that of HT-EV cells (Fig. 3c). Hence, in the cell culture dishes, although HT-CAS cells were smaller in number, they secreted higher protein level into the conditioned media. These results indicate that CAS overexpression increased the secretion of HT-29 cells.

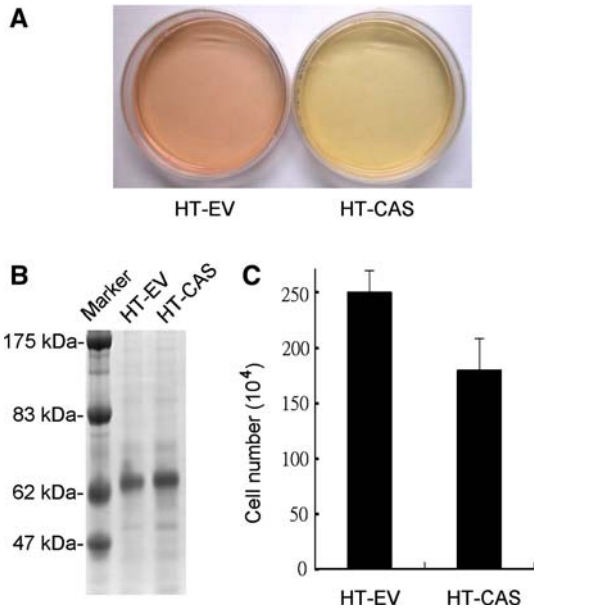

Fig. 3 CAS overexpression increases the secretion of HT-29 cells. **a** A representative photograph of the conditioned media of HT-EV and HT-CAS cells. HT-EV and HT-CAS cells were cultured for 6 days and the media were photographed. **b** The levels of secreted protein in the conditioned media of HT-EV and HT-CAS cells. The conditioned media harvested from serum-starved HT-EV and HT-CAS cells were resolved with SDS-PAGE and stained with Coomassie Blue-250. **c** The numbers of HT-EV and HT-CAS cells in the culture dishes determined by trypan blue exclusion method

CAS overexpression increases the secretion of CEA and cathepsin D from HT-29 cells

The HT-29 cell line is known to secret CEA and cathepsin D [15, 22]. The effects of CAS overexpression on the secretion of CEA and cathepsin D by HT-29 cells were studied. The conditioned media of HT-EV and HT-CAS cells were harvested, and the levels of CEA and cathepsin D in the conditioned media were analyzed with immunoblotting. The results showed that CAS overexpression induced a 4.6-fold increase in the CEA secretion level and induced a 4.2-fold increase in the cathepsin D secretion level from HT-29 cells (Fig. 4). Therefore, CAS regulated the secretion of CEA and cathepsin D by HT-29 cells.

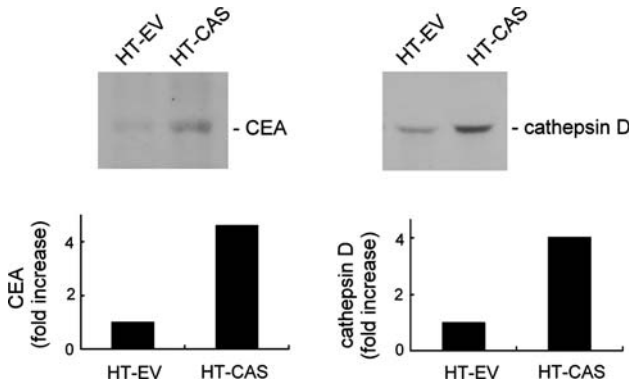

Fig. 4 CAS overexpression increases the secretion of CEA and cathepsin D from HT-29 cells. Conditioned media harvested from HT-EV and HT-CAS cells were subjected to immunoblotting with the anti-CEA and anti-cathepsin D antibodies as described under Materials and methods. The assays were repeated three times and showed similar results; a representative result is shown here

Expression of CAS in human colon and human colorectal cancer tissues

The expression of CAS in human colon was studied by immunohistochemistry with the clone 3D8 anti-CAS antibodies. The results showed that CAS was expressed in the cytoplasm of epithelium cells in human colon. Positive staining of CAS was mainly observed in the goblet cells and columnar cells of mucosa glands as well as cells in the crypts of Lieberkühn of human colon (Fig. 5a–c). The expression of CAS in human colorectal cancer tissue was also examined. CAS was heavily stained in the mucosa

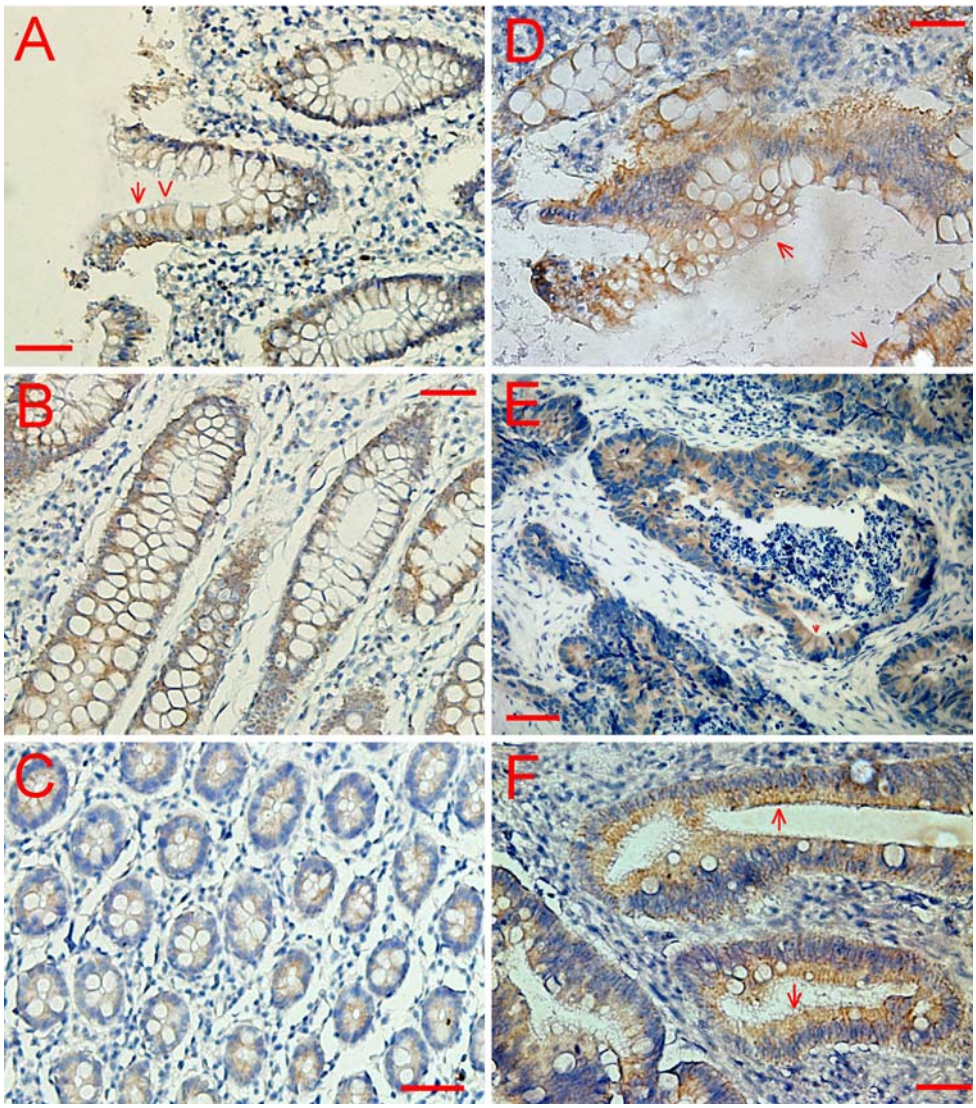

Fig. 5 Expression of CAS in human colon and human colorectal cancer tissues. The distribution of CAS in human colon and metastatic colorectal cancer tissues were analyzed by immunohistochemistry with the clone 3D8 anti-CAS antibodies. Note that CAS was positively stained in the goblet cells (**a**, arrow) and columnar cells (**a**, arrowhead) of colon mucosa as well as cells in the crypts of Lieberkuhn (**b**). Photo (**c**) shows the vertical sections of the crypts of Lieberkuhn. Photos **d–f** show the distribution of CAS in metastatic colorectal cancer tissues. Note that CAS was heavily stained in cancerous glands especially in the cytoplasm facing the gland lumen (arrows). The scale bar represents 50 μm

glands as well as other cancerous glands of the colorectal cancer tissues from patients with metastatic colorectal cancer (Fig. 5d–f). Moreover, heavy staining of CAS was found mostly in the cytoplasm facing the gland lumen (i.e., the secretory face of gland) of the colorectal cancer tissue (Fig. 5d–f, arrows).

Discussion

The establishment and maintenance of epithelial cell polarity are essential for the integrity and function of epithelial organs where vectorial re-absorption and secretion occur. Loss of, or disruption of, cell polarity can result in the formation of various diseases [23–25]. Our data showed that CAS overexpression induced polarization of the HT-29 human colorectal cells (Fig. 1). Therefore, CAS may play an important role in establishing the polarity of cells in colorectal tissue and maintaining the physiological function of human colorectal organ.

The colorectal epithelium has both absorptive and secretory functions, and this is polarity dependent. In colorectal tissues, absorption can be electroneutral via parallel Na^+/H^+ and Cl^-/HCO_3^- exchange [1], therefore has an impact on the electroneutral absorption of NaCl and the regulation of cellular and mucosal pH in the colorectal epithelium and other sections of the gastrointestinal tract [26, 27]. The polarized HT-29 cells have been shown to secrete Cl^- in response to secretagogues [28]. Our data showed that the color of the conditioned media of HT-CAS cells was darker yellow in compared to that of HT-EV cells (Fig. 3a). The change in the medium color of HT-CAS cells might be resulted from the induction of HT-29 cell polarity by CAS, and subsequently resulted in an increase

in the electroneutral absorption of NaCl and the regulation of cellular and mucosal pH of the cells, thereby changing the acidity and color of the media.

Our data showed CAS overexpression increased the secretion of HT-29 cells (Fig. 3). Polarity formation is essential for establishing the selective permeability barriers between biological compartments and the specialized vectorial functions in absorption, transcytosis, and secretion [4, 5]. CAS might increase the secretion of HT-29 cells by inducing the polarity of HT-29 cells. However, our data also showed vesicle-like staining of CAS in cell protrusions, and CAS overexpression increased the translocation of the CAS-stained vesicles to the cytoplasm near cell membrane and cell protrusions (Fig. 2a). Therefore, it is possible that CAS also regulates vesicle translocation and thereby increases the secretion of HT-29 cells.

Glands are the major epithelial components of tubular organs, and they mediate the passage and control of homeostasis by modifying secretion [29]. The crypts of Lieberkühn (or intestinal glands) are glands found in the epithelial lining of the small intestine and colon [30, 31]. Goblet cells and gland cells are highly polarized secretory cells in the colorectal glands and they are also the origins of colorectal carcinomas [32–34]. CAS is highly expressed in the goblet cells of mucosa glands and cells in the crypts of Lieberkühn of the human colon (Fig. 5a–c). And enhanced CAS expression is able to increase the secretion of the HT-29 colorectal cells (Fig. 3). These results possibly suggest that CAS regulates the secretion of human colon.

Pathological studies showed CAS was highly expressed in various cancers and the expression of CAS was positively correlated with high cancer grade, high cancer stage, and worse outcomes in cancer patients [12]. Our data also showed that CAS was heavily stained in the glands of the colorectal cancer tissues from patients with metastatic cancer (Fig. 5d–f). Moreover, we have reported recently that CAS regulates the invasion and metastasis of cancer cells [13]. Therefore, CAS may play an important role in regulating the secretion and the metastasis of colorectal cancer. Taken together, our results suggest that CAS regulates the secretion of colorectal cells and may play important roles in modulating the normal physiological functions of colorectal tissues as well as the development of colorectal diseases such as the metastasis of colorectal cancer.

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