Higher Prevalence of Secretory CSE1L/CAS in Sera of Patients with Metastatic Cancer

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Abstract

Metastatic markers are highly useful diagnostic and prognostic indicators of cancer metastasis. Herein, we report that secretory CSE1L/CAS, a cellular apoptosis susceptibility protein, is a new marker for metastatic cancer. CAS was colocalized with matrix metalloproteinase-2 in vesicles surrounding the outside of MCF-7 cell membranes, and the COOH-terminal domain of CAS was associated with matrix metalloproteinase-2-containing vesicles. Immunohistochemical staining for CAS was positive in the stroma and gland lumens of human metastatic cancer tissues. CAS was also detected in conditioned medium from B16-F10 melanoma cells and more frequently in the sera of patients with metastatic cancer than in sera from patients with primary cancer. Specifically, the prevalence of serum CAS in serum samples from 146 patients was 58.2% (32 of 55), 32.0% (8 of 25), and 12.1% (8 of 66) for patients with metastatic, invasive, and primary cancers, respectively. Our results suggest that CAS is a secretory protein associated with cancer metastasis, which may have clinical utility in metastatic cancer screening and diagnosis. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1570–7)

Introduction

Metastatic cancer cells frequently secrete proteins that enter the circulatory systems of cancer patients. These proteins may be useful for screening, cancer diagnosis and prognosis, assessment of therapeutic responses, and monitoring for cancer recurrence. Thus, secreted cancer cell markers are highly beneficial in the diagnosis and treatment of cancers (1-7).

Matrix metalloproteinases (MMP), including MMP-2, are enzymes involved in the degradation of the extracellular matrix, which show increased expression during the progression of various diseases, including cancer metastasis (8). Experiments have shown that MMP production can be regulated at the level of secretion (9). After cellular protein production, MMPs are stored in vesicles, which can be rapidly secreted in response to angiogenic stimuli (10). Metastatic tumor cells often develop enhanced secretory abilities to enhance MMP secretion, thereby enhancing their metastatic potential (11).

CAS is the human homologue of the yeast chromosome segregation gene, *CSE1* (12). CAS can regulate apoptosis induced by IFN-γ (13) and chemotherapeutic drugs (14, 15). In pathologic studies, CAS expression has been positively correlated with high cancer grade, high cancer stage, and worse patient outcomes (16). Although CAS has been regarded as a proliferation-associated protein (12, 16), our recent studies indicated that CAS regulates metastasis and not proliferation (17). Herein, we report the further characterization of CAS as a secretory protein present in conditioned medium from B16-F10 cells, a highly metastatic cancer cell line, and in the sera of patients with metastatic cancer.

Materials and Methods

Antibodies. The anti-CAS antibodies used in the experiment were clone 24 (BD Biosciences), clone 3D8 (Abnova), C-20 (Santa Cruz Biotechnology), and AP1935a (Abgent). Other antibodies used were anti-MMP-2 (H-76; Santa Cruz Biotechnology), anti-GFP (Ab-1), anti-β-actin (Ab-5; Lab Vision), and goat anti-mouse (or anti-rabbit) IgG secondary antibodies coupled to Alexa Fluor 488 (or Alexa Fluor 568; Molecular Probes).

Cells. MCF-7 breast cancer cells and B16-F10 melanoma cells were from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamate at 37°C in a 5% CO_2 atmosphere (18). Previously reported experimental cell lines included MCF-CAS cells (pcDNA-CAS vector-transfected MCF-7 cells), MCF-EV cells (pcDNA3.1 empty vector-transfected MCF-7 cells), B16-CAS cells (pcDNA-CAS vector-transfected B16-F10 cells), and B16-EV cells (pcDNA3.1 empty vector-transfected B16-F10 cells; ref. 17).

Immunoblotting. Samples were resolved with 8% SDS-PAGE. Proteins were transferred to nitrocellulose

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membranes (Amersham Pharmacia) and immunoblotting was done using specific antibodies and an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia) as described previously (17).

Immunofluorescence. Briefly, cells grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Coverslips were incubated with primary antibodies followed by secondary antibodies coupled to Alexa Fluor 488 or 568. Labeled cells were observed with a Zeiss Axiovert 200 M inverted fluorescence microscope (Carl Zeiss).

MMP-2 and CAS Secretion Analyses. Equal numbers of cells were seeded onto 100 mm culture dishes. Because FBS contains a high level of endogenous MMP-2 and may also have interfered with the assay of CAS in the immunoblotting, cells were grown to subconfluence, washed with PBS, and then incubated in medium without serum for 36 h. The conditioned medium was then collected and centrifuged at 10,000 rpm for 10 min, and supernatants were harvested. Cell numbers were determined and cell number-standardized conditioned medium samples were subjected to immunoblotting with anti-MMP-2 and anti-CAS antibodies.

Matrigel-Based Invasion Assay. The Matrigel-based invasion assay was done using Matrigel (BD Biosciences) and 8 μm pore-sized polyvinylpyrrolidone-free polycarbonate filters (Costar). The filters were soaked in Matrigel diluted 20-fold with DMEM and the invasion assay was done as described previously (17).

Serum Samples. Serum samples from 47 healthy donors were obtained with informed consent. The mean ± SD donor age was 58.5 ± 11.6 years (range, 17-79 years). Cancer serum samples were from 89 patients admitted to Tungs' Taichung MetroHarbor Hospital and 57 colorectal cancer patients admitted to Changhua Christian Hospital. Samples were obtained at the time of diagnosis with informed consent using institutional review board-approved guidelines and included 66 patients with primary cancer (mean age, 56.2 ± 17.0 years; range, 20-91 years), 25 patients with invasive cancer (mean age, 51.3 ± 16.6 years; range, 43-84 years), and 55 patients with metastatic cancer (mean age, 61.9 ± 14.9 years; range, 26-94 years). Samples were collected and the blood was incubated at room temperature for a minimum of 30 min to allow clots to form. Samples were then centrifuged at 1,300 × *g* at 4°C for 20 min, and serum was collected and stored at -80°C for later analyses. All samples were labeled with a unique identifier to protect the confidentiality of the patients. None of the samples were thawed before analysis. To remove possible suspended cells or cell debris, thawed samples were centrifuged at 10,000 rpm for 10 min and the supernatants were used for CAS analysis.

Establishment of the CAS-GFP Fusion Protein-Expressing Cells. The GFP fusion protein expression vectors, GFP-C-CAS, were constructed by PCR amplification of the restriction enzyme-linearized pcDNA-CAS vectors (17) with primers CCTGTCCTTAAAGCTGACGGTATC (sense) and AGTTTAAAGCAGTGTCACACTGGC (antisense). The amplified products were cloned into the pcDNA3.1 GFP fusion protein expression vector using the GFP Fusion TOPO TA Expression Kit (Invitrogen). DNA sequences were verified by DNA sequencing. MCF-7 cells were transfected with GFP-C-CAS vectors using the Lipofectamine Plus reagent (Invitrogen). The transfected cells were selected with a high concentration of G418 for 3 weeks. Multidrug-resistant colonies (>100) were pooled together and expanded in mass culture. The expression of CAS-GFP fusion proteins in transfected cells was evaluated by immunoblotting with anti-GFP antibodies. Transfected cells were maintained in medium containing 200 μg/mL G418. For the experiments described herein, cells were cultured in medium without G418.

Immunohistochemistry. Immunohistochemistry was done on 6-μm-thick, formalin-fixed/paraffin-embedded cancer tissue sections (14 breast cancer tissues and 24 colorectal cancer tissues) using a 50-fold dilution of specific antibodies. Immunohistochemical detection was done using a labeled streptavidin-biotin method with the Histostain kit according to the manufacturer's instructions (Zymed). Sections were developed with diaminobenzidine, washed with distilled water, and counterstained with Mayer's hematoxylin.

GST-CAS Fusion Protein Production and CAS Purification. The GST-CAS fusion protein expression vector was constructed by PCR amplification of the restriction enzyme-linearized pcDNA-CAS vectors with primers *GGATCC*ATGGAACTCAGCGATGCAAATCTG (sense; *Bam*HÉ site is italicized) and *CTCGAG*TTAAAGGCAGTGTCACACTGGCTG (antisense; *Xho*É site is italicized). The amplified products were subcloned into the *Bam*HÉ and *Xho*É sites of pGEX-4T-1 vector (Amersham Pharmacia) to obtain pGEX-CAS vector. The pGEX-CAS vector was transformed into the *Escherichia coli* strain Rosetta (DE3)/pLysS (Novagen) and the GST-CAS fusion protein was induced with 0.1 mmol/L IPTG for 5 h. GST-CAS fusion proteins were purified using glutathione-Sepharose 4B beads and Bulk GST Purification Modules (Amersham Pharmacia) and then cleaved with thrombin (3 units/100 μg fusion protein; Sigma) at 22°C for 16 h. The thrombin and GST were removed using the Amicon Ultra-4 Centrifugal Filter Units (Millipore). The purified CAS protein was verified by immunoblotting with anti-CAS antibodies and the protein concentration was determined with a BCA protein assay kit (Pierce).

ELISA. Anti-CAS (C-20 or clone 24) antibody-coated 96-well plates (Costar) were blocked with 5% bovine serum albumin in PBS for 1 h. Wells were washed with PBS and then incubated with serum samples (3-fold dilution with PBS) for 1 h. After washing with PBS-0.05% Tween 20, wells were incubated with biotin-conjugated rabbit anti-CAS antibodies for 1 h. The biotin-conjugated rabbit anti-CAS antibodies were prepared by biotinylating AP1935a rabbit anti-CAS antibodies using the Biotin Labeling Kit-NH_2 (Dojindo Laboratories). Wells were then washed with PBS-0.05% Tween 20 and reacted with streptavidin-conjugated horseradish peroxidase (R&D Systems) followed by incubation with substrate reagent (R&D Systems). For calibration, three blank wells containing PBS were used as the background value, and three wells that were not coated with the anti-CAS antibodies but did react with all other ELISA reagents

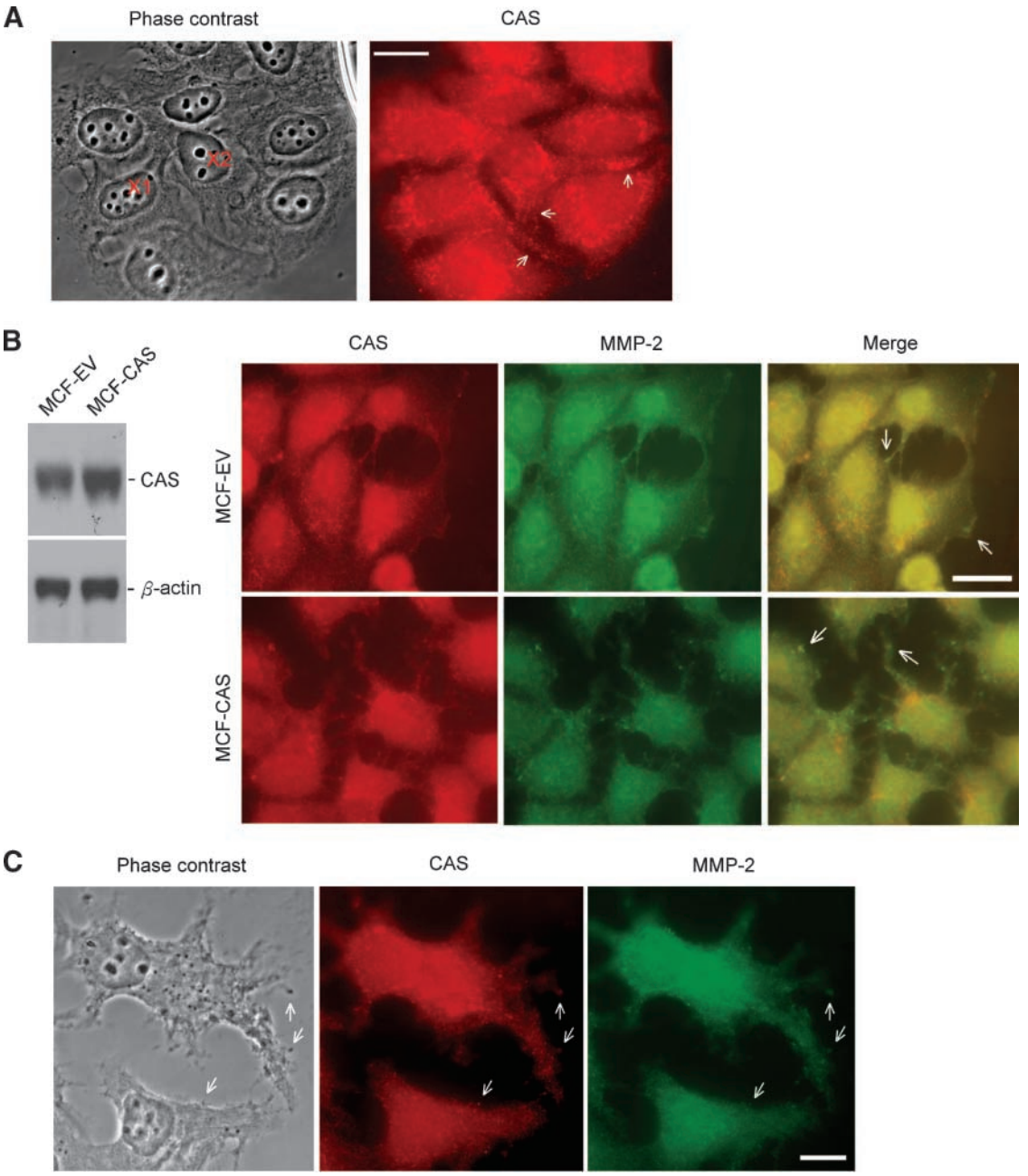

Figure 1. Colocalization of CAS with MMP-2 in vesicles surrounding the outside of MCF-7 cell membrane. **A,** immunofluorescent localization of CAS. Note the vesicle-like staining of CAS (*arrows*) in the protrusions of cells marked with X1 and X2. Bar, 30 μm. **B,** immunofluorescent analysis of CAS and MMP-2 distributions in MCF-EV and MCF-CAS cells. Expression of CAS in MCF-EV and MCF-CAS cells was also analyzed by immunoblotting with anti-CAS antibodies. *Arrows,* some areas of CAS and MMP-2 colocalization. Note the increased distribution of CAS and MMP-2 in the tip and the edge ends of the protrusions of MCF-CAS cells. Bar, 20 μm. **C,** immunofluorescent colocalization of CAS with MMP-2 in vesicles surrounding the outside of MCF-7 cell membranes. *Arrows,* some areas of CAS and MMP-2 colocalization in vesicles surrounding the outside of the cell membrane. Bar, 30 μm.

were used as control wells. The absorbance at 450 nm was measured within 30 min with a Thermo Multiskan EX Microplate Photometer (Thermo Fisher Scientific). Absorbance values of sample wells that were higher than the highest absorbance value of the control wells were considered CAS-positive. Each sample was assayed two times. The C-20 anti-CAS antibody was used in the first assay and the clone 24 anti-CAS antibody was used in the second assay. The results of the two assays were very similar, indicating the accuracy of the assay.

Statistical Analysis. Data were analyzed using SPSS 14.0 statistic software (SPSS). Statistical differences were analyzed by two-tailed χ^2 test. An α level of 0.05 or 0.01 was considered statistically significant.

Results

Colocalization of CAS with MMP-2-Containing Vesicles. The distribution of CAS in MCF-7 cells was studied by immunofluorescence using the clone 24 anti-CAS antibody. CAS can associate with microtubules (12) and the nuclear transport receptor, importin-α (19). Therefore, CAS was predicted to 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. However, in addition to granule-like staining in cytoplasm surrounding the perinuclear areas, CAS also showed vesicle-like staining in the protrusions of MCF-7 cells (Fig. 1A). Cytoplasm vesicles play an important role in regulating the exocytosis and secretion of cells (20). The vesicle-like staining of CAS in cell protrusions indicates that CAS may play a role in regulating cell secretion.

Because MMP-2 is a secretory protein, we studied the effect of CAS overexpression on the cellular distribution of MMP-2 using double-stain immunofluorescence. MMP-2 is reported to be located in cytoplasm. In addition to cytoplasmic localization, our data showed that MMP-2 was also distributed in the nuclei of MCF-7 cells (Fig. 1B). MMP-2 localization in the nuclei of cardiac myocytes has been correlated with the cleavage of poly(ADP-ribose) polymerase (21). Therefore, it is reasonable that MMP-2 might also be present in the nuclei of MCF-7 cells in our study. We also observed that CAS was colocalized with MMP-2 (Fig. 1B). In MCF-EV cells, minor colocalization of CAS with MMP-2 was found in the cell protrusions. However, much greater colocalization of CAS with MMP-2 in MCF-CAS cell protrusions, especially at the tip and the edge ends of the protrusions (Fig. 1B). These results indicate that CAS is associated with MMP-2-containing vesicles and that enhanced CAS expression facilitates the translocation of MMP-2-containing vesicles to cell protrusions.

With high-resolution imaging, colocalization of CAS with MMP-2 was observed in the cytoplasmic areas near the cell membrane as well as in the protrusions of MCF-CAS cells (Fig. 1C). In particular, some CAS was colocalized with MMP-2 in vesicles surrounding the outside of the cell membrane (Fig. 1C, *arrows*). Because MMP-2 is a secretory protein, these results suggest that CAS may be secreted together with MMP-2.

COOH-Terminal Domain of CAS Interacts with MMP-2-Containing Vesicles. The DNA sequence corresponding to the COOH-terminal domain of CAS (1,432-2,919 bp) was cloned downstream of the pcDNA3.1/NT-GFP vector to obtain the GFP-C-CAS vector, which expressed GFP fused to the N-end of COOH-terminal CAS. MCF-7 cells transfected with GFP-C-CAS vectors (the MCF-GFP-C-CAS cells) were used to study the interaction of CAS with MMP-2-containing vesicles. Immunofluorescent staining showed that the GFP-C-CAS fusion protein colocalized with MMP-2-containing vesicles (Fig. 2). In the control assays, MCF-7 cells transfected with the empty pcDNA3.1/NT-GFP control vectors (the MCF-NT-GFP cells) showed no specific colocalization of the GFP protein with MMP-2-containing vesicles (Fig. 2).

Enhanced CAS Expression Increases B16-F10 Cell Invasion. B16-F10 cells were separately transfected with the empty pcDNA3.1 vector or the pcDNA-CAS vector to

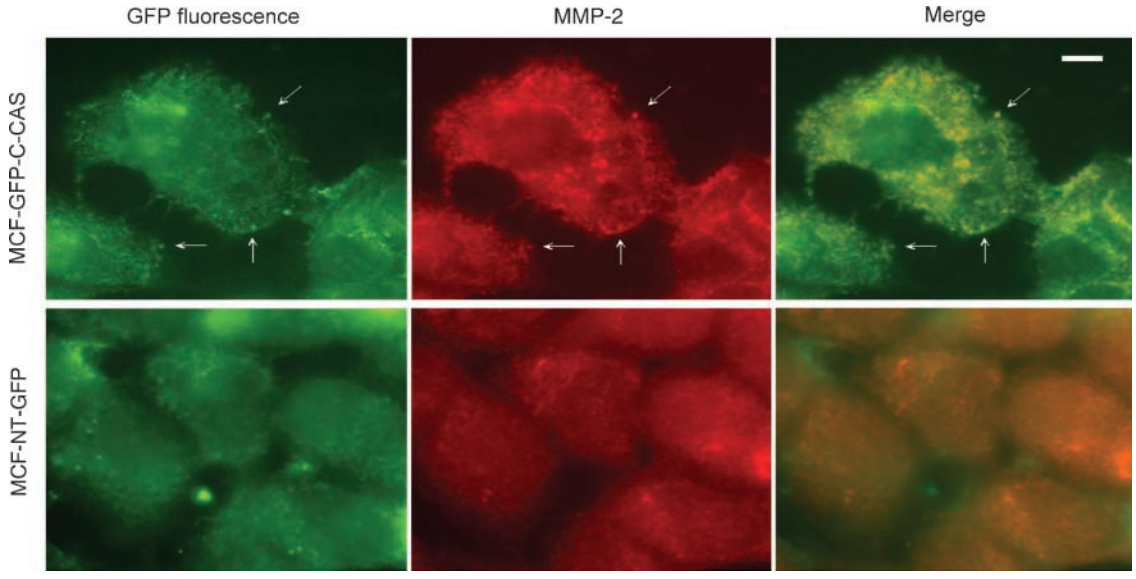

Figure 2. COOH-terminal domain of CAS interacts with MMP-2-containing vesicles. MCF-7 cells stably transfected with GFP-C-CAS vectors were counterstained with anti-MMP-2 antibodies and analyzed by immunofluorescence. Cells transfected with the empty pcDNA3.1/NT-GFP vectors were assayed as the control. *Arrows,* areas of GFP-C-CAS fusion protein and MMP-2 colocalization. Bar, 20 μm.

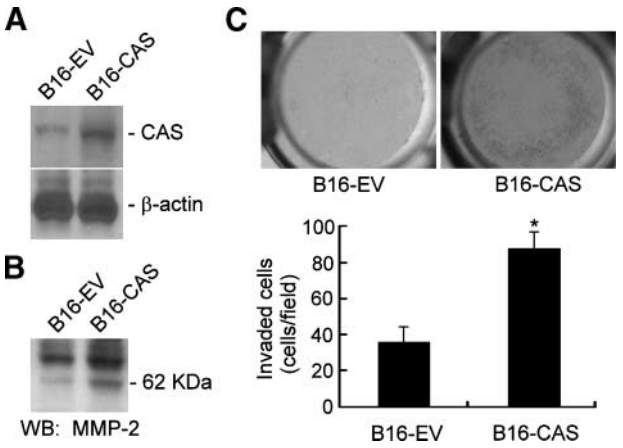

Figure 3. Enhanced CAS expression increases the invasiveness of B16-F10 melanoma cells. **A,** levels of CAS expressed in B16-EV and B16-CAS cells analyzed by immunoblotting with anti-CAS antibodies. **B,** immunoblot analysis of MMP-2 protein in B16-EV and B16-CAS cell conditioned medium. Assays were repeated three times with similar results. Representative immunoblot. **C,** Matrigel-based B16-EV and B16-CAS cell invasion assays. *Top,* representative photograph of the invading cells. Mean of three independent experiments. *, $P = 0.0019$.

obtain B16-EV and B16-CAS cells, respectively (Fig. 3A). Immunoblot analysis of the conditioned medium harvested from B16-EV and B16-CAS cells showed that MMP-2 secretion from B16-F10 cells was enhanced by enhanced CAS expression (Fig. 3B).

The effect of CAS overexpression on the invasive ability of B16-F10 cells was assayed using a Matrigel-based invasion assay. The results showed that CAS overexpression increased B16-F10 cell invasiveness by 249.2%. The mean ± SD number of invasive cells was 35.9 ± 9.4 and 89.5 ± 10.7 cells per field for B16-EV and B16-CAS cells, respectively (Fig. 3C).

CAS Is Positively Stained in the Stroma and Gland Lumen of Cancer Tissues. The distribution of CAS in human metastatic cancer tissues was analyzed by immunohistochemistry. MMP-2 is a secretory protein, and the control assay showed MMP-2 positive staining in the stroma and gland lumen of colorectal cancer tissues (Fig. 4A and B). CAS was mainly localized in the cytoplasm of gland cells in metastatic colorectal (Fig. 4C and D) and breast (Fig. 4E and F) cancer tissues. CAS staining was also present in the stroma of colorectal (Fig. 4C, *arrowhead*) and breast (Fig. 4E, *arrowheads*) cancer tissues. In addition, positive CAS staining was also noted in the gland lumen of colorectal (Fig. 4D, *arrow*) and breast (Fig. 4F, *arrows*) cancer tissues. The presence of CAS in the stroma and gland lumen of colorectal and breast cancer tissues indicates that CAS protein is secreted in cancer tissues.

CAS Is Also a Secretory Protein. Evaluation of CAS as a secretory protein was also assessed by immunoblotting with clone 24 and C-20 anti-CAS antibodies. FBS contains various substances and may have interfered with our assay. Therefore, B16-F10 cells were grown to subconfluence, washed with PBS, and cultured in medium without FBS. The results showed that CAS was present in the conditioned medium of serum-starved B16-F10 cells (Fig. 5A, *lane 11*). Furthermore, the results of immunoblotting showed that the level of secretory CAS was higher in the conditioned medium of serum-starved B16-CAS cells than levels in serum-starved B16-EV cells (Fig. 5B).

Serum samples collected from patients with metastatic cancer were used for assaying the presence of secretory CAS in serum. All of the metastatic cancer cases were pathologically diagnosed as having distant tumor metastases to other organs or lymph nodes. Unlike serum-starved conditioned medium or total cellular protein extracted from cultured cells, human serum contains various substances that might affect immunoblotting. Therefore, in our results, many CAS protein bands were not very sharp. Nevertheless, some samples showed sharp CAS protein bands present in the serum immunoblots (Fig. 5A, *lanes 6* and *8*), indicating that CAS is present in sera of patients with metastatic cancer.

High Prevalence of Secretory CAS in Sera of Patients with Metastatic Cancer. ELISA assays were done to study the prevalence of CAS in the sera of cancer patients. Western blotting showed that the anti-CAS antibodies used in the ELISA are specific to CAS protein (Fig. 6A). The results of ELISA assay showed that serum CAS was detected in 58.2% (32 of 55), 32.0% (8 of 25), and 12.1% (8 of 66) of patients with metastatic, invasive, and primary cancers, respectively (Fig. 6B). The prevalence of serum CAS detected in healthy donors was 6.4% (3 of 47; Fig. 6B). The P values were <0.001 between metastatic group and healthy group, <0.001 between metastatic group and primary group, 0.043 between metastatic group and invasive group, <0.05 between invasive group and healthy group, 0.023 between invasive group and primary group, and 0.431 between primary group and healthy group. With the use of the purified CAS protein as a standard, the cutoff value of CAS in the sera of patients with metastatic cancer was determined to be ≥3 ng/mL.

The presence of secretory CAS in the sera of patients with metastatic cancer was not restricted to a specific cancer type. Analyses of serum samples from patients with metastatic cancer showed that serum CAS was detected in 60.0% (18 of 30) of colorectal cancer, 75% (6 of 8) of breast cancer, 75% (3 of 4) of lung cancer, 75% (3 of 4) of cervical cancer, 100% (1 of 1) of bile duct cancer, 0% (0 of 2) of esophageal cancer, 0% (0 of 1) of ovarian cancer, 0% (0 of 1) of oviduct omental cancer, and 25% (1 of 4) of head and neck cancer (Fig. 6C). For invasive cancers, serum CAS was detected in 20% (3 of 15) of head and neck cancer, 40% (2 of 5) of esophageal cancer, 50% (1 of 2) of breast cancer, 50% (1 of 2) of cervical cancer, and 100% (1 of 1) of ovarian cancer. For primary cancers, serum CAS was detected in 12% (4 of 33) of head and neck cancer, 14.8% (4 of 27) of colorectal cancer, 0% (0 of 3) of lung cancer, 0% (0 of 1) of ribbed cancer, 0% (0 of 1) of liver cancer, and 0% (0 of 1) of esophageal cancer. These results showed that serum CAS was more prevalent in patients with metastatic cancer.

Discussion

CAS is reported to be highly expressed in a variety of cancers (16). Owing to its ability to associate with

microtubules (12), the organelles for cell mitosis, CAS has been thought to regulate the cell cycle progression of cancers by stimulating cancer proliferation and thus has been regarded as a proliferation-associated protein (12, 16). However, there are currently no reports of experiments showing that CAS can stimulate the proliferation of cancer cells. We recently reported that CAS regulates invasiveness and metastasis but not the proliferation of cancer cells (17). The present data showed that CAS is a secretory protein associated with cancer metastasis and that CAS is more frequently detected in the serum of patients with metastatic cancer than in patients with primary cancer. Although CAS undoubtedly plays an important role in cancer development, our findings define a new role for CAS in facilitating cancer development and which may prove useful in the diagnosis and treatment of cancers.

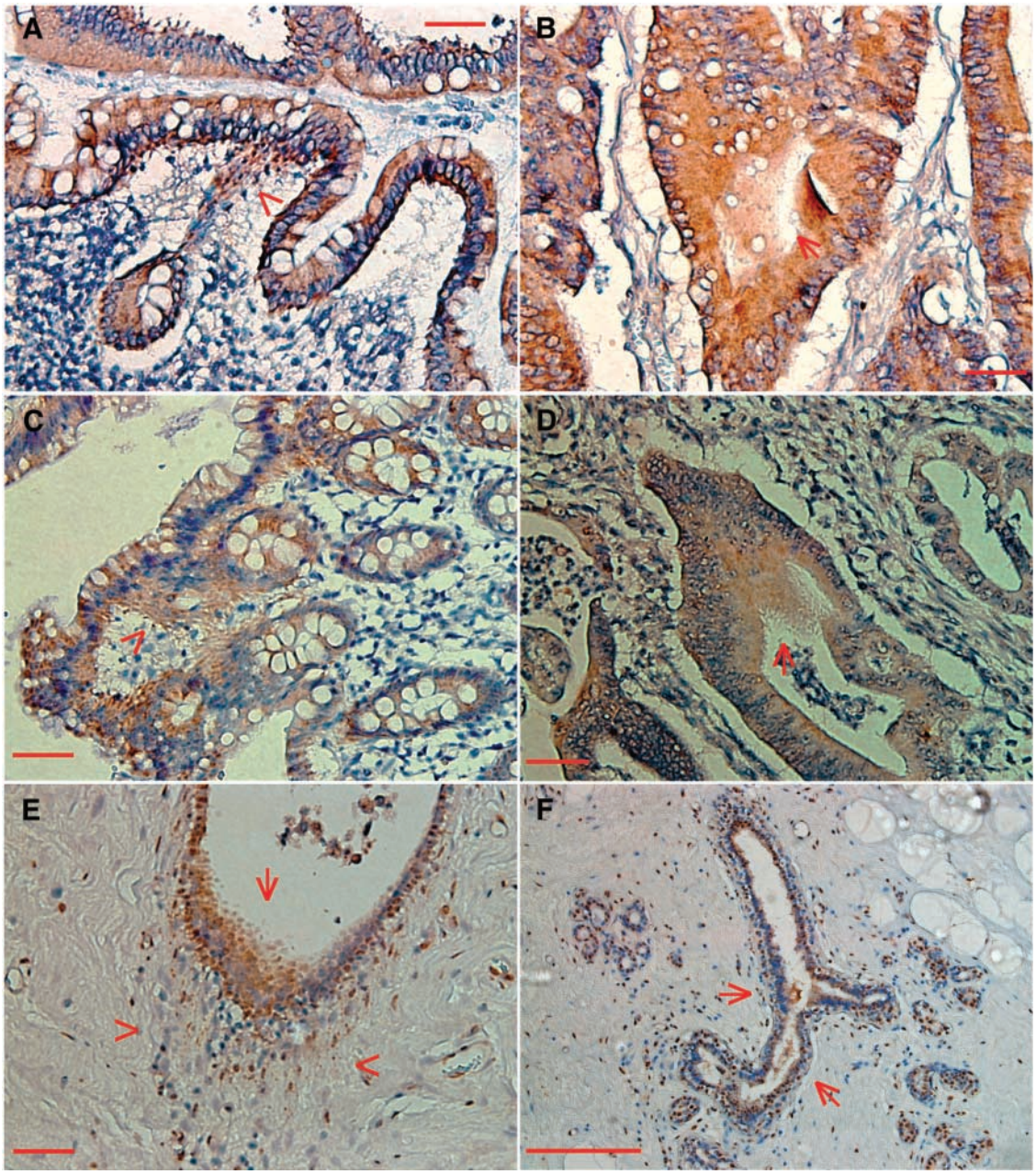

Figure 4. CAS staining in the stroma and gland lumen of cancer tissues. Distributions of CAS in metastatic colorectal (**C** and **D**) and breast (**E** and **F**) cancer tissues were analyzed by immunohistochemistry with clone 3D8 anti-CAS antibodies. Note the positive staining of CAS in the stroma (**C** and **E**, *arrowheads*), and gland lumen (**D** and **F**, *arrows*) of cancer tissues. MMP-2 was positively stained in the stroma (**A**, *arrowhead*) and gland lumen (**B**, *arrow*) of colorectal cancer tissues. Bar, 50 μm.

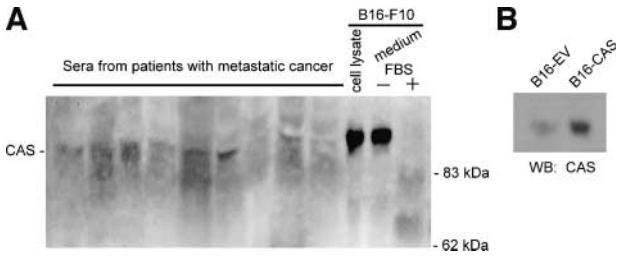

Figure 5. CAS protein in B16-F10 cell conditioned medium and the sera of patients with metastatic cancer. **A,** immunoblot analysis of serum samples collected from patients with metastatic cancer and the conditioned medium of B16-F10 cells with anti-CAS antibodies. Each well was loaded with 30 μL cancer serum sample or 60 μL conditioned medium collected from B16-F10 cells cultured in medium with or without FBS. A well was loaded with the B16-F10 total cell lysate as a control. **B,** enhanced CAS expression increased the secretion of CAS in the conditioned medium. Conditioned medium collected from serum-starved B16-EV and B16-CAS cells were subjected to immunoblotting with anti-CAS antibodies. Each immunoblot was repeated three times and showed similar results. Representative immunoblots.

MMPs, including MMP-2, are involved in several pathologic processes (22). Newly synthesized MMPs are stored in secretory vesicles that are secreted or anchored to the cell surface, thereby maintaining a locally high enzyme concentration and targeting their catalytic activity to specific substrates within the pericellular space surrounding cancer cells (22). Mechanisms regulating the secretion of MMP-containing vesicles are largely unknown. Our studies showed that CAS regulates the translocation and secretion of MMP-2-containing vesicles (Figs. 1 and 3). Hence, CAS may be a potential target for novel therapies developed for treatment of deleterious MMPs-mediated diseases.

Our data showed that CAS was detected in the cytoplasm of gland cells, stroma, and the gland lumen of different cancer tissues (Fig. 4). The tumor microenvironment, or stroma, consists of extracellular matrix and plays an important role in regulating cancer metastasis (23). Glands, the major epithelial components of tubular organs, mediate the passage and control of homeostasis by modifying secretion. Glands in cancer tissues also provide the metastatic cancer cells with a route for invasion of adjacent tissues or other organs (24). CAS staining in the cytoplasm of gland cells, stroma, and the gland lumen of cancer tissues indicate that CAS has play important roles in cancer secretory processes as well as in cancer metastasis. Moreover, substances that are secreted from a gland lumen can ultimately reach blood vessels (25). CAS staining in the gland lumen of metastatic cancer tissues may possibly explain the detection of CAS in the serum of patients with metastatic cancer.

In assaying the presence of secretory CAS, the conditioned medium and serum samples were centrifuged, and only the supernatants were used in the study. Thus, detection of secretory CAS was not the result of contamination by suspended cells or cell debris in the samples. The CAS protein bands on immunoblots were very intense, especially for samples of conditioned

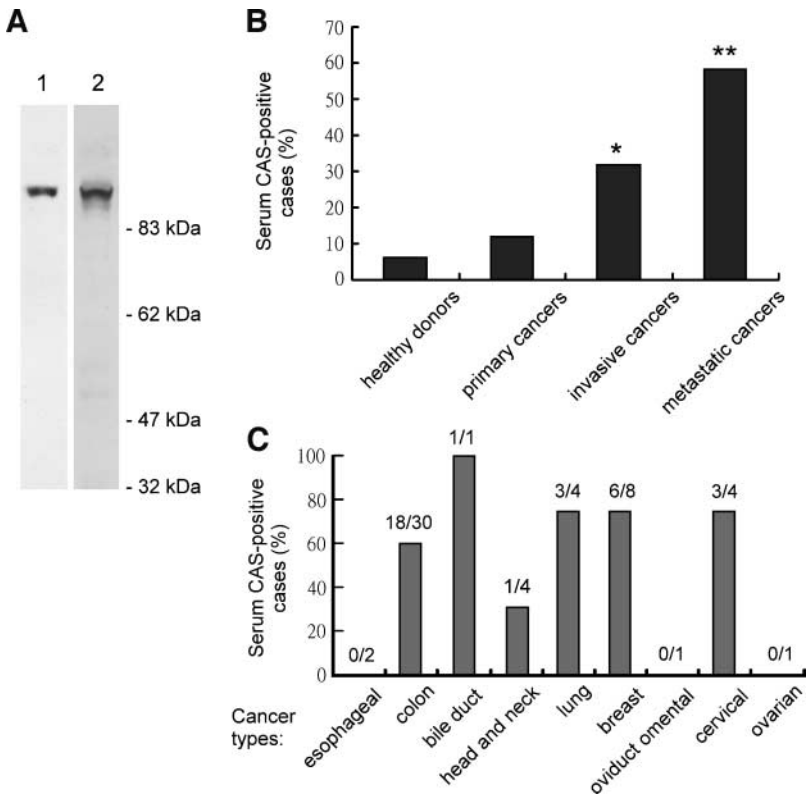

Figure 6. Higher prevalence of CAS in sera of patients with metastatic cancer. **A,** characterization of the specificity of CAS antibodies used in the ELISA assay. Expression of CAS in MCF-7 cells was analyzed by immunoblotting with C-20 (*lane 1*) and AP1935a (*lane 2*) anti-CAS antibodies. **B,** prevalence of CAS detected in the sera of healthy donors and patients with primary, invasive, and metastatic cancers as determined by ELISA. *, **, statistically significant compared with that of the primary cancer group. **C,** ratio of serum CAS detected in metastatic cancers. The number of serum CAS-positive cases to the total number of each cancer type is also indicated.

medium (Fig. 5). The reported molecular weight of CAS (12) and the molecular weight of CAS protein in the conditioned medium and patient serum samples (Fig. 5) were both ~110 kDa. Hence, the CAS protein we identified did not originate from CAS protein released from broken or lysed cells contained in the conditioned medium or cancer serum samples.

In conclusion, CAS is a secretory protein associated with cancer metastasis and is present in sera of patients with metastatic cancer. CAS may have clinical utility in metastatic cancer screening and diagnosis and may also be a potential target for future antimetastasis therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Brenner DE, Normolle DP. Biomarkers for cancer risk, early detection, and prognosis: the validation conundrum. Cancer Epidemiol Biomarkers Prev 2007;16:1918–20.
2. Petricoin EF, Belluco C, Araujo RP, Liotta LA. The blood peptidome: a higher dimension of information content for cancer biomarker discovery. Nat Rev Cancer 2006;6:961–7.
3. Zhang H, Chan DW. Cancer biomarker discovery in plasma using a tissue-targeted proteomic approach. Cancer Epidemiol Biomarkers Prev 2007;16:1915–7.
4. Wang H, Li M, Lin W, et al. Extracellular activity of cyclic AMP-dependent protein kinase as a biomarker for human cancer detection: distribution characteristics in a normal population and cancer patients. Cancer Epidemiol Biomarkers Prev 2007;16:789–95.
5. Skates SJ, Horick NK, Moy JM, et al. Pooling of case specimens to create standard serum sets for screening cancer biomarkers. Cancer Epidemiol Biomarkers Prev 2007;16:334–41.
6. Rodrigues LR, Teixeira JA, Schmitt FL, Paulsson M, Lindmark-Mänsson H. The role of osteopontin in tumor progression and metastasis in breast cancer. Cancer Epidemiol Biomarkers Prev 2007;16:1087–97.
7. Selander KS, Brown DA, Sequeiros GB, et al. Serum macrophage inhibitory cytokine-1 concentrations correlate with the presence of prostate cancer bone metastases. Cancer Epidemiol Biomarkers Prev 2007;16:532–7.
8. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–73.
9. Ben-Yosef Y, Lahat N, Shapiro S, Bitterman H, Miller A. Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res 2002;90:784–91.
10. Nguyen M, Arkell J, Jackson CJ. Active and tissue inhibitor of matrix metalloproteinase-free gelatinase B accumulates within human microvascular endothelial vesicles. J Biol Chem 1998;273:5400–4.
11. Moser TL, Young TN, Rodriguez GC, Pizzo SV, Bast RC Jr, Stack MS. Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma. Int J Cancer 1994;56:552–9.
12. Brinkmann U, Brinkmann E, Gallo M, Pastan I. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. Proc Natl Acad Sci U S A 1995;92:10427–31.
13. Jiang MC, Luo SF, Li LT, et al. Synergic CSE1L/CAS, TNFR-1, and p53 apoptotic pathways in combined interferon-γ/Adriamycin-induced apoptosis of Hep G2 hepatoma cells. J Exp Clin Cancer Res 2007;26:91–9.
14. Liao CF, Luo SF, Shen TY, et al. CSE1L/CAS, a microtubule-associated protein, inhibits Taxol (paclitaxel)-induced apoptosis but enhances cancer cell apoptosis induced by various chemotherapeutic drugs. BMB Rep 2008;41:210–6.
15. Liao CF, Luo SF, Tsai CS, Tsao TY, Chen SL, Jiang MC. CAS enhances chemotherapeutic drug-induced p53 accumulation and apoptosis: use of CAS for high-sensitivity anticancer drug screening. Toxicol Mech Method 2008;18:771–6.
16. Behrens P, Brinkmann U, Wellmann A. CSE1L/CAS: its role in proliferation and apoptosis. Apoptosis 2003;8:39–44.
17. Liao CF, Luo SF, Li LT, Lin CY, Chen YC, Jiang MC. CSE1L/CAS, the cellular apoptosis susceptibility protein, enhances invasion and metastasis but not proliferation of cancer cells. J Exp Clin Cancer Res 2008;27:15.
18. Tsai CS, Luo SF, Ning CC, Lin CL, Jiang MC, Liao CF. Acetylsalicylic acid regulates MMP-2 activity and inhibits colorectal invasion of murine B16F0 melanoma cells in C57BL/6J mice: effects of prostaglandin $F_2\alpha$. Biomed Pharmacother 2008, doi:10.1016/j.biopha.2008.07.094.
19. Kutay U, Bischoff FR, Kostka S, Kraft R, Gorlich D. Export of importin α from the nucleus is mediated by a specific nuclear transport factor. Cell 1997;90:1061–71.
20. Pickett JA, Edwardson JM. Compound exocytosis: mechanisms and functional significance. Traffic 2006;7:109–16.
21. Kwan JA, Schulze CJ, Wang W, et al. Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly (ADP-ribose) polymerase (PARP) *in vitro*. FASEB J 2004;18:690–2.
22. Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol 2007;26:587–96.
23. Zigrino P, Löffek S, Mauch C. Tumor-stroma interactions: their role in the control of tumor cell invasion. Biochimie 2005;87:321–8.
24. Cedermark BJ, Blumenson LE, Pickren JW, Holyoke DE, Elias EG. The significance of metastases to the adrenal glands in adenocarcinoma of the colon and rectum. Surg Gynecol Obstet 1977;144:537–46.
25. Pieper-Bigelow C, Strocchi A, Levitt MD. Where does serum amylase come from and where does it go? Gastroenterol Clin North Am 1990;19:793–810.

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