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## Monoclonal Antibody CC188 Binds a Carbohydrate Epitope Expressed on the Surface of Both Colorectal Cancer Stem Cells and their Differentiated Progeny

Mai Xu,<sup>1</sup> Yuan Yuan,<sup>4</sup> Yang Xia,<sup>2</sup> and Samuel Achilefu<sup>1,3</sup>

**Abstract Purpose:** Recently, cancer stem cells (CSC), undifferentiated cancer progenitor cells, have been successfully isolated from colorectal tumors. Targeting both CSCs and differentiated, rapidly proliferating tumor cells with therapeutic drugs provides a focused strategy to treat cancer. In this study, we isolated the monoclonal antibody (mAb) CC188 and characterized the epitope recognized by mAb CC188, which is useful for developing biological reagents that target CSCs.

**Experimental Design:** We used a hybridoma technique to generate mAbs and an immunomagnetic method to isolate colon CSCs. We characterized mAb CC188 binding epitope and examined the epitope distribution in normal and tumor tissues, particularly in CSCs using tissue arrays and immunofluorescence staining method. We also evaluated the effect of mAb CC188 on invasiveness of NSY tumor cells.

**Results:** mAb CC188 was generated and 98.9% (187 of 189 cases) of colon cancer were positively stained by mAb CC188. "+", "++," and "+++" staining were 25.9%, 28.6%, and 43.4%, respectively. The mAb CC188 binding epitope was identified as a carbohydrate, which was expressed on the surface of colon CSCs (CD133<sup>+</sup>), differentiated colon cancer cells (CD133<sup>-</sup>), and cells from various types of epithelial tumors. In contrast, the expression of the carbohydrate epitope was low in normal prostate muscle and pancreatic acinar cells, as well as in some normal epithelial cells of the breast duct, cervix, and skin. A functional study indicated that mAb CC188 suppressed the invasiveness of NSY tumor cells.

**Conclusion:** mAb CC188 selectively targets a carbohydrate epitope expressed on cancer cells, providing a viable method for specific tumor imaging and targeted therapy.

Colorectal tumor is the third most common form of cancer worldwide (1), with estimated 153,760 new cases and 52,180 deaths in the United States alone in 2007.<sup>5</sup> Improvement of cancer diagnosis and discovery of novel therapeutic approaches are essential to fight the disease. Recent studies have shown that cancer stem cells (CSC) can self-renew and differentiate into mature diversified cancer cells and are responsible for tumor initiation, growth, and metastasis (2–5). In particular, CSCs have been successfully isolated from colorectal tumors (6, 7).

Despite recent advance in cancer treatment, most therapies are targeted to differentiated tumor or highly proliferating cells but not CSCs. In addition, CSCs are resistant to radiation and DNA damage reagents, probably due to the increased capacity

of DNA repair and overexpression of drug-resistant transporter proteins (8, 9). Furthermore, both normal cells and CSCs overexpress antiapoptosis genes such as *BCL2*, the products of which abrogate the sensitivity of cells to chemotherapy and radiotherapy (10–12). In patients, the residual tumor cells, particularly CSCs, often develop metastasis and/or recurrence after treatment. Based on the hypothesis that CSCs are the primary source of the tumor population, complete eradication of CSCs from patients with cancer presents a viable strategy to cure this devastating disease. Toward this goal, identifying biological markers of CSCs will accelerate the development of specific therapeutic reagents targeted to CSCs and efficient approaches for cancer treatment.

Previous studies have identified many colon cancer-associated antigens, with glycoproteins constituting a majority of the biomarkers. A number of these tumor-associated epitopes are normally cryptic antigens and become exposed after incomplete glycosylation that occurs frequently in malignant cells. However, it is unknown whether these epitopes are equally expressed in CSCs.

Currently, many research efforts have focused on identifying biomarkers that are specifically expressed in CSCs and absent in normal stem cells and their differentiated progeny normal cells.

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<sup>5</sup> <http://www.cancer.gov/cancertopics/types/colon-and-rectal>

### Translational Relevance

Cancer stem cells (CSC) can self-renew and differentiate into diversified cancer cells and are responsible for tumor initiation, growth, and metastasis. Despite recent advance in cancer treatment, most therapeutic and imaging reagents are targeted to differentiated or highly proliferating tumor cells but not CSCs. Toward the goal of eradicating primary and metastatic tumors in humans, we have generated a monoclonal antibody designated as mAb CC188, which selectively targets a carbohydrate epitope expressed on CSCs and differentiated cancer cells. A functional study indicated that the epitope suppressed the invasiveness of NSY colon tumor cells. Based on these findings, we envisage the use of mAb CC188 in humans as a carrier for specific delivery of imaging agents and chemotherapeutic drugs to both CSCs and differentiated tumor cells. For example, a comprehensive imaging of colon cancer in humans can be achieved by labeling mAb CC188 with radionuclides for positron emission tomography. Considering that mAb CC188 has an intrinsic ability to suppress invasiveness of colon cancer cells, coupling it with a chemotherapeutic drug could provide an effective therapeutic regimen for the eventual eradication of the devastating disease.

For example, targeting the surface molecule CD44 of CSC using a monoclonal antibody has eradicated human acute myeloid leukemic stem cells in a xenograft animal model while sparing normal stem cells (13). Unfortunately, there are no universal biomarkers for CSCs. Each tumor type possesses a unique combination of cell surface biomarkers that define the cell subpopulation with the highest tumorigenic potential. For example, leukemia stem cells express surface biomarkers CD34<sup>+</sup>/CD38<sup>-</sup> (14) and CD96<sup>+</sup> (15); breast cancer ESA<sup>+</sup>/CD44<sup>+</sup>/CD24<sup>-</sup>/low (16); brain and colon cancer CD133<sup>+</sup> (6, 7, 17, 18); pancreatic cancer ESA<sup>+</sup>/CD44<sup>+</sup>/CD24<sup>+</sup> (19); and prostate cancer CD44<sup>+</sup> (20). Interestingly, most of the CSC biomarkers identified thus far are shared with their normal counterparts. Therefore, identifying CSC-specific biomarkers for each type of tumor is critical for individualized cancer therapies.

In this study, we generated monoclonal antibodies and isolated colon CSCs from human colon adenocarcinoma NSY cells (21) based on CD133 antigen expression. One of the monoclonal antibodies, mAb CC188 was selected for further studies based on preliminary screening assays. We characterized the epitope recognized by mAb CC188 and examined the distribution of the epitope in normal and tumor tissues, with special emphasis on CSCs. In addition, we explored the role of the cell surface antigen in tumor cell metastasis.

### Materials and methods

**Cell lines and cell culture.** Human colon adenoma cell line NSY (21) was cultured in RPMI 1640 supplemented with 10% FCS (Hyclone), 50 units/mL sodium penicillin, and 50 µg/mL streptomycin sulfate (BioWhittaker). Myeloma cells (P3/x63.Ag8) were used as a fusion partner and were maintained in Iscove's modified Dulbecco's

medium supplemented with 20% fetal bovine serum (Hyclone), 50 units/mL sodium penicillin, 50 µg/mL streptomycin sulfate, 4 mmol/L L-glutamine (BioWhittaker), 1 mmol/L sodium pyruvate (BioWhittaker), and 0.0001% β-mercaptoethanol (Sigma Chemical Co.). All cells were cultured in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

**Generation of monoclonal antibodies.** Titermax Gold adjuvant (25 µL; Sigma Chemical Co.) and NSY cells ( $1 \times 10^6$ ) were injected i.p. to 6-wk-old female BALB/c mice once a week for 4 wk. Three days before euthanasia, the mouse was boosted with the same doses of adjuvant and tumor cells as used before. Spleen cells from mouse with serum titer >4,000× were used for fusion. Hybridoma library was established by fusion of both spleen cells from the immunized BALB/c mice and myeloma cells (P3/x63.Ag8) at a 5:1 ratio with polyethylene glycol (PEG)-1500 (Sigma Chemical Co.) following standard procedures (22). Briefly, freshly harvested spleen cells and fusion partner P3/x63.Ag8 cells were copelleted by centrifugation and fused by addition of PEG-1500 solution to the pellet. Fused cells were centrifuged, resuspended in Iscove's modified Dulbecco's medium containing 20% (v/v) FCS and  $1 \times 10^{-5}$  mol/L hypoxanthine-aminopterin-thymidine (Sigma Chemical Co.) and aliquoted into 96-well plates. The hybridomas were allowed to grow for 10 to 15 d, and the supernatants from the hybridomas were collected for screening. Supernatants from the hybridoma culture were screened in the NSY cells by immunofluorescence staining assay as described below. The supernatants containing an antibody that binds to the surface of NSY cells were further tested in the histopathologic section of colon cancer tissues using immunohistochemical staining method. The hybridoma colonies secreting antibodies that reacted with colon cancer tissue were selected for subcloning and expanding the culture for mAb production.

mAbs were purified using protein G Sepharose according to the manufacturer's instruction (Amersham Biosciences). Briefly, the supernatant from the hybridoma culture was centrifuged at  $14,000 \times g$  for 20 min at 4°C, filtered through a 0.22-µm filter to remove fine particles, and the pH was adjusted to 7.0 using equilibrate buffer [1 mol/L Tris (pH 9.0)]. The supernatant was passed through protein G column and the column was washed with binding buffer [50 mmol/L Na<sub>2</sub>PO<sub>4</sub>, 500 mmol/L NaCl (pH 6)], before eluting the antibody with glycine (0.1 mol/L; pH 2.7). The antibody was collected and neutralized in a test tube containing 100 µL of Tris buffer (1 mol/L, pH 9.0).

**Immunofluorescence staining assay, antibody labeling, and microscopy.** For screening hybridoma, we applied immunofluorescence staining assay in cultured cells without fixation and permeation. Under this staining condition, only cell surface molecules can be detected. Briefly, we seeded 5,000 cells per well in 96-well plates and allowed them to continuously culture in an incubator at 37°C for 48 h. After decanting the culture medium, 50 µL supernatant from the hybridoma culture were added and incubated for 40 min at 37°C. The plates were washed thrice with PBS, and 50 µL of 1,000× diluted Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probes) were added to the plates, which was incubated for another 40 min at room temperature. The plates were washed with PBS and observed under a fluorescence microscope. To examine the reactivity of mAb CC188 to colon CSCs, the direct immunofluorescence method was used. mAb CC188 was labeled with Alexa Fluor 488 following the manufacturer's instruction (Invitrogen). Briefly, bicarbonate (50 µL; pH 9.0; 1 mol/L) was added to mAb CC188 (0.5 mL; 2 mg/mL) to optimize the pH for efficient reaction of Alexa Fluor 488 dye and the protein. The antibody solution was transferred to a vial containing the reactive dye and the mixture was incubated for 1 h at room temperature before passing it through a purification column from the labeling kit. The first fluorescence band was collected for immunofluorescence staining. For confocal microscopy, cells cultured on Lab-Tek slides or sedimented by using Cytospin (Thermo Shandon, Inc.) were visualized with an Olympus FV1000 microscope. Dual color, Alexa Fluor 488-conjugated mAb CC188 and phycoerythrin-conjugated mAb 294C3 (Miltenyi) staining slides were analyzed using a sequential program from Olympus FV1000 microscope software.

**Immunohistochemical staining.** Normal and tumor tissue arrays (Biomax), as well as histologic sections of colon cancer tissue, were used in this study. Histopathologic slides were deparaffinized in xylene and gradient alcohol. After rehydration, the slides were immersed in antigen retrieval solution [10 mmol/L sodium citrate, 0.05% Tween 20 (pH 6.0)] preheated to 95°C to 100°C for 20 min. The buffer and the slides were cooled to room temperature and the slides were rinsed twice with PBS before incubation in blocking buffer containing 5% goat serum for 30 min. The slides were further incubated with a primary antibody at a concentration of 10 µg/mL for 1 h at room temperature or overnight at 4°C. After rinsing the slides and blocking with peroxidase blocking solution (30% H<sub>2</sub>O<sub>2</sub> in PBS) for 10 min, the Avidin-Biotin Complex detection system (Vector Laboratory) was used following the manufacturer's instruction. 3,30-Diaminobenzidine tetrahydrochloride dihydrate (Sigma Chemical Co.) was used as chromogen. The stained slides were dehydrated with gradient alcohol and xylene. Finally, the slides were sealed with mounting medium, coverslipped, and scored independently by two researchers with pathology background using a conventional microscope. Based on the staining intensity, all cases were scored as negative (grade 0), weak or faint (grade 1), positive (grade 2), strong positive (grade 3), and very strong positive (grade 4) staining. Based on the cell staining proportion, all cases were classified as no staining (-), <5% (±), 6% to 25% (+), 26% to 70% (++) and >71% (+++). A combination of both staining intensity and percentage resulted in the following classifications: grade 0 or no staining (-); grade 1 or (<5%) ±; and the rest of the cases with positive (grade 2), strong positive (grade 3), and very strong positive staining (grade 4) were classified according to the percentage of positively stained cells.

**Antigen epitope characterization.** To characterize the immunopeptide of mAb CC188, deglycosylation experiment was done as described (23, 24) with some modifications. Briefly, NSY cells were seeded in a 96-well plate, incubated at 37°C for 48 h, and fixed with 4% paraformaldehyde for 30 min. After washing twice with PBS and adding 100 µL of different concentrations of sodium periodate [1, 5, 10, and 20 mmol/L in sodium acetate buffer (pH 4.5)] or trypsin and proteinase K (the initial concentrations were 0.5 and 2.5 mg/mL, respectively, which were diluted 2× in PBS thereafter) into each well (triplicate for each dose), the plates were incubated at 37°C for 2 h for the sodium periodate-treated plate and 1 h for the trypsin- and proteinase K-treated plate. After washing with PBS containing 10% fetal bovine serum, mAb CC188 was added to each well at a concentration of 5 µg/mL and incubated for 1 h at 37°C. The secondary antibody, Alexa Flour 488-conjugated goat anti-mouse, was incubated with the cells in the plates for 45 min. The plates were read with Synergy HT Multi-Detection microplate reader (BioTek, Vermont).

**CSC sorting.** CD133 indirect cell isolation Kit (Miltenyi Biotec) was used to fractionate colon CSCs from human colon carcinoma NSY cells following the manufacturer's standard procedures. Briefly, after trypsinization,  $1 \times 10^7$  cells were resuspended in 100 µL of labeling (separation) buffer [PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, 0.5% bovine serum albumin, 2 mmol/L EDTA (pH 7.2)]. Biotinylated antibody against CD133/1 (10 µL) was added and refrigerated for 10 min. After washing, the cell pellets were suspended in 80 µL of labeling buffer and 20 µL of anti-biotin MicroBeads, and incubated at 4°C for 15 min. The cells ( $1 \times 10^7$ ) were suspended in 500 µL of the separation buffer and loaded onto the column for magnetic separation. To assess the purity of isolated CD133<sup>+</sup> cells, we stained the fraction with phycoerythrin-conjugated 293C3 antibody against CD133/2 (Miltenyi Biotec).

**Effect of mAb CC188 on the invasiveness of NSY colon cancer cells.** To study the function of tumor cell surface antigen recognized by mAb CC188, we performed an invasion assay using BD BioCoat Matrigel invasion chamber (PN:354481) in accordance with manufacturer's instruction. Briefly,  $2.5 \times 10^5$  NSY cells in 2 mL medium with or without 9 µg/mL mAb CC188 were added to each chamber (duplicate for each treatment) after rehydration and cultured in humidified tissue culture incubator at 37°C and 5% CO<sub>2</sub> for 24 and 48 h. Cells were counted under a microscope after removing noninvasive cells by

scrubbing the top surface of the membrane with a dry sterile cotton swab, fixing with 100% methanol and staining with 1% toluidine blue. In addition, mouse IgG (Sigma-Aldrich) was tested in NSY tumor cells for the invasion assay at 9 µg/mL after redialyzing in PBS. The number of invasion cells was comparable with that from the control without treatment.

The invasion index was calculated following the formula

$$\text{Invasion index} = \frac{(\text{no. invading cells in mAb CC188})}{(\text{no. invading cells in control})}$$

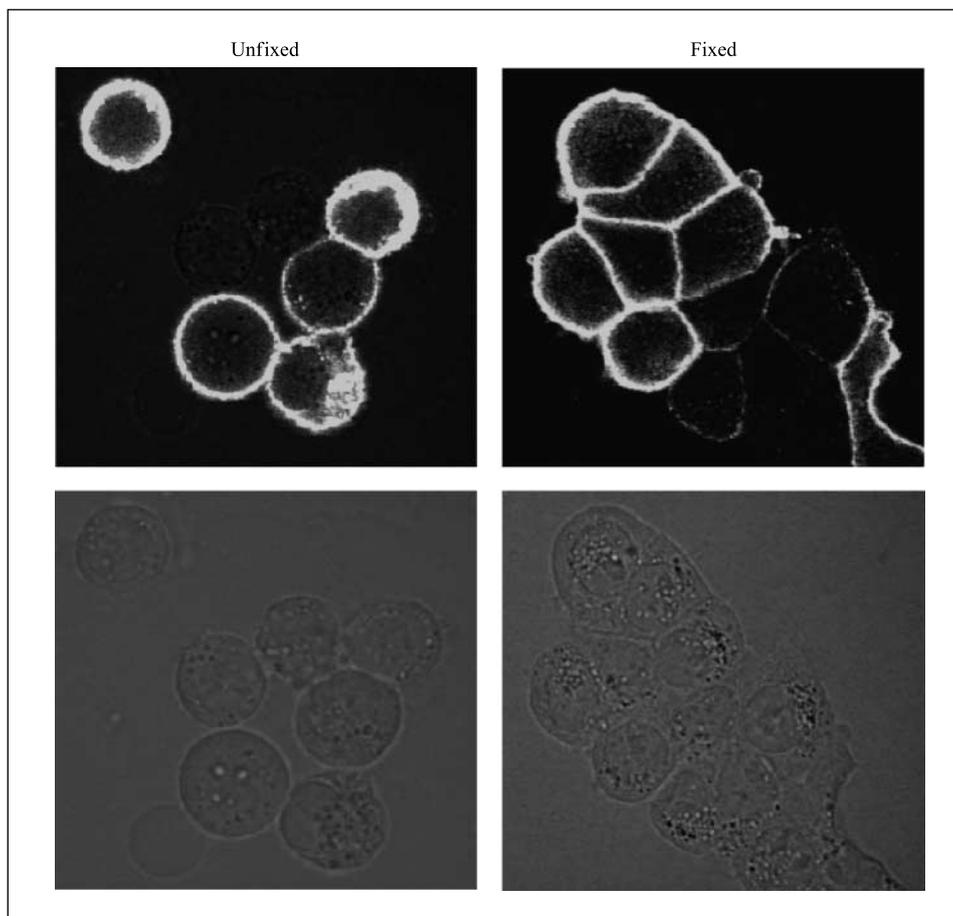
## Results

**Binding of mAb CC188 to the membrane of human colon adenocarcinoma NSY cells.** In this study, we generated a monoclonal antibody designated as mAb CC188 by screening hybridoma libraries. To examine if mAb CC188 binds antigens expressed on cell surface, we performed immunofluorescence staining in cultured cells without fixation and permeation. Very intense cell membrane staining and internalization of mAb CC188 were observed in >80% of the cultured live NSY cells after incubation at 37°C for 40 min (Fig. 1, top left). To further examine the cellular localization of the mAb CC188 binding epitope, we performed immunofluorescence staining in cells fixed and permeated with 4% paraformaldehyde containing 0.2% Triton X-100. mAb CC188 was strongly reactive to the membrane of NSY cells (Fig. 1, top right).

**Human colon cancer tissue array analysis.** To evaluate the usefulness of an antibody for tumor imaging and targeted therapy, it is necessary to test tumor sensitivity, homogeneity, and specificity of the epitope recognized by the antibody in human tumors and normal tissues. At first, we examined the reactivity of the antibody in human colon cancer tissues to test the sensitivity and homogeneity of the epitope targeted by CC188. We found that 98.9% (187 of 189 cases) of colon cancer showed positive staining with mAb CC188. "+", "++", and "+++" were 25.9%, 28.6%, and 43.4%, respectively (Table 1). The results showed that >40% of all colon cancer cases tested exhibited intense homogenous staining, and the epitope recognized by mAb CC188 was expressed in all histologic grades of colorectal cancers, as shown in Fig. 2A. The immunopeptide was also detected in tumor cells that metastasized into lymph nodes (Fig. 2A, c).

**Human normal tissue array analysis.** To test the tumor specificity, we carried out normal tissue array analysis. Our results showed that the immunopeptide was undetectable in most major organs such as the brain, heart, liver, ovary, kidney, and colon, although positive or weak positive staining was observed in pancreatic acinar cells, prostate muscle, some epithelial cells in skin, cervix, and ducts of the breast (Fig. 2B; Table 2).

**Human multiple cancer tissue array analysis.** The epitope recognized by mAb CC188 is distributed in all histologic grades of colorectal cancers and some normal epithelial cells, suggesting that the immunopeptide of mAb CC188 might be shared with other types of cancers originating from epithelial cells. To test this hypothesis, a human multiple cancer tissue array experiment was done. Staining of mAb CC188 was positive in ovarian, stomach, and lung cancers and very strong positive in ovarian cancer. In contrast, the normal tissue



**Fig. 1.** Immunofluorescence staining of mAb CC188 in human colon adenocarcinoma NSY cells. NSY cells (*left*) were unfixed and most of them were intensely stained with mAb CC188. Internalization of mAb CC188 was seen in the cultured cells after incubation for 40 min at 37°C. Cells in the right panel were fixed with 4% paraformaldehyde and 0.2% Triton X-100 and intensely stained on the cell membranes. Top, dark-field images. Bottom, bright-field images that served as controls.

counterparts were negative (Fig. 3). Interestingly, brain tumor (glioma) that came together with the cancer tissue array was also positive (Fig. 3).

**Characterization of the epitope recognized by mAb CC188.** In general, most cell surface antigens are glycoproteins and glycolipids. Therefore, we performed deglycosylation experiments to assess whether the epitope was a carbohydrate. We treated NSY cells with different concentrations of sodium periodate, followed by staining with mAb CC188. Representative images of immunofluorescence staining in the untreated (control) cells and those treated with 10 mmol/L sodium periodate for 2 hours at 37°C are shown in Fig. 4A. A negative staining of mAb CC188 in sodium periodate-treated NSY cells was observed, indicating that the immunoepitope was a carbohydrate. To further confirm this finding, we carried out a fluorometric detection assay. NSY cells were treated with different concentrations of sodium periodate, trypsin, and proteinase K, followed by staining the cells with mAb CC188, as described in Materials and Methods. The reactivity of mAb

CC188 to the cells gradually decreased as the sodium periodate concentration increased. In contrast, the binding activity of the epitope to mAb CC188 was not affected by the treatment with trypsin or proteinase K (Fig. 4B). The fluorometric detection assay was repeated twice (sodium periodate) and thrice (trypsin and proteinase K). Taken together, these findings strongly suggest that the binding epitope of mAb CC188 is a carbohydrate.

**Expression of the epitope recognized by mAb CC188 on the surface of CSCs.** Considering that CSCs are the source of the whole population of tumor, they constitute an important target for tumor imaging and therapy. Because mAb CC188 showed intense staining in colon cancer tissues, we investigated the expression of the immunoepitope in colon CSCs. The colon CSCs were isolated based on CD133 expression (antibody anti-CD133/1 binding site) and stained with mAb CC188 labeled with Alexa Fluor 488 and phycoerythrin-conjugated mAb 293C3 against CD133/2 binding site to confirm the identity of the CSCs. Impressively, mAb CC188

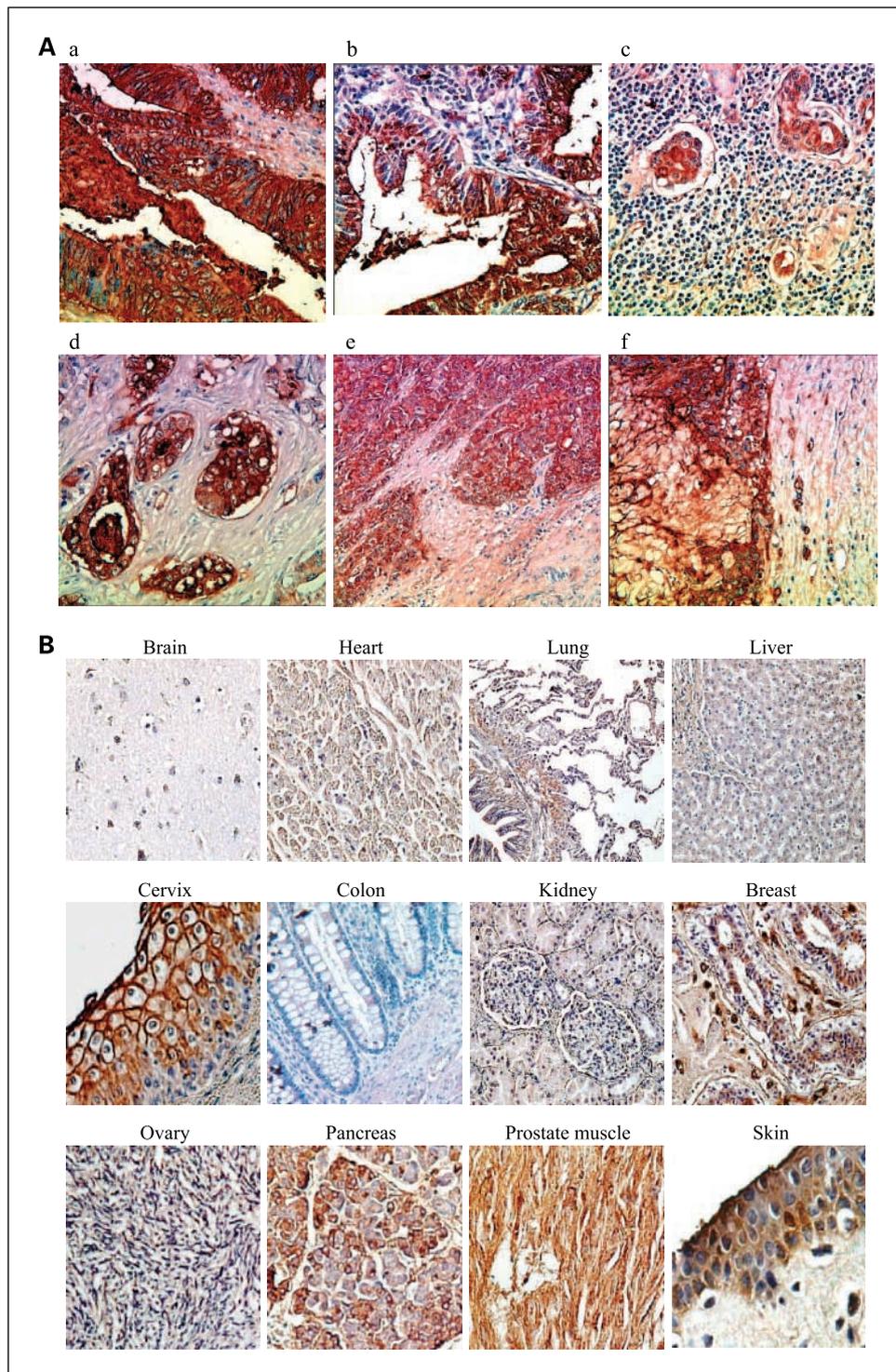
**Table 1.** Summary of mAb CC188 reactivity in human colorectal tumor tissues

| Scoring grade | -        | ±        | +         | ++        | +++       | Total (%) |
|---------------|----------|----------|-----------|-----------|-----------|-----------|
| Cases (%)     | 2 (1.05) | 2 (1.05) | 49 (25.9) | 54 (28.6) | 82 (43.4) | 189 (100) |

not only stained the surface of differentiated (CD133<sup>-</sup>) colon cancer cells but also strongly stained the membrane of colon cancer stem (CD133<sup>+</sup>) cells (Fig. 4C). For tumor molecular imaging and targeted therapy, it is important that the epitope recognized by mAb CC188 is not expressed in peripheral (circulating) blood mononuclear cells. Our result show that the peripheral blood mononuclear cells were negatively stained (Fig. 4D).

**Effect of mAb CC188 on the invasiveness of NSY colon cancer cells.** In this study, we examined the effect of mAb CC188 on the invasiveness of NSY tumor cells. The results showed that mAb CC188 at 9  $\mu\text{g}/\text{mL}$  significantly suppressed tumor cell invasion ( $46 \pm 1.4$  and  $205 \pm 14$  invading cells with mAb compared with  $67 \pm 4.2$  and  $304 \pm 20$  invading cells in controls at 24 and 48 h, respectively). The invasion index was  $\sim 0.69$  and  $0.67$  at 24 and 48 h, respectively. Both invasion

**Fig. 2.** Immunohistochemical staining of mAb CC188 in various histologic grades of colon cancers and human normal tissues. *A*, *a* and *b*, highly differentiated colon cancers. *c*, colon cancer metastasized into lymph node. *d*, moderately differentiated colon cancer. *e*, poorly differentiated colon cancer. *f*, mucinous adenocarcinoma. mAb CC188 stained homogeneously and intensely in tumor cells from various histologic grades of colon cancers. *B*, most normal tissues from major organs showed negative staining with mAb CC188, including the brain, heart, liver, kidney, and ovarian and colon mucosa. Positive staining was observed in prostate muscles, and limited positive staining was observed in epithelial cells in top layer cells of cervix, breast ducts, pancreas, and skin.



**Table 2.** Summary of mAb CC188 binding epitope expression in various human normal tissues

|           |            |            |               |               |            |
|-----------|------------|------------|---------------|---------------|------------|
| Brain (-) | Breast (+) | Cervix (+) | Colon (-)     | Heart (-)     | Kidney (-) |
| Liver (-) | Lung (-)   | Ovary (-)  | Pancreas (++) | Prostate (++) | Skin (+)   |

indexes are significantly different from controls ( $P < 0.05$ ). The results clearly indicated that the mAb CC188 binding antigen plays a role in tumor cell metastasis.

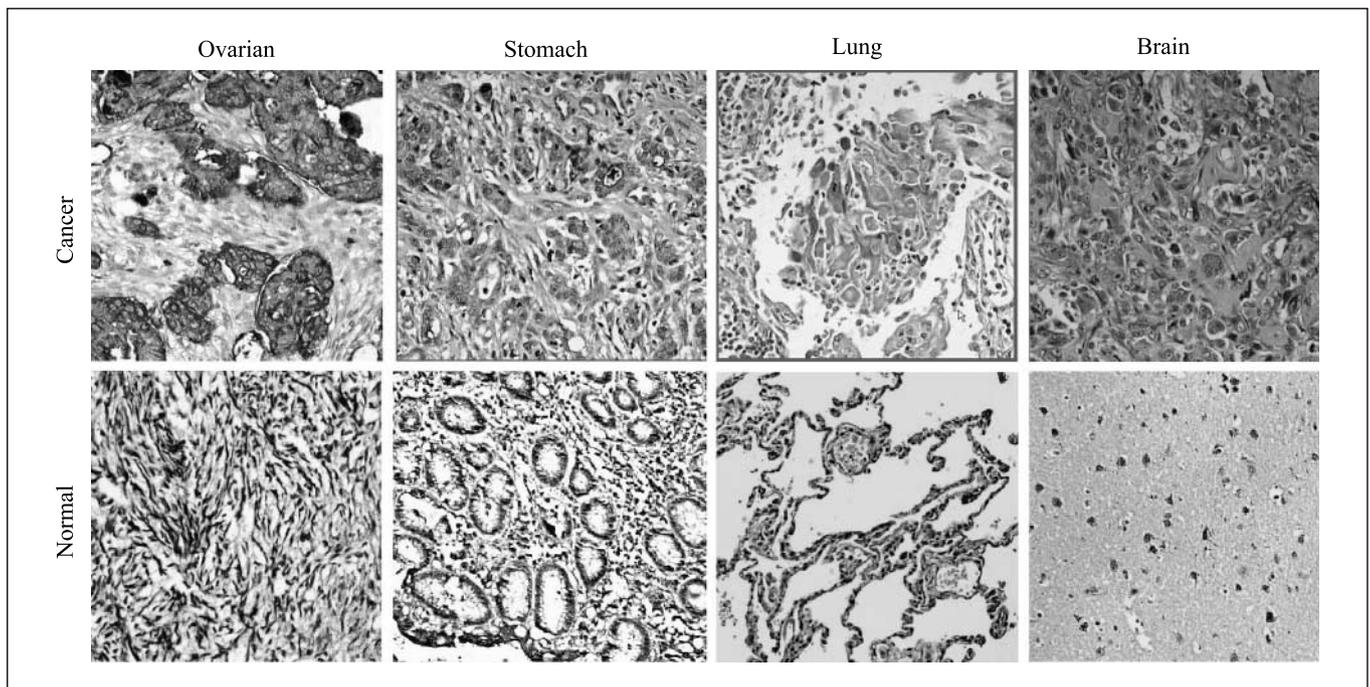
## Discussion

Many tumor-associated antigens have been identified in colorectal cancer. These include CEA (25, 26), TAG72 (27–29), A33 (30, 31), CA19-9 (32), and MUC1 (33). Most of these antigens are glycoproteins that are often abnormal, both in structure and in quantity, in tumor cells. For example, cryptic carbohydrate determinants, T and Tn, are exposed in cancer mucins and serve as tumor-specific biomarkers (34). In this study, mAb CC188 was successfully generated. Initial characterization reveals that the epitope recognized by mAb CC188 is carbohydrate. Analysis of tumor and normal tissues, including peripheral blood mononuclear cells, showed that mAb CC188 had excellent tumor sensitivity, homogeneity, and significant tumor specificity. Even in prostate muscle and some normal epithelial cells of breast, pancreas, cervix, and skin where the epitope was detected, the expression was low. Because mAb CC188 binding epitope is also shared by other types of cancers originating from epithelial cells such as stomach, ovarian, and lung cancers, as well as glioma cells, it provides a versatile approach to diagnose, image, and

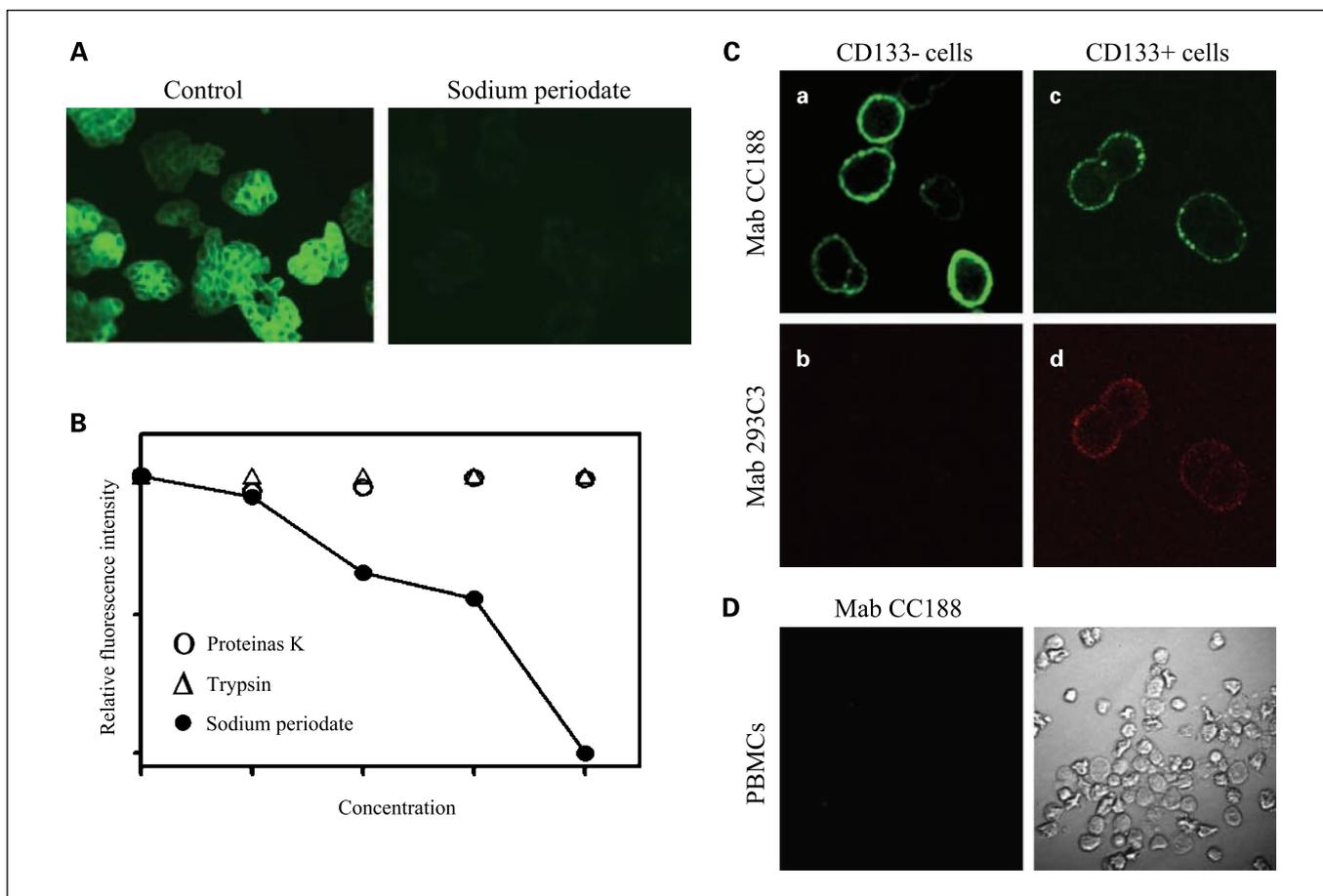
potentially treat a variety of tumors. A similar phenomenon was observed in the mAb CC49 binding epitope in TAG72, a biomarker for tumors of epithelia origin. Its expression extends beyond adenocarcinomas to certain cell lines of hematopoietic origin (35). For these reasons, many antibodies, such as CC49 and B72.3, against TAG72 are widely used in clinical trials (36–38).

Interestingly, the distribution patterns of mAb CC188 binding epitope in tumor tissues are similar to that of mAbs CC49 and B72.3 reactive antigen TAG72 that are broadly expressed in various tumors originating from epithelial cells such as breast, colon, prostate, pancreas, and ovarian cancers. However, differences in specific localization of the antigens exist. For example, TAG72 antigen expression was more prominent in colon mucosa adjacent to the tumor and heterogeneously expressed in tumor cells (39). In contrast, the mAb CC188 antigen was expressed homogeneously in various histologic grades of colon cancer and was undetectable in the colon mucosa adjacent to the tumor. These results suggest that the ability of mAb CC188 to target both CSCs and differentiated tumor cells provides an exciting opportunity to image and treat the whole tumor cell population in a clinical setting.

Our immediate interest is to use CC188 for imaging and treating colon cancer. Antibody-based radioimmunotherapy



**Fig. 3.** Immunohistochemical staining of mAb CC188 in multiple cancer tissues and their normal counterparts. Positive homogeneous staining in all cancer tissues, brain tumor (glioma), ovarian, stomach, and lung cancers but negative staining in their normal counterparts is shown.



**Fig. 4.** Deglycosylation and dual immunofluorescence staining assays. *A*, deglycosylation measured with immunofluorescence staining: NSY cells were brightly stained with mAb CC188 without sodium periodate treatment (*Control*), whereas cells were negatively stained after treatment with sodium periodate. All images were taken with the same settings. *B*, deglycosylation measured with fluorometric detection method: NSY cells were cultured in 96-well plates for 48 h and treated with different concentrations of sodium periodate for 2 h and proteinase K and trypsin for 1 h at 37°C. The numbers labeled in the X axis indicate the concentrations used in the experiments. For sodium periodate, the concentrations are 1, 5, 10, and 20 mg/mL from numbers 1 to 4, respectively. For proteinase K and trypsin, the initial concentrations are 0.5 and 2.5 mg/mL, respectively, and 2× diluted thereafter. 0 on the X axis is control (without treatment). All relative fluorescence intensity values were average from two or three experiments. The binding of mAb CC188 to the cells treated with sodium periodate decreased with increasing concentrations; in contrast, there were no significant changes of mAb CC188 binding to the cells treated with different concentrations of proteinase K and trypsin. *C*, dual immunofluorescence staining of Alexa Fluor 488-labeled mAb CC188 and phycoerythrin-conjugated mAb 293C3 against CD133/2 (secondary binding site of the CD133 molecule) in NSY cancer stem (CD133<sup>+</sup>) cells and differentiated (CD133<sup>-</sup>) cells. NSY differentiated (CD133<sup>-</sup>) cells were intensely stained with mAb CC188 (*a*), but not phycoerythrin-conjugated mAb 293C3 (*b*). NSY cancer stem (CD133<sup>+</sup>) cells were stained with both Alexa Fluor 488-labeled mAb CC188 (*c*) and phycoerythrin-conjugated mAb 293C3 (*d*). *D*, immunofluorescence staining of peripheral blood mononuclear cells (PBMC) with mAb CC188. Left, peripheral blood mononuclear cells were negatively stained; right, bright-field image used as control.

was used in humans in the early 1980s (40) for diagnosing, localizing primary and metastasis tumors, and monitoring therapeutic response and recurrence of colon cancer. Since then, many studies on radioimmunoimaging of colon cancer have been reported (41, 42). Specifically, many tumor cell surface antigens and functional molecules such as carcino-embryonic antigen (25, 26), epidermal growth factor receptor (43, 44), and vascular endothelial growth factor receptor (45) have recently been used as molecular targets in colon cancer-targeted therapy. Remarkable successes have been achieved in clinical trials but it is not known if treatment efficacy is associated with their expression in all tumor cell population, including CSCs. In future studies, it would be interesting to evaluate the expression of established tumor-associated antigens in the CSCs and correlate the results with treatment efficacy.

Presently, colon CSCs are not well characterized, but it is accepted that the cells with the potential to initiate tumors in

mice are in the population of CD133<sup>+</sup> cells. Because CD133 is also expressed in normal stem cells, selective targeting of tumor-associated CD133 is challenging. Therefore, it is necessary to differentiate normal cells from CSCs by identifying CSC-specific molecular biomarkers and defining the subclasses of colon CSCs from the CD133<sup>+</sup> population based on antigen expression profiles and the activity level of signal transduction pathways (46, 47).

Based on the concept of CSCs, cancer treatment targeting neoplastic stem cells is considered indispensable for eradicating the entire tumor and developing curative treatment approaches. Toward the goal of developing targeted therapeutic reagents for colon CSCs, we are evaluating the expression of mAb CC188 epitope in normal stem cells from different normal tissues. There is also a need to know whether all colon CSCs uniformly express the same biomarkers or can be further classified into subpopulations based on antigen expression profile. Therefore, a combination of tumor-targeting reagents,

including antibodies, seems to be a realistic strategy in cancer molecular imaging and targeted therapy. Fortunately, diverse methods to identify CSC-specific biomarkers are available, including the hybridoma method, the phage display technique, and high-throughput assays for screening peptides and chemical libraries using CSCs.

Tumor metastasis is an established cause of death in cancer patients, and tumor cell invasion is an important step in metastasis. Tumor cell surface molecules, particularly adhesion-related glycoprotein, play a critical role in tumor cell invasion. Our results suggest that the mAb CC188 binding antigen plays an important role in tumor cell invasion. However, the mechanism of this biological effect has not been elucidated at this time. Possibly, the antigen interacts with cytoskeletons that provide the driving force for cell migration and affect the release of degradation enzymes that promote tumor cell invasion. In addition to its potential therapeutic effect, investigating the link between antigen expression at different disease stages as well as survival will be the subject of future studies.

In summary, although some contradicting reports about the role of CSCs were recently published (48), evidence supporting the key role of CSCs in tumorigenesis is compelling. We have generated mAb CC188 that has significant tumor sensitivity, specificity, and homogeneity, particularly in human colon cancer. The carbohydrate epitope recognized by mAb CC188 was exposed on the surface of colon CSCs and their differentiated progeny. The results suggest that the carbohydrate epitope is a viable molecular target and mAb CC188 is a potential molecular probe or carrier for molecular imaging and targeted therapy of tumors.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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