Distinct expression of C4.4A in colorectal cancer detected by different antibodies

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Abstract. The metastasis-associated gene C4.4A encodes a glycolipid-anchored membrane protein expressed in several human malignancies. The present study aimed to perform a detailed assessment of C4.4A expression in colorectal cancer tissues, in terms of intra-cellular localization, intra-tumoral location and difference in molecular weight. To advance this goal, we developed three new antibodies against the C4.4A protein (two polyclonal Abs: C4.4A-119 and C4.4A-277 and one monoclonal Ab: C4.4A GPI-M) to use in addition to the two previously produced polyclonal Abs (C4.4A-81, C4.4A GPI-P). Antibody specificities were confirmed by absorption tests. Western blot analysis and immunohistochemistry showed that the C4.4A-119 and C4.4-277 Abs detected 70-kDa C4.4A, mainly in the cytoplasm, irrespective of intra-tumoral location. The C4.4A GPI-P and C4.4A GPI-M Abs reacted with the membranous ~40-kDa C4.4A, exclusively at the tumor invasive front, and each detected an identical tumor cell population. The tested antibodies showed varied C4.4A detection rates in 33 CRC tissues. The C4.4A-277 Ab yielded the highest positive rate in 29 of 33 CRC tissues (87.9%), while the C4.4A GPI-P and C4.4A GPI-M Abs each only showed 33.3% positivity. The present findings suggest that the GPI anchor signaling sequence may be essential for detecting membranous C4.4A at the invasive front of CRC tissues.

Introduction

The C4.4A protein was first identified in a highly metastatic rat pancreatic adenocarcinoma cell line (1,2). The human homologue of rat C4.4A is located on chromosome 19q13.1-q13.2 and was cloned in 2001 (3). Studies of the molecular structure indicate that C4.4A is a glycosylphosphatidyl-inositol (GPI)-anchored membrane protein with 30% homology to the urokinase-type plasminogen activator receptor (4,5). In normal human tissues, C4.4A mRNA is present in placental tissue, skin, esophagus, and leukocytes (3); but the physiological function of the C4.4A protein is largely unknown. C4.4A expression is upregulated in some types of human malignancies, and human C4.4A mRNA has been detected in cancer cell lines, including melanoma, breast, bladder, and renal cell carcinoma, as well as in tumor tissue samples from malignant melanoma, colorectal cancer (CRC), breast cancer, lung carcinoma, and urothelial tumors (5-10).

We previously detected C4.4A protein expression on the plasma membranes of tumor cells at the invasive front in 25.6% of 132 CRCs (11). In that study, we used a polyclonal antibody that recognizes the C4.4A C-terminus containing the GPI anchor signaling sequence. In contrast, Paret *et al* (9) reported that 85.4% of CRC tissues showed distinct C4.4A expression by immunohistochemistry, and they did not mention an invasive front-specific expression pattern. We also observed that another C4.4A polyclonal antibody that was raised against amino acids near the N-terminus did not react with the C4.4A protein in CRC tissue samples, while it did recognize C4.4A in the esophageal squamous epithelium (11). These findings suggest that distinct antibodies detect different species of the C4.4A protein.

In the present study, we further investigated C4.4A expression in CRC tissue samples. We developed three novel antibodies (two polyclonal antibodies: C4.4A-119 and C4.4A-277, and one monoclonal antibody: C4.4A GPI-M) and tested them in addition to the two previously produced antibodies (C4.4A-81, and C4.4A GPI-P) (11). Using these antibodies for immunohistochemistry, we performed a detailed assessment of the C4.4A protein with regard to expression rates in CRC cases, intra-cellular localization in tumor cells (cytoplasm or plasma membrane), and intra-tumoral localization (invasive front, or intermediate portion to superficial portion of the cancer body). Western blot analysis was also performed to determine the molecular weight of the C4.4A protein bound by each antibody. Our results show that GPI anchor signaling sequence may be essential for detecting membranous C4.4A at the invasive front of CRC, which is of clinical significance.

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Abbreviations: Ab, antibody; CRC, colorectal cancer; GPI, glycosyl-phosphatidyl-inositol

Key words: C4.4A, colorectal cancer, GPI anchor, localization

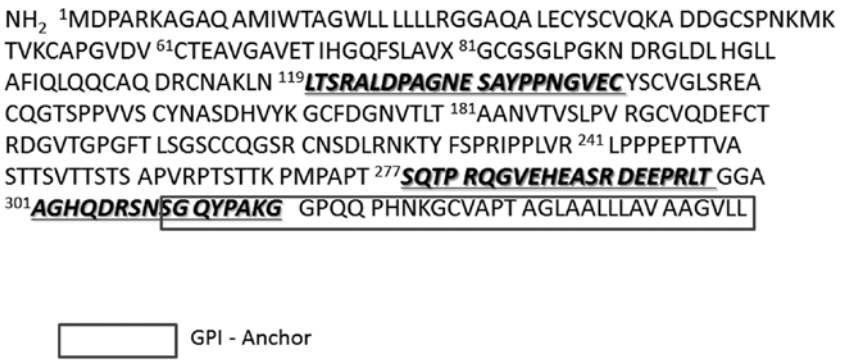

Figure 1. Amino acid sequences of the C4.4A protein with the epitopes employed as immunogens in bold. C4.4A-81 starts at AA 81, C4.4A-119 at AA 119, C4.4A-277 at AA 277, and C4.4A GPI-P and C4.4A GPI-M start at AA 301. The boxed area indicates the GPI anchor signaling sequence. ^{309}SGQYPAKG is destined to be cut off when the C4.4A molecule is anchored onto the plasma membrane.

Materials and methods

Tissue samples and cell lines. All colorectal tissue samples (n=33) were collected during surgery at the Department of Surgery, Osaka University (Osaka, Japan). Samples were fixed in buffered formalin at 4˚C overnight, processed through graded ethanol solutions, and embedded in paraffin. The specimens were appropriately used, with the approval of the Ethics Committee at the Graduate School of Medicine, Osaka University. The human colon cancer cell line HCT116 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37˚C in a humidified incubator, under 5% CO_2 in air.

Antibodies. To generate rabbit polyclonal antibodies, rabbits were immunized with the target peptides bound to thyroglobulin. The C4.4A-specific IgG was purified by passage of the antisera over a peptide column in which the peptide had been coupled to the beads. To generate the anti-human C4.4A monoclonal antibody, mice (BALB/c or BDF1, Charles River, Japan) were immunized weekly with thyroglobulin-conjugated C4.4A peptides (50 μg/mouse). We used partial human C4.4A peptide consisting of amino acid residues 301-316 (AGHQDRSNSGQYPAKG) as an immunogen for the C-terminus containing a portion of the GPI anchor. The cysteine residue was combined in the immunogen beforehand for binding to the carrier protein, bovine thyroglobulin. After four immunizations, the spleen was isolated and fused with X63Ag8 myeloma cells. Through limited dilution and the screening process, the 11A1 clone was selected. The rabbit anti-human actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunohistochemistry. Tissue sections (4-μm thick) were prepared from paraffin-embedded blocks. After antigen retrieval treatment in 10 mM citrate buffer (pH 6.0) at 95˚C for 40 min, immunostaining was carried out using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA) as we have described previously (12,13). The slides were incubated overnight at 4˚C with appropriate antibodies diluted as follows: C4.4A-119, 1:20; C4.4A-278, 1:20; C4.4A GPI-P, 1:200; and C4.4A GPI-M, 1:50. Non-immunized rabbit IgG or mouse IgG (Vector Laboratories) was substituted for the primary antibody as a negative control to exclude possible false-positive responses from the secondary antibody or from non-specific binding of IgG.

Western blot analysis. Western blot analysis was performed as described previously (14,15). Briefly, 20-μg protein samples were separated by 12.5% polyacrylamide gel electrophoresis followed by electroblotting onto a polyvinylidene difluoride membrane (PVDF). The membrane was incubated for 1 h with the primary antibodies at the following concentrations: C4.4A-119, 1:50; C4.4A-278, 1:50; C4.4A GPI-P, 1:500; C4.4A GPI-M, 1:50; and actin, 1:1000. The protein bands were detected using the Amersham Enhanced Chemiluminescence (ECL) Detection System (Amersham Biosciences Corp., NJ, USA).

Absorption test. For absorption testing, an excess amount of immunogen peptide was added to the antibody (20 mol:1 mol), the mixture was incubated overnight at 4˚C and was used instead of the primary antibody.

Results

Generation of C4.4A antibodies. In this study, we generated two novel rabbit anti-human C4.4A polyclonal antibodies, C4.4A-119 and C4.4A-277, and a mouse anti-human C4.4A monoclonal antibody, C4.4A GPI-M. The immunogens used for C4.4A-119 and C4.4A-277 were ^{119}*LTSRALDPAGNE SAYPPNGVEC* and ^{277}*SQTP RQGVEHEASR DEEPRLT*, respectively (Fig. 1). The anti-human C4.4A monoclonal antibody C4.4A GPI-M was raised against ^{301}*AGHQDRSNSGQYPAKG* at the C-terminus containing a portion of the GPI anchor signaling sequence (Fig. 1). The ^{301}*AGHQDRSNSGQYPAKG* immunogen was used in our previous study to generate a GPI-related polyclonal antibody, anti-human C4.4A antibody-2 antibody, which we also used in the present study for comparison (designated as C4.4A GPI-P in this study for simplicity).

Western blot analysis. The C4.4A-119 and C4.4A-277 antibodies in HCT116 each produced a band at around 70 kDa, and the absorbed antibodies eliminated these bands (Fig. 2A and B). On the other hand, the C4.4A GPI-P antibody in HCT116 produced doublet bands at 40 and 52 kDa (Fig. 2C), and the

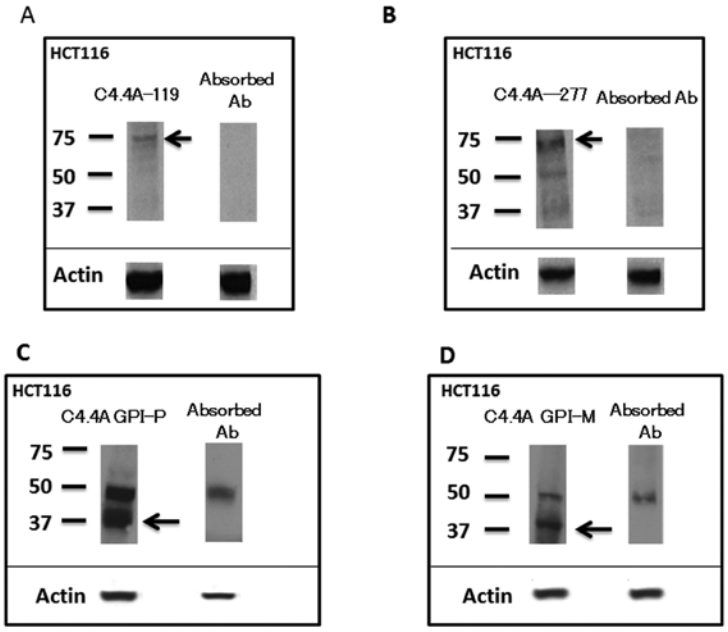

Figure 2. Western blot analysis for the C4.4A protein using lysates from HCT116 colon cancer cells. (A), C4.4A-119 antibody; (B), C4.4A-277 antibody; (C), C4.4A GPI-P antibody; (D), C4.4A GPI-M antibody. The arrows indicate the band corresponding to the C4.4A protein.

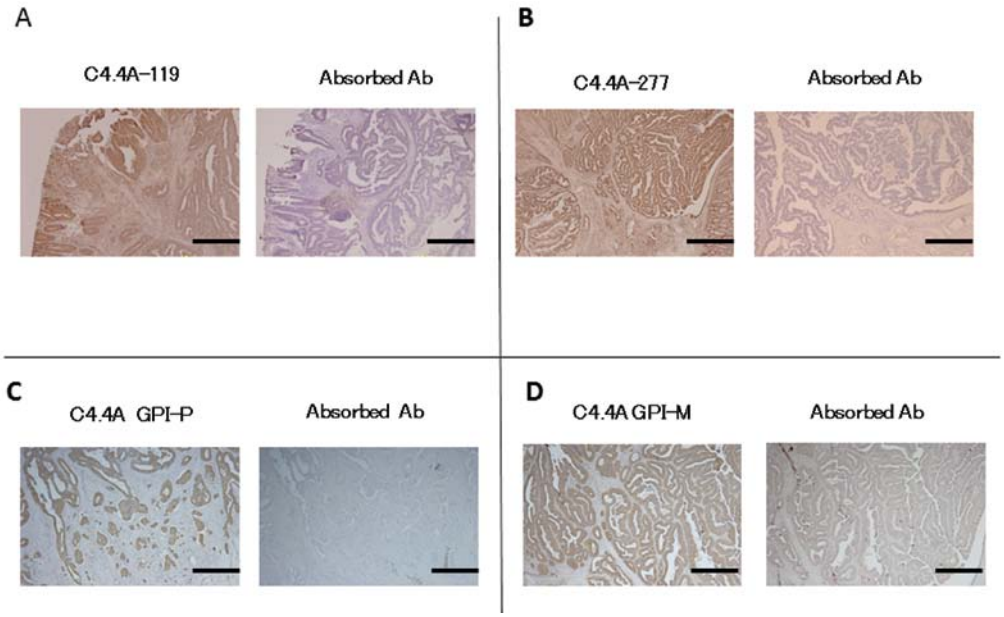

Figure 3. Absorption test on CRC tissues. (A), C4.4A-119 antibody; (B), C4.4A-277 antibody; (C), C4.4A GPI-P antibody; and (D), C4.4A GPI-M antibody. The scale bars indicate 500 μm (A-D).

absorbed antibody abolished a band at 40 kDa, which can be visualized on the tissue sections; similar results were observed with the C4.4A GPI-M antibody (Fig. 2D).

Immunohistochemistry. The C4.4A antibodies yielded positive staining for the C4.4A protein on tumor cells in CRC tissue specimens, and the pre-absorbed antibodies abolished this staining (Fig. 3).

Localization of C4.4A and positive staining rate in CRC tissues with each antibody. Immunohistochemistry revealed that the C4.4A protein was differently detected by each antibody in terms of intra-cellular and intra-tumoral locations, and positive staining rate. Using the C4.4A-119 antibody, positive staining was noted in 17 of 33 CRC cases (51.5%); relatively weak cytoplasmic staining was observed in the tumor cells, irrespective of location in the tumor body, i.e., superficial to intermediate area or invasive front (Fig. 4A). With the C4.4A-277 antibody, 29 of 33 CRC samples (87.9%) were positive for cytoplasmic C4.4A, and staining was observed at both the invasive front and superficial to intermediate portions (Fig. 4A); membranous staining was generally not evident, but weak positive staining

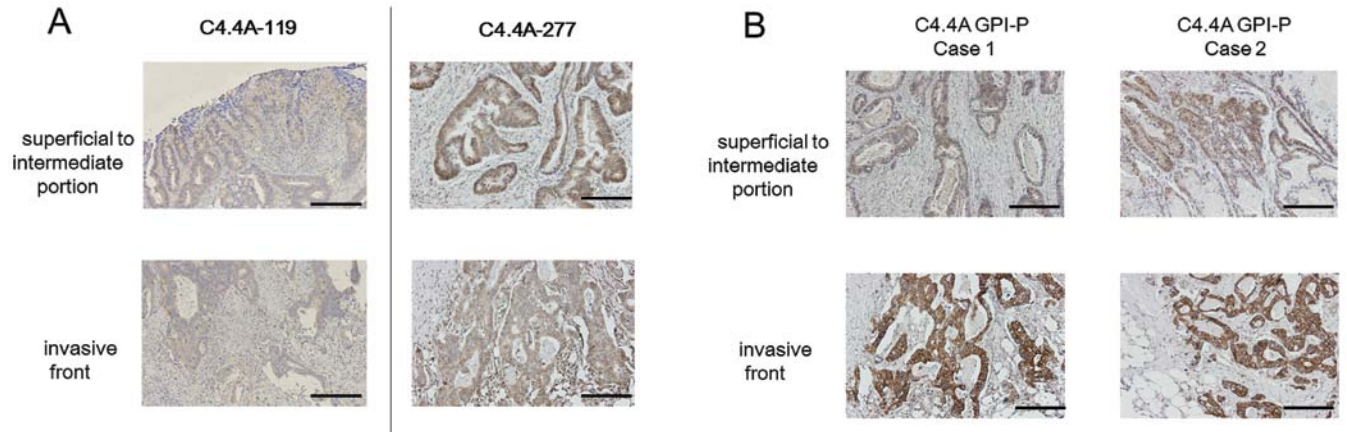

Figure 4. Immunohistochemistry of C4.4A at either a superficial to intermediate portion of the cancer body or at the invasive front of CRC tissue. (A), Representative CRC samples stained with the C4.4A-119 antibody, the C4.4A-277 antibody; and (B), the C4.4A GPI-P antibody. The scale bars indicate 200 μm (A and B).

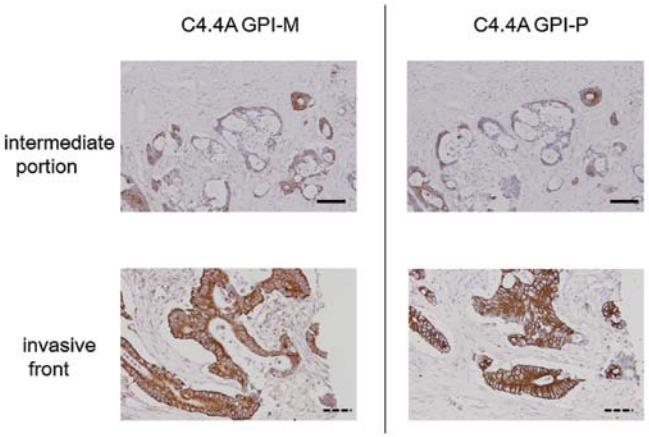

Figure 5. Immunohistochemistry of C4.4A using GPI-related antibodies. Serial sections stained with the C4.4A GPI-M antibody and the C4.4A GPI-P antibody showed identical patterns of C4.4A-positive cells in the cancer glands. Identical heterogeneous C4.4A expression patterns were also noted at the intermediate portion of the cancer body. The scale bars indicate 100 μm. A membranous expression pattern was observed at tumor invasive fronts with each antibody. The dotted scale bars indicate 50 μm.

was occasionally observed in only a portion of the cancer body. On the other hand, the C4.4A GPI-P antibody produced intense membranous staining at the invasive front in 11 of 33 CRC cases (33.3%), but this staining was usually not found at the superficial to intermediate portions (Fig. 4B).

The C4.4A GPI-M antibody showed a staining pattern similar to that produced by the C4.4A GPI-P antibody (Fig. 5). Staining by both GPI-related antibodies in serial sections revealed identical heterogeneous C4.4A expression patterns at the intermediate portion of the cancer body. The C4.4A GPI-M antibody also provided intense membranous staining in the tumor cells, only at the invasive front.

Discussion

In this study, we used four specific antibodies against the C4.4A protein, three of which were newly produced. Antibody specificities were verified by absorption tests, and efficacies by both western blot analysis and immunohistochemistry. The use of these distinct antibodies revealed several isoforms of the C4.4A protein, which differed in terms of molecular weight, intra-tumoral location, and intra-cellular localization. Western blot analysis revealed the existence of at least two isoforms: a long form of ~70 kDa, and a short form of ~40 kDa. This finding is consistent with the report by Paret *et al* that the recombinant C4.4A protein is digested by trypsin treatment from a long form of over 70 kDa to a proteolytic fragment of 40 kDa (9). Hansen *et al* also observed that both a long form (67 kDa) and short form (40 kDa) were present in the normal esophageal epithelium and at the superficial portion of the cancer body of the esophagus, whereas the short form of ~40 kDa was predominant in the invasive front of the cancer body (16).

In the present study, we found that the C4.4A-119 and C4.4A-277 antibodies reacted with the long form of C4.4A (70 kDa) and exhibited mainly cytoplasmic intra-cellular localization, irrespective of intra-tumoral location. In the tested CRC cases,

Table I. Detection of C4.4A expression by C4.4A antibodies.

	C4.4A-119	C4.4A-277	C4.4 GPI-P	C4.4 GPI-M
Positive rate in CRC tissue	51.5%	87.9%	33.3%	33.3%
Intra-cellular localization	Cytoplasm	Cytoplasm (occasionally plasma membrane)	Plasma membrane	Plasma membrane
Intra-tumor localization	All layers	All layers	Limited to invasive front	Limited to invasive front
Molecular weight	70 kDa	70 kDa	40 kDa	40 kDa

the C4.4A-119 and C4.4A-277 antibodies showed positive rates of 51.5% and 87.9%, respectively, indicating that cytoplasmic C4.4A was relatively frequently present in CRC tissues. Paret *et al* (9) previously used an antibody created with immunogens from two different portions of C4.4A (amino acids 278-302 and 119-138), and showed C4.4A expression in more than 80% of CRC tissue samples. Another study on C4.4A expression in esophageal squamous cell carcinoma (ESCC) showed a 100% detection rate (14 of 14 ESCC cases) using an antibody that recognizes the Domain III portion of the C4.4A molecule (5,16). These findings suggest that C4.4A could be a sensitive marker for CRC and ESCC when using the specific antibodies with high C4.4A detection rates.

On the other hand, we found that the C4.4A GPI-P antibody detected mainly membranous C4.4A at the invasive front of CRC tissues, at a lower rate of 33.3%. These findings suggest that, although cytoplasmic C4.4A is commonly detected in CRC tissues, only certain CRCs expressed membranous C4.4A. We hypothesize that the membranous type of C4.4A is functionally important and of clinical significance; it is possible that C4.4A on the plasma membrane could play a crucial role in invasion and metastasis. We previously reported that membranous C4.4A was linked to venous invasion, and associated with poor prognosis (especially hematogenous metastasis) in CRC (11). Moreover, we recently found that membranous C4.4A was tightly linked to EMT (epithelial-mesenchymal transition) change, and associated with tumor budding (17), a putative hallmark of cell invasion of CRC (18,19).

To explore whether the GPI anchor sequence was essential for detecting membranous C4.4A at the invasive front, we developed the novel monoclonal antibody C4.4A GPI-M. The antibody detected a membranous C4.4A expression pattern at the invasive front, similar to that shown by the C4.4A GPI-P antibody. Both C4.4A GPI-P and C4.4A GPI-M antibodies produced a band at around 40 kDa. These findings suggest that the C4.4A protein may exist as a proteolytic fragment on the plasma membrane in a subset of CRC. Based on these results, we concluded that the GPI anchor signaling sequence is essential for detecting membranous C4.4A at the invasive front. The present findings also suggest that C4.4A might be digested into the short form at the invasive front of CRC when it links to the plasma membrane via the GPI anchor.

In conclusion, as summarized in Table I, we found that the majority of CRC tissues expressed cytoplasmic C4.4A, and a subset of CRCs displayed C4.4A on the plasma membrane. Both the C4.4A GPI-M antibody and the C4.4A GPI-P antibody exhibited membranous expression patterns, suggesting that the GPI anchor signaling sequence is essential for detecting membranous C4.4A at the invasive front.

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