Brief Report

Appearance of high-molecular weight sialoglycoproteins recognized by *Maackia amurensis* leukoagglutinin in gastric cancer tissues: A case report using 2-DE-lectin binding analysis

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Summary **Aberrant expression of sialoglycoconjugates has been thought to play an important role in cancer progression. Our previous lectin-histochemical study showed that overexpression of sialoglycoconjugates recognized by α2,3-sialic acid-specific *Maackia amurensis* leukoagglutinin (MAL) was significantly related to the malignancy of gastric cancer. The present study analyzed the sialoglycoproteins in gastric cancer tissues by 2-dimensional electrophoresis (2-DE) in combination with lectin-binding analysis using MAL. Various MAL-positive sialoglycoproteins were detected in cancer tissues but not in non-cancer tissues. The sialoglycoproteins have a high molecular weight of near 200 kDa and over 200 kDa with different pI values for the two. This suggests that the MAL-positive sialoglycoproteins detected in gastric cancer tissues have high molecular weights and may contain different numbers of α2,3-linked sialic acid residues in the carbohydrate moiety.**

Keywords: Sialoglycoproteins, *Maackia amurensis* leukoagglutinin, Lectin, 2-Dimensional electrophoresis, Gastric cancer

1. Introduction

Sialoglycoconjugates, which bear sialic acid residues in their carbohydrate moieties, are essential in various biological events within organisms (*1*). Overexpression of sialoglycoconjugates and structural alteration of the carbohydrate moieties have frequently been detected in various cancer tissues and may be associated with tumor metastasis and progression (*2-5*). In this regard, detection of sialoglycoconjugates in tumor tissues with sialic acid-binding lectins would be helpful in evaluating the metastatic potential of those tumors and predicting patient prognosis. *Maackia amurensis* leukoagglutinin (MAL) recognizes α2,3-linked sialic acid residues (*6*) and has been effectively used for biochemical and histochemical analyses of sialoglycoconjugates (*7-9*). A previous lectin-histochemical study on gastric cancer tissues by the current authors has suggested that MAL-positive sialoglycoconjugates were detected in cancer tissues but not in non-cancer tissues and that overexpression of the MAL-positive sialoglycoconjugates was related to worse prognosis for patients (*10*). However, the nature of MAL-positive sialoglycoconjugates in gastric cancer tissues has yet to be clarified. The present study characterized MAL-positive sialoglycoconjugates in gastric cancer tissues by means of 2-dimensional electrophoresis (2-DE) in combination with lectin binding analysis using MAL.

2. Materials and Methods

2.1. *Tissues*

Fresh gastric cancer and the corresponding non-cancer tissue samples (548 and 258 mg wet weight, respectively)

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were collected from a patient who underwent surgical resection at the Department of Surgery, the University of Tokyo, Japan. Tissue samples were stored in -80°C until use for protein extraction or fixed with formalin followed by embedding in paraffin for lectin-histochemistry.

2.2. *Lectin-histochemistry*

Five-μm-thick sections were cut from formalin-fixed paraffin-embedded tissue blocks and subjected to lectin-histochemistry using 4 μg/mL of biotinylated MAL (Seikagaku Co., Tokyo, Japan) as described elsewhere (*10*). Detection was performed by a biotin-streptavidin-peroxidase complex method using 3,3'-diaminobenzidine as a chromogen and hematoxylin as a counterstain.

2.3. *Protein extraction*

Proteins were sequentially extracted from the tissue samples into 3 fractions based on the different solubility using a ReadyPrep sequential extraction kit (Bio-Rad Laboratories, Richmond, CA, USA). Briefly, the tissue sample was homogenized and sonicated with Reagent 1 (40 mM Tris base) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a hydrophilic protein fraction. Next, the pellet was homogenized and sonicated with Reagent 2 (8 M urea, 4% (w/v) CHAPS, 0.2% SB3-10, 40 mM Tris) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a slightly hydrophobic protein fraction. Finally, the pellet was then homogenized and sonicated with Reagent 3 (5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.2% SB3-10, 40 mM Tris) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a highly hydrophobic protein fraction. The protein in each fraction was quantified with a Protein assay kit (Bio-Rad Laboratories) in accordance with Bradford's method (*11*).

2.4. *Immobilized pH gradient-2-DE*

Individual fractions of protein samples were applied to an immobilized pH gradient (IPG)-2-DE. Briefly, 25 μg of sample protein were subjected to first-dimension isoelectric focusing (IEF) using a PROTEAN IEF system (Bio-Rad Laboratories) and a linear IPG strip (7 cm length, pI 3-10, Bio-Rad Laboratories). After electrofocusing, the gel strip was equilibrated with equilibration buffer I (0.375 M Tris-HCl, pH 8.8, containing 6 M urea, 20% (w/v) glycerol, and 2% (w/v) dithiothreitol) for 20 min and then with equilibration buffer II (0.375 M Tris-HCl, pH 8.8, containing 6M urea, 20% glycerol, 2% SDS, 2.5% (w/v) iodoacetamide) for 10 min. The equilibrated gel strip was placed on a 7.5-15% gradient polyacrylamide gel and then second-dimension SDS-PAGE was carried out at 200 V for 40 min. To detect proteins, the gel was stained with SYPRO Ruby staining solution (Bio-Rad Laboratories).

2.5. *Western blotting and lectin binding assay*

After IPG-2-DE, proteins in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) by using HorizBlot AE-6677 (ATTO, Tokyo, Japan). The membrane was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 3 h at room temperature and then incubated with 5 μg/mL of biotinylated MAL in 1% BSA-TBS for 1 h at room temperature followed by incubation with streptavidin-conjugated horseradish peroxidase solution (Nichirei, Tokyo, Japan) for 1 h at room temperature. Protein spots were detected by an enhanced chemiluminescence (ECL) method (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

3. Results and Discussion

First, expression of MAL-positive glycoconjugates in gastric cancer tissues was examined histochemically. As shown in Figure 1, MAL-positive staining was observed only in cancer tissues (left side) but not in non-cancer tissues (right side), which is consistent with a previous report (*10*).

Next, proteins in the sample tissues were extracted and fractionized as described in Methods and then subjected to 2-DE. Lectin binding analysis using MAL was performed after the proteins were transferred from 2-DE gels to PVDF membranes. As shown in Figure 2A, the hydrophilic protein fraction of gastric cancer tissue yielded two series of MAL-positive spots that were detected at positions with a molecular weight of over 200 kDa and pI range of 4-6 and positions with

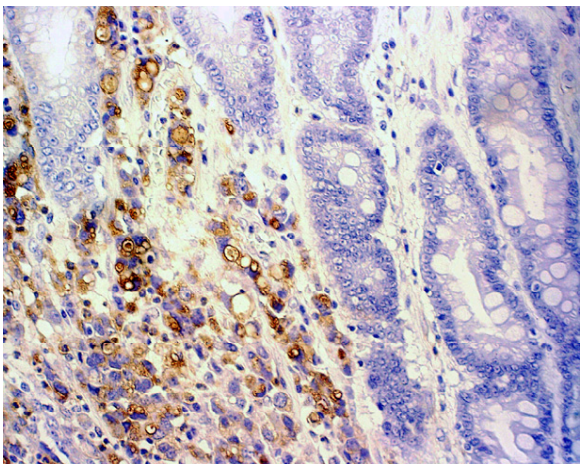

Figure 1. Typical example of lectin-histochemical staining using MAL. The stain was only detected in the cancer region (*left*) but not in the non-cancer region (*right*). Original magnification, ×200.

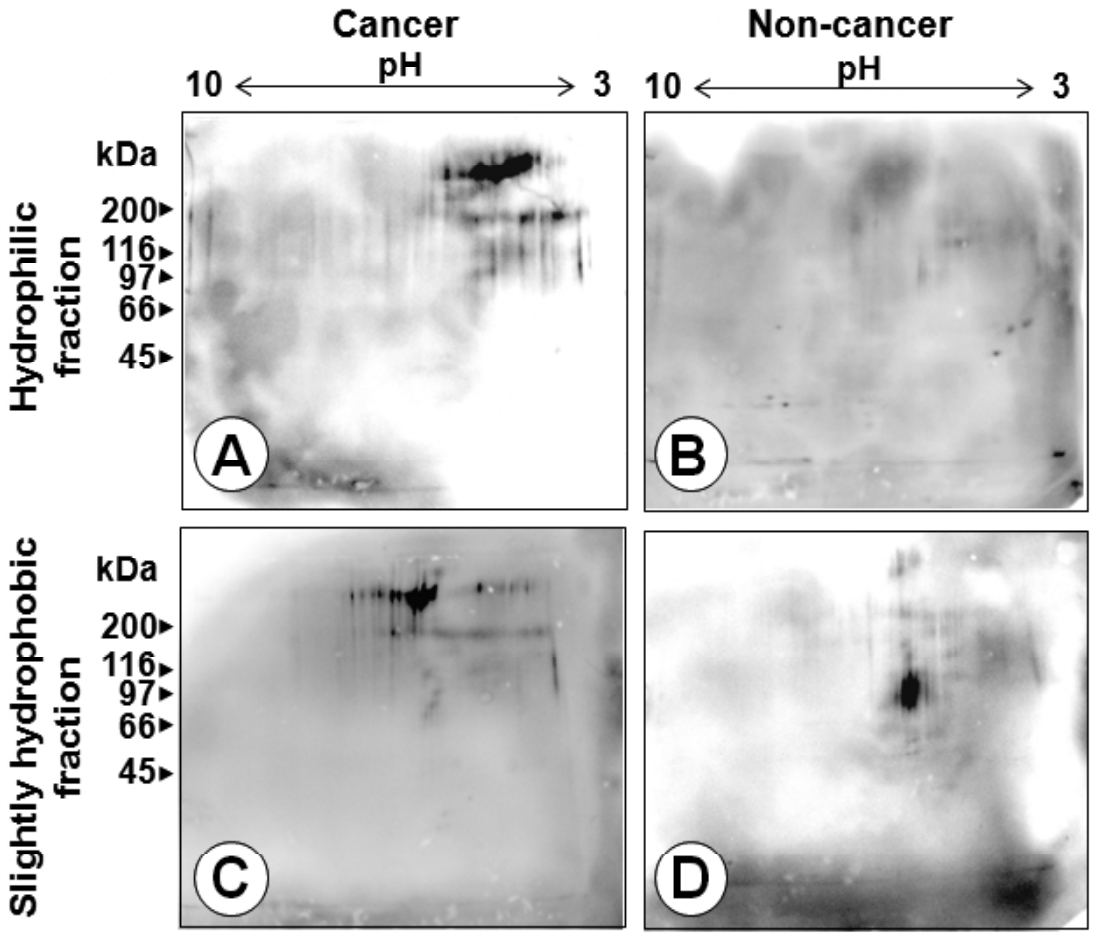

Figure 2. 2-DE-lectin binding analysis using MAL. Proteins in cancer (*A* and *C*) and non-cancer (*B* and *D*) tissues were fractionized into hydrophilic (A and B) and slightly hydrophobic (*C* and *D*) fractions and then subjected to 2-DE-lectin binding analysis using MAL as described in Methods.

a molecular weight of near 200 kDa and pI range of 3.5-5.5. In contrast, distinct MAL-positive spots were not observed in the non-cancer tissue sample (Figure 2B). Two series of MAL-positive spots were also detected in the slightly hydrophobic protein fraction of cancer tissue (Figure 2C). The molecular weight profile of these proteins was similar to that detected in the hydrophilic fraction of cancer tissue, although the pI range of spots with a high intensity appeared to be close to the neutral point (Figure 2C). In contrast, MAL-positive spots were not observed in these areas in the slightly hydrophobic protein fraction from the non-cancer tissue sample (Figure 2D). A MAL-positive protein at a position with ~90 kDa and pI 5.5 detected in this fraction is thought to be derived from contaminated blood components such as leukocytes (*12*). In highly hydrophobic fractions, MAL-positive spots were not detected in both cancer and non-cancer tissues (data not shown).

A previous lectin-histochemical study with gastric cancer tissues by the current authors indicated that MAL-positive glycoconjugates exclusively appear in cancer tissues but not in non-cancer tissues, which was confirmed in the present study (Figure 1), and that the aberrant increase in expression is significantly correlated to invasion and metastasis of cancer cells and a worse prognosis for patients (*10*). Thus, analyzing the property of cancer-specific MAL-positive sialoglycoconjugates is crucial to clarifying the mechanism of cancer progression. A preliminary study by the current authors suggested that MAL-positive sialoglycoconjugates are contained in the protein fraction but not in the glycolipid fraction (data not published). Thus, the present study analyzed the MAL-positive sialoglycoproteins by means of 2-DE and lectin binding analysis.

As described above, two series of cancer-specific MAL-positive sialoglycoproteins with high molecular weights near 200 kDa and over 200 kDa, respectively, were detected in both hydrophilic and slightly hydrophobic fractions. The sialoglycoproteins in each series have similar molecular weights but have different pI values. Therefore, one possibility is that each series of sialoglycoproteins may have a same polypeptide backbone but possess different numbers of α2,3-linked sialic acid residues in its carbohydrate moiety. Although the detailed nature of these glycoproteins has not been determined, the high molecular weight of the MAL-positive sialoglycoproteins detected in gastric cancer tissues suggests that these sialoglycoproteins may be a type of mucins that are known to play a major role in cancer progression (*13*). In addition, the relationship

between clinical characteristics and 2-DE profile of MAL-positive sialoglycoproteins remains unclear. Proteomic analysis with 2-DE is a technique that has often been used to identify specific proteins (*14,15*) and has been developed to identify glycoproteins with a specific carbohydrate structure by using lectins (*16,17*). The current work shows the 2-DE profile of MAL-positive sialoglycoproteins obtained from one patient. Further studies with multiple specimens must be performed to clarify the clinical significance of MAL-positive sialoglycoproteins in gastric cancer tissues and determine their nature.

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