

## Changes to N-linked Oligosaccharide Chains of Human Serum Immunoglobulin G and Matrix Metalloproteinase-2 with Cancer Progression

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**Abstract.** *Background:* Alterations to the sugar chain structure of E-cadherin, a calcium-dependent adhesion molecule, have been shown to influence cancer metastasis. Furthermore, expression of sialyl Le<sup>x</sup> sugar chains on cancer cells has been demonstrated to influence their adhesion to vascular endothelial cells. On the other hand, matrix metalloproteinase-2 (MMP-2) degrades extracellular matrix and is involved in the invasion and metastasis of cancer cells. *Patients and Methods:* N-linked oligosaccharides of human serum immunoglobulin G (IgG) were analyzed in 36 patients with localized or metastatic cancer (12 lung, 12 gastric and 12 prostate cancer) and 10 healthy controls using fluorophore-associated carbohydrate electrophoresis (FACE). MMP-2 levels in the sera were determined by enzyme immunoassay. *Results:* Fr1 (monogalactosyl IgG oligosaccharide) and Fr2 (digalactosyl IgG oligosaccharides) were significantly decreased ( $p < 0.001$ ), while Fr4 (agalactosyl IgG oligosaccharides) were significantly increased ( $p < 0.001$ ) with cancer metastasis. The Fr4/Fr1+Fr2 ratio in localized and metastatic cancer was significantly increased compared to healthy controls ( $p < 0.001$ ), and was significantly higher in metastatic than localized cancer ( $p < 0.001$ ). Serum MMP-2 levels in metastatic cancer were significantly higher than in localized cancer ( $p < 0.001$ ). There was a good correlation between the Fr4/Fr1+Fr2 ratio and serum MMP-2 levels in patients with metastatic cancer ( $p < 0.0001$ ). *Conclusion:* The analysis of serum IgG N-linked oligosaccharide chain structures by FACE may be an auxiliary indicator of serum tumor markers useful for monitoring cancer progression.

Changes in sugar chain structure affect the molecular structure of IgG and the ability of Fc receptors to bind to the macrophage surface (1, 2). A heavy chain of human immunoglobulin G (IgG) has two N-linked oligosaccharide chains at residue Asn 297 in the Fc region (3). Thus, the oligosaccharide chains that bind to glycoproteins help to maintain the three-dimensional structure of those proteins and play an important role in their physiological activity. It was demonstrated by high-performance liquid chromatography that serum IgG oligosaccharide chains lacking galactose (Gla) (agalactosyl IgG oligosaccharides) were frequently found in sera from rheumatoid arthritis (RA) patients (4). It is hypothesized that agalactosyl IgG oligosaccharides activate the complement system via mannose-binding protein, contributing to the pathogenesis of RA (5).

Dosaka-Akita *et al.* demonstrated that expression of N-acetylglucosaminyltransferase V (GnT-V) in cancer cells was increased in patients with non-small cell lung cancer (NSCLC) (6). Kossowska *et al.* showed that fucosylated oligosaccharides were increased in sera from NSCLC patients (7). Furthermore, Span-1 and DU-PAN-2 belong to the same category of carbohydrate structure and are used as serum pancreatic tumor markers (8, 9). Using FACE analysis, we previously reported that agalactosyl IgG oligosaccharides significantly increased during the progression of gastric and lung cancer (10).

On the other hand, it has been demonstrated that cancer cell-derived proteases such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) play an important role in the invasion and metastasis of cancer cells through the degradation of the extracellular matrix (ECM) (11, 12). Of the different MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been shown to degrade type IV collagen, a major component of cellular basement membrane, and to participate in the invasion and metastasis of cancer cells (13).

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*Key Words:* N-linked oligosaccharide chains, matrix metalloproteinase-2, localized, metastatic cancer.

However, there are no reports on the relationships between changes in sugar chain structure and serum MMP-2 levels in patients with localized or metastatic cancer. Therefore, in the present study, we analyzed the serum IgG *N*-linked oligosaccharide chain structures from cancer patients and evaluated the relationship between changes in oligosaccharide chain structure and serum concentrations of MMP-2.

## Patients and Methods

**Patient characteristics.** Serum samples were obtained from 36 cancer patients prior to treatment (18 localized cancer with 6 non-small cell lung cancer (NSCLC), 6 gastric adenocarcinomas and 6 prostate adenocarcinomas, and 18 metastatic cancer also consisting of 6 NSCLC, 6 gastric adenocarcinomas and 6 prostate adenocarcinomas) (mean age 65.6 years, range 52 to 78 years), diagnosed at Kitasato University Hospital. Ten healthy men were enrolled as controls (mean age 62.8 years, range 52 to 76 years). Lung and prostate carcinomas were clinically staged following the TNM classification (14, 15), and gastric cancer following the Japanese classification (16). Serum samples were obtained from these patients and stored at  $-80^{\circ}\text{C}$  until use. Informed consent was obtained from all participants in this study.

**Purification of serum IgG.** Serum (300-500  $\mu\text{l}$ ) was diluted 4-fold with 0.01 M phosphate buffer (pH 7.0), and applied to a Protein G column (Pharmacia Biotech Inc., Uppsala, Sweden). After washing the column with 5 ml of 0.01 M phosphate buffer, protein was eluted with 3 ml of 0.1 M glycine-HCl buffer (pH 3.0) and 0.5 ml of 1 M Tris-HCl (pH 9.0) buffer. The protein was dialyzed against distilled water for 48 h using a dialysis membrane (Sanko Junyaku Inc., Tokyo, Japan) and lyophilized. The purity of the IgG was confirmed by immunoelectrophoresis using anti-human whole serum antibody and anti-human serum IgG antibody.

**Release of *N*-linked oligosaccharide chains from serum IgG.** Purified IgG (250  $\mu\text{g}$ ) was dissolved in 25  $\mu\text{l}$  of distilled water, and 25  $\mu\text{l}$  of 0.1 M phosphate buffer (pH 7.4), 1  $\mu\text{l}$  of 5% SDS and 1.5  $\mu\text{l}$  of 1.44 M 2-mercaptoethanol were added. The mixture was heated at  $100^{\circ}\text{C}$  for 5 min and then treated with 2.5 ml of 7.5% Nonidet P-40 and 2  $\mu\text{l}$  of recombinant peptide *N*-glycosidase F (PNGase F, EC 3.5.1.52; Seikagaku Kogyo Inc., Tokyo, Japan) at  $37^{\circ}\text{C}$  for 2 h. Subsequently, anhydrous ethanol (171  $\mu\text{l}$ ) was added and the mixture cooled for 10 min. After centrifugation of the mixture at 15,000 rpm for 5 min at  $4^{\circ}\text{C}$ , the supernatant containing the released oligosaccharides was evaporated to dryness and recovered.

**Fluorescence labelling of *N*-linked oligosaccharide chains from serum IgG.** Five  $\mu\text{l}$  of 0.15 M 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) in 15% acetic acid and 5  $\mu\text{l}$  of 1.0 M sodium cyanoborohydride in 1.0 M dimethyl sulfoxide (DMSO) were added to the oligosaccharides in the residue and the mixture was incubated at  $37^{\circ}\text{C}$  for 16 h.

**Electrophoresis and imaging analysis.** The ANTS-labelled oligosaccharides were separated by electrophoresis (SDS-PAGE) on a FACE-*N*-linked-oligosaccharide gel (Glyko Inc., Novato, CA, USA) at a constant current of 15 mA for 90 min. After the

termination of electrophoresis, the gel was imaged with a FACE IMAGER scanner (Glyko Inc.), and the fluorescent fraction (Fr) patterns were analyzed in five fractions by FACE Imaging Software version 2.47 (Glyko Inc.). Oligo ladder standard (Glyko Inc.), containing ANTS-labeled glucose polymers composed of 1 to 20 glucose residues, was applied to the gel as the marker. The determination of each Fr band was calculated compared with a standard degree of polymerization (DP) of G4, composed of 4 glucose residues, and is shown as a percentage (%).

**Sugar chain structures of the respective fractions.** We analyzed healthy controls using exoglycosidase and categorized the sugar chain structures into the five fractions (17): Fr1, IgG oligosaccharides with two Gla (digalactosyl IgG oligosaccharides); Fr2, with one Gla (monogalactosyl IgG oligosaccharides); Fr3 with one sialic acid (Sia) (monosialyl oligosaccharides); Fr4 without Gla (agalactosyl IgG oligosaccharides); and Fr5 with two Sia (disialyl oligosaccharides). The pattern of serum IgG *N*-linked oligosaccharide chains by SDS-PAGE was illustrated in a previous report (10).

**The determination of MMP-2 levels in serum.** Serum MMP-2 levels were determined by measuring pro-MMP-2 using a one-step sandwich enzyme immunoassay for MMP-2 (Fuji Chemical Industries, Toyama, Japan) according to the manufacturer's instructions.

**Statistical analysis.** The gel images were extracted from the FACE imaging software into Adobe PhotoShop version 5.5 after converting them to PICT files. The Mann-Whitney *U*-test was used for statistical analysis and  $p < 0.001$  was considered statistically significant.

## Results

**Structure of IgG *N*-linked oligosaccharide chains.** Figure 1 shows the serum IgG *N*-linked oligosaccharide chain structures for the respective fractions.

**The percentage of each fraction of serum IgG *N*-linked oligosaccharide chains.** Table I shows the percentage of each fraction of serum IgG *N*-linked oligosaccharide chains present in healthy controls, localized and metastatic cancer patients. The most abundant fraction according to the DP value was Fr2 (34.8%), followed by Fr1 (23.2%), Fr3 (22.7%), Fr4 (11.6%) and finally Fr5 (7.8%) in healthy controls. Serum IgG oligosaccharide chains of cancer patients were separated into five fractions as in healthy controls. In cancer patients, Fr1 significantly decreased in localized and metastatic cancer compared to healthy controls ( $p < 0.001$ ), and was significantly lower in metastatic than localized cancer ( $p < 0.001$ ). Fr2 slightly increased in localized cancer compared to healthy controls, but significantly decreased in metastatic cancer ( $p < 0.001$ ). Fr2 in metastatic cancer was significantly lower than in localized cancer ( $p < 0.001$ ). Fr3 significantly decreased in both localized and metastatic cancer compared to healthy controls ( $p < 0.001$ ), but was lower in metastatic than localized cancer. Fr4 significantly increased in localized and metastatic cancer compared to healthy controls ( $p < 0.001$ ), and was significantly higher in metastatic than

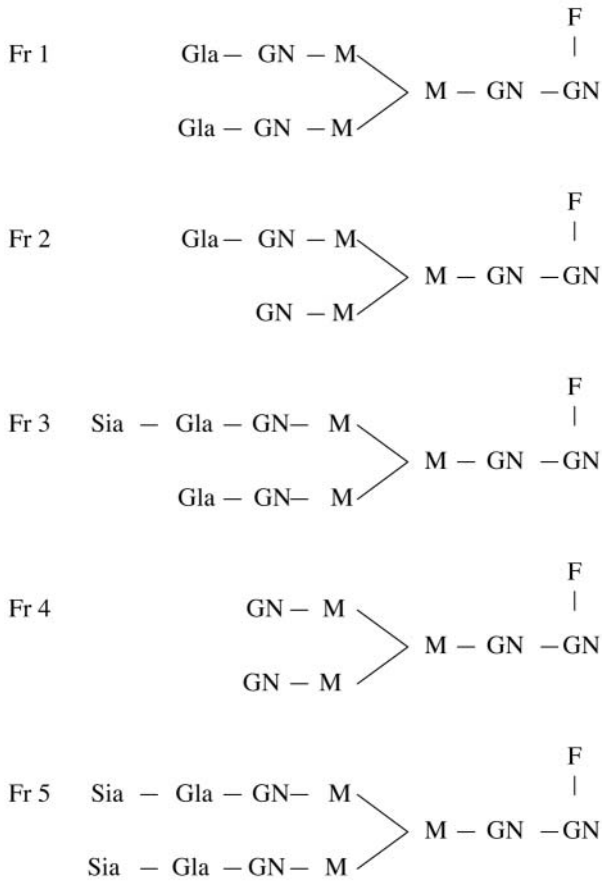


Figure 1. Structure of IgG oligosaccharide chains in five fractions. Sia, sialic acid; Gla, galactose; GN, N-acetylglucosamine; M, mannose; F, fucose.

localized cancer ( $p < 0.001$ ). Fr5 also increased in localized and metastatic cancer compared to healthy controls, and was again higher in metastatic cancer. We calculated the ratio of Fr 4 without Gla to Fr 1 and Fr2 that have Gla (Fr4/Fr1+Fr2 ratio). The Fr4/Fr1+Fr2 ratio significantly increased in localized and metastatic cancer compared to healthy controls ( $p < 0.001$ ), and was significantly higher in metastatic than localized cancer ( $p < 0.001$ ).

**Serum MMP-2 levels.** Serum MMP-2 levels slightly decreased in localized cancer compared to healthy controls, but significantly increased in metastatic cancer ( $p < 0.001$ ). Serum MMP-2 levels in metastatic cancer were significantly higher than in localized cancer ( $p < 0.001$ ) (Table II).

**Relationship between changes in serum IgG N-linked oligosaccharide chains and concentrations of serum MMP-2.** We also evaluated the relationship between changes in serum IgG N-linked oligosaccharide chains and concentrations of serum MMP-2 in localized and metastatic cancer. There was

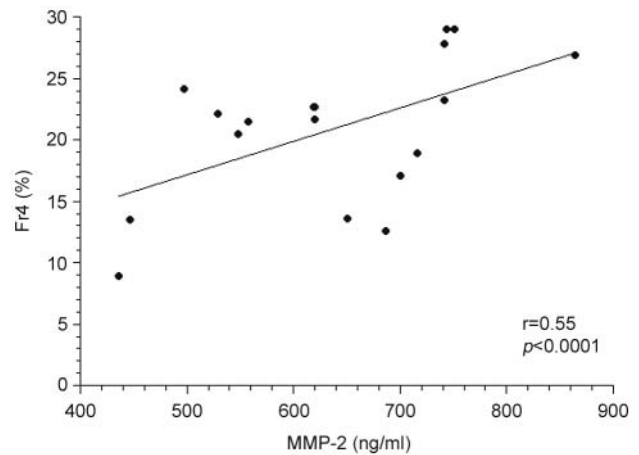


Figure 2. Correlation between serum MMP-2 levels and Fr4 in patients with localized cancer. There was a positive correlation and a significant association between serum MMP-2 levels and Fr4.

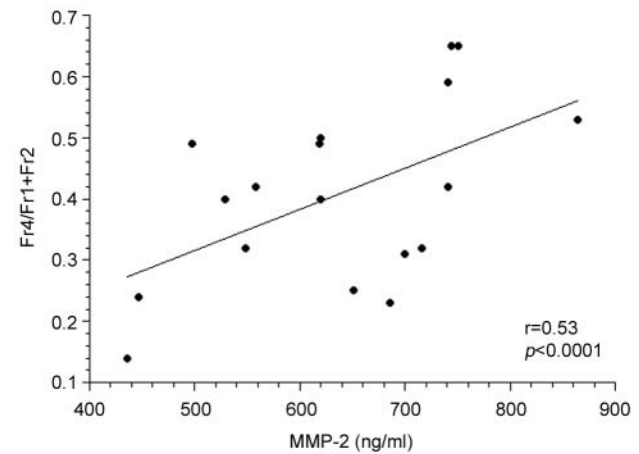


Figure 3. Correlation between serum MMP-2 levels and the Fr4/Fr1+Fr2 ratio in patients with localized cancer. There was a positive correlation and a significant association between serum MMP-2 levels and the Fr4/Fr1+Fr2 ratio.

a positive correlation between serum MMP-2 levels and Fr4 ( $r = 0.55$ ,  $p < 0.0001$ ) (Figure 2) as well as the Fr4/Fr1+Fr2 ratio ( $r = 0.53$ ,  $p < 0.0001$ ) (Figure 3), in localized cancer patients. There was also a positive correlation between serum MMP-2 levels and Fr4 ( $r = 0.56$ ,  $p < 0.0001$ ) and the Fr4/Fr1+Fr2 ratio ( $r = 0.68$ ,  $p < 0.0001$ ) in metastatic cancer patients as well (Figures 4 and 5, respectively).

**Discussion**

It has been demonstrated that alterations in the sugar chain structures of E-cadherin, a calcium-dependent adhesion molecule, and sialoyl Le<sup>x</sup> levels, which increase in cancer

Table I. Serum IgG N-linked oligosaccharide fractions (% of total, mean ±SD) in healthy controls and patients with localized and metastatic cancer as determined using FACE analysis.

	Healthy controls (n=10)	Localized cancer (n=18)	Metastatic cancer (n=18)	P-value of healthy controls vs. localized cancer	P-value of healthy controls vs. metastatic cancer	P-value of localized cancer vs. metastatic cancer
Fr1	23.18±3.67	16.50±4.34	11.06±3.58	<0.001	<0.001	<0.001
Fr2	34.83±6.60	36.04±3.27	29.60±4.53	NS	<0.001	<0.001
Fr3	22.63±2.46	17.69±2.86	15.84±2.90	<0.001	<0.001	NS
Fr4	11.55±4.61	20.79±6.19	33.50±10.51	<0.001	<0.001	<0.001
Fr5	7.76±0.88	8.88±2.08	9.73±3.58	NS	NS	NS
Fr4/Fr1+Fr2	0.21±0.09	0.41±0.15	0.90±0.51	<0.001	<0.001	<0.001

NS: No statistical significance.

Table II. Serum MMP-2 levels (ng/ml, mean ±SD) in healthy controls, and patients with localized and metastatic cancer.

	Healthy controls (n=10)	Localized cancer (n=18)	Metastatic cancer (n=18)	P-value of healthy controls vs. localized cancer	P-value of healthy controls vs. metastatic cancer	P-value of localized cancer vs. metastatic cancer
MMP-2	704±62.40	643.69±122.39	860.24±97.32	NS	<0.001	<0.001

NS: No statistical significance.

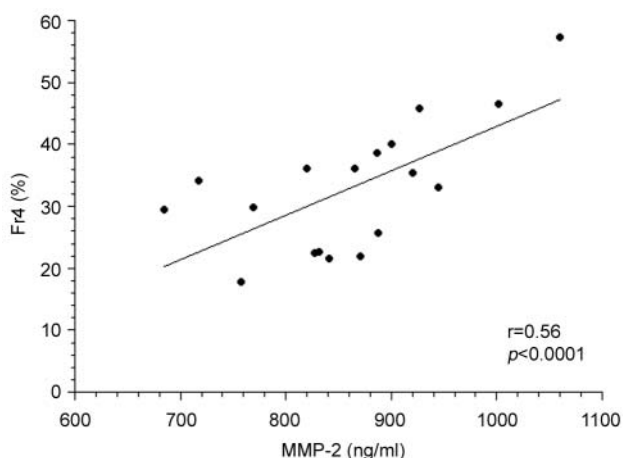


Figure 4. Correlation between serum MMP-2 levels and Fr4 in patients with metastatic cancer. There was a positive correlation and a significant association between serum MMP-2 levels and Fr4.

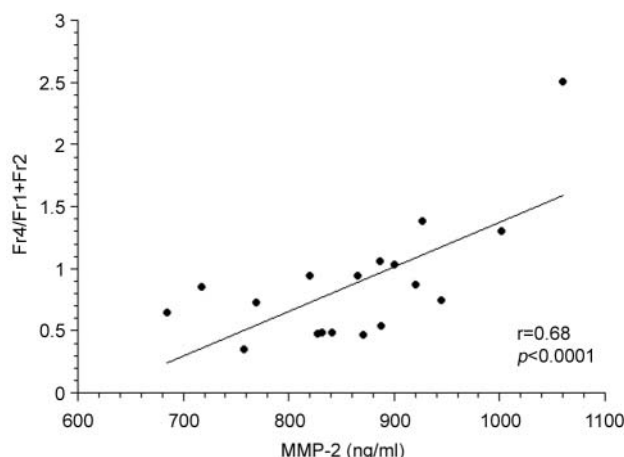


Figure 5. Correlation between serum MMP-2 levels and the Fr4/Fr1+Fr2 ratio in patients with metastatic cancer. There was a positive correlation and a significant association between serum MMP-2 levels and the Fr4/Fr1+Fr2 ratio.

cells, influence cancer metastasis (18). Oyama *et al.* showed a high rate of sialoyl Lewis X-i antigen (SLX) expression on two key sugar chains in lung adenocarcinoma (19). Regarding changes in sugar chain structure with tumor progression, it has been shown that fucosylated oligosaccharides were increased in sera from NSCLC patients (7). Furthermore,

another study demonstrated that there was an increase in the amount of one outer arm fucosylation (A3G3F0) in sera from patients with NSCLC (20). We have previously reported that analyzing serum IgG N-linked oligosaccharides by FACE may be useful for evaluating the diagnosis and prognosis of patients with gastric and lung cancer (10).

MMP-2 degrades a major component of the cellular basement membrane, type IV collagen. Of the different MMPs, MMP-2 as well as MMP-9 are thought to be involved in the invasion and metastasis of cancer cells (13). Gelatin zymography has shown that activated MMP-2 and MMP-9 can both be detected in the cerebrospinal fluid of patients with metastatic brain tumors or carcinomatous meningitis; moreover, this may be useful in differential diagnosis of cancer with or without brain metastasis (21). Increased expression of pro-MMP-2 and activated MMP-2 has been found in invasive urothelial cancer tissues, where their levels are significantly higher than in superficial urothelial cancer (22). These reports are consistent with the idea that MMP-2 and MMP-9 are involved in invasion and metastasis of cancer. We previously demonstrated that serum MMP-2 levels may be an auxiliary indicator of serum PSA for monitoring progression of prostate cancer (23).

In the present study, we analyzed the serum IgG *N*-linked oligosaccharide chain structure in patients with cancer progression and evaluated the relationship between changes in these structures and serum concentrations of MMP-2.

The serum IgG *N*-linked oligosaccharide chains from cancer patients could be separated into the same five fractions as healthy controls. Fr1 and Fr2 that have Glc were significantly decreased, while Fr4 without Glc was significantly increased with cancer metastasis. The Fr4/Fr1+Fr2 ratio in localized and metastatic cancer significantly increased compared to healthy controls, and was significantly higher in metastatic than localized cancer. Thus, the structure of serum IgG *N*-linked oligosaccharide chains changed and agalactosyl IgG oligosaccharides significantly increased with cancer metastasis. The sugar chain structure of IgG is formed after the addition and/or repair of sugar chains by glycosyltransferase. In plasma cells, this process initiates in the endoplasmic reticulum and is transmitted to the Golgi body. It is believed that Glc is linked to IgG sugar chains by the action of galactosyltransferase (Gal-T) (24). Although the exact mechanism responsible for altered glycosylation in carcinogenesis and tumor progression remains to be discovered, it is thought that Gal-T activity in plasma cells is down-regulated during the process of carcinogenesis. Thus, tumor progression causes a significant increase of agalactosyl IgG oligosaccharides (Fr4).

MMP-2 is involved in invasion and metastasis of cancer cells and is a useful marker indicating cancer metastasis. We previously demonstrated that serum MMP-2 levels in metastatic prostate cancer were significantly higher than in localized disease. In the present study, serum MMP-2 levels in metastatic cancer were significantly higher than in localized cancer. There was a good correlation and a significant association between the Fr4/Fr1+Fr2 ratio and serum MMP-2 levels in patients with metastatic cancer.

In conclusion, it is thought that alterations to serum IgG *N*-linked oligosaccharide chains in cancer progression are caused by abnormal glycosylation associated with carcinogenesis. The Fr4/Fr1+Fr2 ratio was significantly increased in metastatic cancer, and there was a good correlation between this ratio and serum MMP-2 levels. Therefore, the analysis of serum IgG *N*-linked oligosaccharide chain structures by FACE may be an auxiliary serum tumor marker useful for monitoring cancer progression.

### Acknowledgements

This study was supported by grants from the Parent's Association Grant of Kitasato University, School of Medicine.

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*Received September 4, 2007*

*Revised November 9, 2007*

*Accepted December 11, 2007*