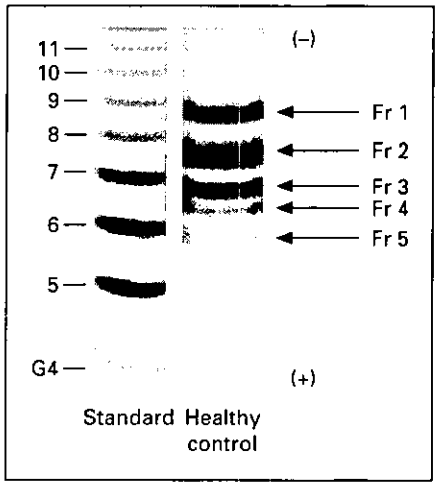

Fig. 1. Pattern of IgG oligosaccharide chain in normal individuals by SDS-PAGE. Oligo Ladder standard consists of a mixture of glucose polymers and 50 pmol of maltotetraose fraction (G4).

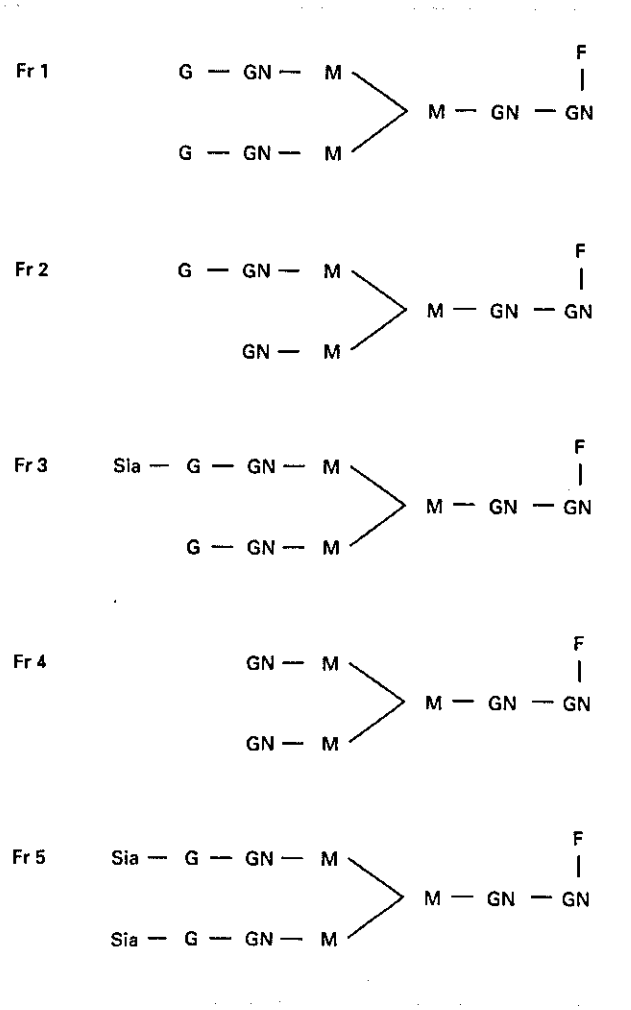

Fig. 2. Structure of IgG oligosaccharide chain in the fractions separated by PAGE. Sia = Sialic acid; G = galactose; GN = N-acetylglucosamine; M = mannose; F = fucose.

healthy controls, and decreased in metastatic lung cancer compared to localized lung cancer (table 1, fig. 3). The pattern of IgG oligosaccharide chains in different tissue type of lung cancer was almost identical.

In gastric cancer patients, Fr 1 and 3 had a tendency to be decreased, and Fr 4 to be significantly increased ($p < 0.01$–0.05) as found in lung cancer. Fr 5 had a tendency to be increased with gastric cancer progression. Fr 2 increased in localized gastric cancer compared to healthy controls and decreased in metastatic gastric cancer compared to localized gastric cancer (table 2, fig. 4).

Table 1. Percentage of each fraction in healthy controls, localized and metastatic lung cancer patients

	Healthy controls (n = 10)	Localized lung cancer (n = 5)	Metastatic lung cancer (n = 5)	p value of healthy controls vs. localized lung cancer	p value of localized lung cancer vs. metastatic lung cancer
Fr 1	23.18±3.67	14.68±1.23	11.66±4.28	<0.01	NS
Fr 2	34.83±6.60	34.34±1.88	26.02±6.54	NS	<0.05
Fr 3	22.63±2.46	18.24±2.35	15.1±2.97	<0.05	NS
Fr 4	11.55±4.61	24.6±2.69	39.94±13.15	<0.01	<0.05
Fr 5	7.76±0.88	8.12±2.21	7.3±4.28	NS	NS

F4 (agalactosyl IgG oligosaccharide) was markedly increased with tumor progression. NS = No statistical significance.

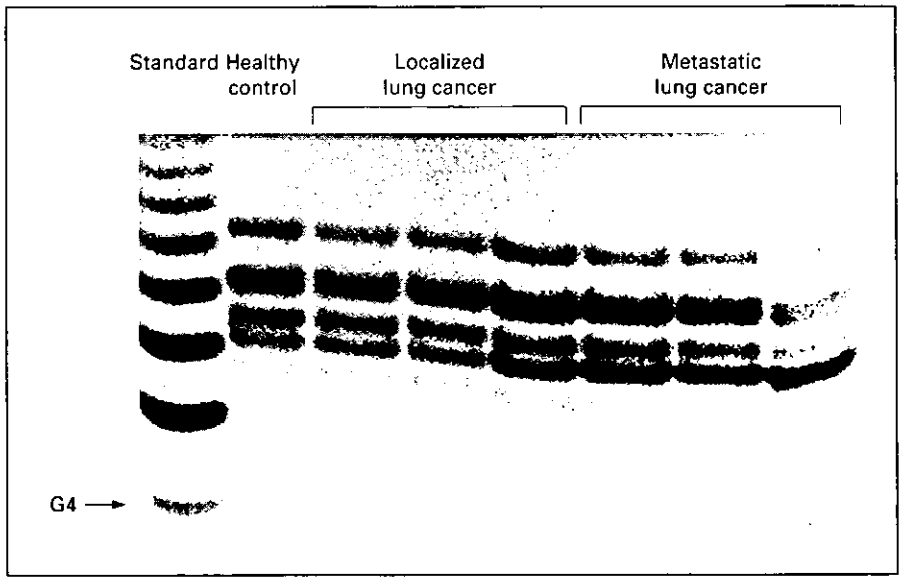

Fig. 3. Pattern of IgG oligosaccharide chain in healthy controls, localized and metastatic lung cancer patients.

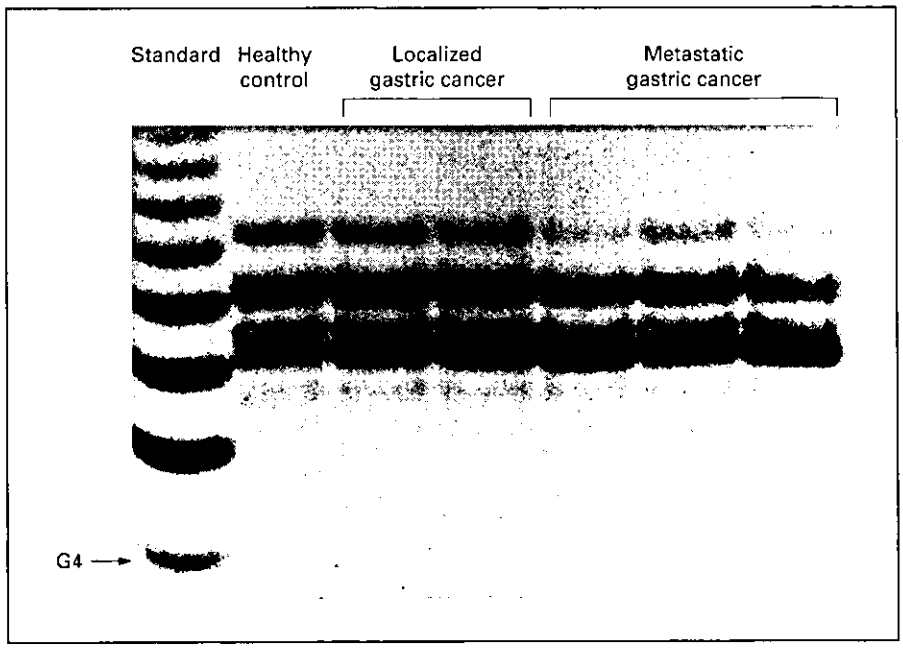

Fig. 4. Pattern of IgG oligosaccharide chain in localized and metastatic gastric cancer patients.

Discussion

It has recently been demonstrated that cancer cell-derived proteases such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator play an important role in the invasion and metastasis of cancer cells through the degradation of extracellular matrix composed of collagen fibers and glycoproteins [12–14]. The biological activity of these proteases is downregulated by protease inhibitors such as α_2-macroglobulin, plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinases (TIMPs) produced by fibroblasts and other types of cells. Therefore, it is documented that a quantitative imbalance between proteases such as MMP-2 (gelatinase A), MMP-9 (gelatinase B) and protease inhibitors such as TIMP-1 and TIMP-2 is a causative factor in the invasion and metastasis of cancer cells [15–17]. We have previously reported that serum MMP-2 levels may be an

Table 2. Percentage of each fraction in healthy controls, localized and metastatic gastric cancer patients

	Healthy controls (n = 10)	Localized gastric cancer (n = 6)	Metastatic gastric cancer (n = 6)	p value of healthy controls vs. localized gastric cancer	p value of localized gastric cancer vs. metastatic gastric cancer
Fr 1	23.18±3.67	16.96±6.71	8.92±2.17	<0.01	NS
Fr 2	34.83±6.60	37.76±4.05	30.13±2.29	NS	<0.01
Fr 3	22.63±2.46	17.6±3.38	15.78±2.21	<0.05	NS
Fr 4	11.55±4.61	19.04±7.87	35.7±6.08	<0.01	<0.01
Fr 5	7.76±0.88	8.7±0.86	9.47±1.58	NS	NS

F4 was markedly increased with tumor progression. NS = No statistical significance.

auxiliary indicator of serum PSA to detect the progression of prostate cancer [18]. On the other hand, it has been reported that the sugar chains of cancer cell-derived adhesion molecules such as integrins and CD44 affect the metastatic ability of cancer cells [19]. Presently, CA50 and CA19-9 (cancer-associated sugar chains) are used as serum tumor markers in pancreatic and biliary tract cancer. Furthermore, previous studies have clearly documented that the β1–6 branched oligosaccharide increases in breast cancer tissue [20]. However, the relationship between tumor progression and changes of serum IgG oligosaccharide chain structure, as assessed by FACE, has not been explored in cancer patients. This is the first report that analyzes serum IgG oligosaccharides associated with tumor progression.

Serum IgG oligosaccharide chains of cancer patients were separated into the same five fractions as in healthy controls. Mashiko et al. [11] reported that Fr 1 consisted of IgG oligosaccharide with two Gla (digalactosyl IgG oligosaccharide), Fr 2 had one Gla (monogalactosyl IgG oligosaccharide), Fr 3 had one sialic acid (Sia; monosialyl oligosaccharide), Fr 4 was without Gla (agalactosyl IgG oligosaccharide), and Fr 5 consisted of IgG oligosaccharide with two Sia (disialyl oligosaccharide).

In the present study, Fr 1 and 3 had a tendency to be decreased with lung and gastric cancer progression. There was a significant difference between the healthy controls and localized cancer ($p < 0.01$–0.05), but no significant difference between the localized and the metastatic cancer. On the other hand, it is noteworthy that Fr 4, agalactosyl IgG oligosaccharide, showed the significant increase in the metastatic cancer compared to localized cancer (lung cancer, $p < 0.05$; gastric cancer, $p < 0.01$), as well as in the localized cancer compared to healthy controls (lung cancer, $p < 0.01$; gastric cancer, $p < 0.01$).

The sugar chain structure of IgG is formed after the addition and/or repair of sugar chains by glycosyltransferase through a process transmitted to the Golgi body from the endoplasmic reticulum in plasma cells. It is believed that Gla is linked to IgG sugar chains by the action of galactosyltransferase (Gal-T) [21]. Nakao et al. [9] found out that there was an increase of agalactosyl oligosaccharide in Castleman's disease, and speculated that the hypogalactosylation of IgG resulted from the decrease of Gal-T activity in plasma cells. Nishiura et al. [8] revealed that the Gal-T/N-acetylglucosaminyltransferase III activity ratio decreased in multiple myeloma cells with a large amount of agalactosyl IgG oligosaccharide. Axford et al. [22] demonstrated that the decrease of Ga-T activity in B lymphocytes caused the increase of agalactosyl IgG oligosaccharide in RA and other rheumatic diseases. In addition, Martin et al. [7] examined serum IgG oligosaccharide chains in the patients with autoimmune diseases by the same analytical method as we employed. Although the ranges of each fraction overlapped to some extent, they concluded that agalactosyl IgG oligosaccharide significantly increased in patients with RA, ankylosing spondylitis and psoriatic arthritis. Their results are almost consistent with those obtained from our study.

It is very interesting that the similar changes of serum IgG oligosaccharide chains found in the B lineage cell tumors and autoimmune diseases were also detected in carcinomas. Although the exact mechanism of altered glycosylation in carcinogenesis and tumor progression remains to be solved, it is thought that the Gal-T activity in plasma cells is downregulated in the process of carcinogenesis and tumor progression causes the significant increase of agalactosyl IgG oligosaccharide. Further study will clarify the role of altered glycosylation in carcinogenesis.

In conclusion, it has clearly been shown that serum IgG oligosaccharide chain structure changed and agalactosyl IgG oligosaccharide significantly increased in carcinogenesis and tumor progression of lung and gastric cancers. Therefore, the analysis of serum IgG oligosaccharide chain structure by FACE may be useful for evaluating the diagnosis and prognosis in patients with these carcinomas.

Acknowledgments

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Changes in Serum IgG Oligosaccharide Chains with Prostate Cancer Progression

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Abstract. *Background: Changes of serum IgG oligosaccharide chain structure have been found in B cell lineage tumors and autoimmune diseases. Currently, the cancer-associated carbohydrate epitopes CA72-4 and CA15-3 are used as serum tumor markers. In the present study, we analyzed the structure of serum IgG oligosaccharide chains in prostate cancer (PCa) patients using the simple new method of fluorophore-associated carbohydrate electrophoresis (FACE). We also evaluated the relationship between changes of serum IgG oligosaccharide chain structure and serum concentration of prostate-specific antigen (PSA). Materials and Methods: The structure of serum IgG oligosaccharide chains from 12 PCa patients (6 localized cancer, 6 metastatic cancer) and 10 healthy controls was evaluated by FACE. PSA levels in serum were determined by enzyme immunoassay. Results: Fr 1 (monogalactosyl oligosaccharide) and Fr 2 (digalactosyl oligosaccharide) decreased significantly ($p<0.05$), while Fr 4 (agalactosyl IgG oligosaccharide) increased with PCa tumor progression. The Fr 4 / Fr 1+2 ratio in metastatic PCa patients was significantly higher than in healthy controls ($p<0.05$), and there was a significant correlation ($r=0.84$, $p<0.05$) between serum PSA levels and the Fr 4 / Fr 1+2 ratio in all patients with PCa. Conclusion: The changes of serum IgG oligosaccharide chain structure with PCa progression are based on the abnormality of glycosylation in PCa metastasis. Therefore, the analysis of serum IgG oligosaccharide chain structure by FACE may be an auxiliary indicator of PSA for monitoring PCa progression.*

One heavy chain of human immunoglobulin G (IgG) is associated with two N-linked oligosaccharide chains at the ^{297}Asn site in the Fc region (1). Axford *et al.* showed that changes in the serum IgG oligosaccharide chain structure affect the whole molecular structure of IgG and its ability to bind to the macrophage surface Fc receptor (2,3). It is reported that serum IgG oligosaccharide chains lacking galactose (agalactosyl IgG oligosaccharide) are frequently found in the serum of rheumatoid arthritis (RA) patients by using high-performance liquid chromatography (HPLC) (4,5). Furthermore, it is hypothesized that agalactosyl IgG oligosaccharide activates the complement system *via* mannose-binding protein, contributing to the pathogenesis of RA (6). The increase of agalactosyl IgG oligosaccharide was considered to be a specific change to RA (4,5,7), but Nishiura *et al.* found that agalactosyl IgG oligosaccharide increases in the monoclonal IgG of advanced multiple myeloma (stage II, III) or polyclonal IgG of Castleman's disease (8). Although HPLC was used as the conventional method for analyzing protein-binding sugar chains, fluorophore-assisted carbohydrate electrophoresis (FACE) was developed as a new, easier method. Using this technique, we first reported that agalactosyl IgG oligosaccharide in serum increases significantly with lung and gastric cancer progression (9).

Prostate-specific antigen (PSA) is a 32 kDa glycoprotein secreted by glandular epithelial cells of the prostate which functions to liquidize semen (10,11). PSA is a useful specific marker for the diagnosis, prognosis and monitoring of treatment efficacy in PCa (12-14). There are no reports on the relationship between PCa, tumor progression and changes of serum IgG oligosaccharide chain structure as assessed by FACE. Therefore, in the present study, we analyzed the serum IgG oligosaccharide chain structure of PCa patients, and evaluated the relationship between changes of serum IgG oligosaccharide chain structure and serum PSA levels.

Materials and Methods

Patient characteristics. Untreated serum samples were obtained from 12 prostate cancer (PCa) patients (mean age 64.8 years old, range 54 to 78), diagnosed at Kitasato University Hospital, Japan. Six patients had localized (stage T1 and 2) PCa and 6 patients had metastatic (stage M1b) PCa. Ten healthy men were enrolled as

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Key Words: IgG oligosaccharide chains, prostate-specific antigen, prostate cancer.

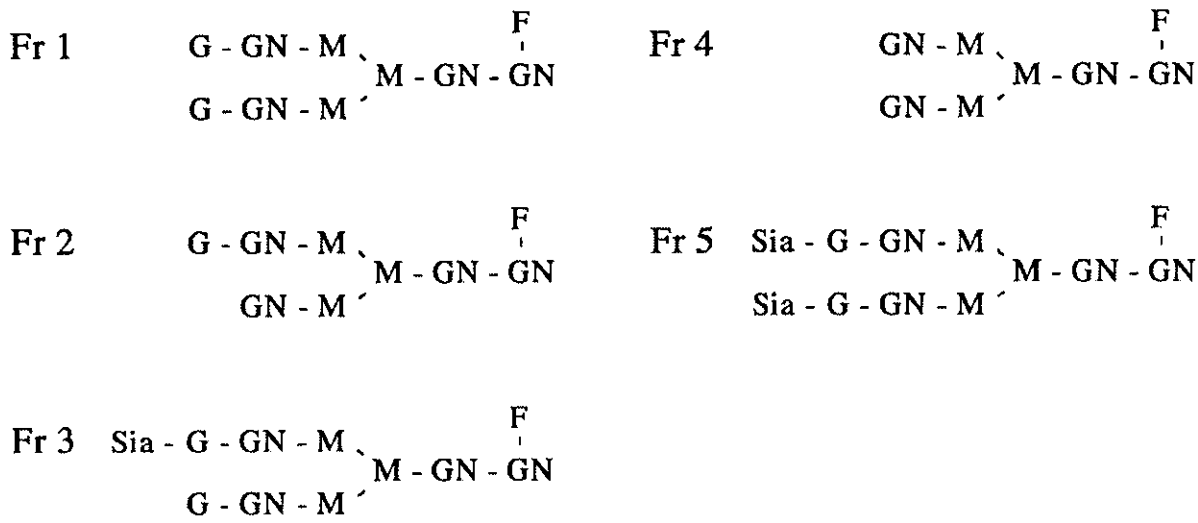

Figure 1. *Structure of IgG oligosaccharide chain in the fractions separated by PAGE. Sia : sialic acid, G : galactose, GN : N-acetylglucosamine, M : mannose, F : fucose.*

controls (mean age 62.6 years old, range 52 to 70). We obtained informed consent from all subjects for this study.

Histopathology of prostate cancer. Histopathology was confirmed by six-sextant biopsy and/or transurethral resection in all cases. PCa was staged clinically following the TNM classification (15). Briefly, stage T1 is defined as tumor not clinically recognizable and identifiable only by histopathological examination of prostatic tissue. Stage T2 tumors are palpable but confined within the prostate. Stage T3 tumors are palpable and extend through the prostatic capsule with unilateral or bilateral extension. M1 stage is defined by the presence of distant metastasis and M1b by bone metastasis. Serum samples were obtained from these patients and stored at -80°C until use.

Purification of serum IgG. Serum (300~500 μl) 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 μg) was dissolved in 25 μl of distilled water, and 25 ml of 0.1 M phosphate buffer (pH 7.4), 1 ml of 5% SDS and 1.5 ml of 1.44 M 2-mercaptoethanol were added. The mixture was heated at 100°C for 5 min, and then treated with 2.5 ml of 7.5% Nonidet P-40 and 2 ml of recombinant peptide N-glycosidase F (PNGase F, EC 3.5.1.52, Seikagaku Kogyo Inc., Tokyo, Japan) at 37°C for 2 h. Subsequently, anhydrous ethanol (171 μl) was added and cooled for 10 min. After centrifugation of the mixture at 15,000 rpm for 5 min at 4°C, the supernatant containing the released oligosaccharides was evaporated to dryness and recovered.

Fluorescence labelling of N-linked oligosaccharide chains from serum IgG. Five ml of 0.15 M 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) in 15% acetic acid and 5 μl 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°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-labelled 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 (%).

The determination of PSA levels in serum. PSA levels in serum were determined by enzyme immunoassay (TOSOH, AIA-600, Tokyo, Japan).

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 $p<0.05$ was considered statistically significant.

Results

Sugar chain structures of the respective fractions. Figure 1 shows the serum IgG oligosaccharide chain structure of the respective fractions (Fr).

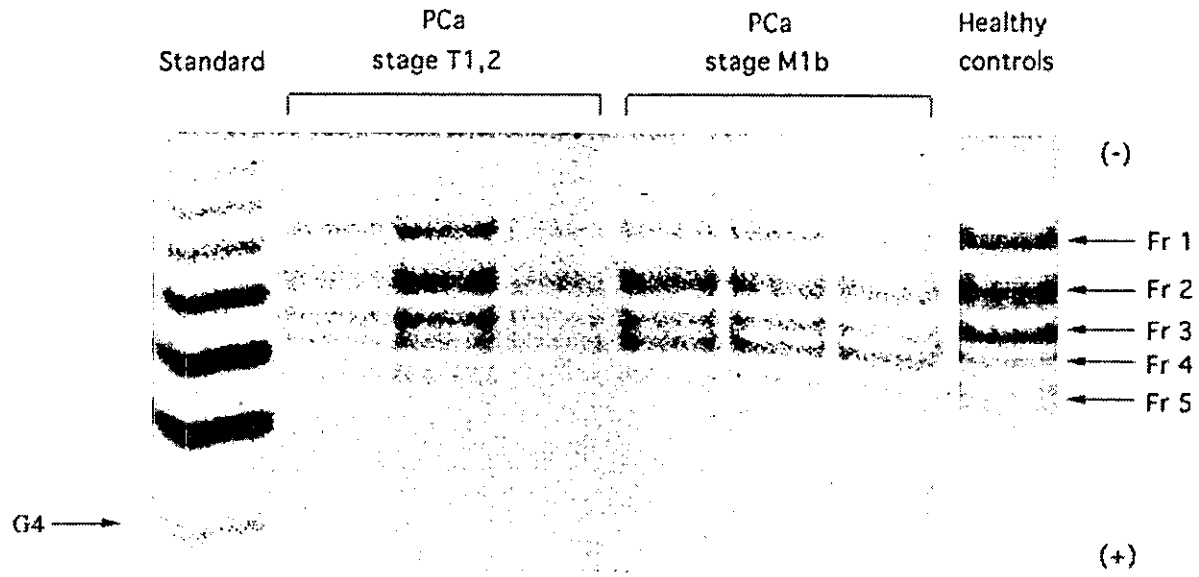

Figure 2. *Pattern of IgG oligosaccharide chain in healthy controls and prostate cancer (PCa) patients.*

Table I. *Percentage of each fraction in healthy controls and PCa patients.*

	Healthy Controls (n=10)	PCa stage T1,2 (n=6)	PCa stage M1b (n=6)	*p* value of healthy controls *vs* T1,2	*p* value of healthy controls *vs* M1b	*p* value of T1,2 *vs* M1b
Fr 1	23.18±3.67	19.0±2.04	13.36±3.51	NS	<0.05	<0.05
Fr 2	34.83±6.60	36.46±3.39	32.66±2.14	NS	NS	<0.05
Fr 3	22.63±2.46	17.06±3.57	16.46±4.16	NS	NS	NS
Fr 4	11.55±4.61	17.08±4.27	23.88±6.76	NS	<0.05	NS
Fr 5	7.76±0.88	10.1±2.78	12.7±3.04	NS	<0.05	NS

NS ; No statistical significance.

Serum IgG oligosaccharide chains of healthy controls. The serum IgG oligosaccharide chains of healthy controls were separated into five fractions, designated Fr 1-5 from the cathode side (Figure 2). The most abundant fraction according to DP value was Fr 2 (34.9%), followed by Fr 1 (23.2%), Fr 3 (22.7%), Fr 4 (11.6%) and finally Fr 5 (7.8%).

Serum IgG oligosaccharide chains of prostate cancer patients. Serum IgG oligosaccharide chains of PCa patients were separated into the same five fractions as in the healthy controls. There was a tendency for Fr 1 and 3 to decrease and Fr 2, 4 and 5 to increase in localized PCa (T1, 2) compared to healthy controls. Fr 1 decreased significantly ($p<0.05$), Fr 2 and 3 tended to decrease and Fr 4 and 5 increased significantly ($p<0.05$) in metastatic PCa (M1b) compared to healthy controls. Fr 1 and 2 decreased significantly ($p<0.05$), Fr 3 tended to decrease and Fr 4 and 5 tended to increase in metastatic PCa (M1b) compared to localized PCa (T1, 2) (Figure 2, Table I). We calculated the ratio of agalactosyl IgG oligosaccharide (Fr 4) to digalactosyl IgG oligosaccharide (Fr 1) and monogalactosyl IgG oligosaccharide (Fr 2) (Fr 4 / Fr 1+2 ratio) in order to evaluate any changes of galactosyl IgG oligosaccharide with PCa progression. The Fr 4 / Fr 1+2 ratio tended to increase with PCa progression, and the ratio in metastatic PCa patients differed significantly from healthy controls ($p<0.05$) (Figure 3). Furthermore, there was a strong correlation between serum PSA levels and the Fr 4 / Fr 1+2 ratio in all PCa patients ($r=0.84, p<0.05$) (Figure 4).

Discussion

It is reported that the sugar chains of adhesion molecules such as the β-integrin, CD44 influence the metastatic ability of cancer cells (16). Furthermore, CA72-4 (17,18) and CA15-3 (19,20) are used as serum tumor markers in ovarian and breast cancer, respectively. We previously reported that the analysis

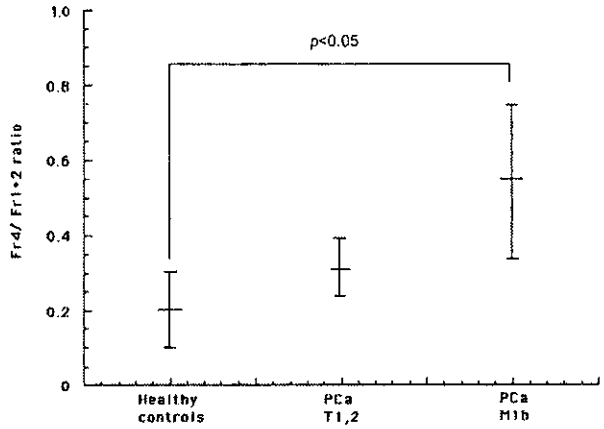

Figure 3. *Fr 4 / Fr 1+2 ratio in healthy controls and PCa patients. Fr 4 / Fr 1+2 ratio increased with PCa disease progression.*

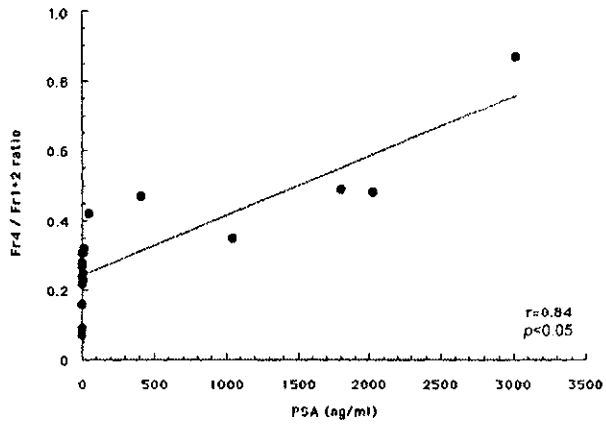

Figure 4. *Correlation between serum PSA levels and Fr4 / Fr 1+2 ratio in PCa patients. A significant association was found between serum PSA levels and Fr4 / Fr 1+2 ratio.*

of serum IgG oligosaccharide chains using FACE may be a useful indicator for the diagnosis and prognosis of lung and gastric cancer (9). Here, we analyzed the serum IgG oligosaccharide chain structure in each stage of PCa, and evaluated the relationship between changes of IgG oligosaccharide chains and serum levels of PSA, *i.e.* serine protease derived from the cancer cells. Although this study includes only a limited number of patients with PCa, it is the first report to suggest the possibility that analysis of serum IgG oligosaccharide chains by FACE could be developed into an indicator of the degree of PCa progression.

Serum IgG oligosaccharide chains of PCa patients were separated into the same five fractions as healthy controls. The author *et al.* (9) reported that Fr 1 consisted of IgG oligosaccharide with two Gla, Fr 2 had one Gla, Fr 3 had one sialic acid (Sia), Fr 4 was without Gla, and Fr 5 consisted of IgG oligosaccharides with two Sia.

In the present study, the serum IgG oligosaccharide chain structure was altered with PCa progression. It was demonstrated that galactosyl IgG oligosaccharide (Fr 1 and 2) decreased, and that agalactosyl IgG oligosaccharide (Fr 4) increased with PCa progression. Furthermore, the Fr 4 / Fr 1+2 ratio significantly increased with metastasis of PCa. Although the mechanism of altered glycosylation in tumor progression is not clear, the possibility discussed below can be investigated as a cause of the increases of agalactosyl IgG oligosaccharide seen with cancer progression.

The sugar chain structure of IgG is formed after the addition and/or repair of sugar chains by glycosyltransferase, through a process transmitted to the Golgi body from the endoplasmic reticulum in plasma cells. Fujji *et al.* reported that Gla was linked to IgG sugar chains by the action of galactosyltransferase (Gal-T) (21,22). Nakao *et al.* found that there was an increase of agalactosyl IgG oligosaccharide in Castleman's disease, and speculated that the hypogalactosylation of IgG resulted from the decrease of Gal-T activity in plasma cells (23). It was demonstrated that this decrease in B lymphocytes caused the increase of agalactosyl IgG oligosaccharide in rheumatoid arthritis and other rheumatic diseases (24,25). Thus, it was demonstrated that the glycosylation of IgG oligosaccharide is based on the action of glycosyltransferase. It is speculated that Gal-T activity in plasma cells is down-regulated during tumor progression and that this causes the significant increase of agalactosyl IgG oligosaccharide.

PSA exists in blood either as a free molecule or a complex form with α2 macroglobulin and α1 antichymotrypsin (α1ACT) (26-28). It has been reported that the relative proportions of free and complexed forms of PSA are different in patients with PCa and benign prostate hypertrophy (BPH) (29,30). We have previously reported that the relative proportion of free and complex-type PSA was different in PCa and BPH, and that the complex-type PSA with α1ACT increased with PCa disease progression (31). It has also been demonstrated that the ratio of free PSA to total PSA is useful to differentiate PCa from BPH in patients with intermediate levels of PSA (4-10 ng/ml) (32,33). Thus, PSA is a specific marker for PCa and has been widely used for the diagnosis and evaluation of treatment efficacy in PCa.

It is very interesting that the Fr 4 / Fr 1+2 ratio increased with metastasis of PCa, and that there was a strong correlation between this ratio and serum PSA levels in all patients with PCa. In conclusion, the changes of serum IgG oligosaccharide chain structure with PCa progression is based on the abnormality of glycosylation in PCa metastasis. Therefore, the analysis of serum IgG oligosaccharide chain structure by FACE may be an auxiliary indicator of serum PSA for monitoring PCa disease progression.

Acknowledgements

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