Abstract. Background: It has been demonstrated that increased expression of the sialyl Lea sugar chains on cancer cells influences cellular adhesion to vascular-endothelial cells. Therefore, it was thought that alterations in the sugar chain structure of E-cadherin, a calcium dependent adhesion molecule, influence the metastasis of cancer cells. Materials and Methods: In this study, N-linked oligosaccharides of human serum immunoglobulin G (IgG) were analyzed in 12 patients with non-small cell lung cancer (NSCLC) (6 localized cancer: 3 adenocarcinomas and 3 squamous cell carcinomas; 6 metastatic cancer: 3 adenocarcinomas and 3 squamous cell carcinomas) and 10 healthy controls using fluorophore-associated carbohydrate electrophoresis (FACE). The relationship between changes in sugar chain structure and serum concentrations of carcinoembryonic antigen (CEA) and cytokeratin 19 fragment (CYFRA21-1) were evaluated. CEA levels in the sera were determined using an enzyme immunoassay, and CYFRA21-1 levels were determined using an enzyme chemiluminescent immunoassay. Results: Fr1 (monogalactosyl IgG oligosaccharides) and Fr2 (digalactosyl IgG oligosaccharides) decreased, while Fr4 (agalactosyl IgG oligosaccharides) significantly increased (p<0.01-0.05) with NSCLC progression. The Fr4/Fr1+Fr2 ratio increased with NSCLC progression, and the ratios in localized and metastatic NSCLC were significantly higher than in healthy controls (p<0.01 and p<0.01, respectively). There was a strong correlation between serum CEA levels and Fr4 (r=0.91) and a significant correlation between serum CEA levels and the Fr4/Fr1+Fr2 ratio (r=0.83, p<0.05) in patients with lung adenocarcinoma. There was a significant correlation between serum CYFRA21-1 levels and Fr4 (r=0.88, p<0.001) and a positive correlation between serum CYFRA21-1 levels and the Fr4/Fr1+Fr2 ratio (r=0.38) in patients with lung squamous cell carcinoma. Conclusion: The analysis of serum IgG N-linked oligosaccharide chain structures by FACE may be an auxiliary indicator of serum tumor markers for monitoring NSCLC progression.

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

On the other hand, it was reported that expression of N-acetylgalcosaminyl-transferase-V (GnT-V) in cancer cells increased in patients with NSCLC (6). In addition, it was demonstrated that fucosylated oligosaccharides increased in sera from NSCLC patients (7). As previously reported, agalactosyl IgG oligosaccharides significantly increased during the progression of gastric and lung cancer using FACE analysis (8). However, there are no reports on the relationship between changes in sugar chain structure and serum tumor markers in patients with NSCLC. Therefore,
in the present study, the serum IgG N-linked oligosaccharide chain structures from NSCLC patients were analyzed and the relationship between changes in oligosaccharide chain structure and serum concentrations of tumor markers was evaluated.

Materials and Methods

Patient characteristics. Serum samples were obtained from 12 NSCLC patients who had no previous treatment (6 localized cancer: 3 adenocarcinomas and 3 squamous cell carcinomas; 6 metastatic cancer: 3 adenocarcinomas and 3 squamous cell carcinomas) (mean age 64.2 years old, range 54 to 76 years), diagnosed at Kitasato University. Ten healthy men were enrolled as controls (mean age 62.8 years old, range 52 to 72 years). NSCLC was clinically staged following the TNM classification (9). Serum samples were obtained from these patients and stored at –80°C until use. Informed-consent from all patients was obtained.

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 μl of 0.1 M phosphate buffer (pH 7.4), 1 μl of 5% SDS and 1.5 μl 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 μl 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 the mixture was 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 labeling of N-linked oligosaccharide chains from serum IgG. Five μl 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-labeled 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 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, shown as a percentage (%).

The determination of CEA and CYFRA21-1 levels in serum. CEA levels in serum were determined using an enzyme immunoassay (TOSOH, AIA-600, Tokyo, Japan) and CYFRA21-1 using an enzyme chemiluminescent immunoassay (Hitachi, modular analytics).

Statistical analysis. The Mann-Whitney U-test was used for statistical analysis and p<0.05 was considered statistically significant.

Results

Figure 1 shows the serum IgG N-linked oligosaccharide chain structures for the respective fractions. The serum IgG oligosaccharide chains of healthy controls were separated into five fractions as follows: Fr1, monogalactosyl; Fr2, digalactosyl; Fr3, monosialyl; Fr4, agalactosyl; and Fr5, disialyl.
The pattern of serum IgG N-linked oligosaccharide chains was determined using SDS-PAGE illustrated in a previous report (8). The percentage of each fraction of serum IgG N-linked oligosaccharide chains in healthy controls and NSCLC patients is shown in Table I. The most abundant fraction according to its DP value was Fr2 (34.8%), followed by Fr1 (23.2%), Fr3 (22.7%), Fr4 (11.6%) and Fr5 (7.8%). In NSCLC patients, Fr1 and Fr3 decreased with NSCLC progression, though both fractions from localized and metastatic NSCLC were significantly lower than healthy controls (Fr1: \( p < 0.01 \) and \( p < 0.05 \), respectively; Fr3: \( p < 0.05 \) and \( p < 0.05 \), respectively). Fr2 tended to decrease with NSCLC progression. It significantly decreased in metastatic NSCLC compared to localized NSCLC (\( p < 0.05 \)). Fr4 increased with NSCLC progression, and significantly increased in localized and metastatic NSCLC compared to healthy controls (\( p < 0.01 \) and \( p < 0.01 \), respectively). Fr4 significantly increased in metastatic NSCLC compared to localized NSCLC (\( p < 0.05 \)). While Fr5 increased in localized NSCLC in comparison to healthy controls, it decreased in metastatic NSCLC compared to localized NSCLC, though not significantly. The ratio of Fr4 no Gla to Fr1 and Fr2 with Gla (Fr4/Fr1+Fr2 ratio) was also calculated. The ratios in localized and metastatic NSCLC were significantly higher than in healthy controls (\( p < 0.01 \) and \( p < 0.05 \), respectively). Furthermore, the Fr4/Fr1+Fr2 ratio increased with NSCLC progression, but not significantly.

The relationship between changes in serum IgG N-linked oligosaccharide chains and concentrations of serum tumor markers in localized and metastatic NSCLC was also evaluated. There was a strong correlation between serum CEA levels and Fr4 (\( r = 0.91, \ p = 0.11 \)) (Figure 2) and a significant correlation between serum CEA levels and the Fr4/Fr1+Fr2 ratio (\( r = 0.83, \ p < 0.05 \)) (Figure 3) in patients with lung adenocarcinoma. On the other hand, there was a

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Table I. Serum IgG N-linked oligosaccharide fractions (% of total) in healthy controls and non-small cell lung cancer (NSCLC) patients as determined using FACE analysis.

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n=10)</th>
<th>Localized NSCLC (n=6)</th>
<th>Metastatic NSCLC (n=6)</th>
<th>p-value of healthy controls vs. localized NSCLC</th>
<th>p-value of healthy controls vs. metastatic NSCLC</th>
<th>p-value of localized NSCLC vs. metastatic NSCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr1</td>
<td>23.18±3.67*</td>
<td>14.05±1.89</td>
<td>11.28±3.93</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Fr2</td>
<td>34.83±6.60</td>
<td>4.27±1.69</td>
<td>26.52±5.97</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fr3</td>
<td>22.63±2.46</td>
<td>18.28±2.11</td>
<td>15.38±2.74</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>F4</td>
<td>11.55±4.61</td>
<td>25.3±3.00</td>
<td>39.32±11.86</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fr5</td>
<td>7.76±0.88</td>
<td>8.05±1.99</td>
<td>7.52±3.87</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fr4/Fr1+Fr2</td>
<td>0.21±0.09</td>
<td>0.53±0.08</td>
<td>1.17±0.71</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: No statistical significance.
Localized NSCLC: 3 adenocarcinomas and 3 squamous cell carcinomas. Metastatic NSCLC: 3 adenocarcinomas and 3 squamous cell carcinomas. *mean±SD.

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Figure 2. Correlation between serum CEA levels and Fr4 in lung cancer (adenocarcinoma) patients. There was a strong correlation, but no significant association between serum CEA levels and Fr4.

Figure 3. Correlation between serum CEA levels and the Fr4/Fr1+Fr2 ratio in lung cancer (adenocarcinoma) patients. There was a strong correlation and a significant association between serum CEA levels and the Fr4/Fr1+Fr2 ratio.
significant correlation between serum CYFRA21-1 levels and Fr4 ($r=0.88$, $p<0.001$) (Figure 4) and a positive correlation between serum CYFRA21-1 levels and the Fr4/Fr1+Fr2 ratio ($r=0.38$, $p=0.25$) in patients with lung squamous cell carcinoma (Figure 5).

**Discussion**

There is much evidence correlating disease progression with changes in the carbohydrate chains of glycoproteins. It was reported that alterations in the sugar chain structures of E-cadherin, a calcium dependent adhesion molecule, and of sialyl Le$^\alpha$ whose levels increase in cancer cells, influence cancer metastasis (10). Span-1 and DU-PAN-2 belong to the same category of carbohydrate structure and are used as serum pancreatic tumor markers (11,12). Oyama et al. showed a high rate of sialyl Lewis X-i antigen (SLX) on two key sugar chains in lung adenocarcinoma (13). Further, Dosaka-Akita et al. demonstrated the relationship between GnT-V and metastasis of NSCLC (6). As previously reported, analyzing serum IgG N-linked oligosaccharides using FACE may be useful for evaluating the diagnosis and prognosis of patients with gastric and lung cancers (8). In this study, the serum IgG N-linked oligosaccharide chain structure in patients with NSCLC progression was analyzed, and the relationship between changes in serum IgG N-linked oligosaccharide chain structure and concentrations of serum tumor markers was evaluated. The change of serum IgG N-linked oligosaccharide chains determined using FACE analysis had no significant difference among different tissue types of NSCLC. On the other hand, there was a good or strong correlation between changes in serum IgG N-linked oligosaccharide chains and serum tumor marker levels in patients with NSCLC. Although this study includes only a limited number of patients with NSCLC, it is the first report evaluating this relationship with NSCLC progression. The serum IgG N-linked oligosaccharide chains of healthy controls using exoglycosidase was analyzed and the sugar chain structures were categorized into five fractions (14). In the present study, the serum IgG N-linked oligosaccharide chains from NSCLC patients were separated into the same five fractions as those of healthy controls, but in a different rank order (Table I).

Regarding the changes in sugar chain structure with tumor progression, Kossowska et al. showed that fucosylated oligosaccharides were increased in sera from NSCLC patients (7). Another study demonstrated that there was an increase in the amount of one outer arm fucosylation (A3G3F0) in sera from patients with NSCLC (15). In the present study, Fr1 and Fr2 with Gla decreased, while Fr4 no Gla significantly increased with NSCLC progression. The Fr4/Fr1+Fr2 ratio increased with NSCLC progression, and the ratios in localized and in metastatic NSCLC significantly increased compared to healthy controls. Thus, the structure of serum IgG N-linked oligosaccharide chains changed, and agalactosyl IgG oligosaccharides (Fr4) significantly increased during the progression of NSCLC. 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 Gla is linked to IgG sugar chains by the action of galactosyltransferase (Gal-T) (16). Although the exact mechanism behind altered glycosylation in carcinogenesis and tumor progression remains to be solved, it is thought that the Gal-T activity in plasma cells is down-regulated.

Figure 4. Correlation between serum CYFRA 21-1 levels and Fr4 in lung cancer (squamous cell carcinoma) patients. There was a strong correlation and a significant association between serum CYFRA 21-1 levels and Fr4.

Figure 5. Correlation between serum CYFRA 21-1 levels and the Fr4/Fr1+Fr2 ratio in lung cancer (squamous cell carcinoma) patients. There was a positive correlation, but no significant association between serum CYFRA 21-1 levels and the Fr4/Fr1+Fr2 ratio.
during the process of carcinogenesis. Thus, tumor progression causes a significant increase in agalactosyl IgG oligosaccharides (Fr4).

CEA is a useful marker specifying adenocarcinoma (17, 18) and CYFRA21-1 is a useful marker in identifying squamous cell carcinoma (19, 20). Both tumor markers are used for diagnosis, prognosis and monitoring treatment efficacy of NSCLC. It is very interesting that there was a strong correlation between serum CEA levels and Fr4, and a significant correlation between serum CEA levels and the Fr4/Fr1+Fr2 ratio in patients with lung adenocarcinoma. On the other hand, there was a significant correlation between serum CYFRA21-1 levels and Fr4 and a positive correlation between serum CYFRA21-1 levels and the Fr4/Fr1+Fr2 ratio in patients with lung squamous cell carcinoma. Although this study includes only a limited number of patients with NSCLC, these results may be translated into valuable clinical applications.

In conclusion, it is thought that alteration of serum IgG N-linked oligosaccharide chains in NSCLC progression is caused by abnormal glycosylation associated with lung cancer progression. The Fr4 and Fr4/Fr1+Fr2 ratio increased during the progression of NSCLC, and there was a good correlation between those Fr and serum tumor markers of lung cancer. Therefore, the analysis of serum IgG N-linked oligosaccharide chain structures using FACE may be an auxiliary indicator of serum tumor markers useful for monitoring NSCLC progression.

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References


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