serum α1,3fucosyltransferase activity [38]. In fact, the patient was determined to possess the *pf* gene homozygously (*pf*/*pf*) indicating occurrence of lethal mutation in the *FUT6* gens which resulted in the absence of fucosylated glycans in AGP [37]. However, a couple of fucosylated glycans were still detected as less than several % of the total glycans in AGP (Fig 4b). Accordingly, it remains to be examined a significance of poorly expressed fucosylated glycans in such a cancer patient with FUT6-deficiency (details of molecular and enzymatic studies on fucosylation of AGP in patient N with FUT6-deficiency will be published elsewhere).

Discussion

Proteins are frequently modified post-translationally and more than half of all proteins in nature are glycosylated [39] resulting in being present as glycoproteins with covalently attached glycans having highly heterogeneous and diverse structures. It must therefore be plausible that these glycans have significant implication and responsibility for the structure and function of such glycoproteins. It has been widely recognized that glycans play important roles in not only protein folding and clearance but also cell to cell interaction, recognition and binding together with frequent occurrences of modifications in glycans themselves [40–42]. With the aid of detailed analyses of glycan structures in well characterized glycoproteins, it has been demonstrated that aberrant and novel glycans are synthesized associated with various biological processes, in particular, malignancies [2,43]. Hence, cancer-associated changes of glycan molecules in glycoproteins as well as in other glycoconjugates such as glycolipids [44,45] have been widely demonstrated. Further, such changes are recognized as one of the most promising targets to improve existing tumor markers by means of predicting prognosis of cancer patients more precisely [46].

Whereas, since all these glycans are synthesized enzymatically through catalytic actions of a series of glycosyltransferases, such enzymes could be an important target to detect glycan-based changes in malignancies involving possible establishment of a novel biomarker for diagnosis of cancer. In fact, our previous studies demonstrated the usefulness of analyzing α1,2- [47–49], α1,3- [20,21,37,38,50–54], α1,4- [55–58] and α1,6fucosyltransferase [59] activities using specific sugar-acceptors, which emphasized the specific role of the fucosylated antigens appeared to play on carcinogenesis [1,47–49]. In particular, significantly elevated activities of α1,3fucosyltransferase in plasma and/or serum have been observed in patients with diverse cancers [20,21,51–54]. It has, therefore, been suggested that measurement of serum α1,3fucosyltransferase activity has some superiority over diagnosis with tumor-associated antigen levels in the same samples as a novel biomarker for patients in earlier clinical stages and with smaller size of tumors. Although the possibility cannot be excluded that the enzyme with elevated activities is originated from tumor itself as observed in patients with colorectal carcinoma [60], the origin of α1,3fucosyltransferase in serum has been considered to be the liver. It has also been determined that α1,3fucosyltransferase in serum is encoded by the *FUT6* gene and the missense mutation that occurs in the *FUT6* gene results in the deficiency of serum α1,3fucosyltransferase activity [37,38,61]. Therefore, tumor-associated elevation of serum α1,3fuclsyltransferase activities is assumed to occur in the liver but not originated from tumors formed.

Human AGP is generally recognized as the major serum protein with highly glycosylated glycans and to be originated from the liver. Glycans in AGP consist of five complex type glycans including di-, tri- and tetraantennary structures [25,33]. It has also been determined by our recent mass spectrometric analysis that almost all the terminal galactose residues in every glycan chains are sialylated and the α1,3fucosyl residues are present on the lactosamine structures in both tri- and tetraantennary glycans and elongated tetraantennary ones with repeating lactosamine structure. Hence, our previous investigations convincingly demonstrated both

presence of a significant correlation between activities of α1,3fucosyltransferase and total amounts of fucosylated glycans in AGP using the same serum samples [34] and absence of fucosylated glycans in AGP obtained from FUT6-deficent individuals [37]. Furthermore, such afuco-glycans of AGP obtained from FUT6-deficient individuals could be fucosylated on their tri- and tetraantennary structures using the recombinant *FUT6* enzyme or purified plasma α1,3fucosyltransferase under the presence of GDP-fucose as a sugar-donor [S. Yazawa, unpublished observation]. It could, therefore, be strongly suggested that tumor-dependent, increased α1,3fucosylated glycans in AGP are synthesized by an action of the hepatic *FUT6* gene encoded α1,3fucosyltransferase with highly elevated activities.

There have been many reports indicating that analyses of changes occurred in both glycan structures and concentrations of AGP in serum could be useful for the diagnosis and successful management of various diseases including diverse cancers [31,62–74]. Our previous studies suggested that fucosylation of AGP implied significant potential as a novel biomarker for diagnosis and prognosis of cancer patients, which must be strongly associated with tumor-dependent changes of serum α1,3fucosyltransferase activity. Indeed, a strong correlation of levels between fucosylated AGP and α1,3fucosyltransferase activity in the same serum samples was demonstrated previously. Further, the fucosylation index of AGP glycoforms and the relative abundance of fucosylated glycans in AGP (FUCAGP) determined by means of CAIE method [34] and mass spectrometric analysis [35], respectively, were found, for the first time, to be useful for predicting postoperative cancer patients.

In the present study, we first focused on levels of FUCAGP and serum AGP concentrations in patients with various cancers in different clinical stages. Levels of FUCAGP and serum AGP were analyzed in relation to their clinicopathological features in patients (n = 30) who received surgical interventions and/or various chemotherapy treatments. As demonstrated previously [34,35], significant elevated levels of both FUCAGP and serum AGP concentrations were observed in cancer patients at preoperative periods when compared with those in healthy controls. No significant correlation was found between levels of FUCAGP and AGP in both cancer patients and healthy controls, respectively. Interestingly, a very low level of FUCAGP was found in a patient who underwent neoadjuvant chemotherapy. Whereas, as demonstrated previously [34,35], increases of relative amount of diantennary glycans rather than those of tri- or tetraantennary glycans in AGP occurred primarily along with a new synthesis of AGP shortly after operation. This rapid response seemed to occur commonly in patients at the time of operation, and to involve as a consequence of the acute phase reaction toward surgical treatment. During the periods, fucosylated glycans were scarcely detected in AGP, since as described above, only tri- and tetraantennary glycans in AGP were fucosylated through α1,3linkages.

Previous studies on glycoforms and corresponding glycan structures also indicated that, secondarily, AGP with highly fucosylated and branched glycan chains increased specifically in patients with poor prognosis but not in patients with good prognosis. Further, levels of FUCAGP were suggested to become a clinically relevant biomarker over existing diagnostic tumor markers. However, levels of FUCAGP and AGP followed in our previous studies [34, 35] were determined for a limited duration up to one and a half year after operation, and in particular, validation of the fucosylation index of AGP glycoforms and levels of FUCAGP was scarcely performed for predicting treatment outcome in therapeutic settings. Accordingly, we then focused on analyzing variability of serum FUCAGP in patients who had been followed for several years after operation and had undergone various chemotherapies during the period in connection with patients' responses to medication and various chemotherapy treatments. Seventeen patients including ten patients who died due to the recurrence and/or metastasis of tumors were followed up by analyzing levels of FUCAGP and AGP concentrations in their serum samples together with general surveillance with CT scans and tumor marker diagnosis.

Results from follow-up studies of FUCAGP levels conducted depending on patients' prognoses indicated that elevated levels of FUCAGP were retained and no striking drop of the level was observed in patients who seemed not to respond to any repeated treatments of chemotherapy and died with a short survival after operation. Whereas, patients who survived for a longer period (up to 1639 POD) under the therapeutic settings with repeated chemotherapies showed partly different changes of FUCAGP levels. First, patients whose FUCAGP levels were found to extremely elevate shortly after operation and survived for a longer period with repeated chemotherapy seemed to respond to the first line of adjuvant chemotherapy resulting that the level fell and in some cases dropped below the cut-off level. However, either recurrence and/or metastasis or no experience of complete remission occurred, and subsequently they died due to the progress of disease accompanying constantly elevated levels of FUCAGP. Second, patients whose FUCAGP levels had been retained at relatively low and around the cut-off level under receiving repeated chemotherapy treatments showed an incomplete response to the latest line treatment of chemotherapy resulting in progress of disease and constant elevation of FUCAGP levels.

Whereas, patients who had been survived during the observation period with and without chemotherapy treatment showed either clear response to repeated chemotherapy treatments or no clinical recurrence during the period of observation. It was of particular interest that in these patients with good prognosis, elevated levels of FUCAGP once after receiving the previous line treatment of chemotherapy fell around the cut-off level by receiving the latest line treatment of chemotherapy and low levels of FUCAGP after operation were retained around the normal range without adjuvant chemotherapy treatment. Therefore, these follow-up studies of FUCAGP levels in patients with different prognosis suggested that patients with poor prognosis possessed extremely elevated levels in the latest period but patient with good prognosis seemed to retain relatively low levels around the normal range. Further, in patients who seemed to respond well to chemotherapy treatment and survived, elevated levels found in association with the recurrence and/or metastasis of tumors dropped to the normal range after treatment with the latest line treatment of chemotherapy against such clinical events. It is more noteworthy that all such changes in FCUAGP levels seemed to occur frequently in advance before detection of recurrence and/or metastasis of tumors and respective tumor markers coming up positive.

One of the obvious evidences to provide that fucosylation of the AGP molecule is accompanied solely by the *FUT6* gene-encoded hepatic α1,3fucosyltransferase has been obtained from molecular and enzymatic studies on FUT6 deficient individuals. Lack of serum α1,3fucosyltransferase due to the FUT6-deficiency has scarcely been found so far and only restricted numbers of individuals whose AGP molecule has no fucosylated glycan have been reported previously [37,38,75]. The patient N was supposed to possess the lethal mutation in the *FUT6* genes resulting in possessing the mutant *pf* gene homozygously as demonstrated previously [37]. While, mass spectrometric analysis of AGP glycans in this patient showed that up to several % of glycans in AGP were still fucosylated and a couple of fucosylated glycans were constantly detected during the period of observation (Fig 4b). One of the seven individuals with FUT6-deficiency in our previous study showed to possess a small amount of fucosylated glycoforms in AGP [37]. These fucosylated glycans might be synthesized by the action of a certain α1,3fucosyltransferase other than *FUT6*-encoded α1,3fucosyltransferase. CEA levels in the patient N were continuously above the normal range even though CT scans revealed no significant remarks during the period. Accordingly, CEA levels in this patient might be subjected to be false-positive range as demonstrated recently [76]. It has been widely recognized that CA 19–9 could not be used as tumor marker in Lewis-negative cancer patients because individuals with Lewis negative genotypes lack the key enzyme to synthesize Fucα1,4GlcNAc linkage, part

of its antigenic determinant [57,77]. Hence, the results suggest that FUCAGP level could not be useful in patients with FUT6 deficiency as a clinically relevant biomarker.

The function of plasma AGP and its potential physiologic significance as an acute phase protein have generated profound interest [33,78–81]. Generally, AGP has highly glycosylated *N*-glycan chains on the molecule with more than 45% content and with abundant heterogeneity. It has therefore been demonstrated that despite expression of different genomic variants and mRNA levels of AGP, drastic changes occur frequently in not only serum concentrations but also their glycan structures including branching, sialylation and, in particular, fucosylation degrees as posttranslational modification involving in rapid responses to various biological phenomena [25–35,78–84]. It was also shown that each AGP glycoform was renewed at a rate of 15% per day of the plasma pool [85]. A wide variety of changes and a large amount of replacements were also observed in AGP glycans from various cancer patients who received surgical interventions and chemotherapies [34].

Glycoproteomic approach to investigate tumor-associated alteration of glycans in glycoproteins has been developed recently. As one of the most powerful tools, mass spectrometric analyses through MALDI TOF or LC-MS/MS together with specific enrichment methods such as the use of lectin-affinity toward glycans targeted have been applied for a comprehensive analysis of large amounts of glycans. Indeed, findings of usefulness of *Aleuria aurantia* lectin (AAL) for isolation of fucosylated antigens [86,87] and characterization of its detailed binding specificity [88] have been carried on into further applications of this lectin to recent glycoproteomic studies using human plasma and/or serum samples [34,71,73,89–92]. Whereas, AGP is a major serum protein and due to its original aspect as an acute phase protein, changes of AGP concentrations in serum waver depending on the degree of inflammation caused by a series of diseases and biological events. As expected from our previous studies [34,35], serum AGP levels were found to change differently from FUCAGP ones during the follow-up period in most of the patients. It was also suggested that AGP level changed indistinguishably in patients with poor and good prognosis after operation. Further, it was not unusual observation to see aberrantly elevated AGP levels in serum samples from cancer patients up to several times higher than those in healthy controls. It should therefore be important for targeting serum AGP molecule to focus on not only the fucosylated AGP glycans but also relative abundance of fucosylated AGP glycans after exclusion of large amounts of background noise.

In conclusion, in the present study, FUCAGP could be a clinically relevant biomarker of cancer progression as well as cancer prognosis. The FUCAGP level was found to closely relate to patients' response to various chemotherapies. Further, instead of our previous CAIE technique for measuring AGP glycoforms which was not simple or convenient for assaying large numbers of samples at attempt, the evaluation of AGP glycans in the present study, by means of an AGP-prep-DOCK and mass spectrometric analyses, was aimed for a quick determination of serum AGP glycans. Additionally, recently we developed and improved AGPAS software for very rapid determination of FUCAGP levels in serum samples. These reflect our long standing view that rapid and accurate measurement of FUCAGP in serum samples might advance diagnosis of better treatment outcomes of cancer patients. We believe that changes in FUCAGP level in a cohort of patients followed up while undergoing various chemotherapeutic regimens warrant further investigation.

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Author Contributions

Conceived and designed the experiments: SY TA. Performed the experiments: SY RS. Analyzed the data: SY RT TY RS AM ARS TA. Contributed reagents/materials/analysis tools: SY RT TY RS AM ARS HK TA. Wrote the paper: SY.

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