Clinica Chimica Acta 478 (2018) 120–128

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

A new enzyme immunoassay for the determination of highly sialylated and fucosylated human α_1-acid glycoprotein as a biomarker of tumorigenesis

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ARTICLE INFO

Keywords:
α1-Acid glycoprotein
Enzyme immunoassay
Fucosylated glycans
Biomarker
Tumorigenesis
Treatment outcomes

ABSTRACT

Background: Upon initiation and progression of cancer, α_1-acid glycoprotein (AGP) possessing highly sialylated and fucosylated glycans appears in the serum, and recently has attracted a great deal of attention, as a potential biomarker of tumorigenesis in humans.
Methods: To establish a rapid and precise method for the quantitative assay of fucosylated AGP in serum samples, we developed an enzyme immunoassay (EIA) bearing an anti-AGP antibody and a fucose-binding lectin, *Aleuria aurantia* (AAL) with additional endeavor to improved sample handling, and antibody preparations.
Results: The amounts of fucosylated AGP could be determined by the present method with a good performance feature in all tested samples from both cancer patients and healthy controls. From cancer patients under chemotherapy we show that fucosylated AGP could be a clinically relevant biomarker for cancer progression or prognosis as well as for an early assessment of clinical response and treatment outcomes. Furthermore, in a different setting, fucosylated AGP also showed relevance in patients who received immunotherapy with an anti-programmed cell death-1 (PD-1) antibody.
Conclusions: α1,3fucosylated AGP is a potential biomarker of cancer initiation, progression and response to treatment in cancer patients.

1. Introduction

Proteins in nature are frequently modified post-translationally and more than half of them are glycosylated and presented as glycoproteins [1]. It has been widely recognized that each glycan attached to proteins plays crucial roles in not only protein folding and clearance but also cell to cell interaction, recognition, adhesion and binding [2]. Among glycans consisting of glycoproteins, fucose (Fuc) residues attach to the terminal galactose (Gal) residues through α1,2 linkage, the sub-terminal *N*-acetylglucosamine (GlcNAc) residues through α1,3 or α1,4 linkages and the inner core GlcNAc residues through α1,6 linkages, respectively, and these fucosylated glycans are widely expressed on the cell surface and in secretions [3,4]. It has also been demonstrated that such fucosylated glycans are frequently modified associated with various biological processes and occurrence of diseases, in particular, malignant transformation [5,6]. These fucosylated glycans are usually detectable with the aid of antibodies and/or fucose-binding lectins. Thus, our first applications of *Aleuria aurantia* lectin (AAL) [7,8] and YB-2 monoclonal antibody [9–11] proved their usefulness for isolation, purification and detection of certain fucosylated glycoproteins.

A series of glycan-based antibodies and lectins have also been demonstrated to be promising tools and widely applied to develop as analytical reagents for detection and quantification of targeting glycans [12,13]. Further, recently developed techniques such as mass spectrometric analyses through MALDI-TOF-MS and LC-MS/MS technology in glycoproteomics have contributed a great deal to innovation for comprehensive analyses of glycans expressed in glycoproteins [14–16]. Likewise, some lectins and glycan-based antibodies have been used effectively as reagents for enrichment of samples to be analyzed [17,18].

Human α_1-acid glycoprotein (AGP) is one of the major plasma glycoproteins and has a molecular weight of 41 to 43 KD with a highly

Abbreviations: AGP, α_1-acid glycoprotein; AS-AGP, asialo-α_1-acid glycoprotein; EIA, enzyme immunoassay; AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; BSA, bovine serum albumin; PD-1, programmed death-1; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; CAIE, crossed affinoimmunoelectrophoresis; CT, computed tomography; PB, phosphate buffer; PBS, phosphate buffered saline; $NaOI_4$, sodium metaperiodate; POD, postoperative days; FUCAGP, relative abundance of α1,3fucosylated AGP glycans

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https://doi.org/10.1016/j.cca.2017.12.030
Received 18 November 2017; Received in revised form 6 December 2017; Accepted 20 December 2017
Available online 21 December 2017
0009-8981/

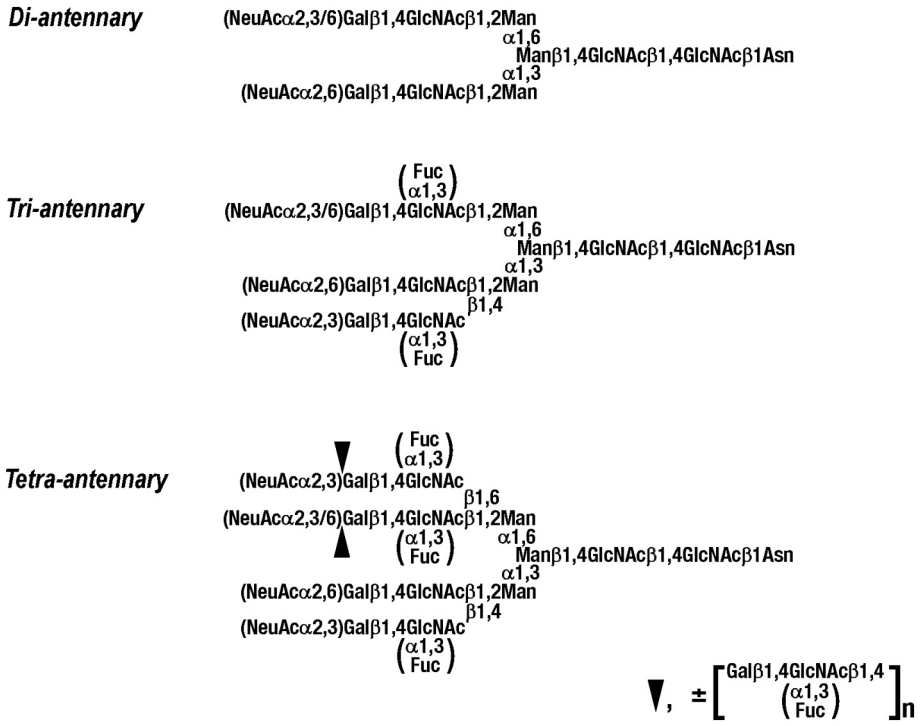

Fig. 1. Differently branched N-glycans in α_1-acid glycoprotein (AGP). One molecule of AGP possesses 5 glycan chains including di-antennary, tri-antennary and tetra-antennary glycans. All possibilities are shown in brackets. Repeated lactosamine (Galβ1, 4GlcNAc) structures were also shown in some cases (at the position of the black triangle). GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal, galactose; NeuAc, *N*-acetylneuraminic acid; Fuc; fucose and Asn, asparagine.

branched N-linked glycoprotein which consists of 5 complex type glycan chains including diantennary, triantennary and tetraantennary glycans (Fig. 1). *N*-Acetylneuraminic acid (NeuAc) residues attach to most of the terminal Gal residues in these 5 chains through α2,3 or α2,6 linkages and elongated glycans in the tetraantennary glycans with repeating lactosamine (Galβ1,4GlcNAc) structures are also seen in some cases [19,20]. Furthermore, the (sialyl) Le^x determinant ((NeuAcα2,3)Galβ1,4[Fucα1,3]GlcNAc), which is present in both tri- and tetraantennary glycan chains, has been demonstrated to be a specialized glycan structure highly expressed in AGP during the inflammation process [19,20]. The physiological significance of AGP has been investigated as an acute phase protein with diverse immunomodulating effects accompanying by increased attentions directed to changes of its complex glycan chains as well as concentrations in plasma. As a result, information has been collected through studies on not only quantitative changes of AGP with significantly increased concentrations in plasma but also qualitative changes of AGP glycans with occurrence of highly sialylated and fucosylated glycans in association with inflammation, pregnancy, estrogen treatment, liver diseases, malignancies and autoimmune diseases like rheumatoid arthritis [19–27].

We previously investigated AGP glycoforms in a large number of serum samples from patients with various cancers as well as healthy controls by using a crossed affinoimmunoelectrophoresis (CAIE) with Con A lectin, AAL and anti-AGP antibody [28]. As a result, patients with advanced malignancies who had glycoforms containing highly branched and α1,3fucosylated glycans for long periods after surgical operation were found unexceptionally to have a poor prognosis. Whereas, patients who had AGP glycoforms without such changes were expected to have a good prognosis irrespective of their clinical stages and types of malignancies. Furthermore, although serum AGP concentrations in preoperative patients were significantly high compared with those in healthy controls, no clear difference was found between serum AGP concentrations in patients and their prognosis after operation.

Recently, we also established a novel system for comprehensive analysis of serum AGP glycans. The system consisted of a reliable purification method for serum AGP, a specifically labeling method for enzymatically cleaved glycans, and a mass spectrometer together with a newly developed software (AGPAS) allowing very rapid determination of primary structures of AGP glycans [29]. The results obtained from 30 patients with diverse cancers following for a long period after operation clearly showed that changes in α1,3fucosylated AGP glycans after operation could be used as a novel biomarker for monitoring and predicting patients' prognosis. Accordingly, amounts of relative abundance of α1,3fucosylated tri- and tetraantennary glycans in AGP (FUCAGP) were found to significantly elevate in cancer patients when compared in healthy controls. It was also demonstrated that amounts of FUCAGP changed depending on patients' prognosis; highly increased amounts of FUCAGP were found only in patients with poor prognosis but not in patients with good prognosis.

More recently, amounts of FUCAGP in serum samples from 13 various cancer patients who had undergone chemotherapy were followed for several years post operation [30]. Amounts of FUCAGP determined by means of a MALDI-TOF-MS together with improved methods for purification and determination of AGP glycans were found to be a clinically relevant biomarker of cancer prognosis or progression. In addition, interestingly, changes of the amount showed to be closely involved in patients' responses to chemotherapy treatments.

In the present study, we developed and evaluated an enzyme immunoassay system to establish a rapid and precise method for the qualitative assay of α1,3fucosylated AGP together with additional endeavor to improve sample handling and antibody preparations. The method was also applied for following cancer patients who had been received chemotherapies. Furthermore, in a different setting, a preliminary evaluation of the fucosylated AGP as a biomarker for early response and outcome was conducted for the first time in patients with advanced lung cancer who received an immunotherapy targeting the programmed death-1 (PD-1) and its ligand, PD-1L checkpoint signaling inhibition [31] after repeated treatments with unsuccessful chemotherapies.

2. Materials and methods

2.1. Materials

Human serum AGP, bovine serum albumin (BSA), Tween 20, sodium metaperiodate, D-sorbitol and borane-dimethylamine complex