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N-glycan profiles as tools in diagnosis of hepatocellular carcinoma and prediction of healthy human ageing

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ABSTRACT

Protein glycosylation, the most common form of co-translational modification of proteins, is the enzymatic addition of sugars or oligosaccharides (glycans) to proteins. Protein glycosylation increases the diversity of the functions of proteins encoded in the genome. The result is that different glycomes of the same protein may have different functional, kinetic or physical properties. The glycosylation pathway is largely regulated by the condition of the cells, which means that the sugar chains can be altered by the physiological or pathophysiological condition of the cell. Thus, the type of glycans produced by cells, tissues, or organism could reflect their current physiological state. We determined the N-glycan profiles of serum proteins by using DNA sequencer-based carbohydrate analytical profiling technology. We show that two N-glycan structures (NGA2F and NA2F) present in human blood glycoproteins change with ageing, and that one triantennary glycan (NA3Fb) is correlated with tumor stage in HCC patients. Therefore, examining alterations in serum glycan fingerprint by using our platform could be a suitable tool for monitoring the healthiness of ageing and for the follow-up of pathophysiological conditions such as liver cancer.

1. Introduction

Protein glycosylation is the enzymatic addition of sugars or oligosaccharides (glycans) to proteins. It is the most common form of co-translational modification of proteins, with over half of all proteins estimated to contain one or more glycan chains (Apweiler et al., 1999). Glycosylation occurs primarily in the endoplasmic reticulum (ER) and Golgi apparatus. For example, N-glycosylation starts co-translationally in the ER, where a lipid-linked precursor oligosaccharide is attached to the protein, followed by initial trimming. This precursor oligosaccharide and the initial trimming in the ER are almost universal in eukaryotes. Species-, tissue- and cell-specific modifications occur later in the Golgi apparatus. Once the proteins are fully glycosylated, they are embedded in the plasma membrane or secreted (Weerapana and Imperiali, 2006). Glycosylation increases the diversity of the functions of the proteins encoded in the genome. Different glycans on the same protein might have different functional, kinetic or physical properties. Unlike phosphorylated proteins, most glycosylated proteins (i.e. glycoproteins) are associated with extra-cellular functions.

The glycome by definition comprises all glycan structures produced by an organism, whereas glycomics encompasses the study of glycan structure, biosynthesis, and function (von der Lieth et al., 2004). The roles of glycans are diverse. They contribute to the folding and conformational stability of many proteins (Helenius and Aebi, 2004; Wang et al., 1996), mediate host–pathogen interactions and aspects of innate immunity (Smith and Helenius, 2004), and serve as ligands for glycan-binding proteins that mediate cell trafficking, adhesion, and signaling (Rabinovich et al., 2002). Glycomics is as essential as proteomics and genomics to the understanding of the complexity of human life. However, until now, direct use of glycosylation in diagnosis or evaluation of physiological conditions has been limited, because of the unavailability of appropriate analytical techniques. Analysis of N-glycans

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Abbreviations: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; DSA-FACE, DNA sequencer-assisted (DSA), fluorophore-assisted carbohydrate electrophoresis (FACE); GnT-III, -IV, -V, β1,4-*N*-Acetylglucosaminyltransferase III, IV, V.

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poses particular difficulties. For example, N-glycans do not have strong absorbance at wavelengths preferred for spectrophotometry. Consequently, sensitive detection of glycans requires labeling before separation. Another difficulty is that an important subclass of glycans is not charged at physiological pH, and so their separation by electrophoresis requires the use of strongly anionic tags (Laroy et al., 2006).

Our recently developed "DNA sequencer adapted-fluorophore assisted carbohydrate electrophoresis" (DSA-FACE) is a high-throughput technology platform for profiling N-glycans from proteins in serum and other body fluids (Callewaert et al., 2001; Laroy et al., 2006). It is based on capillary electrophoresis (electrophoresis in very narrow-bore channels), it is highly sensitive and quantitatively reliable, and it can resolve isobaric glycan stereoisomers. Samples are prepared with ease in 96-well plates, and the only piece of specialized equipment needed is a DNA sequencer of the type found in almost every molecular biology laboratory.

Here, we describe the use of the N-glycan profiles as a tool in diagnosis of liver cancer and in assessment of the healthiness of ageing.

2. Materials and methods

2.1. Italian volunteers

Plasma samples were obtained from 239 Italian volunteers grouped by age as follows: 28 of <40 years with mean age of 32.5; 33 of 41–70 years with mean age of 57; 59 of 71–90 years with mean age of 79.7; 119 centenarians (>90 years) with mean age of 100.1. All volunteers were in general good health (free of relevant acute or chronic disease affecting the immune system), and gave informed consent for the studies described here.

2.2. Patients

The study was approved by the Ethics Committee of Peking University Health Science Centre, and by the Ethics Committee of Renji Hospital, Shanghai Second Medical University. Informed consent was obtained from each patient.

Patients were recruited from the hospitals in China. A HCC group ($n = 44$) and a cirrhosis group ($n = 55$) with HBV infection and AFP level of 1–400 ng/ml were included. Grounds for exclusion were metastasis, autoimmune liver disease, drug-related hepatitis, alcoholic hepatitis, or obstructive jaundice. All patients were negative for antibodies against HAV, HCV and HDV (Abbott EIA), EBV and CMV (EIA, Human Co. Ltd., Germany), and HEV (EIA, Genelabs, Singapore).

2.3. Clinical stage and tumor stage

The liver cirrhosis patients and HCC patients had been extensively studied and their clinical data had been published previously by Liu et al. (2007). All patients had cirrhosis and were infected with hepatitis B virus (HBV) as diagnosed by serological detection of HBsAg, anti-HBsAg (HBsAb), HBeAg, anti-HBeAg (HbeAb), anti-HBcAg (HBcAb) and HBV DNA. The diagnosis of liver cirrhosis was made by histological examination, imaging, and several liver function tests. Liver samples were evaluated independently by two experienced hepatopathologists who were unaware of the glycomics results. Liver cirrhosis patients were staged according to the Child–Pugh classification (Child and Turcotte, 1964; Pugh et al., 1973). Cirrhosis patients with HCC were diagnosed histologically by biopsy and clinically by ultrasonography and/or computed tomographic scanning during routine examinations, as well as by measurement of AFP. The tumor stages were ranked according to the TNM criteria (Afdhal, 2004): T1 = solitary tumor without vascular invasion; T2 = solitary with vascular invasion, multiple, ≤5 cm; T3 = multiple, >5 cm, invading major branch of portal or hepatic veins; T4 = invading adjacent organs other than gallbladder, perforating visceral peritoneum. All blood samples were obtained before any treatment or operation.

2.4. Processing blood samples for protein N-glycome analysis

The N-glycans present on the proteins in 2 μl of serum were released, labeled, and analyzed as described previously (Laroy et al., 2006; Vanhooren et al., 2008). Labeled N-glycans were analyzed by DSA-FACE technology, using a capillary electrophoresis (CE)-based ABI3130 sequencer. Data were analyzed with the GeneMapper v3.7 software (Applied Biosystems, Foster city, CA). We measured the heights of the peaks that were detected in all the samples to obtain a numerical description of the profiles, and analyzed these data with SPSS 15.0 statistical software.

3. Results

3.1. N-glycan fingerprint in human serum

The human serum N-glycan fingerprint can be examined by using DSA-FACE technology. The most abundant N-glycans in serum and serum immunoglobulins (Igs) are illustrated in Fig. 1. Each peak represents a different glycan structure, and its relative concentration is determined by normalizing its height to the sum of the heights of all peaks in the profile. We found that the serum glycan profile is stable and is not affected by either diminished or excessive nutritional intake over a short period of time, e.g. 1 week (data not shown).

3.2. Glycomics in diagnosis of liver cancer

Hepatocellular carcinoma (HCC), also known as primary liver cancer, is the fifth most common cancer worldwide (Bruix et al., 2004). It develops most commonly in patients with chronic viral hepatitis (in 20% of Hepatitis B or C) or with cirrhosis (in about 80%) (Liaw, 2005). Age is certainly a risk factor for liver cancer. In the United States and Europe, liver cancer is diagnosed on average at about the age of 60 years. So far, no fully accurate screening test for liver cancer exists. To screen people at high risk of liver cancer, physicians sometimes use a blood test to check for the presence of alpha-fetoprotein (AFP), a protein not normally found in adults (Koike, 2005). Though measurement of serum AFP is important in screening for HCC, studies have indicated that it is of limited use in detecting HCC in liver cirrhosis patients due to frequent mild elevation of AFP levels in cirrhosis (Colli et al., 2006). In practice, one has to use a much higher cutoff value for AFP (400 ng/ml) to maintain high specificity, with concomitant reduction in the sensitivity of HCC detection. Thus, it is necessary to have complementary marker(s) to detect HCC when the AFP level is <400 ng/ml.

To enable specific HCC detection on a cirrhosis background, we recently used DSA-FACE to identify glycan structures whose abundance does not increase in cirrhosis patients but is elevated in HCC patients. We previously found an elevated level of a branch

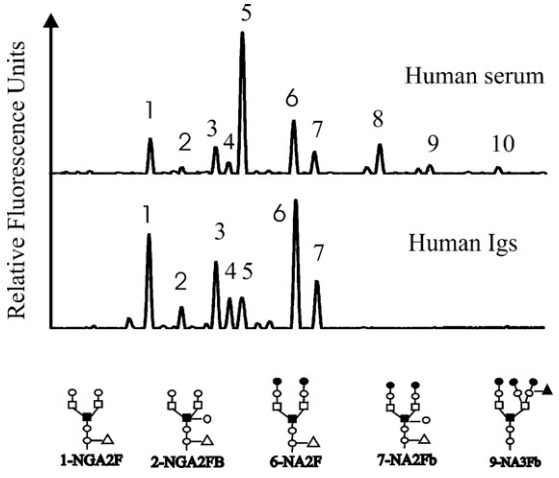

Fig. 1. A typical desialylated N-glycan profile from human total serum protein (upper panel) and from immunoglobulins (lower panel). The structures of the N-glycan peaks are shown below the panels. Peak 1 is an agalacto, core-α-1,6-fucosylated biantennary glycan (NGA2F), peak 2 is an agalacto, core-α-1,6-fucosylated bisecting biantennary (NGA2FB), peak 6 is a bigalacto, core-α-1,6-fucosylated biantennary (NA2F), peak 7 is a bigalacto, core-α-1,6-fucosylated bisecting biantennary (NA2FB), peak 9 is a branching α-1,3-fucosylated triantennary (NA3Fb). The symbols used in the structural formulas are (○) β-linked N-acetylglucosamine (GlcNAc); (●) β-linked galactose; (▲ or △) α-1,3/6-linked fucose; (□ or ■) α/β-linked mannose.