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Differential proteomic approach for identification and verification of aberrantly glycosylated proteins in adenocarcinoma lung cancer (ADLC) plasmas by lectin-capturing and targeted mass spectrometry

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ABSTRACT

To investigate quantitative differences in aberrant glycosylation of target glycoproteins between noncancerous group and patient group with adenocarcinoma lung cancer (ADLC), differential proteomic approach was developed by cooperatively using comparative lectin-capturing, targeted mass spectrometry (MRM MS), and antibody/lectin sandwich ELISA. Plasma samples comparatively prepared from 3 ADLC patients and 3 controls, with and without lectin-fractionation using fucose-specific *Aleuria aurantia* lectin (AAL), were trypsin-digested and analyzed for target glycoproteins, alpha-1-acid glycoprotein (AGP) and ceruloplasmin (CP), by MRM MS. From the MRM MS data the abundance levels of AAL-captured glycoforms of both targets were significantly higher in ADLC cases compared to controls, although the levels in total protein abundance were comparable between ADLC and control groups. This difference between ADLC and control groups in the fucosylated glycoform levels was originated mainly from aberrant fucosylation on the targets in ADLC plasmas rather than change in total protein abundance of the targets, and also confirmed by sandwich ELISA. AGP and CP were further verified to be biomarker candidates by MRM-based analysis of AAL-captured plasmas (30 ADLC cases, 30 controls), with AUROC 0.758 and 0.847 respectively. This differential proteomic approach can be useful for identifying and verifying biomarker candidate involved in aberrant protein glycosylation.

Biological significance
The present paper introduces an efficient differential proteomic method to investigate quantitative differences in aberrant protein glycosylation of serological glycoproteins

Abbreviations: AAL, *Aleuria aurantia* lectin; ADLC, adenocarcinoma lung cancer; MRM, multiple reaction monitoring; AGP, alpha-1-acid glycoprotein; CP, ceruloplasmin.

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between noncancerous group and lung cancer patient group. This differential proteomic approach consisting of the targeted MRM MS of comparatively lectin-captured plasma fractions and the antibody/lectin sandwich ELISA-based assay was evaluated to be useful for identification of aberrantly fucosylated glycoproteins AGP and CP in lung cancer plasmas. In addition, we have demonstrated that the MRM MS-based differential proteomic approach is also useful for high-throughput verification of the aberrantly fucosylated glycoproteins AGP and CP using the large number of individual plasmas. Therefore, the present MRM MS-based differential proteomic strategy with lectin-capturing can be a powerful tool for high-throughput verification of aberrantly glycosylated biomarker candidates, identified preliminary by mass profiling experiments in proteomic fields but requiring further validation using a large number of cohorts.

1. Introduction

Mass spectrometry (MS) featuring a simple, high-throughput, and cost-effective technique, has played a central role in identifying cancer biomarker candidates, mainly based on qualitative protein profiling experiments by tandem MS (MS/MS) analysis of serological proteomic samples [1–4]. Although large numbers of serological proteins have been identified as potential cancer biomarker candidates through the comparative MS/MS-based protein profiling method, further validation studies are necessary based on quantitative analytical platform technology, as well as biological examination related to tumorigenesis. The protein profiling method based on data-dependent MS/MS analysis shows features of run-to-run variability during data acquisition of a given sample and poor reproducibility between laboratories. Multiple reaction monitoring MS (MRM-MS) using a stable isotope-coded internal standard is attractive to quantitatively verify potential biomarker candidates identified by the nonquantitative MS/MS profiling method. Therefore, the MRM-MS approach, a multiplexed, targeted proteomics platform, has been recognized as a high-throughput and cost-effective method for protein quantification, and can be applied for quantitative preclinical verification of newly identified protein biomarker candidates that have not yet been verified as biomarkers [5–7]. This quantification ability of the MRM technique, with high specificity and sensitivity, can also be useful for differentiating the quantitative difference in a target protein between samples to be compared.

Nonetheless, as exemplified in the case of alpha-fetoprotein (AFP)-L3, the aberrantly fucosylated glycoforms of AFP known as a hepatocellular carcinoma biomarker [8–10], aberrant protein glycosylation that may be closely involved in pathological mechanisms during cancer progression has been discovered with features such as increased fucosylation or specific branching [11–14]. Each aberrant glycoform of a glycoprotein, including aberrantly fucosylated glycoforms, is normally present in sub-stoichiometric levels due to glycan microheterogeneity. Thus, the abundance of aberrant glycoforms of a target glycoprotein of interest is far lower than the total protein abundance of the target protein. Therefore, correct monitoring of quantitative variations in the aberrant protein glycoforms of the target glycoprotein aberrantly glycosylated in cancer is unachievable by using only the MRM technique due to the glycan microheterogeneity on the target glycoprotein. Fractionation of the sub-stoichiometric, aberrant glycoforms of target glycoproteins involved in cancer progression is essential prior to quantitative MRM mass analysis, such as lectin-capturing during the sample manipulation step [15].

A variety of lectins has been employed for capturing protein glycoforms containing a specific glycan structure from a complex glycoproteome mixture [16–21]. Since a variety of lectins is available and many lectins exhibit narrow, specific affinity for a given glycan structure, lectin can be used for capturing aberrant protein glycoforms with cancer-related specific glycan structures in the sample prefractionation step. Lectin-captured fractions can be analyzed subsequently by the MRM-based quantitative mass spectrometric method. Usefulness of the MRM-based assay of lectin-captured glycoproteomes has been evaluated in our previous studies using liver cancer blood samples [22–24]. Blood samples exhibit high complexity with a wide dynamic range of

Table 1 – Target glycoproteins selected for investigating aberrant protein glycosylation in ADLC plasmas using AAL-assisted MRM analysis.

Gene name	Protein name	Peptides sequence [a]	Q1 (m/z)	Charge state (+)	Transition 1		Transition 2		Transition 3		RT (min)	LOQ [d] (fmol)
					Q3 [b] (m/z)	C.E. [c]	Q3 [b] (m/z)	C.E. [c]	Q3 [b] (m/z)	C.E. [c]		
AGP	Alpha-1-acid glycoprotein	TEDTIFLR	497.8	2	**764.4**	11	649.4	14	548.4	13	9.7	9
		TEDTIFL*R	501.2	2	**771.4**	11	656.4	14	555.4	13	9.7	
CP	Ceruloplasmin	GAYPLSIEPIGVR	686.4	2	1080.6	19	**870.5**	27	783.5	16	12.6	9
		GAYPLSIEPIGV*R	689.4	2	1086.6	19	**876.5**	27	789.5	16	12.5	

[a] * indicates a stable isotope-coded site of a heavy internal standard peptide.
[b] Among three pairs of the selected transitions (Q3) in each target peptide, the transition pair noted in bold was employed for MRM data processing.
[c] Optimized collision energy used for fragmentation of the precursor ion (Q1) of each target peptide.
[d] Based on triplicate MRM measurements with S/N > 100 and CV < 15%, showing linear concentration ranges.