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Research paper

Proteomic analysis of Tn-bearing glycoproteins from different stages of melanoma cells reveals new biomarkers

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ABSTRACT

Cutaneous melanoma, the most aggressive form of skin cancer, responds poorly to conventional therapy. The appearance of Tn antigen-modified proteins in cancer is correlated with metastasis and poor prognoses. The Tn determinant has been recognized as a powerful diagnostic and therapeutic target, and as an object for the development of anti–tumor vaccine strategies. This study was designed to identify Tn-carrying proteins and reveal their influence on cutaneous melanoma progression. We used a lectin-based strategy to purify Tn antigen-enriched cellular glycoproteome, the LC-MS/MS method to identify isolated glycoproteins, and the DAVID bioinformatics tool to classify the identified proteins. We identified 146 different Tn-bearing glycoproteins, 88% of which are new. The Tn-glycoproteome was generally enriched in proteins involved in the control of ribosome biogenesis, CDR-mediated mRNA stabilization, cell-cell adhesion and extracellular vesicle formation. The differential expression patterns of Tn-modified proteins for cutaneous primary and metastatic melanoma cells supported nonmetastatic and metastatic cell phenotypes, respectively. To our knowledge, this study is the first large-scale proteomic analysis of Tn-bearing proteins in human melanoma cells. The identified Tn-modified proteins are related to the biological and molecular nature of cutaneous melanoma and may be valuable biomarkers and therapeutic targets.

1. Introduction

Despite all the effort invested in understanding the molecular biology of malignant melanoma, the prognoses of patients suffering from metastatic melanoma remain far from satisfactory. No effective treatment for this life-threatening disease is currently available [1]. Discovering the molecular alterations associated with tumorigenesis and metastasis formation is an essential task in the development of new strategies for cancer treatment. Changes in the glycosylation profile of proteins are a hallmark of ongoing neoplastic transformation. The unique sets of tumor-associated carbohydrate antigens expressed by malignant cells offer promising diagnostic and therapeutic targets [2–5]. It is known that proteins with altered glycosylation affect the growth, proliferation and survival of those cells, and that they contribute to their acquisition of the ability to migrate and invade [6,7]. One of the most commonly observed alterations is the expression of truncated Tn (GalNAc-α1-*O*-Ser/Thr) mucin-type antigen, also called pan-carcinoma antigen or oncofetal antigen [8]. The expression of Tn antigen is highly correlated with cancer progression and metastasis [9,10]. Generally, Tn structures are very rare in human normal tissues; they are observed as uniquely appearing in tumor tissue where generate novel *O*-glycopeptide epitopes composed of a truncated *O*-glycan and a short peptide sequence; these may be relevant cancer-specific antigens recognizable by drug-loaded antibodies [9]. In the present study we used *Vicia villosa* agglutinin

Abbreviations: CBB, Coomassie Brilliant Blue; CRD, Coding Region instability Determinant; GalNAc, N-acetylgalactosamine; GO, Gene Ontology; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LC-MS/MS, liquid chromatography combined with tandem mass spectrometry; PVDF, polyvinylidene fluoride; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tn, GalNAc-α1-*O*-Ser/Thr; VVA, *Vicia villosa* agglutinin.

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(VVA), which has high affinity for capturing Tn antigen-bearing proteins [8,11], to identify the Tn-enriched subfraction of the melanoma glycoproteome and to reveal changes in the Tn-glycoproteome during cancer progression. Using the DAVID Functional Annotation Tool (https://david.ncifcrf.gov/), all identified proteins were characterized according to Gene Ontology annotations in terms of cellular component, molecular function and biological process [12,13]. To our knowledge, this study is the first large-scale proteomic analysis of Tn-bearing proteins in human melanoma cells. The study yielded findings about this group of proteins which may prove useful in work aimed at proposing new melanoma biomarkers and therapeutic targets.

2. Materials and methods

2.1. Reagents

Biotinylated and agarose-bound lectin VVA as well as GalNAc were purchased from Vector (USA). Immobilon-P transfer membrane was obtained from Millipore (USA). RPMI 1640-GlutaMAX-I medium (Gibco), foetal bovine serum (Gibco) and the Total Protein Kit (Micro Lowry, Peterson's Modification) were obtained from Sigma (St. Louis, MO). All other chemicals were analytical grade and were purchased from Sigma (St. Louis, MO).

2.2. Cell lines

The ESTDAB Melanoma Cell Bank (Tübingen) supplied human cutaneous primary melanoma cell lines WM115 (ESTDAB-066; VGP), FM-55-P (ESTDAB-014), IGR-39 (ESTDAB-080; VGP) and acral lentiginous WM3211 (ESTDAB-079; VGP); and metastatic melanoma cell lines FM-55-M2 (ESTDAB-013; subcutaneous), COLO-794 (ESTDAB-001; subcutaneous), WM1205Lu (ESTDAB-067; lung) and FM-3 (ESTDAB-007; inguinal lymph node).

2.3. Cell culture conditions and cell extract preparation

Cells were maintained in RPMI 1640-GlutaMAX-I medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown in monolayers in a 5% CO_2 atmosphere at 37 °C in a humidified incubator. Cell extract proteins were prepared as described previously [14]. The protein concentration was determined with the Total Protein Kit (Sigma).

2.4. Isolation and mass spectrometry analysis of VVA-positive glycoproteins

2.4.1. Lectin precipitation

Lectin precipitation was performed as described previously [14]. Briefly, each cell extract (600 μg total protein) was diluted to 300 μl with precipitation buffer (10 mM HEPES, 0.15 M NaCl, 0.1 mM Ca^{2+}, 0.01 mM Mn^{2+}, pH 7.5) and incubated overnight at 4 °C with 40 μl VVA-agarose. All precipitates were washed three times with incubation buffer and once with PBS, with centrifugation each time (1500 × g, 3 min). Glycoproteins bound to VVA-agarose were liberated by boiling (100 °C, 8 min) in Laemmli sample buffer (80 μl) containing 5% β-mercaptoethanol and 1 mM EDTA.

2.4.2. SDS-PAGE, lectin blotting and mass spectrometry analysis

After boiling, each supernatant was divided as follows: 3/4 (450 μg total protein) for in-gel protein staining, and 1/8 (75 μg total protein) for on-blot lectin probes, and electrophoresed on 8% SDS-polyacrylamide gel according to Laemmli [15]. One part of the gel was stained with Brilliant Blue G Colloidal (CBB, Sigma) and the other was electrotransfered on a PVDF membrane and probed with active and inhibited VVA-biotin lectin (1:125 dilution). After washing, the membranes were incubated with ExtrAvidin-AP conjugate (1:4000 dilution, Sigma) for 1 h at RT. The conjugated alkaline phosphatase was detected by NBT/X-phosphate staining. VVA lectin inhibition was achieved by lectin preincubation with 0.2 M GalNAc (2 h, RT). Individual protein bands corresponding to the VVA staining pattern were excised from the gel, dried in a vacuum centrifuge (SpeedVac) and sent for mass spectrometry analysis (Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, http://mslab-ibb.pl/en/). In the Laboratory of Mass Spectrometry, the gel bands were chopped into small pieces, destained with 50% acetonitrile in 50 mM ammonium bicarbonate, pH 7.8, reduced with 10 mM DTT (30 min at 56 °C), alkylated with 50 mM iodoacetamide (45 min in a dark, RT) and finally digested with 12.5 ng/μl of trypsin (Sequencing Grade Modified Trypsin– Promega V5111; Madison, USA) in 50 mM ammonium bicarbonate, pH 7.8 (18 h, 37 °C). Extraction of peptides was done with 0.1% TFA in 2% acetonitrile. Peptide mixtures were analyzed by LC-MS-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using the Nano-Acquity (Waters) UPLC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA, USA; instrument type: ESI-FTICR-MS). Proteins were identified by matching the tryptic peptides with the National Center for Biotechnology Information (NCBI) database, using the Mascot database search engine (http://www.matrixscience.com). Search parameters were as follows: peptide mass tolerance ±40 ppm; fragment mass tolerance ±0.8 Da; maximal number of missed cleavage sites allowed: 1; fixed modification of cysteine by carbamidomethylation; variable modification of lysine by carbamidomethylation and methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to <5% False Positive Rate, calculated by the Mascot procedure, were considered to be positively identified. Some representative TIC chromatograms are shown in Supplementary Information, Fig. S1.

2.5. Bioinformatics analysis

The functional annotation tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID ver. 6.8 Oct. 2016; https://david.ncifcrf.gov/) was used to validate the identified proteins in terms of Gene Ontology (GO) function enrichment. The EASE score (p-value) was set to below 0.001 or 0.01.

3. Results and discussion

3.1. Selective enrichment of Tn antigen-modified glycoproteome and protein identification

In this study we used a strategy for effective enrichment of the Tn antigen-modified cellular glycoproteome. For this purpose we chose VVA, a GalNAcα/β1-specific lectin, which strongly recognizes the unmasked Tn antigen located on threonine, serine or tyrosine residues [16,17], and is used to detect glycoproteins carrying these glycoepitopes in cancer biopsies and for follow-up of cancer progression and evolution [18]. Clustered Tn epitopes significantly enhance affinity with VVA as compared to single Tn epitope; however, the arrangement of GalNAc residues also influences the affinity between lectin and Tn antigens [19]. In addition, more selective binding of VVA to the glycopeptides carrying Tn-threonine than Tn-serine residues was reported [20]. Using VVA binding properties, we ran lectin affinity experiments employing VVA-conjugated agarose to capture lectin-positive proteins in eight human cutaneous melanoma cell lines. For each cell line, one part