ELSEVIER

Archives of Medical Research 46 (2015) 642–650

Archives of Medical Research

ORIGINAL ARTICLE

Exploring Glycan Markers for Immunotyping and Precision-targeting of Breast Circulating Tumor Cells

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Received for publication July 22, 2015; accepted November 24, 2015 (ARCMED-D-15-00543).

Background and Aims. Recognition of abnormal glycosylation in virtually every cancer type has raised great interest in exploration of the tumor glycome for biomarker discovery. Identifying glycan markers of circulating tumor cells (CTCs) represents a new development in tumor biomarker discovery. The aim of this study was to establish an experimental approach to enable rapid screening of CTCs for glycan marker identification and characterization.

Methods. We applied carbohydrate microarrays and a high-speed fiber-optic array scanning technology (FAST scan) to explore potential glycan markers of breast CTCs (bCTCs) and targeting antibodies. An anti-tumor monoclonal antibody, HAE3-C1 (C1), was identified as a key immunological probe in this study.

Results. In our carbohydrate microarray analysis, C1 was found to be highly specific for an O-glycan cryptic epitope, gp^{C1}. Using FAST-scan technology, we established a procedure to quantify expression levels of gp^{C1} in tumor cells. In blood samples from five stage IV metastatic breast cancer patients, the gp^{C1} positive CTCs were detected in all subjects; ~40% of bCTCs were strongly gp^{C1} positive. Interestingly, CTCs from a triple-negative breast cancer patient with multiple sites of metastasis were predominantly gp^{C1} positive (92.5%, 37/40 CTCs).

Conclusions. Together we present here a practical approach to examine rare cell expression of glycan markers. Using this approach, we identified an O-core glyco-determinant gp^{C1} as a potential immunological target of bCTCs. Given its bCTC-expression profile, this target warrants an extended investigation in a larger cohort of breast cancer patients.

Key Words: Carbohydrate microarrays, *O*-glycan cryptic antigens, Circulating tumor cells, Metastatic breast cancer, Tumor immunotyping.

Introduction

Breast cancer (BCa) is among the most prevalent cancers and accounts for the highest number of cancer-related deaths among women worldwide. Identifying biomarkers of immunological significance is important in developing precision diagnostic and therapeutic strategies to advance current BCa healthcare (1). Recognition of abnormal glycosylation in virtually every cancer type has raised great interest in exploration of the tumor glycome for biomarker discovery (2–5). Potential glycan markers of BCa identified may include, but are not limited to, mucin-1 (CA 15-3) (6), carcinoembryonic antigen (CEA) (7), sialyl Lewis x (sLe^x/CD15s) (8,9), and glycoforms of a number of serum acute phase proteins such as α1-acid glycoprotein, α1-antichymotrypsin, and haptoglobin β-chain (10). Because carbohydrate moieties are often surface-exposed and easily

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0188-4409/$ - see front matter.
http://dx.doi.org/10.1016/j.arcmed.2015.11.007

accessible by antibodies, some targets have been employed for antibody therapeutics (11−14).

Exploring glycan markers of breast circulating tumor cells (bCTCs) represents a new development in tumor biomarker discovery. Although bCTCs are rare in blood, they play a key role in tumor metastasis (15,16). Detection of CTCs has been explored as a non-invasive "liquid biopsy" for tumor diagnosis and prognosis (17−19). Glycan markers of bCTCs may have unique value in BCa healthcare, especially in the personalized therapy that targets specific immunotypes of BCa. Thus, our team has worked to identify potential glycan markers of bCTCs (20).

A key immunological probe of this investigation is an anti-tumor glycan monoclonal antibody (mAb), HAE3. This antibody was raised by a murine mammary tumor antigen epiglycanin (EPGN) (21) but was found to cross-react with a number of human epithelial tumors in tissues including the lung, prostate, bladder, esophagus, and ovaries (22−24). In a recent flow-cytometry-based screening for tumor cell surface biomarkers, we found that HAE3 also strongly cross-reacts with human breast tumors (20).

This striking cross-species tumor-binding profile suggests the possibility that HAE3 may recognize a conserved tumor glycan marker that is co-expressed by both mouse- and human-derived epithelial cancers. We therefore explored the potential natural ligands of HAE3 in the repertoire of carbohydrate-based autoantigens. By scanning a large collection of carbohydrate antigens using carbohydrate microarrays, we found that HAE3 is highly specific for a blood group precursor cryptic epitope normally hidden in the cores or internal chains of blood group substances (20). In this study, we further investigated whether this target is applicable for detection and immunotyping analysis of CTCs in patients with metastatic breast cancer. To ensure the observed cross-species antigenic reactivities are not owing to the unexpected presence of oligoclonal populations in the original HAE3 hybridoma cell line, we further subcloned HAE3 and produced antibody from a single clone, HAE3-C1 (C1), for this study. As summarized below, antibody C1 was verified by carbohydrate microarrays and a glycan-specific enzyme-linked immunosorbent assay (ELISA) to be highly specific for a conserved O-glycan cryptic glyco-epitope gp^{C1} in human blood group precursors. With this key reagent, we further established a FAST-scan-based method for monitoring gp^{C1} expression in bCTCs.

Materials and Methods

Patient Samples

CTCs analyzed were from patients undergoing treatment for metastatic breast cancer at the City of Hope Cancer Center. Blood samples were collected and used under protocols approved by the Institutional Review Boards of the City of Hope Cancer Center, Palo Alto Research Center, and SRI International. All patients gave their written, voluntary, informed consent (www.clinicaltrials.gov: NCT01048918 and NCT00295893). Patient demographics and clinical characteristics are described in the Results section.

Carbohydrate Antigens, Anti-glycan Antibodies, and Tumor Cell Lines

Carbohydrate antigens for carbohydrate microarray analysis are listed in Supplementary Table 1. Antibody C1 (IgM) was produced in this study by cell line HAE3-C1, which is a subclone of the parent murine hybridoma, HAE3 (22). Tumor cell lines used include lung (A549)- and breast (T47D and SKBR3)-derived epithelial tumor cell lines. Both T47D and SKBR3 were derived from metastatic sites in breast cancer patients. All tumor cell lines were acquired from the American Type Culture Collection (ATCC), Manassas, VA.

Carbohydrate Microarrays and ELISA

Carbohydrate antigens of various structural compositions were dissolved in phosphate-buffered saline (PBS) (glycoprotein conjugates) or saline (polysaccharides) and spotted onto SuperEpoxy 2 Protein slides (ArrayIt Corporation, Sunnyvale, CA) by a high-precision robot designed to produce cDNA microarrays (Cartesian Technologies PIXSYS 5500C). Immediately before use, the printed microarray slides were washed in 1X PBS at room temperature for 5 min and blocked with 1% bovine serum albumin (BSA)-PBS at room temperature for 30 min. They were then incubated at room temperature with C1 (IgM) antibody at 5.0 μg/mL in 1% (wt/vol) BSA in PBS containing 0.05% (wt/vol) NaN_3 and 0.05% (vol/vol) Tween 20. An R-phycoerythrin (R-PE)-conjugated affinity-purified F(ab') fragment of goat anti-mouse IgM secondary antibody preparation (Rockland Immunochemicals, Inc., Limerick, PA) was applied at 2.0 μg/mL to reveal the C1-specific staining signal. The stained slides were rinsed five times with PBS with 0.05% (vol/vol) Tween 20, air-dried at room temperature, and then scanned for fluorescent signal using a ScanArray5000A Microarray Scanner (PerkinElmer Life Science). The SAS Institute JMP-Genomics software package (http://www.jmp.com/) was used for further microarray data standardization and statistical analysis. Results of the microarray assay are shown as microarray scores, i.e., means of the log2-transformed fluorescent intensities (MFIs) of multiple detections of a given antigen preparation (Figure 1A and Supplementary Table 1). Glycan-specific ELISA was performed as described in Figure 1 legend following our standard protocol (20).

Fiber-optic Array Scanning Technology (FAST Scan)

An established procedure for multiple marker measurement using the FAST scan (17,25,26) was followed with minor