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Molecular identification of lectin binding sites differentiating sialylation of epidermal growth factor receptor

The epidermal growth factor receptor (EGFR), family of receptor tyrosine kinases, also known as ErbB1 or HER1, plays crucial roles in the development of multicellular organisms. Mutations and over-expression of the EGF-receptors have been implicated in a variety of diseases. The extracellular, ligand binding regions of EGFR are quite heavily sialylated. Here we designed a lectin-metabolic assay that allows direct comparison of cell surface sialylation between health and disease states. We utilize two human mammary epithelial cell lines, HB4A (breast normal cells) and T47D (breast cancer cells) as a model system for the assessment of differential sialylation extracellular domain of ErbB1. Under starved condition, sialic acid treatment of both cells resulted increased lectin fluorescence signals that indicated the accumulation of more sialic acids in the metabolic state as demonstrated by Western blotting and immuno-precipitation. Furthermore, lectin-precipitation showed a very strong MAL-I (Maackia amurensis agglutinin I) binding on the EGF-receptors of sialic acid treated T47D cells suggesting an increase of Neu5Acα2→3Gal on the cell surface. The MAL-I lectin can be used for discrimination at molecular level between healthy and diseased cells.
Keywords: cancer biomarkers, epidermal growth factor receptor, sialylation, lectins, starvation.

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Молекулярная индентификация лектин-связывающих сайтов при дифференциальном сиалилировании эпидермального фактора роста

Семейство рецепторов эпидермального фактора роста (EGFR) играет важнейшую роль в развитии многоклеточных организмов. Мутации и гиперэкспрессия EGF-рецепторов может вызывать различные заболевания. Известно, что внеклеточные домены EGFR, отвечающие за связывание с лигандами, довольно сильно сиалилированы. Мы провели дизайн лектин-метаболического исследования, позволяющего провести прямое сравнение по уровню сиалилирования внеклеточного домена рецептора ErbB1 (или HER1) между культурами эпителиальных клеток молочной железы, представляющих норму (HB4A) и рак (T47D). Обработка клеточных культур HB4A и T47D сиаловой кислотой в условиях голода вызывала увеличение связывания сиаловой кислоты с внеклеточным доменом ErbB1, что продемонстрировано Вестерн-блоттингом и иммуннопреципитацией. Кроме того, иммунопреципитация 3-х лектинов выявила, что наиболее сильную реакцию в отношении раковых клеток проявлял лектин MAL-I (Maackia amurensis agglutinin I). Мы предполагаем, что обработанные сиаловой кислотой T47D клетки увеличивают активность Neu5Acα2 → 3Gal на клеточной поверхности. Таким образом, MAL-I лектин можно использовать для визуализации молекулярных различий между здоровыми и больными клетками.
Ключевые слова: биомаркеры рака, рецептор эпидермального ростового фактора, сиалилирование, лектины, голодание.

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Эпидермальды өсу факторының дифференциальды сиалилденуінде лектин-байланыстырушы сайттарды молекулалық идентификациялау

Көп клеткалы организмдерде эпидермальды өсу факторы (EGFR) рецепторларының тұқымдасы үлкен рөл атқарады. Организмде EGF-рецепторларының мутациясы және гиперэкспрессиясы әртүрлі ауруларды тудыруы мүмкін. Әртүрлі лигандтардың байланысуына жауапты клеткааралық EGFR домендері сиалилденген болып табылады. Бұл жұмыста сүт бездерінің қалыпты (HB4A) және ісік (T47D) эпителиальды клеткалары арасында ErbB1 (немесе HER1) клеткааралық домен рецепторларында сиалилдену деңгейін тексеру мақсатында лектин-метаболитті зерттеулер жүргізілді. HB4A және T47D клеткаларын сиал қышқылымен өңдеу клеткааралық

ErbB1 доменінде сиал қышқылының деңгейінің үлкен болатынын көрсетті. Ол нәтижелер вестерн-блот және иммунопреципитация әдістерімен анықталды. Онан басқа, 3 түрлі лектиннің иммунопреципитациясынан ісік клеткаларында MAL-I лектинінің (Maackia amurensis agglutinin I) күшті реакциялық қасиетке ие болатыны анықталды. Сиал қышқылымен өңделген T47D клеткаларында Neu5Acα2 → 3Gal активтілігі өседі, яғни осыған қарап MAL-I лектинді ауру және сау клеткалардағы молекулалық айырмашылықтарды анықтауға қолдануға болады деп тұжырымдауға болады.

Түйін сөздер: ісік аурулардың биомаркерлері, эпидермалды өсу фактордың рецепторы, сиалилдеу, лектиндер, аштық.

The cell surface of mammalian cells contains a variety of glycoconjugates, which are involved in modulating and mediating events such as cell-cell, cell-matrix, or cell-molecule interactions that are critical for the function of a complex organism [1]. N-glycans are covalently attached to asparagine residues within a specific consensus motif of proteins and share a common GlcNAc2Man3 core structure, which can be processed and/or elongated with additional monosaccharaides such as galactose, GlcNAc, fucose, sialic acids, and rarely by GalNAc [2]. Well-studied members of the cell-surface sugars are the N-acetyl neuraminic acids (abbreviated as Neu5Ac), also alias sialic acids (Sias), which are ninecarbon monosaccharides, bear a negative charge and are most commonly found at the non-reducing terminal positions of N- and O-glycans. These prominent positions as well as their negative charge reveal the scope and scale of their functional roles and their impact on cell disease [3] and various constituents of the sialylated glycans are recognized by a group of sugar-binding proteins called lectins [4]. The goal of the present work was to develop a novel lectin-based metabolic assay for studying glycan sialylation changes in the extracellular, ligand binding regions of EGF-receptors to be used in disease characterization. We expected that the sialic acid treatment would result in enhanced glycan sialylation alterations, which can be especially relevant in diseases such as cancer and widen diagnostic-therapeutic windows in cancer applications. We chose to study these alterations by lectin characterization to terminal **α**2,6-linked Sias (*Sambucus nigra* lectin, SNA) and Sia-**α**2,3-Gal residues (*Maackia amurensis* lectin I, MAL-I) on the cellular surface [5].

Materials and Methods

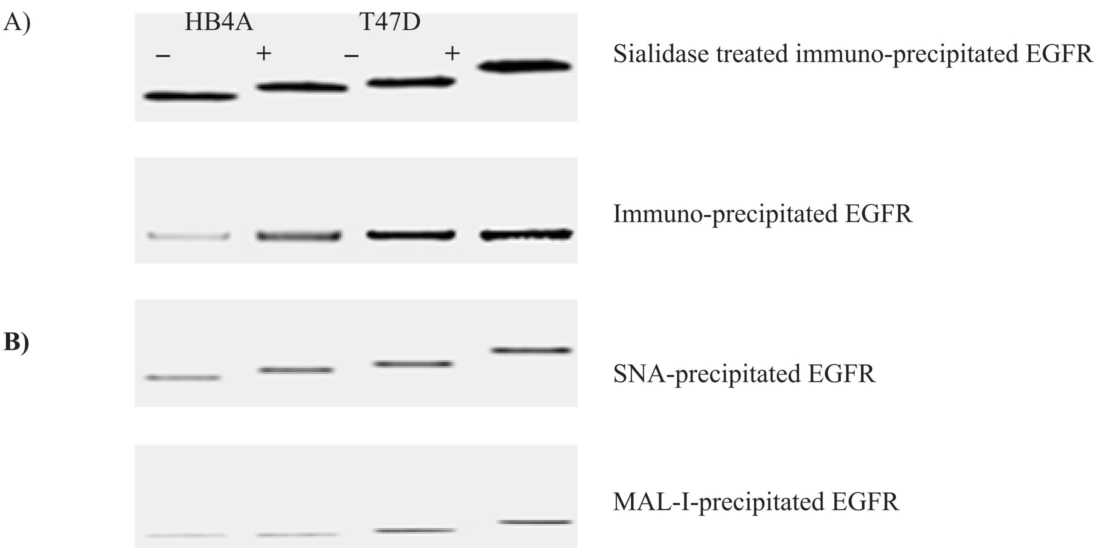
Cell lines and culture conditions. Human normal mammary epithelial cell line HB4A and breast cancer cell line T47D (American Type Culture Collection, USA) were cultured in RPMI1640 medium (without add antibiotics to avoid its inhibition to the activity of sialyltransferases), supplemented with 1% FBS (to minimize the interference degree of BSA sialylation) at 37 °C under 5% CO2. For normal cells, the medium also contained 10 μg/ml insulin and 5 μg/ml hydrocortisone. For all experiments, HB4A and T47D cells were used within the first three passages and cells were harvested by treatment with 5 ml of buffer containing 0.54 mM EDTA, 154 mM NaCl and 10 mM N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic acid (HEPES), pH 7.4 for □5 min at 37 °C.

Sialic acid metabolic assay. Cells were cultured in 175 cm2 flasks and incubated 48 h to reach mid-exponential growth phase, harvested in the indicated conditions, then resuspended in serum-free RPMI1640 medium. After washing 3 times with warm (37 °C) PBS, 20-mL of cell suspension were starved in PBS supplemented with 10 mM sialic acid for 2 hours in shake flasks in a 37 °C incubator under 5% CO_2. Control cells included only PBS. The cells were plated at a density of 3 × 105 cells mL−1. After washing, the cells were fixed in 75% ice-cold ethanol for 15 minutes and maintained at 4°C for experiments. For negative controls, cells were also treated with sialic acid in serum containing medium and harvested as described above.

Western blotting, immuno-precipitation and lectin-precipitation. Cells were lysed in Triton X-100 lysis buffer (10 mM Tris–HCl [pH 8.0], 5 mM EDTA, 320 mM sucrose, 1% Triton X-100, 1 mM PMSF, 2 mM DTT, 1 μg/mL leupeptin, 1 μg/mL aprotinin) and then incubated on ice for 15 min. Following centrifugation, the supernatant was collected and protein concentrations were determined by BCA protein assay kit (Pierce). For each sample, 50 μg total lysate was separated by SDS-PAGE and transferred onto PVDF membranes (Pierce) following standard procedures. After incubation with primary antibody specific for EGFR (Cell Signaling), the blots were incubated with corresponding secondary antibody-horseradish peroxidase (HRP) conjugate (Santa Cruz) and signals were detected by ECL system (Pierce). For immuno-precipitation, each cell extract (100 μg of total protein) was incubated with 1 μg of anti-EGFR antibody. The precipitated protein was subjected to SDS-PAGE and Western blotting followed by the detection with the corresponding antibody as described above. For precipitation of α2→3/6-sialylated glycoproteins with SNA and MAL-I, each cell extract (100 μg of total protein) was incubated with 1 μg lectin. The precipitated protein was separated on SDS-PAGE followed by Western blot detection with anti-EGFR antibody as described above. Precipitation experiments were also performed with the desialylated protein extract and similar blots were prepared. For desialylation, the cell extract was incubated with neuraminidase (100 mU/ml) for 1 h at 37 °C.

Results

Sialic acid treatment under nutrient deprivation increases sialylation of EGF-receptors. It has been reported that glycosylation regulates the function of glycoproteins by inducing conformational changes or by affecting intramolecular interactions [6]. Many membrane-bound proteins, such as receptors, are glycosylated, and it has been reported that glycosylation status is crucial for their function [7]. All the members of ErbB family are glycoproteins. For example, EGF-receptor contains 11 potential N-glycosylation sites in its extracellular domain, and glycosylation is essential for its function [8]. Sialylation is required for the proper sorting of EGF-receptor to the membrane as well as for ligand binding. To verify if EGF-receptors were over-expressed or over-sialylated after sialic acid treatment, EGF-receptors were immuno-precipitated from same amount of each cell extract followed by Western blot analyses. As shown in Figure [1a], anti-EGFR antibody immuno-precipitated increased amounts of EGFR from T47D malignant cell lysates compared to the HB4A normal cell lysate. Higher expression of these glycoproteins is more evident in the sialic acid treated T47D malignant cells. EGF-receptors from the sialic acid treated malignant cells appeared to be over-sialylated as they moved slower on SDS-PAGE compared to those from the untreated cells. Over sialylation of EGF-receptors was confirmed as sialidase treated EGF-receptors shifted down to the same level as those from the untreated normal cells.



A. Equal amount (100 μg) of each cell extract was subjected to immuno-precipitation with anti-EGFR antibody and the precipitated proteins were subjected to Western blot and immuno-detection. In parallel, equal amount of each crude protein extract was desialylated and similar precipitation was carried out.
B. Equal amount (100 μg) of immuno-precipitated proteins (Muc1 and EGFR) as described above was subjected to lectin (SNA and MAL-I) precipitation and detected on W. blot as described above. (+) Sialic acid treated cells and (-) Sialic acid untreated cells.
Figure 1 – Examination of sialylation of EGFR on HB4A normal and T47D malignant cells after sialic acid treatment under nutrient deprivation.

Sialic acid treatment under nutrient deprivation promotes differential sialylation of EGF-receptor. Equal amount of immunoprecipitated EGF-receptors were allowed to precipitate with SNA and MAL-I and subjected to Western blot followed by immunodetectection. Similar to immunoprecipitated EGF-receptors, lectin-precipitated EGF-receptors from sialic acid treated malignant cells moved slower on SDS-PAGE due to over sialylation (Fig. 1b). Moreover, over sialylation of EGF-receptors appeared to be linked via **α**2→3 and **α**2→6 as these immunoprecipitated glycoproteins were further precipitated by MAL-I and SNA.

Overall, data demonstrated that sialic acid treatment of nutrient deprived malignant cells could differentially decorate cell surface EGF-receptors with **α**2→3(6) sialylation.

Discussion

The formation of complexes between proteins and ligands is fundamental to biological processes at the molecular level. Manipulation of molecular recognition between ligands and proteins is therefore important for basic biological studies and has many biotechnological applications, including the construction of enzymes, biosensors, genetic circuits, signal transduction pathways and chiral separations. The systematic manipulation of binding sites remains a major challenge [9]. The metabolic derangements in cancer cells known arise either from the selection of cells that have adapted to the tumor microenvironment or from aberrant signaling due to oncogene activation. Accordingly, the metabolism of cancer cells differs from that of normal cells and opportunities to specifically target these differences have been accelerated in recent years. Of particular interest the epidermal growth factor receptor family which belongs to subclass I RTKs and consists of four members; EGFR (also known as ErbB1 or HER1), ErbB2 (Neu or HER2), ErbB3 (HER3) and ErbB4 (HER4). The ErbB receptor contains an extracellular ligand-binding domain, a single-pass transmembrane α-helix and an intracellular domain that encodes a tyrosine kinase followed by a regulatory region. The glycosylation plays critical role in the four ErbB family members which recognize at least 11 different but structurally related growth factors including epidermal growth factor (EGF), transforming growth factor α (TGF-α) and neuregulins (NRGs) [10]. Our approach is useful and logical when seeking to differential sialylation that is quantitative or linkage-specific as sialylation differences on tumor cells may arise from altered metabolism. Differential cell surface sialylation during cancer transformation and progression is known [11, 12] and perhaps occurs when malignant cells are deprived from nutrient. Increased sialylation on the cell surface promote cell detachment from primary tumors through charge repulsion, thereby inducing tumor proliferation and migration [13]. Previous studies have been reported that MAL-I, SNA, and WGA recognize sialic acid on the terminal branches. In particular, MAL-I detects glycans containing Neu5Ac-Gal-GlcNAc with sialic acid at the 3 position of galactose, while SNA binds preferentially to sialic acid attached to terminal galactose in an (α2→6) and an (α2→3) linkage to a lesser degree. WGA binds to almost all the isomers of sialylated glycans where its signal translates into a general level of sialylation [14]. This is consistent with the observation that EGF-receptors of both untreated cells normally show abundant α2→6 linked sialic acid, whereas terminal α2→3 linked sialic acid residues are highly restricted. More importantly, comparison of the EGF-receptors from sialic acid treated cells profiles revealed that EGF-receptors of T47D cells enforced a much broader range of (α2→3) and (α2→6) sialylated glycans compared with those in HB4A cells which expressed high level of (α2→6) sialylated glycans, and retained restrictions of (α2→3) sialylated glycans. Thus, the results of the current study suggest that Neu5Ac treatment reflect the variations in renewal of the (α2→3) sialylated glycans structures on the EGF-receptors of cancer cells rather than normal cells. To our knowledge, this is the first report showing that lectins, in particular MAL-I, can be used in a metabolic-specific manner to discriminate between normal and cancer glycans at a molecular level positioning this approach for rapid translation to clinical settings.

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