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[1,2,3]Haitham A. Badr*, [1,2]Leyla B. Djansugurova, [3]Hafiz Ahmad
[1]Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology,
Al-Farabi Kazakh National University, Kazakhstan, Almaty
[2]Laboratory of Molecular Genetics, Institute of General Genetics and Cytology, Baltimore, USA
[3]Department of Biochemistry and Molecular Biology, University of Maryland, School of Medicine
*E-mail: h.badr@kaznu.kz

A solid-phase lectin-binding assay for the characterization of glycan terminator on cell surface

Cell surface proteins in mammals are typically elaborated with a complex array of glycans. N-acetyl neuraminic acids (abbreviated as Sialic acids), are usually found at the non-reducing terminal position of these glycans. This terminal glycan sialylation imparts a negative charge at physiological pH values and mediates many biological functions. Here, we utilize two human mammary epithelial cell lines, MCF10A (breast normal cells) and MCF7 (breast cancer cells) as a model system to show differential glycan terminator when treated with sialic acid under nutrient deprivation. Under starved condition, sialic acid treatment of both cells resulted increased activities of α2→3/6 sialyltransferases as demonstrated by lectin solid phase assay. The presence of increased sialyltransferase expression is corroborated by stronger binding with sialic acid-specific lectins such as (Sambucus nigra agglutinin, SNA) and (Maackia amurensis agglutinin I, MAL-I). However, MAL-I binding discriminates malignant cells from normal cells suggesting a preferential increase of Neu5Acα2→3Gal on the SA-treated malignant cell surface.

Keywords: cancer biomarkers, sialyltransferases, glycan terminator, lectins, starvation.

Бадр Хайсам А., Джансугурова Лейла Б., Хафиз Ахмед

Твердофазный анализ связывания лектинов для характеристики терминальных гликанов на клеточной поверхности

У млекопитающих белки клеточной поверхности, как правило, содержат массивные комплексы гликанов. N-ацетил нейраминовые кислоты (сиаловые кислоты) обычно находятся на концевых позициях гликанов. Сиалилирование гликанов придает клеточной поверхности отрицательный заряд при физиологических значениях pH и опосредует многие биологические функции. В данной работе мы используем 2 культуры клеток, представляющие нормальные (MCF10A) и раковые (MCF7) эпителиальные клетки молочной железы человека, в качестве модельной системы для демонстрации различий по сиалилированию концевых позиций гликанов после обработки клеток сиаловой кислотой в условиях голода. Такая обработка привела к увеличению активности α2→3/6 сиалилтрансфераз, что показано анализом лектинов в твердой фазе. Усиление сиалилтрансферазной активности подтверждено повышением уровня связывания с клеточной поверхностью специфичных к сиаловой кислоте лектинов, таких, как Sambucus nigra agglutinin (SNA) и Maackia amurensis agglutinin I (MAL-I). Отмечено, что связывание обработанных сиаловой кислотой клеток с MAL-I сильнее увеличивает различия между нормальными и раковыми клетками благодаря предпочтительному усилению активности сиалилтрансфераз Neu5Acα2 → 3Gal на поверхности злокачественных клеток.

Ключевые слова: биомаркеры рака, сиалинтрансферазы, гликановый терминатор, лектины, голодание.

Бадр Хайсам А., Джансугурова Лейла Б., Хафиз Ахмед

Клетка бетіндегі терминальды гликандарды сипаттау үшін лектиндерді қаттыфазада талдау

Сүтқоректілерде клетка үсті белоктарының құрамында гликандар комплексі өте көп болады. N-ацетил нейрамин қышқылы (сиал қышқылы) негізінен гликандардың соңғы жағдайында тұрады. Сиалилденген гликандар клетка үстіне теріс заряд береді және көптеген биологиялық процестерге қатысады. Бұл жұмыста сүт бездерінің қалыпты (MCF10A) және ісік (MCF7) эпителиальды клеткаларында гликандардың сиалилденуін сиал қышқылымен өңдеуден кейінгі аштық жағдайында қарастырылды. Мұндай өңдеу α2→3/6 сиалилтрансфераза ферментінің активтілігінің өсуіне әкелді. Сиалилтрансфераза ферментінің активтілігінің өсуі сиал қышқылына сезімтал клетка үсті лектиндерінің, оның ішінде Sambucus nigra agglutinin (SNA) және Maackia amurensis agglutinin I (MAL-I) дәлелденді. MAL-I агглютининін сиал қышқылымен өңдегенде қалыпты және

ісік клеткаларында айтарлықтай айырмашылық пайда болды. Яғни ол ісік клеткаларының үстінде Neu5Acα2 → 3Gal сиалилтрансфераза ферментінің активтілігі жоғары болуына байланысты деп қорытуға болады.

Түйін сөздер: ісік аурулардың биомаркерлері, сиалинтрансферазалар, гликанды терминатор, лектиндер, аштық.

It has long been known that cancer cells often express more heavily sialylated glycans on their surface and that this feature sometimes correlates with invasion. It is now well established that specific sialylated structures, such as the Thomsen-Friedenreich-related antigens, the sialyl Lewis antigens, the sialyl alpha2-6 lactosaminyl structure, the polysialic acid or some gangliosides, can mediate cellular interactions and are altered in cancer cells [1]. Changes in glycan terminator, including increases in level of sialylation and changes to the sialic acid modifications have been identified in cancer cells [2]. The increase in sialylation in cancer may be due to overexpression of sialyltransferases [3]. Studies showed that sialylation changes resulted from deregulation in sialyltransferases enzymatic activity involved in their biosynthesis, but the precise molecular mechanisms remain unknown [4]. Usually, sialic acid residues are linked to the inner sugar residue galactose (Gal) via α2,6 or α2,3-linkage or linked to galactosamine or Nacetylgalactosamine (GalNAc) via α2,6-linkage. Altered sialylation is very common and important in cancers, including cancer transformation and cancer metastasis. More understandings of the role in glycosylation, especially sialylation, for cancers will offer a new vision in managing cancers in the near future [5]. The goal of the present work was to develop a lectin solid phase assay for studying glycan terminator on the cell membrane as reliable biomarkers in women with breast cancer. We expected that the sialic acid treatment would result in enhanced glycan sialylation, which can be attractive targets in the search for novel cancer biomarkers. We used the following plant lectins and their specificity for particular sugars for the characterization of the glycan terminator: *Sambucus nigra* agglutinin (SNA) binding **α**2,6-Sia residues; and *Maackia amurensis* agglutinin I (MAL-I) binding to Sias linked to galactose (Gal) with an **α**2,3-linkage [6].

Materials and Methods

Cell lines and culture conditions. Human normal mammary epithelial cell line MCF10A and breast cancer cell line MCF7 (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, MCF10A and MCF7 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.

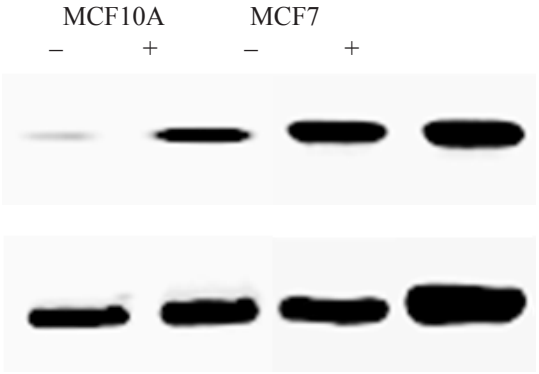
Detection of sialyltransferase activity. The activity of α2→3-sialyltransferase (α2→3-ST) and α2→6-sialyltransferase (α2→6-ST) to galactose was determined by solid phase assay using asialofetuin precoated plates as previously described [7]. Briefly, various cell lysates containing equal amount of protein were taken into the wells and CMP-Neu5Ac was then added to initiate the reaction. After washing and blocking, the sialylated fetuin was allowed to interact with either biotinylated lectin (MAL-I or SNA) followed by binding with streptavidin-horseradish peroxidase. The negative control includes only lectin binding to asialofetuin. After binding and washing, the reaction was developed with 100 μl of substrate (0.03% H2O2, 2 mg/ml o-phenylenediamine in 0.1 mM citrate buffer, pH 5.5) for ~10 min and terminated with 1 M H2SO4. The absorbance at 492 nm was measured using an automatic multi-well spectrophotometer (Bio-Rad, USA).

Western blot of sialyltransferase. 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 antibodies [specific for ST3Gal-I (Abnova), ST6Gal-I (Abnova), and β-actin (Santa Cruz)], the blots were incubated with corresponding secondary antibody-horseradish peroxidase (HRP) conjugate (Santa Cruz) and signals were detected by ECL system (Pierce).

Statistical analysis. The statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparisons (Graphpad Instat, version 3). The differences were considered significant when $p<0.05$.

Results

Sialic acid treatment under nutrient deprivation increases sialyltransferase expression. Aberrant glycosylation is a common feature of malignant change. Overexpression of sialylated antigens, including sialyl-Tn, sialyl-T, sialyl-Le^a, and sialyl-Le^x, at the surface of cancer cells has been widely reported [8]. This has been shown, in part, to be due to changes in the expression of glycosyltransferases, including the up-regulation of some sialyltransferases (STs) [9]. Sialyltransferases can be classified into four families according to the glycan linkage they synthesize: the ST3Gal (α2,3-ST), ST6Gal (α2,6-ST), ST6GalNAc, and ST8Sia (α2,8-ST) families [10]. To verify if sialyltransferases were over-expressed after sialic acid treatment, sialyltransferases were immuno-precipitated from same amount of MCF10A and MCF7 cell extract followed by Western blot analyses.



Equal amount (100 μg) of each cell extract was subjected to immuno-precipitation with anti-ST3Gal-I and anti-ST6Gal-I antibodies and the precipitated proteins were subjected to Western blot and immuno-detection.

(+) Sialic acid treated cells and (-) Sialic acid untreated cells.

Figure 1 – Investigation of sialyltransferase on MCF10A normal and MCF7 malignant cells after sialic acid treatment under nutrient deprivation.

As shown in (Fig. 1), anti-sialyltransferases antibodies immuno-precipitated increased amounts of ST3Gal-I (α2→3-ST) and ST6Gal-I (α2→6-ST) from MCF7 malignant cell lysates compared to the MCF10A normal cell lysates. Higher expression of these sialyltransferases is more evident in the sialic acid treated MCF7 malignant cells. Sialyltransferases from the sialic acid treated malignant cells appeared to be over-expressed as they moved slower on SDS-PAGE compared to those from the untreated cells.

Sialic acid treatment under nutrient deprivation promotes sialyltransferase activity and glycan terminator. To further investigate if sialic acid treatment under nutrient deprived condition influences sialyltransferase activity and subsequently glycan terminator, cell extract was incubated with asialofetuin (acts as acceptor) and the resultant sialo-fetuin was allowed to bind with linkage specific

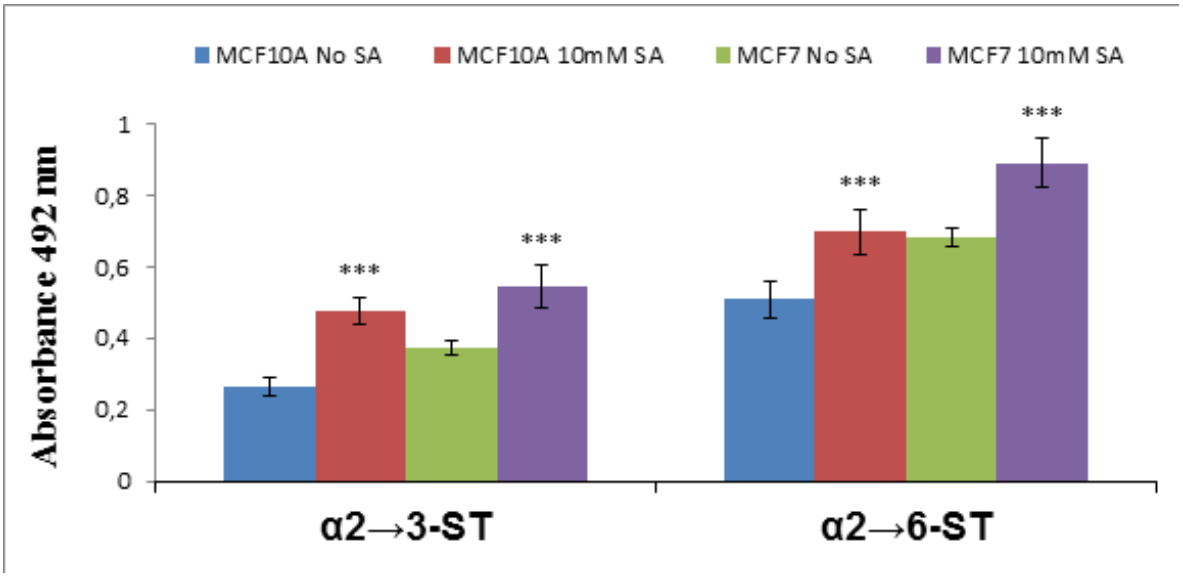


Figure 2 – Detection of ST3Gal-I and ST6Gal-I activities.

lectins as described in the experimental procedures. Both α2→3-ST and α2→6-ST activities in all cells tested were increased by 29-79% (Fig. 2).

Cells were lysed and the same amounts of proteins were used on solid phase assays. The α-2,3 and α-2,6 sialylated glycans of resultant fetuin was detected with biotinylated MAL-I and biotinylated SNA, respectively. The data are representative of four independent experiments with S.D. indicated by error bars. *P* values are determined by two-tailed student *t*-test and □0.05, <0.01 and <0.001 are indicated by one, two and three asterisks, respectively.

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

Discussion

The oligosaccharide chains of glycoproteins and glycolipids are often decorated by sialic acids, a family of nine carbon sugars derived from neuraminic acid. In humans, sialylation of glycoconjugates is mediated by different sialyltransferase enzymes which, depending on their nature, may establish different types of linkages [linkage through an α2-3- or an α2-6-bond to galactose (Gal); through an α2-6-bond to N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc); or through an α2-8-bond to another sialic acid, forming polysialic acids [11]. During neoplastic transformation, the activity of sialyltransferases may be altered and, as a consequence, cancer cells express more heavily sialylated glycans at the surface [12]. This aberrant sialylation may mediate key pathophysiological events during the various steps of tumor progression, including invasion and metastasis formation. This is due to the fact that sialylated structures can prevent cell-cell interactions through non-specific charge repulsion, but they can be specifically bound by cell adhesion molecules, such as selectins [13]. On the other hand, the addition of sialic acids may mask the underlying sugar structure, thus avoiding recognition by other specific glycan binding molecules, such as galectins [14]. The metabolism of cancer cells differs from that of normal cells and opportunities to specifically target these differences have been accelerated in recent years [15]. However, there is no early sensing method that can be ubiquitously applied to detect glycan sialylation in diverse clinical samples with high selectivity and sensitivity [16] 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 [17]. Previous studies have been reported that MAL-I and SNA 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 [18]. This is consistent with the observation that sialyltransferases 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 sialyltransferases from sialic acid treated cells profiles revealed that sialyltransferases of MCF7 cells enforced a much broader range of (α2→3) and (α2→6) sialylated glycans compared with those in MCF10A 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 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 glycan terminator positioning this approach for rapid translation to clinical settings.

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