RESEARCH ARTICLE

Metabolic Labeling of Malignant Breast Cells Resistance to Chemotherapy with N-Glycolylneuraminic Acid Allows Differential Surface Detection

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Abstract

The ultimate goal of cancer science is to learn how to select a cancer cell surface. Glycans in cancer cells are frequently at varying quantities or have fundamentally various structures than those seen in normal cells. The presence of sialic acid (Neu5Ac), which is prevalent at the end of glycan chains, is significant. N-glycolylneuraminic acid (Neu5Gc) is a “non-human” sugar type that intercept into Neu5Ac natural biosynthetic machinery, presaging as a perspective biomarker. Malignant breast cells resistance to chemotherapy and their non-malignant counterparts, when treated with the Neu5Gc under nutrient deprivation, display cell surface tumor-associated Neu5Gc-rich glycans, which is capture with Neu5Gc linkage specific plant lectins such as Triticum vulgaris agglutinin (WGA), Sambucus nigra agglutinin (SNA), and Maackia amurensis agglutinin I (MAL-I). Nevertheless, MAL-I binding differentiates surface of malignant breast cell line resistance to chemotherapy MCF-7/H compared to normal mammary epithelial cell line MCF-10/A. These findings emphasize the importance of one oxygen atom in Neu5Gc; it’s supplementing of nutrient-depleted cancer cells’ resistance to chemotherapy and facilitates the way for the implementation of better diagnostic and prognostic approaches.

Keywords: oxygen atom, neuraminic acids, glycan metabolism, plant lectins, nutrient deprivation.

Introduction

Avian haemoparasitic infections are known to cause pathogenic effects on their hosts resulting in anaemia, emaciation, retardation of growth, reproductive failure, reduced productivity, high mortalities, and may exert negative effects on behavior and community structure [1-3]. More than 200 species of avian haemoparasites have been reported worldwide, with Aegyptinella species, Eperythrozoon spp., Haemobartonella spp., Haemoproteus spp., Leucocytozoon spp., Plasmodium spp., and Trypanosoma spp. described as the most common [4-6]. Parasitic infections of avian species vary in their degree of host specificity, and a broad host range might even increase the general transmission rate resulting in a high prevalence in several host species [7]. Most of the avian haemoparasites are closely related in their life cycle as they require mosquitoes, midges, Simulium, Culicoides, or hippoboscid flies as their vectors [3].

Avian species refers basically to domestic birds such as chickens (including broilers, cockerels, indigenous chickens, and layers), ducks, guineafowls, peasants, pigeons, turkeys, and more recently ostriches that are...
kept for meat or egg production [8, 9]. Poultry production contributes meaningfully to the socio-economic development of many developing countries of the world including Nigeria [10]. Poultry is an important component of the livestock subsector in Nigeria, and it has developed to the level of a commercial enterprise involving thousands of birds which provides income, employment, and animal protein for urban and rural dwellers as well as manure for crop production [9, 11]. The population of poultry in Nigeria is estimated to be about 160 million; with chickens comprising about 72.4 million [8, 12]. Poultry is one of the most accepted major sources of animal protein for humans [4]. Most poultry small flocks are kept in a free-range system, thus exposing them to so many parasitic infections [4, 13].

The investigation of avian haemoparasites may be done using microscopic identification of the parasites in blood smears [14], as well as through the use of molecular diagnostic techniques (amplification and sequencing of DNA) [15, 16]. Some studies have shown that both methods can have similar sensitivities for the detection of avian haemoparasites [15, 17].

The aim of this study is to determine the diversity, prevalence, and risk factors associated with the occurrence of haemoparasitic infections in chickens and guineafowls in Kwara Central, Nigeria.

Material and methods

Materials

Santa Cruz Biotechnology provided N-glycolylneuraminic acid (Neu5Gc; sc-202234-10MG) (USA). Sigma-Aldrich provided the ribonuclease-A (RNase A; R5125-100MG) (USA). Life Technologies Corporation provided the Roswell Park Memorial Institute 1640 Medium (RPMI1640 culture medium; 11875093-500mL), fetal bovine serum (FBS; 10082147-500mL), N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES; 15630049-20mL), and carbocyanine monomer nucleic acid stain with far-red fluorescence (TO-PRO™-3 stain; T3605-1mL) (USA). Invitrogen provided phosphate-buffered saline (PBS, pH 7.4; 10010002-500mL) (USA). Vector Laboratories provided fluorescein lectins conjugates of Maackia amurensis agglutinin I (MAL-I; FL-1311-10MG particular to Neu5Gc2,3Gal), Sambucus nigra agglutinin (SNA; FL-1301-10MG particular to Neu5Gc2,6Gal), and Triticum vulgaris agglutinin (WGA; FL-1021-10MG particular to Neu5Gc and GlcNAc) [23], (USA). All additional compounds were bought in analytical grade quality from Sigma-Aldrich.

Cell lines and culture conditions

MCF-10/A, a human normal mammary epithelial cell line, and MCF-7/H, a chemotherapy-resistant breast cancer cell line, were a generous gift from the lab of Prof. Chen-Zhong Li (Miami, USA) and cultivated in 175 cm² flasks in RPMI1640 culture medium (no antibiotics given to prevent sialyltransferase inhibition), enriched with 1% FBS (to reduce BSA glycan-sialylation interference), at 37 °C under 5% CO₂ [24, 25]. The media for MCF-10/A cells also included 10 g/ml insulin and 5 g/ml hydrocortisone. In all studies, MCF-10/A and MCF-7/H cells were used within the first three passages, cultured for 73 hours to reach mid-exponential growth phase, and gathered by incubating for 5 minutes at 37 °C in a solution containing 0.54 mM EDTA, 154 mM NaCl, and 10 mM N-2-Hydroxy-Ethyl-Piperazine-N-2-Ethane Sulfonic Acid (HEPES), pH 7.4 for < 5 min at 37 °C.

Nutrient deprivation and Neu5Gc treatment

Cells were split and cultivated in RPMI1640 culture media enriched with 1 percent heat-inactivated human-serum (RPMI/HUS) rather than FBS prior to nutrient deprivation tests, culminating with the existing chasing-out Sia5Gc [26]. Following that, cells were either fed 10 mM Neu5Gc in RPMI/HUS medium or starved as described previously [27], briefly: Protocol for nutrient deprivation of cells in suspension: Cells were cultivated in Step 1 according to Section 2.2. Stage 2: cells were resuspended in RPMI/HUS medium and pelleted for 5 minutes by centrifugation at 1000 x g; cells were deprived of nutrients for...
15 minutes in serum-free media lacking Neu5Gc during this step. Stage 3: After centrifuging the cells two times in 37 °C PBS for 5-min, 1 mL aliquots of 10^5 cells mL\(^{-1}\) were pipetted slowly into 20 mL BD-Falcon tubes; the cells' metabolism was further nutrient-deprived for an extra 30-min throughout this step. Step 4: Equilibrate 10^5 cell mL\(^{-1}\) cell suspension aliquots in Neu5Gc-PBS buffer tubes in a humidified incubator at 37°C and 5% CO\(_2\) with constant shaking at 30 rmps for 45 minutes; negative controls were kept in non-enriched PBS during this Neu5Gc-supplementation step. Step 5: In the absence of supplemental Neu5Gc, the Neu5Gc-PBS solution was decanted, and the BD-Falcon tubes were gently tapped to loosen the gravity-pelleted cells before being washed twice in warm (37 °C) PBS and pelleted by 5 minutes centrifugation each time; this process added an adequate 30 minutes of nutrient-deprivation metabolism. With the exception of lectin staining, which used nutrient-deprived cells under adherent circumstances, the preceding 5-step technique resulted in 2 hours of nutrition deprivation metabolism. With the exception of lectin staining, which used nutrient-deprived cells under adherent circumstances, the preceding 5-step technique resulted in 2 hours of nutrition deprivation, after which the cells were investigated using the techniques listed below.

**Cell viability assay**

Without fixation, cells were collected as stated above. Cell pellets were resuspended in PBS consisting of 1 mg/ml propidium iodide and incubated for 5 minutes at room temperature. Flow cytometry was used to examine the cells using a CyAnTM ADP flow cytometer (Beckman Coulter Inc., USA). The proportion of propidium iodide stained cells was used to calculate the number of dead cells.

**Flow cytometric analysis**

Ethanol-fixed cells were cultured on 96-well plates (1*10^4 cells per well) after being rinsed twice with cold PBS. The fluorescein isothiocyanate (FITC)-labeled lectins were subsequently used to stain the plated cells (MAL-I, SNA, and WGA). The equipment parameters for fluorescence and compensation were the same for all studies when comparing mean fluorescence peaks intensity. For each specimen, data was obtained from at minimum 10,000 cells.

**Lectin staining and cell imaging**

Cells were cultured on a cover-slip surface, and attached cells were fixed in 70% ice-cold ethanol for 15 minutes. Cells were stained with FITC-labeled lectins (MAL-I, SNA, and WGA, 5-g/mL) for 1 hour after being washed with PBS. After staining, cells were medicated with Ribonuclease-A (10 g/mL) and TO-PRO-3 was used to counterstain the nuclei.

**Statistical analysis**

The independent t-test (SPSS version 19) was used to compare the means of the investigated parameters between the experimental groups. The differences were considered significant when p<0.05.

**Results and discussion**

**Neu5Gc treatment does not affect the cell viability**

The propidium iodide (PI) flow cytometric test has been widely used to evaluate apoptosis in several experimental approaches since its introduction. It is predicated on the notion that apoptotic cells are differentiated by DNA fragmentation and, as a consequence, reduction of nuclear DNA content, among other things. The use of a fluorochrome has the ability to bind and label DNA, such as PI, enables for a rapid and exact measurement of cell viability using flow cytometric analysis [28]. The proportion of viable cells for untreated MCF-10/A and MCF-7/H cells (PBS control) were 92.45 percent ±1.9 and 95.27 percent ±2.8, respectively, utilizing a constant count of cells (3*10^5 cells mL\(^{-1}\)). The cell
viability of Neu5Gc-treated MCF-10/A and MCF-7/H cells was 94.16 percent ±1.7 and 96.05 percent ±2.1, consecutively (Fig. 1 A and B), indicating that exposure to Neu5Gc at the given conditions had no effect on cell viability. **Sia5Gc treatment promotes differential expression of cell surface sialo-glycoconjugates**

To investigate if Neu5Gc medication could influence display of cell surface glycans, particularly sialoglycans, flow cytometry using Neu5Ac linkage specific lectins (MAL-I, SNA, and WGA) was performed. As shown in Figure 2, the shared profile of untreated cells indicated an increase in the binding of SNA and WGA lectins. In contrast, the MAL-I lectin showed weak binding to the surface of untreated cells. Conspicuously, the Neu5Ac treated cells responded similarly but with a marked increase in the binding of SNA and WGA lectins whereas, to our surprise, the MAL-I force elevated binding to MCF-7/H cells but not for MCF-10/A cells (Fig. 2 A and B). We next conducted lectin-mediated staining accompanied by fluorescence microscopy evaluation. As shown in Figure 3, SNA and WGA lectins showed consistently strong fluorescence signals that demonstrated the more Neu5Gc-mediated sialoglycans' accumulation in the metabolic state of treated MCF-10/A and MCF-7/H cells contrasted with those of untreated MCF-10/A and MCF-7/H cells. Lower staining patterns were noted with MAL-I lectin on the surface of untreated MCF-10/A and MCF-7/H cells whereas, the treated MCF-10/A and MCF-7/H cells indicated conversely elevated levels of fluorescence (Fig. 3). Although the examined lectins are distributed in the Golgi region of normal cells, the malignant cells show diffuse cytoplasm or diffuse cytoplasm/membrane distribution (Fig. 3). The high molecular mass of the MAL-I and SNA lectins renders a passive entrance unlikely, and fluorescence microscopy accepted a membrane-associated fluorescence. As a result, an increase in Neu5Gc-mediated sialoglycans on the outer leaflet of cellular membranes can be ascribed to an increase in SNA and MAL-I staining [29]. Because sialyltransferases associated with the biosynthesis of Neu5Ac(s) have been proven to be effective in the creation of Neu5Gc in mammals, involving humans, Neu5Gc may theoretically exist in any sialoglycans-producing mammalian cell if Neu5Gc is present. However, glycan-bound Neu5Gc is rarely noted in normal mammalian cells due to the extremely low effect of CMP-Neu5Ac-Synthetase catalyzed formation of CMP-Neu5Gc, although a minute amount of Neu5Ac-rich glycan was detected during bioactive synthesis of some types of sialoglycoproteins in normal cells when the free Neu5Gc level in the cell.

Thus, the presence of Neu5Gc in human cells and/or bodily fluid could indicate a problem with Neu5Gc metabolism. Other research studies have been noted that MAL-I, SNA, and WGA diagnose Neu5Gc-sialylated glycans kinds on the terminal branches [30-33]. MAL-I, in particular, identifies glycans composed of Neu5Gc-Gal-GlcNAc with Neu5Ac at the 3 position of galactose, whereas SNA attaches preferentially to Neu5Gc-sialylated glycans bonded to terminal galactose in a (α2→6) and (α2→3), to a lesser extent a linkage. WGA adheres to almost all Neu5Gc-sialylated glycan isomers, in which its signal translates into a general degree of glycan-sialylation. This is in line with the results that both untreated cells have an abundance of α2→6 connected Neu5Gc-sialylated glycans, but terminal α2→3 linked Neu5Gc residues are extremely restricted. More crucially, a contrasting of the treated cell profiles showed that MCF-7/H cells imposed a much wider variety of (α2→3) and (α2→6) Neu5Gc-containing sialylated-glycans than MCF-10/A cells, which demonstrated an extreme level of (α2→6) Neu5Gc-containing sialylated-glycans while retaining restrictions of (α2→3) Neu5Gc-mediated sialylated-glycans. Thus, the current findings imply that Neu5Gc medication reflects differences in new-synthesis of the (α2→3) Neu5Gc-containing sialylated-glycan structures on the surface of cancer cells resistant to chemotherapy rather than normal cells.
Figure 1: Exclusion of propidium iodide was used to determine MCF-10/A and MCF-7/H cells viability. On ice for 20 minutes, cells were stained with 1 mg/ml propidium iodide (PI) and examined by flow cytometry in the PE-Texas Red setup. The fraction of PI stained cells was utilized to calculate the number of dead cells. The living and dead cells for MCF-10/A and MCF-7/H cells are displayed in cytograms in the exitance or lack of Neu5Gc (A), and the live cells are depicted in a bar diagram (B). The data are from three different tests, with standard deviation indicated by error bars.
Figure 2: Lectin binding analysis of MCF-10/A and MCF-7/H cells using flow cytometry. Flow cytometry was utilized to record the fluorescence attributed to FITC-lectin binding to -2,6 and -2,3 sialylated surface moieties in the exitance or lack of 10 mM Neu5Gc, and the fluorescence attributed to FITC-lectin binding to α-2,6 and α-2,3 sialylated surface moieties was measured using bivariate sideward scattering (SS Lin) vs. FITC log variables. The data comes from three separate experiments, and the error bars express the standard deviation. p value <0.001 is indicated by three asterisks.
Because of their flexibility, glycan structures that differ in their asialotopes have substantial topographic features in common and, as a result of this similarity, will bind to the same lectin molecule [34]. On the other hand, different types of lectins specific for the same asialotopes structure may recognize different binding regions of its surface [35]. To date, there are no antibodies have been identified to bind structural sialylated-glycan, such as α2,3– and α2,6–linked terminal sialic acid residues, whereas lectins have this specificity [36]. To the best of our knowledge, this is the first report demonstrating that Neu5Ac-specific plant lectins, specifically MAL-I, can be utilized in a metabolic-specific manner to distinguish between normal and cancer-bearing Neu5Gc-containing sialylated-glycans, paving the way for rapid translation to clinical surroundings. When compared to Neu5Ac, Neu5Gc biomolecule amplifies the critical influence (by adding a single oxygen atom) in determining the activity of many diverse sialoglycans because all transporters and enzymes that operate on Neu5Ac can also operate on Neu5Gc. Given how naturally Neu5Ac chemical engineering occurs via these enzymatic approaches, Neu5Gc differential cell surface glycan expression may seem to be a promising approach at first, as the approach is focused on controlling the influx rate or modifying the Neu5Gc synthesis enzymes.

![Figure 3: Confocal imaging of MCF-10/A and MCF-7/H cells after lectin-mediated staining. Cells were treated for 2 hours with 10 mM Neu5Gc in the existence or lack of FITC-labeled lectins (WGA, SNA, and MAL-I) at a concentration of 5 µg/ml and stained for 1 hour with FITC-labeled lectins (WGA, SNA, and MAL-I) at a concentration of 5 µg/ml during food deprivation (green fluorescence). The nuclei were counter-stained with TO-PRO-3 after the cells were medicated with 50 µg/ml ribonuclease A. (blue fluorescence). The images have been ten times magnified.](image-url)
Therefore, insight gained from the study of “nonhuman” Neu5Gc nutrient-deprived environment, utilizing breast normal and BC cells as a model system, can offer a proof-of-concept endeavors to unravel novel glycobiology biomarkers. In future, this biomarker can be used as a tool for both diagnostic and therapeutic, so called ‘theranostic’. Ultimately, our results will pave the way for metabolic engineering methods to decorate cell surfaces with specific glycan epitopes, which is crucial for the implementation and optimization of BC diagnostic products.

**Conclusion**

The major focus of this study is that differential uptake of exogenous Neu5Gc could be used as a diagnostic test to identify BC cells resistance to chemotherapy. The evidence presented shows some differences between BC normal and malignant cells, but the additional studies required to demonstrate that this work could have predictive power for breast cancer prognosis.

**Conflict of Interest**

GlycoMantra, Inc. of the United States filed a non-provisional complete patent on August 16, 2016, with a priority date of August 17, 2015, entitled "Exploiting nutrient deprivation for modification of glycosylation in research, diagnostic, therapeutic, and biotechnological applications." One of the three inventors named was Haitham A. Badr. There are no other financial interests that are competing with this one.

**References**


المخصص العربي

خلايا الأورام السرطانية المقاومة للعلاج الكيميائي عند المعالجة بحمض جليكوسَّيَاليك تشكل زياده في المتمزجة الأيضية مقتارنات سكرية تثير ينجم خارقة من أسطح الخلايا بالفترة كدالة حيوية. دينا م. الصادق, هيثم ع. بدر, حفيظ أحمد 1, ديانا م.م. الصادق بقسم الأنسجة والخلايا- كلية الطب البيطري- جامعتي الزقازيق. المصرية.3

الهدف النهائي من البحث في علم السرطان هو تعلم كيف يتم تمييز سطح الخلية السرطانية. تتم عملية تقدم السرطان على خطوات عديدة منها ما يقوم على الإرتباط بأنواع محددة من المقتتات السكرية وهو ما يدير مفتاحاً يسمح السرطان بالنفور من الخلية السليمة بواسطة أبواب تكتسب وحدات حمض السَّيَال "أبواب" الكليّة السرطانية. تكتسب وحدات حمض جليكوسَّيَاليك لخلايا الأورام السرطانية و"باب شكلت خارقة من أسطح الخلايا السرطانية. وتعبر أكثر تحديداً عن أنواع السكرات الموجودة في الشبكة "غير شائعة في الخلايا البشرية" لحمض جليكوسَّيَاليك تعزى وسيلة الخلايا السرطانية. في هذا البحث تم استخدام نوعين من الخلايا السرطانية مكرر معالفة مع حمض جليكوسَّيَاليك (MCF-10A) وخلايا السرطانية المقاومة لعلاج الكيميائي (MCF-7/H).3

１- شركة جليكومانتران- ميرلاند- الولايات المتحدة 3

2- قسم الكيمياء الحيوية- كلية الزراعة- جامعتي الزقازيق. المصرية

محتويات المقتتات السكرية على أسطح هذه الخلايا عند المعالجة بحمض جليكوسَّيَاليك تحت ظروف خاصة من الحرمان من التغذية. وجد زيادة في نشاط الإنزيمات التي تتحكم في تخلخل jaws الأنسجة الحاملة لخلايا السرطانية محض جليكوسَّيَاليك أو تفكيكها عند ظروف الحرمان من التغذية. تعد مقتتات السكرية في كل النوعين من الخلايا وذلك طبقاً لنتائج التجربة تجارب الاتصال باللكتوبات النباتية مثل (WGA, SNA, MAL-I). أظهرت نتائج التجربة في خلايا السرطانية (MCF-10/AlA) وخلايا السرطانية مكرر مائية بحمض جليكوسَّيَاليك (MCF-7/H) وضعياً بنحو احترافي خلايا السرطانية، والخلايا السرطانية المكررة. 1, 3

مثلاً، تظل علاج معنوي جليكوسَّيَاليك من نوع (2) و (3). يعد هذا أول تقدير ينطبخ في استخدام انواع المقتتات في الأطوار النباتية في الأستهداف الانتقائي للخلايا السرطانية. هذه الطرق تتيح للاختيار في الأطوار السكرية مدة نهائية بصورة حيدوية في النكنين المبكر للسرطان ولتحسين طرق التشخيص.