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Cancer-Type Expression of Tn Epitopes and LacdiNAc Structures: Human Cancer Cells Exhibit Distinctly Varying Levels of Heterogenous Ser/Thr Bearing Polypeptides, a Neutral β Galactosidase Converting T-Hapten to Tn, Ser/Thr: αGalNAc- and GlcNAc: β1-3/β1-4 GalNAc Transferase Activities

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Abstract

Terminal sugar-alteration in carbohydrate chains such as GalNAc replacing Gal in prostate and pancreatic cancers and Gal3-O-sulfation in breast, colon and gastric tumors could play a crucial role in cancer pathogenesis. We found the activities of cancer cell GalNAc transferases (GalNAc-Ts) as 0%, 0% <20%, 20-50% and 20-120% respectively towards Galβ1-3GalNAcα-O-Bn, 4-FGlcNAcβ1-6 (Galβ1-3)GalNAcα-O-Bn, LacNAcβ-O-Bn, GlcNAcβ1-4 GlcNAcβ-O-Bn and GlcNAcβ1-6GalNAcα-O-Bn as compared to GlcNAcβ-O-Bn. α-[6-^{3}H]GalNAc-ylated endogenous cancer cells Ser/Thr polypeptides by the corresponding cancer cell αGalNAc-T were at variable levels, heterogenous, and exhibited complete binding to VVL-agarose and non-binding to WGA-, WFL- and ConA- agarose. PNA-agarose binding and non-binding radioactive products from [6-^{3}H] GalNAc-ylated exogenous acceptor GlcNAcβ1-6(Galβ1-3) GalNAcα-O-Al indicated cancer type variable β1-3Galactosidase activities at neutral pH. TLC analysis identified two radioactive products by confirming PNA-agarose data. WGA-agarose tight binding and VVL-agarose weak binding respectively of the products [6-^{3}H] GalNAcβ1-4 and β1-3GlcNAcβ-O-Bn isolated from the exogenous acceptor GlcNAc-β-O-Bn by Sep-Pak C18 method were used to quantitate β1-4 and β1- 3GalNAc-T activities in cancer cells. DU4475, MDA-MB-435S, PA-1, LNCaP, PC3, DU145, EG7 and GL261-OVA over-expressed β1-3GalNAc-T activity. Tumorigenic MDA-MB-435/LLC6 as compared to non-tumorigenic MDA-MB-435S contained ~2-fold each of αGalNAc-T and β1-4GalNAc-T. The breast cancer DU4475 uniquely expressed 10-fold β1-3GalNAc-T with respect to β1-4GalNAc-T. HPLC identified negligible β1-6GalNAc-T in cancer cells and high-level β1-3GalNAc-T in pancreatic and gastric tumors. It is known that Tn epitopes correlate with cancer progression and metastasis and β-galactosidase is a senescence-biomarker and molecular-target for ovarian cancer. It is apparent that βGalNAc-T, Tn polypeptides, αGalNAc-T and neutral β1-3galactosidase could play a crucial role in cancer pathogenesis.

Keywords: Cancer cell O-glycans; Tn polypeptides; T-hydrolyzing neutral β1-3 galactosidase; αGalNAc- and β1-3/1-4GalNAc transferases; Lectin-agarose; HPLC and TLC

Abbreviations

AL: Allyl; Bn: Benzyl; BSA: Bovine Serum Albumin; ConA: Concavalin A; GalNAc-T: GalNAc Transferase; HPLC: High Performance Lipid Chromatography; NEU: Sialidase; PNA: Peanut Agglutinin; RM: Reaction Mixture; SA-β Gal: Senescence-Associated β Galactosidase; ST: Sialyltransferase; TLC: Thin Layer Chromatography; Tn GalNAcα-O-Ser/Thr: T Galβ1-3 GalNAcα-O-Ser/Thr Type-I LDN GalNAcβ1-3GlcNAc (LacdiNAc) Type-II LDN GalNAcβ1-4GlcNAc (LacdiNAc); VVL: Vicia Villosa Lectin; WFL: Wisteria Floribunda Lectin; WGA: Wheat Germ Agglutinin

Introduction

The glycoproteins containing complex glycan structures serve as the communication interface between cells and intracellular environment [1]. Several studies indicate that glycans and glycosylation of cellular proteins participate in the process of cancer cell adhesion, dissemination, and metastasis [2-5]. An unique expression of fucosyltransferase FT VI by colon cancer cell lines was

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identified by using GlcNAcβ1-4 GlcNAc as the specific acceptor [6]. The pattern of glycosyl- and glycan: Sulfotransferase activities in a wide range of human cancer cell lines was shown to be able to predict individual cancer associated signature carbohydrate structures [7]. A significant role for glycosyltransferases in invasion and intractability of pancreatic cancer became evident from a high-level over-expression of glycosyltransferases in pancreatic tumor [8]. Distinct changes in glycosyltransferases- specificities and lectin-binding by replacing terminal Gal with GalNAc in carbohydrate chains indicated that terminal sugar alteration could play a major role in cancer pathogenesis [8].

The LacdiNAc group is found mainly in N-glycans but also occurs in O-glycans [9-11]. The expression of the LacdiNAc group in N-glycans was reported to vary in human breast, prostate, ovarian and pancreatic cancers [12]. LacdiNAc-glycosylated PSA was better than the conventional PSA in identifying patients with clinically significant prostate cancer [13]. The NeuAcα2-6 GalNAcβ1-4GlcNAc sequence specifically found in secretory glycoproteins [14]. Recently mammalian glycoproteins were shown to carry GalNAcβ1-3GlcNAc on N-glycans in contrast to the presence of GalNAcβ1-4GlcNAc structures in N- and O- glycans of many mammalian glycoproteins, suggesting that GalNAcβ1-3GlcNAc and GalNAcβ1-4GlcNAc terminal units in glycans may have different roles *in vivo* [15].

The O-glycans impart unique features to mucin glycoproteins [16-19]. The first committed step in O-glycan biosynthesis is the addition of GalNAc to Ser/Thr [20]. Some αGalNAc-transferases such as T2 and T4 accomplish high-density glycosylation of certain protein substrates probably through binding as lectins [21]. Unsubstituted Tn epitopes occur in human cancers of colon, breast, bladder, prostate, liver, ovary and stomach and their presence correlate with cancer progression and metastases [22-24]. The expression of αGalNAc glycoconjugates detected by binding of HPA was found to be associated with metastatic competence and poor prognosis in a range of human adenocarcinomas [25]. ST6GalNAc1 mediated sialylation of Tn antigen and the frequent mutation of the cosmc chaperone that is required for the galactosyltransferase activity results in incomplete glycan structures [26].

The present study examined the acceptors-specificities of GalNAc transferases by using a variety of chemically synthesized compounds and identified by lectin-agarose affinity chromatography the levels of GlcNAc: β1-3 and β1- 4 GlaNAc tranferase activities in several human cancer cell lines. We found in these cell lines distinctly different levels of Tn epitope generating Ser/Thr containing small polypeptides (2-6 KD) and αGalNAc transferase activities. The present study found significant levels of a β galactosidase capable of converting T-glycotope to Tn at neutral pH in human cancer cell lines.

Materials and Methods

Cancer cell lines

T47D, MDA-MB-231, MCF-7, ZR-75-1, DU4475, MDA-MB-435S, MDA-435/LCC6 (breast), COLO 205, SW1116, LS180 (colon), SW626, PA-1 (ovarian), HL60 (leukemic), Hep G2 (hepatic), LNCaP, PC3, DU145 (prostate), U87GB (glioblastoma), EG7 (lymphoma), RIF (fibrosarcoma) and GL261-OVA (glioma) were cultured as recommended by ATCC (Manassas, VA) and as reported in earlier studies [6,7,27]. All cell samples were homogenized with 0.1M Tris-Maleate pH 7.2 containing 2% Triton X-100 using a Dounce glass, hand-operated homogenizer. The homogenate was centrifuged at 16,000g for 1h at 4°C. Protein was measured on the supernatants by the BCA micro method (Pierce Chemical Co) with BSA as the standard. The supernatants were adjusted to 5mg protein/mL by adding the necessary amount of extraction buffer and then stored frozen at -20°C until use.

Tissue specimens

The tissue specimens were obtained from the tissue procurement facility of Roswell Park Cancer Institute. All tissue specimens were stored frozen at -70°C until processed as reported earlier [6,7,28,29). The tissue samples were homogenized at 4°C with 4 volumes (1ml/per g tissue) of 0.1M Tris-Maleate pH 7.2 using Kinematica. After adjusting the concentration of Triton-X100 to 2%, these homogenates were mixed in the cold room for 1h using Speci-Mix (Thermolyne) and then centrifuged at 20,000g for 1h at 4°C. The clear fat free supernatant was adjusted to 10mg/ml protein by adding 0.1M Tris-Maleate pH 7.2 containing 2% TritonX100 and stored frozen at -20°C until use.

Acceptor compounds

The chemically synthesized compounds have already been used as acceptors for glycosyltransferases in our earlier studies and thus are well documented acceptor compounds for the study of glycosyltransferases [8,30-32].

Column chromatography

Biogel-P2 column or Biogel-P6 column (Fine Mesh; 1.0x116.0 cm) chromatography was carried out with 0.1 M pyridine acetate (pH5.4) as the eluent at room temperature. Void volume of this column is 30mL. The peak fraction containing [6-^{3}H] GalNAc radioactivity were pooled, lyophilized to dryness, dissolved in a small volume of water and stored frozen at -20°C for further experimentation. Lectin-agarose affinity chromatography was carried out using columns of 7ml bed volume of ConA-, PNA-, WGA-, WFL- and VVL- agarose (Vector Lab, Burlingame, CA) under conditions recommended by supplier [6,32]. The radioactive sample was applied to the column in 1.0ml of the running buffer. After entry of the sample into the column bed, the sample remained in contact with the gel for 20min before starting elution with the running buffer. Fractions of 1ml were collected. The bound material from WGA-agarose was eluted with 0.5M GlcNAc and from WFL-agarose and VVL-agarose were eluted with 1.0M Gal. PNA-agarose and ConA agarose bound materials were eluted with 0.2M Gal and 0.1M methylα-D-mannoside respectively. Depending on the time of elution, the lectin binding interactions of WGA, VVL and WFL were classified into four categories; non-binding, weak binding, regular binding and tight binding as explained in our earlier report [32]. TLC was carried out on Silica gel GHLF (250μm scored 20X20cm; Analtech Newark DE). The solvent system 1-propanol/NH4OH/H2O (12/2/5 v/v) was used [31]. The [6-^{3}H] GalNAc products were located by scraping 0.5cm width segments of silica gel and soaking in 2.0ml water in vials followed by liquid scintillation counting. Pronase digestion of Biogel P-2 [6-^{3}H] GalNAc containing peak 1 fraction was carried out in 600μl reaction mixture containing 4mg pronase CB, 0.1M Tris. HCL pH 7.0, 2mM $CaCl_2$ and 2% ethanol at 37°C for 18h and then subjected to Biogel P-6 chromatography.

Table 1A: Specificity of plant lectins PNA, VVL and WGA utilized in the present study as detailed in this table A.

PNA- and VVL binding specificity as revealed by Hapten inhibition using enzyme linked lectin assay [33]				
	PNA		VVL	
	mM	I(%)	mM	I(%)
Galβ1-3GalNAcα-O-Al	0.23	50	4	12
Galβ1-3 (6-O-Me) GalNAcα-O-Al	0.18	50	2	0
Galβ1-3(6-O-Sulfo) GalNAcα-O-Al	0.71	50	2	0
Galβ1-3 (NeuAcα2-6) GalNAcα-O-Bn	1.75	50	2	0
3-O-Sulfo Galβ1-3GalNAcα-O-Al	4	4	4	5
6-O-Sulfo Galβ1-3GalNAcα-O-Al	2	7	2	0
NeuAc Galβ1-3GalNAcα-O-Bn	2	0	2	0
GalNAcα-O-Al	4	9	1	50
6-O-Sulfo GalNAcα-O-ONP	2	10	2	6
NeuAcα2-6GalNAcα-O-Bn	2	0	2	5
	μM		μM	
Galβ1-3GalNAcα-O-Al/acrylamide copolymer	0.05	50	8.3	0
GalNAcα-O-Al/acrylamide copolymer	8.3	6	0.02	50
Antifreeze glycoprotein	1.9	50	19.6	13

Note: The lectin binding data presented in A from our earlier studies in order to achieve a meaningful understanding of the results obtained in the present study.

Table 1B: Specificity of plant lectins PNA, VVL and WGA utilized in the present study as detailed in this table B.

WGA- and PNA- agarose binding characteristics revealed by affinity chromatography [32]		
WGA-agarose		
NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	NB	
Galβ1,4(Fucα1,3)GlcNAcβ-O-Bn	NB	
GalNAcβ1,3(Fucα1,4)GlcNAcβ-O-Bn	NB	
NeuAcα2,6GalNAcβ1,4GlcNAcβ-O-Bn	NB	
NeuAcα2,3Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al	RB	
6-O-SulfoGlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Al	RB	
3-O-SulfoGalβ1,4GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Bn	RB	
GalNAcβ1,4GlcNAcβ1, 6(Galβ1, 3) GalNAcα-O-Bn	TB	
GalNAcβ1,4(Fucα1,3)GlcNAcβ-O-Bn	TB	
Galβ1,4GlcNAcβ1,6(9-^{3}H NeuAcα2,3 Galβ1,3)GalNAcα-O-Bn	TB	
GalNAcβ1,4(Fucα1-3)GlcNAcβ1,6(9-^{3}H NeuAcα2,3 Galβ1,3)GalNAcα-O-Bn	TB	
PNA-agarose		
Galα1,3Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn	NB	
Galβ1,4GlcNAcβ1,6 (NeuAcα2,3 Galβ1,3)GalNAcα-O-Bn	NB	
NeuAcα2,6Galβ1,4(6-O-Sulfo)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me	NB	
Galα1,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	RB	
Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	RB	
	WGA-agarose	VVL-agarose
GalNAcβ1-3GlcNAcβ-O-Bn	NB	WB
GalNAcβ1-4GlcNAcβ-O-Bn	TB	RB

Note: The lectin binding data presented in B from our earlier studies in order to achieve a meaningful understanding of the results obtained in the present study. NB: Non-Binding; RB: Regular Binding; TB: Tight Binding.

[6-^{3}H] labelling of GalNAc β1-3GlcNAcβ-O-Bn, GalNAc β1-4GlcNAcβ-O-Bn and GalNAc β1-6GlcNAcβ-O- Bn

These synthetic compounds (1.5μmol) were mixed separately with 20U of galactose oxidase and 200U of horse radish peroxidase in 0.1M Na-phosphate buffer pH 7 in 160μl reaction volume and incubated at 37°C for 21h and the oxidized GalNAc product was isolated by Sep-Pak method. The methanol eluates (5ml each) were concentrated to dryness and dissolved in 200μl of 0.05M Na-phosphate buffer pH 7,

and then mixed with 100µl NaB [^{3}H]4 (5mCi/500µl of 0.05M Na-phosphate pH 7.0) and left at room temperature for 2h. Then 100µl NaBH4 (100mg/ml water) was added, mixed well intermittently, and left at room temp for 1h. Then these three solutions were neutralized by adding drops of acetic acid, left in the cold room overnight and the [6-^{3}H] labelled compounds were isolated by Sep-Pak method.

Assay of GalNAc Transferases [8]

The incubation mixture (1.6ml) contained 0.1M Hepes pH 7.0 containing protease inhibitors (Calbiochem), exogenous synthetic acceptor Galβ1, 3 (GlcNAcβ1,6)GalNAc-o-Allyl (3.0µmol), 20mM Mn acetate, 7mM ATP, 3mM Na azide, UDP-GalNAc (Sigma Chemical Co. St. Louis, MO; 0.2µmol), UDP-(6-^{3}H) GalNAc (American Radio Labeled Co. St. Louis, MO; 20µCi) and 1.0ml of Triton X100 solubilized cell extract. The final concentration of UDP-GalNAc and the exogenous acceptor were 0.125mM and 1.9mM respectively. After incubation at 37°C for 20h, the incubation mixture was fractionated on a Biogel P2 column for the separation and quantitation of the radioactive products arising from endogenous and exogenous accetors. For the isolation of [6-^{3}H] GalNAc-yl product from GlcNAcβ-O-Bn, 200µl of the incubation mixture contained 0.6µm of GlcNAcβ-O-Bn and 100µl of cell or tissue extract and other components in the same proportion as above. The radioactive products from benzylglycosides were separated by hydrophobic chromatography on a Sep-Pak C18 cartridge (Waters, Milford, MA) and elution of the product was done with 3mL methanol. The methanol eluate was concentrated to dryness by flash evaporation, dissolved in a small volume of water and stored frozen at -20°C for experimentation.

HPLC

The HPLC separation [31] was performed on a C18 reverse-phase column using a gradient of acetonitrile in 10mM ammonium formate (pH 4.0). The sample injection volume was 20µl.

Results

Lectin specificities

The present study utilized the specificities of PNA, VVL and WGA for characterizing N-acetyl galactosaminyl products from the endogenous and exogenous acceptors. Table 1 explains the specificities of these lectins using hapten inhibition assay showed that Galβ1-3GalNAcα-O-Al and its acrylamide copolymer are the most effective compounds for PNA. Antifreeze glycoprotein containing Galβ1-3GalNAcα-O-Ser/Thr chains was also highly effective. Any substitution on Gal in Galβ1-3GalNAcα- abolishes the inhibitory activity. The hapten inhibition of PNA binding is reduced considerably by methyl, sulfate or NeuAc substituent on C_6-OH whereas they almost abolished the VVL binding. GalNAcα-O-Al and its acrylamide copolymer are effective in inhibiting VVL binding. Lectin agarose chromatography indicates NeuAcα2-3Galβ1-3 (GlcNAcβ1-6) GalNACα is the unit for regular binding of WGA whereas GalNAcβ1-4GlcNAc unit as such imparts tight binding to WGA. PNA-binding is inhibited by NeuAc and sulfate group on the β1-6 linked chain in mucin core 2 structure but not by αGal or Fuc.

Characterization of [6-^{3}H] GalNAc containing product from endogenous acceptor

When Galβ1-3(GlcNAc β1-6) GalNAcα-O-Al was incubated with LNCaP cell extract and subjected to Biogel P2 column chromatography, the [6-^{3}H] GalNAc-yl product from this acceptor emerge as Peak II fraction whereas the [6-^{3}H] GalNAc-yl product from endogenous acceptor emerge first designated as Peak I fraction (Figure 1A). When 4-Fluoro GlcNAc β1-6 (Galβ1-3) GalNAcα-O-Bn) was subjected to the same treatment, there was only Peak I radioactive product (Figure 1B) indicating that there was no transfer of [6-^{3}H] GalNAc to 4-FluoroGlcNAc as anticipated. When Peak I and Peak II fractions were treated with β N-acetyl hexosaminidase (Jack bean) and then subjected to Biogel P2 chromatography, Peak I fraction was not affected (Figure 1C) whereas Peak II was hydrolyzed for a complete release of [6-^{3}H] GalNAc (Figure 1D). Further, Peak I radioactive material did not bind at all to ConA- agarose (Figure 1E), WGA-agarose (Figure 1F) and also mostly to WFL-agarose (Figure 1G), but bound completely to VVL- agarose (Figure 1H) indicating its identity as Tn epitope bearing Ser/Thr polypeptides. When Peak I radioactive material was subjected to Biogel P6 column chromatography before and after Pronase digestion, the conversion of endogenous αGalNAc-ylated polypeptides to αGalNAc containing small peptides was evident as shown in (Figure 1I).

Characterization of [6-^{3}H] GalNAc containing product from exogenous acceptor Galβ1-3(GlcNAc β1-6) GalNAcα-O-Al

It is evident that [6-^{3}H] GalNAc is transferred to β1-6 linked GlcNAc in β linkage since it is completely released by Jack bean β N-Acetyl hexosaminidase digestion (Figure 1D) and GalNAc is transferred likely to C-4 hydroxyl group of GlcNAc since 4 Fluoro GlcNAc containing acceptor is inactive (Figure 1B). The synthetic [6-^{3}H] GalNAc compounds namely [6-^{3}H] GalNAc β 1-3 and β 1-4 GlcNAC-β-O-Bn showed respectively non-binding (Figure 1J) and tight binding to WGA-agarose (Figure 1K) whereas they exhibited respectively weak binding (Figure 1L) and regular binding (Figure 1M) to VVL-agarose and both radioactive compounds showed complete regular binding to WFL-agarose (data not shown). The [6-^{3}H] GalNAc-yl product from Galβ1-3(GlcNAc β1-6) GalNAcα-O-Al (Biogel P2 Peak II fraction) surprisingly showed only 73% binding to PNA-agarose (Figure 1N). It showed 80% binding to WGA-agarose (Figure 1O) indicating that the binding product contains β 1-4 linked GalNAc. It is interesting to note that complete binding of this radioactive product (Biogel P2 Peak II fraction) was seen with WFL-agarose (Figure 1P) which binds GalNAc linked β 1-3 as well as β 1-4 to GlcNAc whereas αGalNAc containing polypeptides (Biogel P2 Peak I fraction) did not bind to WFL-agarose (Figure 1G). It shows further that Biogel P2 Peak II fraction is not contaminated by αGalNAc containing polypeptides.

Carbohydrate specificities of βGalNAc-transferase activities in cancer

As reported in Table 2, we used chemically synthesized compounds as acceptors and the Triton-X100 solubilized extract of 2 different cancer cells T47D (breast) and LS180 (colon) as enzyme source. Galβ 1-3GalNAc α-O-Bn and D-Fuc β 1-3GalNAc α-O-Bn, 3-O-MeGalβ 1-4GlcNAcβ 1-6 (Galβ 1-3)GalNAc-α-O-Bn and 4-F GlcNAc β1-6 (Gal β1-3)GalNAcα-O-Bn did not serve as acceptors for GalNAc-T of T47D and LS180 indicating that non-transfer of GalNAC to T-hapten and also to 4-F GlcNAc whereas Galβ 1-3GlcNAc β-O-Bn, Galβ 1-4GlcNAc β-O-Bn and Galβ 1-4GlcNAcβ 1-6 (3-O-MeGalβ1-3) GalNAc served as acceptors to some extent indicating the possibility

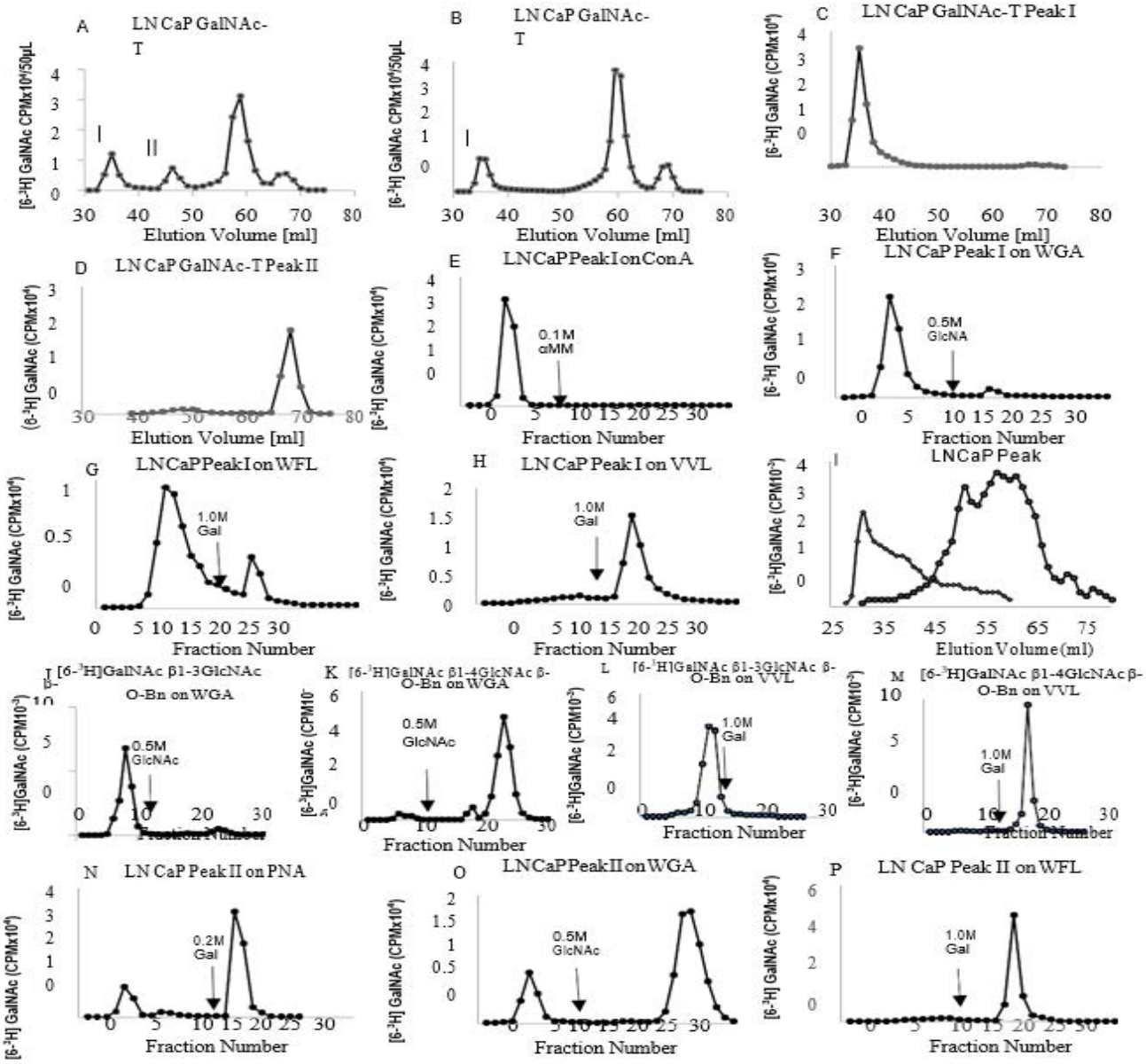

Figure 1: Initial characterization of cancer associated α- and β- GalNAc transferase activities using prostate cancer LNCaP cells.
Biogel P2 chromatography of RM containing the exogenous acceptor: A. GlcNAc β1-6 (Galβ1-3) GalNAcα- O-Al; B. 4Fluoro GlcNAc β1-6 (Galβ1-3) GalNAcα-O-Al; Biogel P2 chromatography after Jack bean β-N-Acetylexosaminidase treatment: C. Peak I fraction from A; D. Peak II Fraction from A. Affinity chromatography of Peak I Fraction from A: E. Con A-agarose; F. WGA -agarose; G. WFL- agarose; H. VVL-agarose; Biogel P6 chromatography of Peak I Fraction from A: I. before and after pronase treatment; Affinity chromatography of radioactive synthetic compounds [6-^{3}H] GalNAcβ1-3 GlcNAcβ-O-Bn and [6-^{3}H] GalNAcβ1-4 GlcNAcβ-O-Bn: J, K. WGA-agarose; L, M. VVL-agarose; Affinity chromatography of Biogel P2 Peak II Fraction from A: N. PNA-agarose; O. WGA-agarose; P. WFL-agarose.

of minor extension of LacNAc chain by GalNAc. The other acceptors containing the GlcNAc terminal namely Galβ 1-3/D- Fuc β 1-3/Fuc α1-2Galβ 1-3 (GlcNAcβ 1-6) GalNAcα-O-Bn acted as acceptors for GalNAc T. GlcNAc B-O-Bn was the best acceptor in both cases indicating that both β 1-3 and β 1-4 GalNAc transferases can use this acceptor since this acceptor contains no other sugar residues for exerting specificity related constraints.

Cancer cell βGalNAc transferase activities towards three synthetic acceptors

All cancer cells (Table 3) showed less activity towards GlcNAc linked β1-4 to GlcNAc β-O-Bn as compared to GlcNAc β-O-Bn in the range of 21.1-38.9% (breast cancer), 37.0-49.2% (colon cancer), 33.3-41.2% (ovarian cancer), 33.3% (leukemia), 25.9% (hepatic cancer) and 30.2-37.2% (prostate cancer). As GlcNAcβ1-4 GlcNAcβ-O- Bn may serve as an acceptor favorable to β1-3GalNAc transfer, these values would suggest a higher level of β1- 4GalNAc transferase activity as compared to β1-3GalNAc transferase activity in cancer cells. GlcNAcβ-O-Bn would act as a favorable acceptor for both β1-3 and β1-4 GalNAc transferases whereas GlcNAcβ1-6GalNAcα-O-Bn would be a more favorable acceptor for β1-4GalNAc transferase as suggested by the inactivity of 4-FGlcNAc β1-6 (Galβ1-3)GalNAc

Table 2: Specificities of βGalNAc transferase activities present in two different cancer cells as revealed by chemically synthesized compounds tested as acceptors.

Synthetic Compounds	Incorporation of [6-^{3}H] GalNAc (CPM x10^{-4}) into the acceptors catalyzed by 1mg protein of solubilized cell extract	
	T47D (Breast cancer)	LS180 (Colon cancer)
GlcNAcβ-O-Bn	10.6	3.4
Galβ1-3GalNAcα-OBn	0	0
D-Fucβ1-3GalNAcα-OBn	0	0
D-Fucβ1-3 (GlcNAc β1-6) GalNAcα-OBn	3.5	2.1
Galβ1-3 (GlcNAc β1-6) GalNAcα-OBn	3.9	2.1
Galβ1-4GlcNAcβ-O-Bn	1.2	0.6
Galβ1-3GlcNAcβ-O-Bn	2.1	0.1
Fucα1-2 Galβ1-3 (GlcNAc β1-6) GalNAcα-OBn	4.3	2.5
4-FGlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Bn	0	0.1
3-O-MeGalβ1-3 (GlcNAcβ1-6) GalNAcα-O-Bn	3.7	2.3
3-O-MeGalβ1-4 GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Bn	0	0
Galβ1-4 GlcNAcβ1-6 (3-O-MeGalβ1-3) GalNAcα-O-Bn	1.6	0.3

Table 3: Human cancer cell line GalNAc T activities as measured with three synthetic acceptors namely GlcNAC β-O-Bn, GlcNAc β1-6GalNAcα-O-Bn and GlcNAc β1-4GlcNAcβ-O-Bn.

Cancer Cells	Incorporation of [6-^{3}H] GlaNAc(CPM x 10^{-4}) into the acceptor catalyzed by 1mg protein of solubilized cell extract		
	GlcNAC β-O- Bn	GlcNAc β1-6GalNAc α-O-Bn	GlcNAc β1-4GlcNAc β-O-Bn
Breast Cancer			
T47D	4.28	3.88 (90.7)	1.54 (36.0)
MDA-MB-231	3.22	3.20 (99.4)	0.88 (27.4)
MCF-7	2.9	2.54 (87.5)	1.13 (38.9)
ZR-75-1	12.06	7.35 (61.0)	3.02 (25.0)
DU4475	9.5	2.28 (24.0)	2.05 (21.6)
MDA-MB-435S	1.1	0.62 (56.4)	0.23 (21.1)
MDA-435/LCC6	1.54	1.23 (79.6)	0.57 (37.1)
Colon Cancer			
COLO205	2.82	3.11 (110.4)	1.39 (49.2)
SW1116	2.65	2.55 (96.4)	1.01 (38.1)
LS180	3.43	2.38 (69.4)	1.27 (37.0)
Ovarian Cancer			
SW626	3.94	3.10 (78.8)	1.62 (41.2)
PA-1	1.72	1.14 (66.5)	0.57 (33.3)
Leukemia			
HL60	4.02	2.74 (68.3)	1.34 (33.3)
Hepatic Cancer			
HepG2	7.14	7.03 (98.5)	1.85 (25.9)
Prostate Cancer			
LNCaP	4.52	5.27 (116.6)	1.68 (37.2)
PC3	3.68	2.84 (77.2)	1.21 (32.9)
DU145	7.56	5.86 (77.5)	2.28 (30.2)
Miscellaneous			
U87GB (Glioblastoma)	5.44	ND	ND
EG7 (Lymphoma)	2.22	ND	ND
RIF (Fibrosarcoma)	5.6	ND	ND
GL261-OVA (Glioma)	3.64	ND	ND

Note: ND: Not Determined Values in parenthesis are the activities towards the two acceptors GlcNAc β1-6GalNAcα-O-Bn, GlcNAc β1-4GlcNAc β-O-Bn in percentage with respect to the activity towards GlcNAc β-O-Bn.

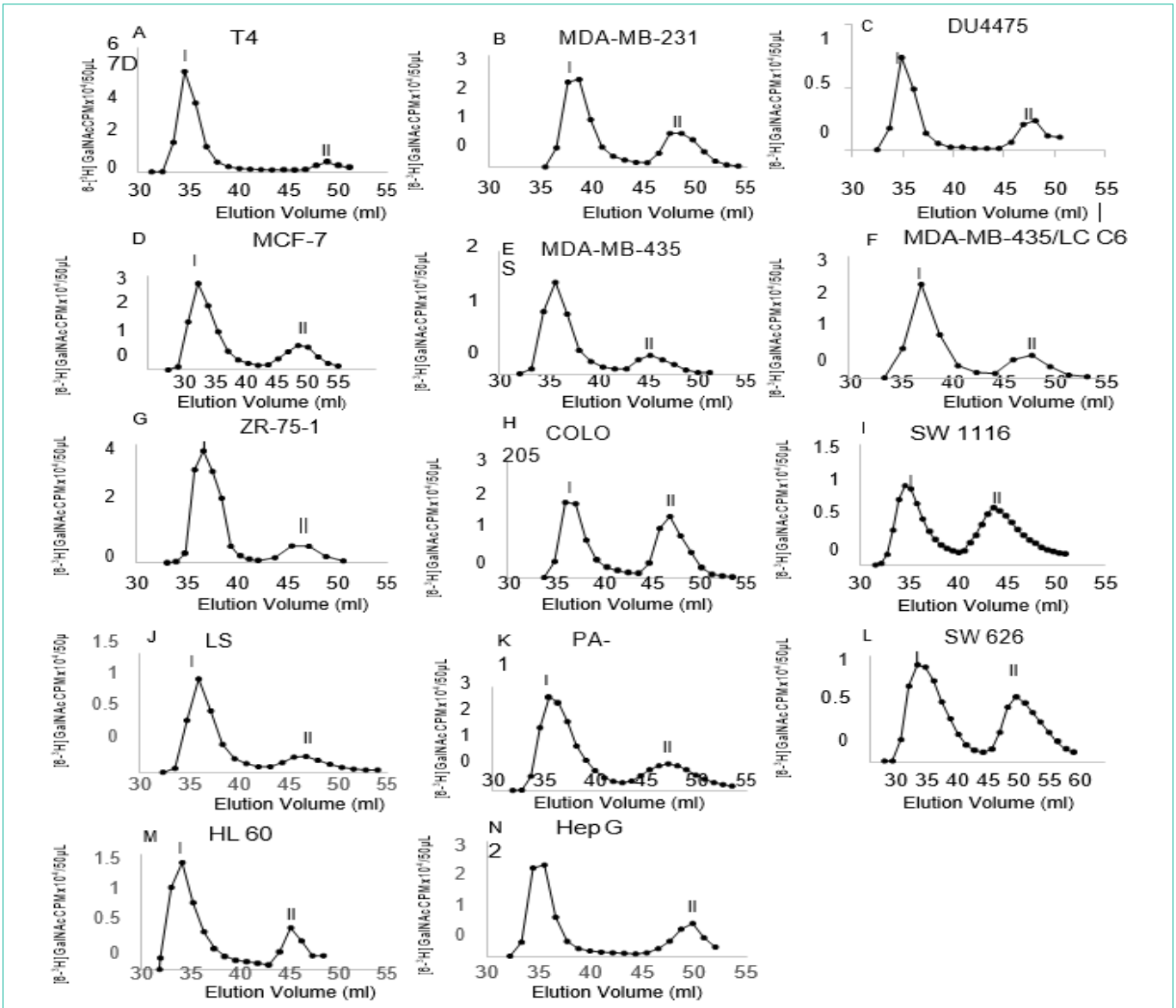

Figure 2: Separation of radioactive products from GalNAc transferase reaction mixtures from cancer cells containing the exogenous acceptor GlcNAc β1-6 (Galβ1-3) GalNAc α-O-Al by Biogel P2 column chromatography.
A) T47D; B) MDA-MB-231; C) DU4475; D) MCF-7; E) MDA-MB-435 S; F) MDA-MB-435/LCC6; G) ZR-75-1; H) Colo 205; I) SW 1116; J) LS 180; K) PA-1; L) SW 626; M) HL 60; N) HepG2.

α-O-Bn as an acceptor. The activity towards GlcNAcβ1-6GalNAcα-O-Bn as compared to GlcNAcβ-O-Bn is slightly less in most cases except for considerably lower values in ZR-75-1 (61.0%), DU4475 (24.0%) and MDA-MB-435S (56.4%) and slightly higher values in Colo205 (110.4%) and LNCaP (116.6%). The exceptionally low level of GalNAc transferase activity towards β1-6 linked GlcNAc exhibited by DU4475 appears to be an unique situation that would possibly indicating the predominance of β1-3GalNAc transferase activity in DU4475.

The levels of [6-^{3}H] GalNAc-yl products from endogenous Ser/Thr containing polypeptides and exogenous acceptor namely mucin core 2 trisaccharide Galβ1-3 (GlcNAcβ1-6) GalNAcα-O-Al from various cell lines

The chemically synthesized compound GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al was used as the exogenous acceptor for GlcNAc: βGalNAc-transferases. The purity of this compound was established by its mobility as a single spot in TLC (located on the TLC plates by spraying with sulfuric acid in ethanol and heating at 100o C) using two different solvent systems 1-propanol/NH4OH/H2O (12/2/5, V/V) and CHCL3/CH3OH/H2O (5/4/1, V/V). Further after [9-^{3}H] sialylation with cloned ST3 Gal II, this compound moved as a single radioactive compound on TLC in the above two solvent systems, being located by scrapping 0.5cm width segments of silica gel and soaking them in 2ml water in vials followed by liquid scintillation counting. Mass spectral analysis confirmed the identity and purity of these non-sialylated and sialylated compounds [31].

Figure 2 presents the Biogel P2 column chromatographic profiles of [6-^{3}H] GalNAc-yl products (Peak I and Peak II) by the action of

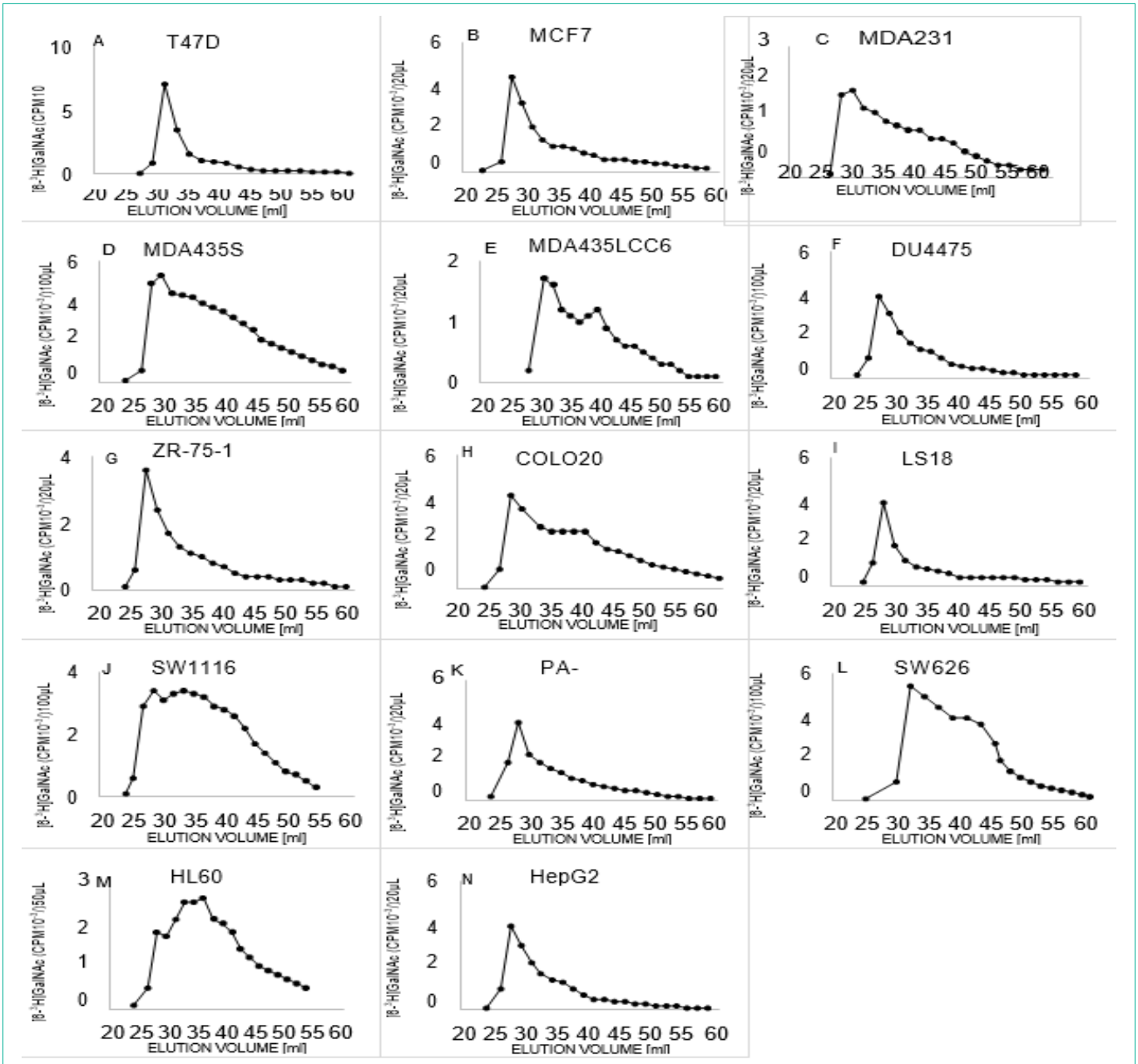

Figure 3: Demonstrating the heterogeneity of Biogel P2 Peak I Fraction containing αGalNAc-yl polypeptides by utilizing Biogel P6 column chromatography. A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) ZR-75-1; H) Colo 205; I) LS 180; J) SW 1116; K) PA-1; L) SW 626; M) HL 60; N) HepG2.

GalNAc-transferases present in TritonX-100 solubilized extracts from various cancer cell lines.

When Peak I endogenous [6-^{3}H] GalNAc-yl products from the various cell lines were examined by Biogel P6 column chromatography (Figure 3), it was evident that they contained quite heterogenous Tn epitope bearing small poly peptides. Tn polypeptides from T47D, MCF-7, DU4475, ZR75-1, LS180, PA-I and HepG2 (Figure 3A,3B,3F,3G,3I,3K and 3N respectively) are comparatively larger than those from remaining cell lines (Figure 3C,3D,3E,3H,3J,3L and 3M respectively). We have already demonstrated that [6-^{3}H] GalNAc-yl endogenous material (Biogel P2 Peak I) can be digested to smaller Tn peptide fragments by pronase digestion (refer Biogel P6 chromatography Figure 1I).

In all cases (Table 4) except for LS180 and LNCaP, the level of endogenous product exceeded the product from exogenous acceptor. It is quite remarkable that all breast cancer cell lines exhibited a high level of Tn containing polypeptides in the range of 1.9 - 8.8-fold that of corresponding exogenous product. SW116, PA1, HL60 and HepG2 also showed a high level of Tn polypeptides 3.9, 2.2, 3.5 and 2.5- fold respectively. Further, it is noteworthy that βGalNAc- transferase activity towards GlcNAc in Galβ-3(GlcNAcβ-6)GalNAcα-O-Al was found in higher level in MDA-MB-231 (6.7), ZR-75-1 (7.1), Colo

Table 4: Ser/Thr: αGalNAc- and GlcNAc: β1-3 and β1-4 GalNAc-Transferase activities in human cancer cell lines.

Cancer Cells	Incorporation of [6-^{3}H] GalNAc into the acceptor (CPM x 10^{-4}) catalyzed by1mg protein of the solubilized cell extract		
	αGalNAc-T: Ser/thr peptides (Biogel P2 Peak I fraction)	βGalNAc-T: Galβ1-3 (GlcNAc β1-6) GalNAcα-O-Al (Biogel P2 Peak II fraction)	Enzyme level in folds: αGalNAc- T vs βGalNAc-T
Breast Cancer			
T47D	11.4	1.3	8.8
MDA-MB-231	13	6.7	1.9
MCF-7	9.8	3.7	2.7
ZR-75-1	23.4	7.1	3.3
DU4475	5.8	2.6	2.2
MDA-MB-4355	7.7	2.1	3.7
MDA-435/ LCC6	12.5	4.4	2.8
Colon Cancer			
COLO205	8.7	7.2	1.2
SW1116	15	3.9	3.9
LS180	5.7	6.4	0.9
Ovarian Cancer			
SW626	8.2	5.6	1.5
PA-1	14.6	6.7	2.2
Leukemia			
HL60	9.9	2.8	3.5
Hepatic Cancer			
HepG2	9.1	3.6	2.5
Prostate Cancer			
LNCaP	15.8	18.1	0.9

205 (7.2), LS180 (6.4), SW626 (5.6), PA-I (6.7) and LNCaP (18.1) as compared to other cell lines (Table 3).

GlcNAc: β1-4GalNAc transferase activities of cancer cell lines towards the synthetic acceptor GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al as determined by WGA-Agarose tight binding and also compared with VVL- Agarose binding

Figure 4 and 5 present the affinity chromatography profiles of Biogel P2 fraction II containing [6-^{3}H] GalNAc-yl product from GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al from the fractionation on WGA-Agarose and VVL-Agarose column, respectively. The data obtained from these fractionations are given in Table 5. In this context it is important to note that for the conversion of UDP-GalNAc to UDP-GlcNAc by UDP-Glc-4-epimerase, Daenzer et al. (2012) used the reaction mixture containing 100mM glycine buffer pH 8.7, 1.6mM UDP-GalNAc and 0.5mM NAD. As our reaction conditions are quite different, there is no possibility of UDP-GalNAc being converted to UDP-GlcNAc.

In all cases except for Colo 205, VVL-Agarose binding gave higher values as compared to the corresponding values of WGA-Agarose binding. This discrepancy is explainable by the fact that WGA-Agarose binds tightly and strictly only GalNAcβ1-4GlcNAc moiety whereas VVL-Agarose binds, in addition to GalNAcβ1-4GlcNAc, also αGalNAc containing small peptides that could be present as a very minor contaminant in Biogel P2 Peak II fractions. From the highly reliable values from the WGA-Agarose binding, it is evident that the breast cell lines ZR-75-1, DU4475 and MDA-MB-435S and the ovarian cell lines PA-1 express respectively lower GlcNAc: β1-4GalNAc transferase activities as follows: 26.6%, 33.6%, 30.7% and 22.1% whereas other cell lines exhibit this activity in the range 54.0% to 89.6%.

Detection of β1-3 Galactosidase activity at neutral pH

When Biogel P2 Peak II fractions containing [6-^{3}H] GalNAc β1-3/β1-4 GlcNAc β1-6 (Galβ1-3) GalNAcα-O-Al were subjected to PNA-Agarose chromatography with the anticipation of 100% binding of the [6-^{3}H] GalNAc-yl product to the PNA-Agarose column, it was found that it contained both PNA binding and non-binding fractions indicating that β1-3 linked Gal had been hydrolyzed during incubation with the cell extract to a variable extent in the case of all cell lines examined. As Galβ1-3GalNAcα-OBn and 3-O-MeGalβ1-4 GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Bn were 100% inactive as acceptors for cancer cells βGalNAc transferases, the β1-3 Gal is available for the action of cancer cell β-galactosidase. The fractionation profiles on PNA-Agarose are presented in Figure 6. The data on PNA-Agarose binding and non-binding fractions from Figure 6 are given in Table 6. Further, the separation of the products presents in Biogel P2 Peak II fraction was also carried out by thin layer chromatography. Quantitation of the two radioactive products was made by scrapping the silica gel (0.5 cm width segments) and soaking in 2ml water

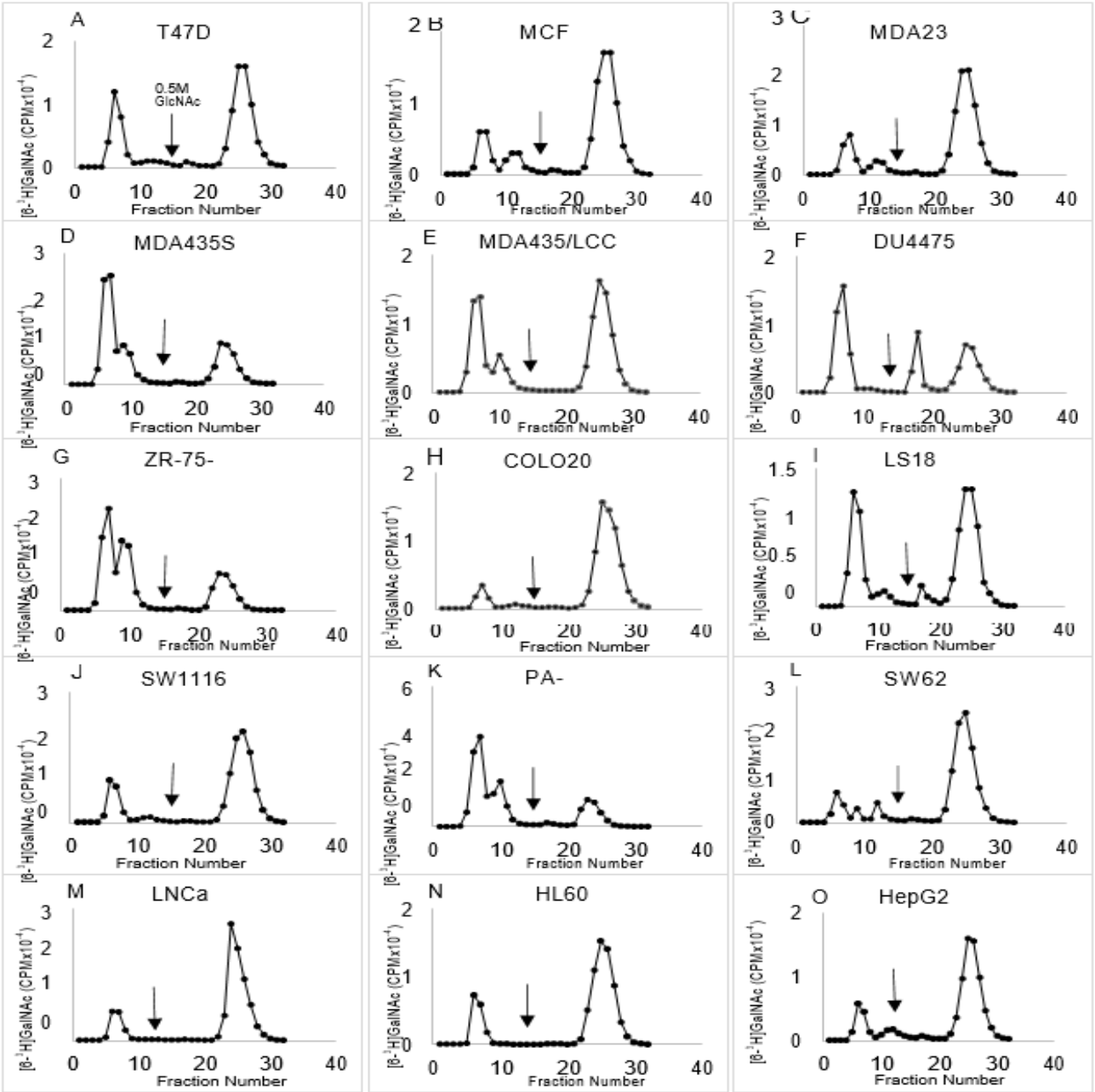

Figure 4: WGA-agarose affinity chromatography of Biogel P2 Peak II Fraction.
A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) ZR-75-1; H) Colo 205; I) LS 180; J) SW 1116; K) PA-1; L) SW 626; M) HL 60; N) HepG2.

followed by liquid scintillation counting. The TLC separation of the products is presented in Table 8 and the data obtained from this table is presented in Table 6 as percent of the radioactivity present in each component from the total CPM of the two components of each cell lines. These TLC values closely resembled the percentage of PNA-Agarose binding and non-binding fractions in spite of the fact that the TLC data is not expected to be 100% quantitative as compared to the PNA-Agarose data. We identified component C2 as [6-^{3}H] GalNAc β1- 3/4GlcNAcβ1-6GalNAcα-O-Al as follows: When Galβ1-3(GlcNAcβ1-6) GalNAcα-Ol-O-Al was treated with recombinant β1-3galactosidase (Calbiochem) for complete conversion to GlcNAcβ1-6 GalNAcα-Ol-O-Al and then subjected to [6-^{3}H] GalNAc-ylation by using separately PA-1 and LNCaP extracts, the radioactivity moved as a single component in TLC with the mobility of component C2 in both cases (not shown). The conversion of T- epitope to Tn at neutral pH catalyzed by the β-Galactosidase present in the cell extracts (Table 6 last column) was calculated from the PNA non-binding data and considering that the incubation mixture contained 3 μmol of the acceptor GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al and 5mg protein from each cell extract. The human cancer cell lines thus contained

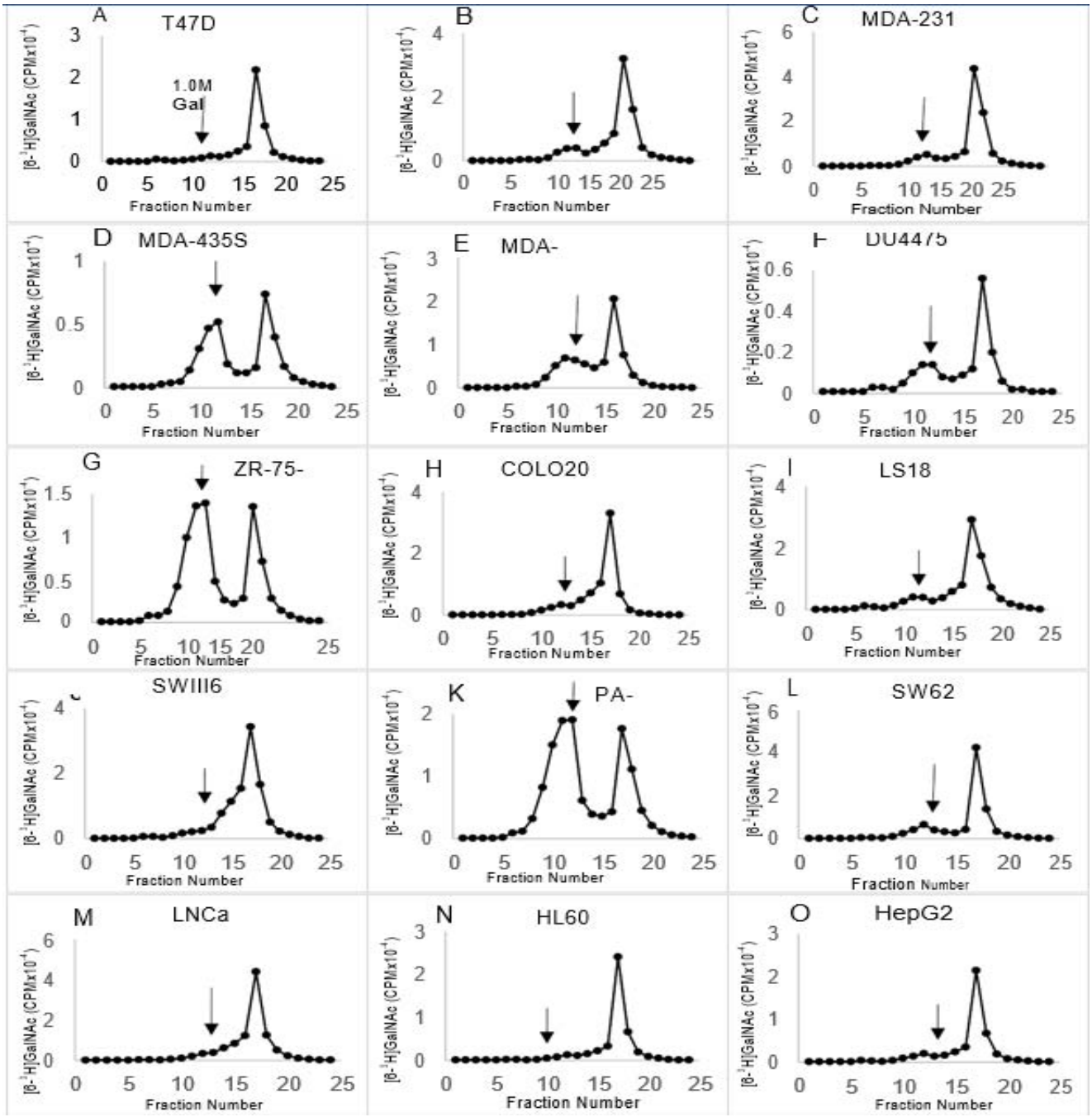

Figure 5: VVL-agarose affinity chromatography of Biogel P2 Peak II Fraction.
A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) ZR-75-1; H) Colo 205; I) LS 180; J) SW 1116; K) PA-1; L) SW 626; M) HL 60; N) HepG2.

quite a high level of neutral β-Galactosidase activity exhibiting the ability to catalyze the hydrolysis of 0.18-0.59 μmol T-epitope per mg protein of the cell extracts (e.g., T47D: 0.6μmol X 98.7% (PNA-Agarose non-binding) = 0.59μmol; MDA-MB-231: 0.6μmol X 46% = 0.28μmol).

Determination of GlcNAc

β1-3GalNAc and GlcNAc: β1-4GalNAc transferase activities utilizing the universal acceptor GlcNAcβ-O-Bn for both enzymes, the tight binding of GalNAcβ1-4GlcNAc to WGA-agarose and the weak binding of GalNAcβ1-3GlcNAc to VVL-agarose: The WGA-agarose tight binding and VVL-agarose weak binding respectively of the products [6-^{3}H] GalNAcβ1-4- and β1-3-GlcNAc β-O-Bn from the exogenous acceptor GlcNAcβ-O-Bn, isolated by Sep-Pak C18 method were used for quantitating β1-4 and β1-3 GalNAc-T activities. When [6-^{3}H] GalNAcβ1-3/β1-4GlcNAcβ-O-Bn isolated by using PA-1 and LNCaP extracts as enzyme sources were subjected to galacdose oxidase-horse-radish peroxidase treatment, these products

Table 5: Determination of GlcNAc: β1-4GalNAc-transferase activity in cancer cell lines by utilizing the binding of [6-^{3}H] GalNAc-yl product from GlcNAcβ1-6 Gal (β1-3) GalNAcα-O-Al to WGA-Agarose as well as VVL- Agarose affinity columns.

Cancer Cells	[6-^{3}H] GalNAc-yl product from GlcNAcβ1-6 (β1-3) GalNAcα-O-Al (Biogel P2 Peak II fraction)	
	WGA-Agarose binding (%)	VVL-Agarose binding (%)
Breast Cancer		
T47D	64.1	90.6
MDA-MB-231	74.2	84.2
MCF-7	72.4	82.9
ZR-75-1	26.6	37.6
DU4475	33.6	66.6
MDA-MB-4355	30.7	48
MDA-435/LCC6	54	54.4
Colon Cancer		
COLO205	89.6	84.9
SW1116	75.8	98.2
LS180	56.1	81.5
Ovarian Cancer		
SW626	68.3	75.3
PA-1	22.1	37.1
Leukemia		
HL60	77.4	90.2
Hepatic Cancer		
HepG2	75.9	84.5
Prostate Cancer		
LNCaP	82.9	99

Table 6: Identification of β1-3 Galactosidase activity converting T-antigenic structure (Galβ1-3 GalNAc α-) to Tn (GalNAc α-) in human cancer cell lines.

Cancer Cells	GalNAc β1-3/4GlcNAc β1-6 GalNAcα-O-Al		GalNAc β1-3/4GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al		[a]Conversion of T epitope to Tn at neutral pH catalyzed by β-Galactosidase present in 1mg protein of the cell extract (μmol)
	PNA-Agarose non-binding (%)	TLC Component C2 (%)	PNA Agarose binding (%)	TLC Component C2 (%)	
Breast Cancer					
T47D	98.7	88	1.3	12	0.59
MDA-MB-231	46		54		0.28
MCF-7	67.6		32.4		0.41
ZR-75-1	55.5	47.7	44.5	52.3	0.35
DU4475	45		55		0.27
MDA-MB-435S	46.3		53.7		0.28
MDA-435/LCC6	41.8		58.2		0.25
Colon Cancer					
COLO205	29.3	26.6	70.7	73.4	0.18
SW1116	70.1	61.6	29.9	38.4	0.42
LS180	55.1	35.8	44.9	64.2	0.33
Ovarian Cancer					
SW626	94.9	89.6	5.1	10.4	0.57
PA-1	53.2	40.1	46.8	59.9	0.32
Leukemia					
HL60	20.3	11.6	79.7	88.4	0.12
Hepatic Cancer					
HepG2	80	74.4	20	25.6	0.48
Prostate Cancer					
LNCaP	29.7	19.5	70.3	80.5	0.18

[a]Calculation was based on 3μmol Galβ1-3 (GlcNAcβ1-6) GalNAcα-O-Al and 5mg protein present in the incubation mixture of each cell extract and PNA-Agarose non-binding material in percent as reported in column 2 of this table.

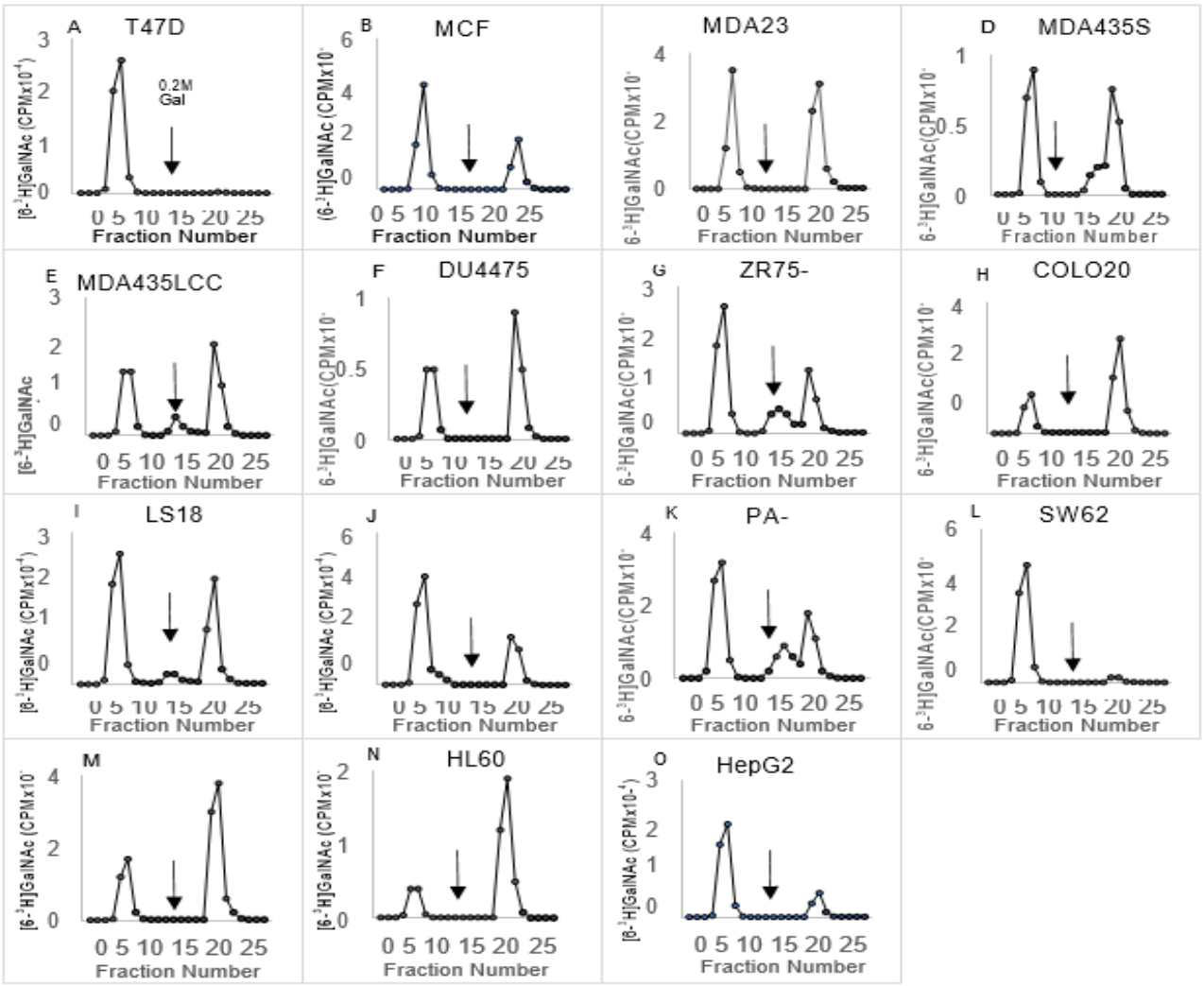

Figure 6: Separation of [6-^{3}H] GalNAc-yl products present in Biogel P2 Peak II Fraction by affinity chromatography on PNA-agarose.
A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) ZR-75-1; H) Colo 205; I) LS 180; J) SW 1116; K) PA-1; L) SW 626; M) HL 60; N) HepG2.

lost C6-^{3}H label showing the absence epimerase reaction under our incubation conditions for GalNAc-T activity.

WGA-Agarose and VVL-Agarose affinity chromatographic profiles of [6-^{3}H] GalNAc-yl products from GlcNAcβ- O-Bn are presented in Figure 7 and 8 respectively. The product [6-^{3}H] GalNAcβ1-4GlcNAcβ-O-Bn exhibited tight binding to WGA-agarose whereas [6-^{3}H] GalNAcβ1-3 GlcNAcβ-O-Bn showed weak binding to VVL-agarose. The values for the binding obtained from these Figures are given in Table 7. The fold of activity for β1-4GalNAc transfer as compared to β1-3GalNAc transfer are presented in Table 6 last Column. These values for DU-4475 and MDA- MB-435S (breast cancer), PA-1 (ovarian cancer), LNCaP, PC3 and DU145 (prostate cancer), EG7 (lymphoma), and GL261-OVA (Glioma) were 0.1, 0.6, 0.3, 0.2, 0.4, 0.3, 0.3 and 0.5 respectively indicating the predominance of β1- 3GalNAc transferase activity in these cell lines. Among the remaining cell lines, T47D, MDA-MB-231 and MCF-7 (breast cancer), Colo205 and SW1116 (colon cancer) and U87GB (Glioblastoma) showed high β1-4GalNAc transferase activity which exceeded 2-fold of β1-3GalNAc transferase activity in the range 2.3-4.9. It needs to be mentioned that in Sep-Pak C18 fractionation, most of the αGalNAc bearing polypeptides present in the incubation mixture containing the [6-^{3}H] GalNAc-Ylated product from the exogenous acceptor GlcNAc-B-O-Bn were eliminated in the exhaustive water washings of Sep-Pak C18 cartridge.

Measuring the level of β1-3 and β1-4 GalNAc transferase activities present in some cancer cell lines and tissue specimens by using HPLC for the separation of [6-^{3}H] GalNAc-yl products resulting from the acceptor GlcNAcβ-O-Bn

The HPLC data is presented in Figure 9. The separation of synthetic compounds in HPLC is shown in Figure 9A. GalNAcβ1-6GlcNAcβ-O-Bn, GalNAcβ1-3GlcNAcβ-O-Bn and GalNAcβ1-4GlcNAcβ-O-Bn are emerging respectively from the column in the same sequence. As shown by the analysis with WGA-Agarose and

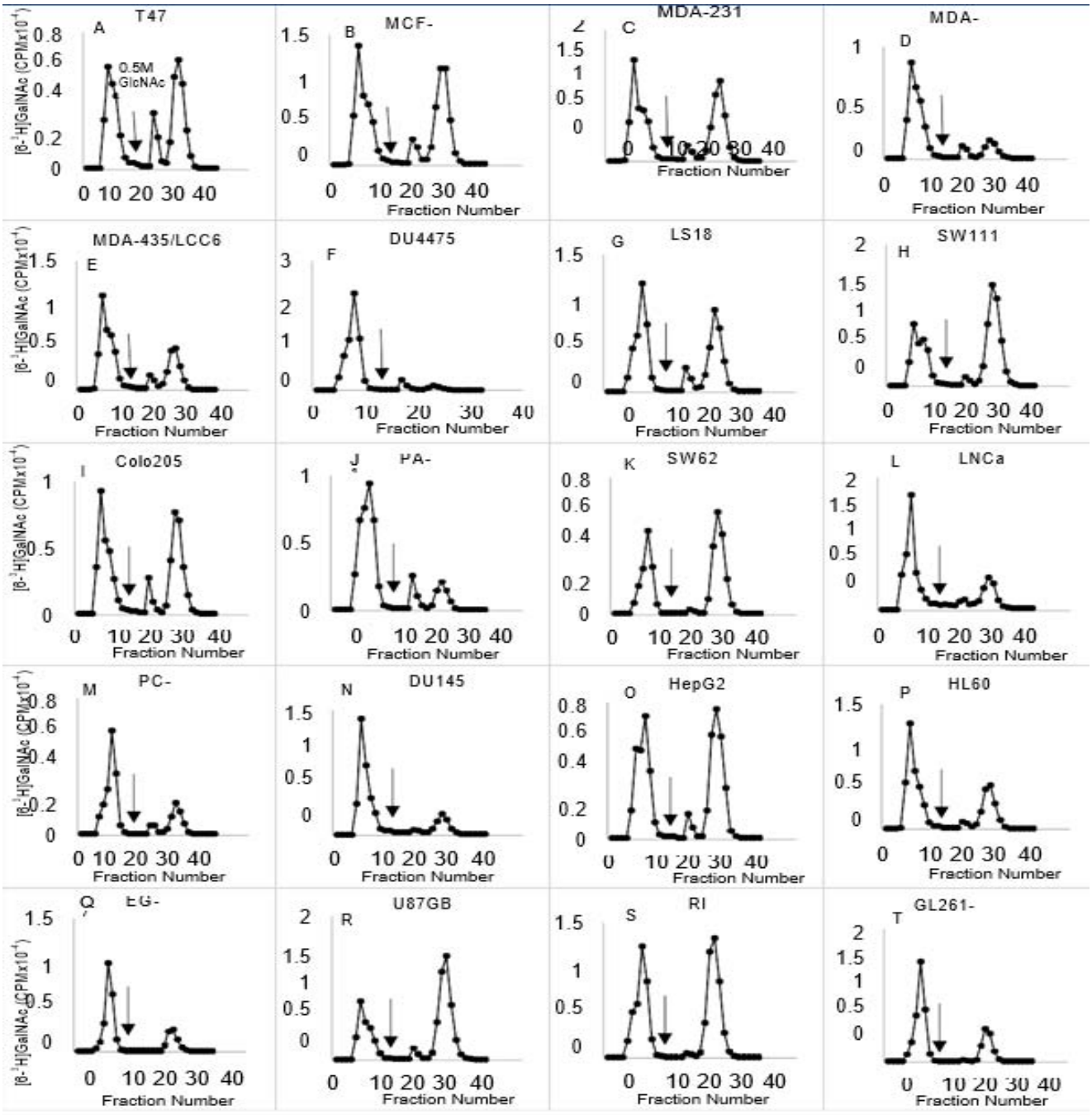

Figure 7: WGA- agarose affinity chromatography of [6-^{3}H] GalNAc-yl products from GlcNAcβ-O-Bn.
A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) LS180; H) SW1116; I) Colo 205; J) PA-1; K) SW 626; L) LNCaP; M) PC-3; N) DU145; O) HepG2; P) HL60; Q) EG-7; R) U87GB; S) RIF; T) GL261-OVA.

VVL- Agarose affinity chromatography, the HPLC data indicate that the prostate cancer cell lines LNCaP, PC3 and DU145 (Figure 9B-9D), the breast cell lines DU4475 and MDA-MB-435S (Figure 9E and 9G) and the ovarian cell lines PA1 and SW626 (Figure 9I and 9J) contained significant β1-3GalNAc transferase activity in contrast to the predominant β1-4GalNAc transferase activity in T47D and Colo205 (Figure 9F and 9H). The HPLC analysis of some tumor specimens for β1-3/β1-4 GalNAc transferase activities indicated that the two pancreatic tumor specimens (Figure 9N and 9O) and one gastric tumor specimen (Figure 9P) express significantly high level of β1-3GalNAc transferase activity as compared to its level in three prostate tumor specimens (Figure 9K and 9L) and one normal testes specimen (Figure 9M). The HPLC profiles (Figure 9) further showed either negligible or extremely low level of GalNAcβ1-6GlcNAcβ-O-Bn in all cases examined.

Table 7: Determination of the products [6-^{3}H] GalNAcβ1-3GlcNAc β-O-Bn and [6-^{3}H] GalNAcβ1-4GlcNAcβ-O-Bn resulting from GlcNAc β-O-Bn by the action of GalNAc-transferases in human cancer cell lines.

Cancer Cells	WGA-Agarose Tight Binding (%) GalNAcβ1-4 GlcNAcβ-O-Bn	VVL-Agarose Weak Binding (%) GalNAcβ-3 GlcNAcβ-O-Bn	Enzyme levels in fold: β1-4GalNAc-T vs β1-3 GalNAc-T
Breast Cancer			
T47D	44.8	15	3
MDA-MB-231	44	19.1	2.3
MCF-7	44.2	16.3	2.7
DU4475	5.3	63.7	0.1
MDA-MB-435S	15.7	25.6	0.6
MDA-435/LCC6	30	24.6	1.2
Colon Cancer			
COLO205	44.4	15.1	2.9
SW1116	58.6	17.6	3.3
LS180	36.7	34.3	1.1
Ovarian Cancer			
SW626	58	32.1	1.8
PA-1	14.1	44.2	0.3
Leukemia			
HL60	32.1	22.6	1.4
Hepatic Cancer			
HepG2	48.1	25.5	1.9
Prostate Cancer			
LNCaP	16.9	76.1	0.2
PC3	24.5	56	0.4
DU145	18.7	66.5	0.3
Miscellaneous			
U87GB	61.7	12.6	4.9
(Glioblastoma)			
EG7 (Lymphoma)	24.5	70.5	0.3
RIF	52.7	34.7	1.5
(Fibrosarcoma)			
GL261-OVA	28.2	60.9	0.5
(Glioma)			

Discussion

Multiple sequential and competitive enzymatic pathways govern the synthesis of glycan structures of the cell surface and secreted molecules [35,36]. The glycans change dynamically responding to small variation in the extracellular environment and intracellular events [37]. Changes in glycan structures, generally but not uniformly, correlate with alteration in transcript abundant for the corresponding biosynthetic enzymes [37]. The present study was aimed to find any distinct variation and correlation in the expression of GalNAc-Ts in conjunction with other glycosyltransferases-activities associated with O-glycan biosynthesis among various cancers by examining a wide range of human cancer cell lines.

Specificities of GalNAc-T

The present study showed in cancer cells that Galβ1-3GalNAc (T-hapten) and 4-FGlcNAc do not serve as acceptors for GalNAc-T but the enzymatic transfer of GalNAc to LacNAc occurs to some extent indicating the possible minor extension of LacNAc chain by GalNAc. GlcNAcβ1-4GlcNAc was shown as a poor acceptor for GalNAc-T due to the fact that β1-4GalNAc transfer is not favored at all due to the structural specificity related constraint as evident from the structure of polylactosamine containing alternate β1-3 and β1-4 linkages. Further, GlcNAcβ1-4GlcNAcdid not accept more GalNAc by the action of β1-3GalNAc-T which is present predominantly in some cancer cell lines as compared to other cancer cell lines (Table 2). It appears that internal βlinked GlcNAc in general inhibits enzymatic transfer of GalNAc to terminal GlcNAc. While GlcNAcβ-(O)-Bn could act as an acceptor for both β1-3 and β1-4 GalNAc transferases, GlcNAcβ1-6GalNAcβ-(O)-Bn could be a favorable acceptor for β1-4GalNAc-T as evident from the known structure of mucin core2

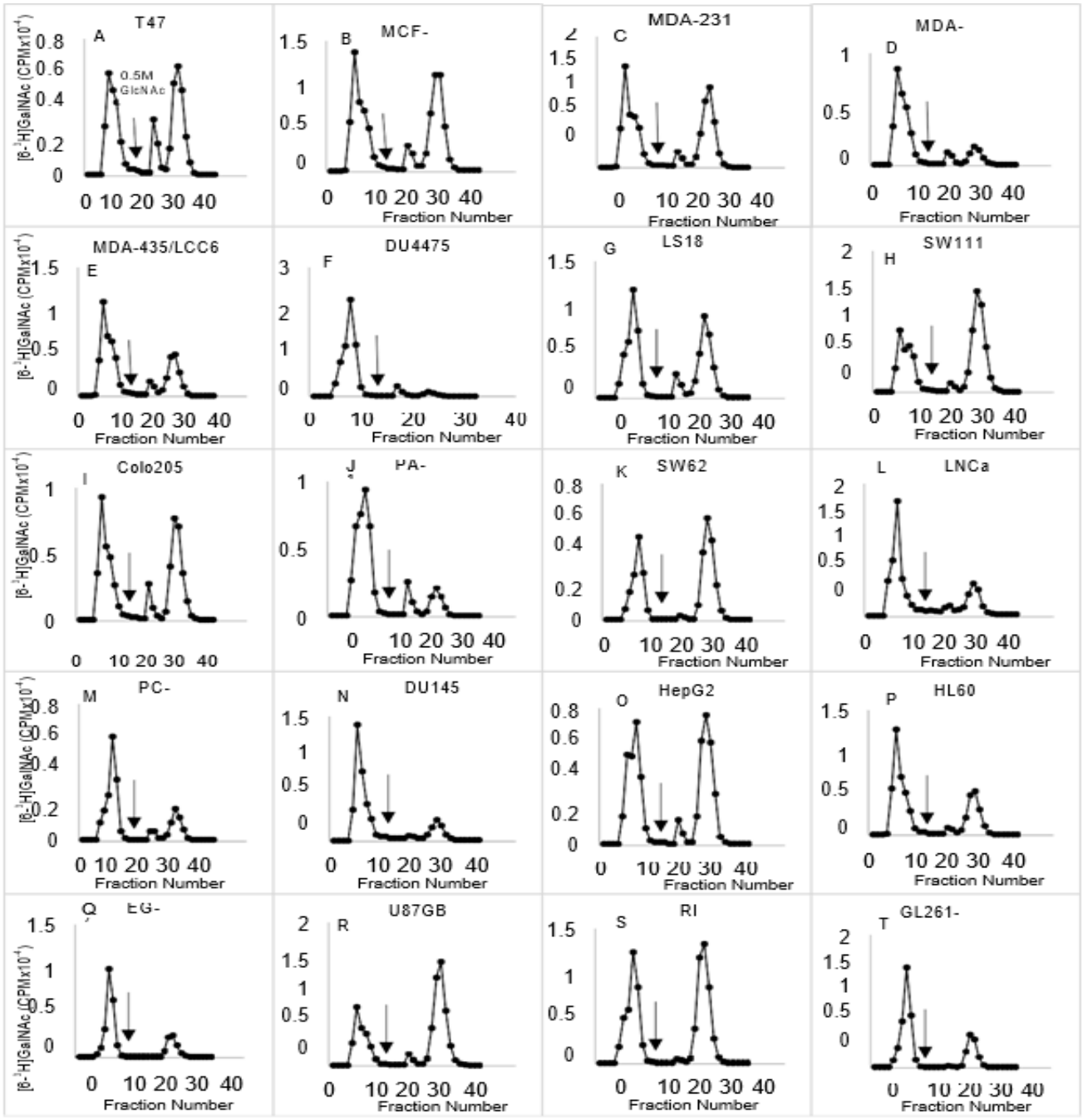

Figure 8: VVL-agarose affinity chromatography of [6-^{3}H] GalNAc-yl products from GlcNAcβ-O-Bn.
A) T47D; B) MCF-7; C) MDA-MB-231; D) MDA-MB-435 S; E) MDA-MB-435/LCC6; F) DU4475; G) LS180; H) SW1116; I) Colo 205; J) PA-1; K) SW 626; L) LNCaP; M) PC-3; N) DU145; O) HepG2; P) HL60; Q) EG-7; R) U87GB; S) RIF; T) GL261-OVA.

tetrasaccharide namely Galβ1-4GlcNAcβ1-6 (Galβ1-3) GalNAcα-, where Gal is attached β1-4 to GlcNAc.

Breast cancer cells: The enzymatic transfer of 1.9-8.8 fold of GalNAc to endogenous Ser/Thr containing polypeptides as compared to βGalNAc transfer to exogenous acceptor GlcNAcβ1-6(Galβ1-3) GalNAcα-O-Al would indicate both the high activity of αGalNAc-T and predominant occurrence of Ser/Thr containing polypeptides in breast cancer cells. DU4475 and MDA-MB-435S were unique among breast cancer cells by expressing a high level of GlcNAc: β1-3GalNAc-T as compared to β1-4GalNAc-T activity. Very low level of α2-3(O)sialyltransferase activity was present in DU4475 (0.9) whereas ZR-75-1 (36.8), MDA-MB-435S (25.7) and MDA-MB-435/LCC6 (17.2) had high level of this enzyme activity. αGalNAc: β1-3Gal-T activity in DU4475 was considerably high (4.7) among breast cancer cell lines. In this context it is to be noted that DU4475 was derived from breast cancer metastatic cutaneous nodule whereas

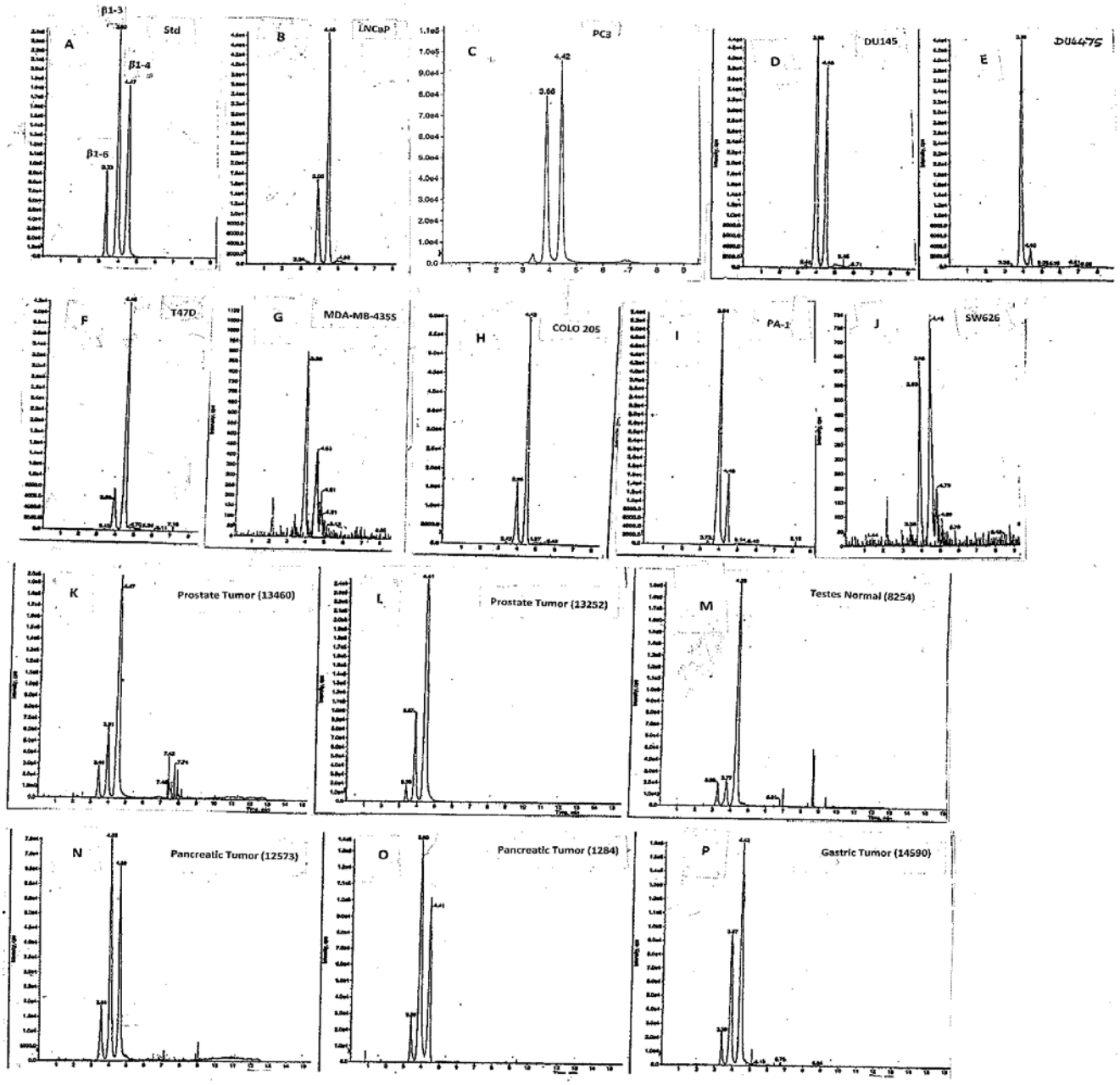

Figure 9: HPLC separation of GalNAcβ1-3 GlcNAc β-O-Bn, GalNAcβ1-4 GlcNAc β-O-Bn and GalNAcβ1-6 GlcNAc β-O-Bn formed from GlcNAc β-O-Bn by βGalNAc-T present in cancer cells and tissue specimens.
A) Separation of standards [6-^{3}H] GalNAcβ1-3 GlcNAcβ-O-Bn, [6-^{3}H] GalNAcβ1-4 GlcNAcβ-O-Bn and
[6-^{3}H] GalNAcβ1-6 GlcNAcβ-O-Bn; B) LNCaP; C) PC3; D) DU145; E) DU4475; F) T47D; G) MDA-MB-435S; H) Colo205; I) PA-1; J) SW626; K) Prostate tumor (13460); L) Prostate tumor (13252); M) Testes normal (8254); N) Pancreatic tumor (12573); O) Pancreatic tumor (1284); P) Gastric tumor (14590).

other breast cancer cells were established by cultivation of cells from the pleural effusion of breast cancer patients. MDA-MB-435S which is not tumorigenic in athymic nude mice had low β1-3Gal-T (1.1) and β1-4Gal-T (8.0) activities in contrast to high activities of these enzymes (4.5 and 40.0) in MDA-MB-435/LCC6, which grow as both malignant ascites and solid tumors *in vivo* in nude mice and nude rats.

Further it is interesting to note that MDA-MB-435/LCC6 exhibited more αGalNAc transfer than MDA-MB435S (12.5 *vs.* 7.7). Thus, it is conceivable that in general, breast cancer cells exhibit a significant elevation in the levels of Ser/Thr:αGalNAc-T, αGalNAc:β1-3Gal-T, GlcNAc:β1-4Gal-T, neutralβ1-3 galactosidase converting T to Tn and decreased level of α2-3(O)ST.

Colon cancer cells: Although all colon cancer cells showed similar levels of βGalNAc-T activity, only SW1116 contained a high level of αGalNAc-T activity (15.0; Figure 10C) and highly heterogenous Ser/Thr containing polypeptides (Figure 3J). The levels of β1-3Gal-T, α2-3(O)ST and β1-4Gal-T as well as neutral β1-3 galactosidase activities

Table 8: Thin layer chromatography of [6-3H] GalNAc-Yl products from Galβ1-3 (GlcNAcβ1-6) GalNAcα- O-Al (Biogel P2 Peak II Fraction).

20	478	211	201	61	20	98	87	111	445	20	131	94
19	458	205	364	103	19	129	75	114	512	19	221	155
18	342	196	268	85	18	95	105	139	437	18	253	167
17	258	184	202	113	17	116	91	125	313	17	282	217
16	326	185	214	91	16	113	112	121	424	16	252	238
15	320	194	207	142	15	138	116	109	460	15	260	230
14	368	243	226	139	14	98	148	143	375	14	285	250
13	941	328	240	205	13	130	200	123	3274	13	243	311
C2 12	3568	6234	875	601	12	215	2524	1857	7803	12	762	274
11	3087	7580	3542	2781	11	115	3485	1699	1440	11	2524	546
10	397	1392	2131	336	10	92	431	283	372	10	412	1516
9	298	271	239	136	9	99	152	153	384	9	201	441
8	3357	461	569	135	8	839	389	1536	2936	8	765	203
C1 7	6594	938	4308	242	7	2304	1521	7213	3786	7	10227	1283
6	1409	373	2304	131	6	346	379	1867	688	6	3797	2740
5	312	204	272	97	5	110	150	188	283	5	442	471
4	446	298	277	220	4	148	206	212	314	4	321	291
3	275	225	151	107	3	117	207	125	236	3	331	215
2	222	180	115	109	2	76	100	73	219	2	136	176
1	552	259	162	175	1	88	170	90	347	1	159	446
	PA-1	SW626	ZR-75-1	T47D		HL60	HepG2	Colo205	SW1116		LNCaP	LS180
C1%	59.9	10.4	52.3	12		88.4	25.6	73.4	38.4		80.5	64.2
C2%	40.1	89.6	47.7	88		11.6	74.4	26.6	61.6		19.5	35.8
PNA- NB%	53.2	94.9	55.5	98.7		20.3	80	29.3	70.1		29.7	33

A) PA-1; B) SW626; C) ZR-75-1; D) T47D; E) HL60; F) HepG2; G) Colo205; H) SW1116; I) LNCaP; J) LS180.

(6.2, 9.6, 50.3 and 0.42) respectively were also higher when compared to these activities in Colo205 and LS180 (Figure 10B and 10C). Thus, some colon cancer cells express higher activities of αGalNAc-T, β1-3Gal-T, β1-4Gal-T, and α2-3(O)ST and highly heterogenous Ser/Thr polypeptides.

Ovarian cancer cells: SW626 contained quite heterogenous Ser/Thr bearing polypeptides (Figure 3L) and higher βGalNAc-T activity (Figure 10A) whereas PA1 had higher αGalNAc-T activity and larger molecular size Ser/Thr polypeptides (Figure 3K). Further β1-3GalNAc activity was higher (3 fold) with respect to β1-4 GalNAc-T activity in PA1 (Figure 10A). PA1 had much lower β1-3Gal-T, β1-4Gal-T, and neutral β1-3 galactosidase activities (Figure 10B and 10C). Both SW626 and PA1 expressed very low level of α2-3(O)ST (Figure 10C). It is apparent that the expression of β1- 3Gal-T, β1-4Gal-T, neutral β1-3galactosidase and α2-3(O)ST may vary in ovarian cancer cells.

Prostate cancer cells: These cells expressed predominantly higher β1-3 GalNAc-T activity as compared to other cancer cell lines with exception of DU4475 and MDA-MB-435S (Figure 10A). Neutral β1-3 galactosidase activity was low in LNCaP. β1-3Gal-T and β1-4Gal-T activities were high in DU145. While α2-3(O)ST activity was at high level in LNCaP (62.3) and PC3 (80.8) with respect to other cancer cell lines, it was found extremely low (0.7) in DU145 (Figure 10C). It seems that prostate cancer cells can be expected to contain a high level of β1-3GalNAc-T, α2- 3(O)ST and αGalNAc-T and most likely a low level of β1-3galactosidase activity.

HL60, HepG2 and other cell lines: HepG2 as compared to HL60 expressed higher levels of GlcNAc: βGalNAc-T (2 fold), neutral β1-3 galactosidase (4 fold), β1-3Gal-T (>3 fold), α2-3(O)ST (>3 fold) and β1-4Gal-T (2 fold) (Figure 10). HL60 had more heterogenous Ser/Thr containing polypeptides whereas the molecular size of these peptides was distinctively larger in HepG2 (Figure 3M and 3N). U87GB and RIF expressed 4.9 and 1.5 fold whereas EG7 and G261-OVA had 0.3 and 0.5 fold β1-4GalNAc-T as compared to β1-3GalNAc-T. Hepatic cancer cells HepG2 express high levels of neutral β1-3galactosidase, β1-3Gal-T, β1-4Gal-T and α2-3(O)ST and high molecular size Ser/Thr polypeptides. Lymphoma cells (EG7) and Glioma (GL261-OVA) express significantly very high activity of β1-3GalNAc-T as compared to Glioblastoma (U87GB) and Fibrosarcoma (RIF).

The HPLC analysis of [6-^{3}H] GalNAc-yl products arising from GlcNAcβ-O-Bn by the action of βGalNAc-T from nine cancer cell lines and six tissue specimens showed that GlcNAc:β1-3GalNAc-T activities in prostate cancer cells LNcaP, PC3 and DU145, breast cancer cells DU4475 and MDA-MB-475S, ovarian cancer cells PA-1 and SW626 and pancreatic tumor specimens were present at higher level than β1-4GalNAc-T.

Neutral β1-3galactosidase converting T-glycotope to Tn in cancer cells

Several human cells express a β-galactosidase histochemically

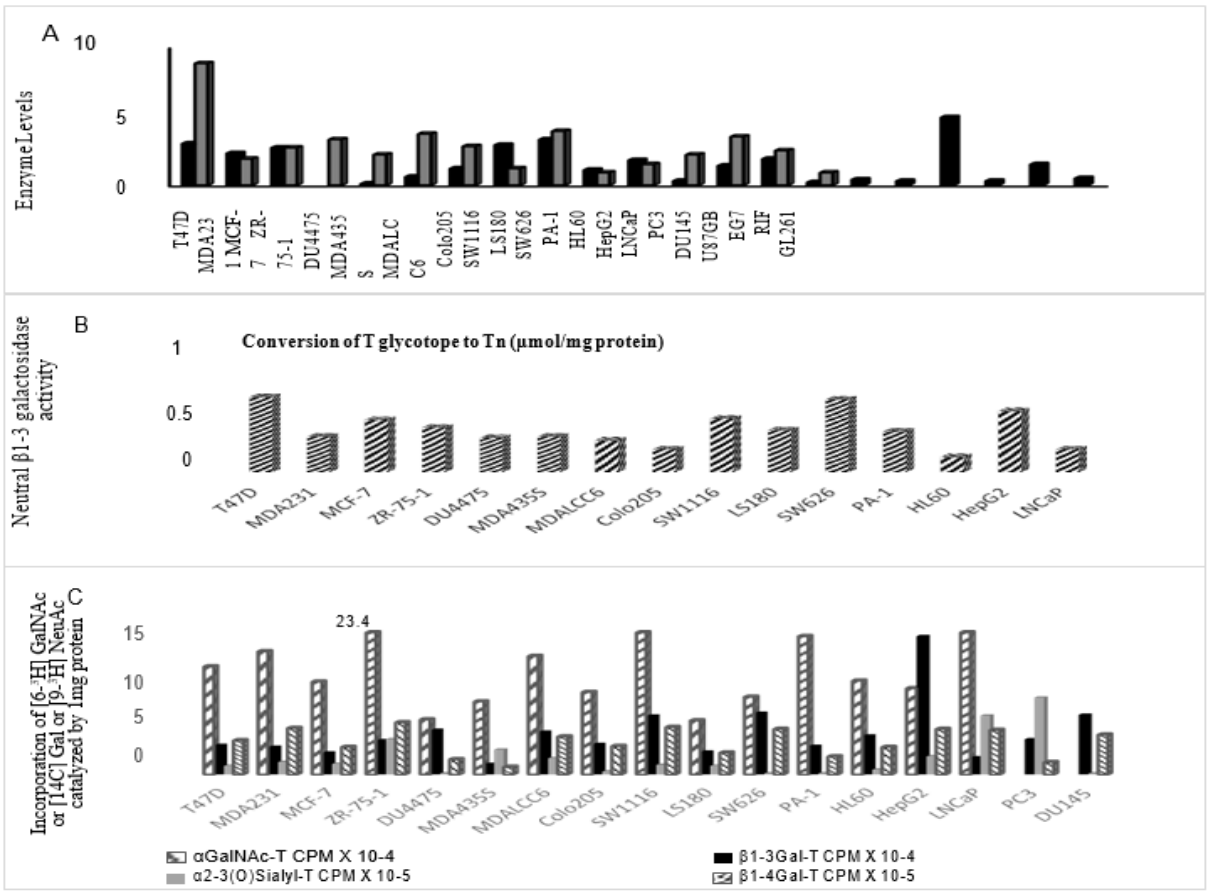

Figure 10: The levels of α and β N-Acetylgalactosaminyltransferases and neutral β1-3Galactosidase in human cancer cell lines.
Panel A: The fold of GlcNAc: β1-4GalNAc-T activity with respect to GlcNAc: β1-3GalNAc-T activity by measuring the activities with the acceptor GlcNAcβ-O-Bn followed by WGA-agarose and VVL-agarose affinity cheomatography. The fold of endogenous acceptor polypeptide Ser/Thr: αGalNAc-T activity with respect to GlcNAc: β1-3/4 activity by using GlcNAcβ1-6 (Galβ1-3) GalNAcα-O-Al as exogenous acceptor and followed by Biogel P2 column chromatographic separation and quantitation of Peak I and Peak II Franctions.
Panel B: For calculating the conversion of T-glycotope to Tn by neutral β1-3 galactosidase activity in cancer cell lines, the percentage of PNA-agarose non-binding fractions were used.
Panel C: A comparison of the levels of αGalNAc-T ( ), αGalNAc: β1-3 Gal-T ( ), α2-3 (O) ST ( ), GlcNAc: β1-4 Gal-T ( ) in human cancer cell lines. For a meaningful understanding of the participating glycosyltransferases in O-glycans biosynthesis, the data obtained by using the same batch of cells on α2- 3 (O) ST, αGalNAc: β1-3Gal-T and GlcNAc: β1-4Gal-T were used from earlier publications.

detectable at pH 6 upon senescence in culture. In skin samples from human donors of different age, there was an age-dependent increase in this marker in dermal fibroblasts and epidermal keratinocytes. Thus β-galactosidase is a bio marker that identifies senescent human cells in culture and in aging skin *in vivo* [38]. As senescent cells expressed 3-5-fold lysosomal β-galactosidase mRNA, there is a possibility of the pH optimum of lysosomal β-galactosidase being altered due to localization. Lysosomal β- galactosidase cleaves β-linked terminal galactosyl residues from gangliosides, glycoproteins and glycosaminoglycans. Mutations in the β-galactosidase locus at chromosome 3 cause deficient or reduced enzyme activity [39].

The studies of βgalactosidases in senescent human cells [38], rat mammary gland [40], rat milk [40], bovine testes [41], bovine liver [42], human liver [43] and ovarian tumor tissue cells [44,45] indicate a lysosomal origin of β galactosidase with an optimum pH 4.0 and most of them are quite inactive at neutral pH. The present study used the [6-^{3}H] GalNAc containing product from the enzymatic transfer of [6-^{3}H] GalNAc from UDP [6-^{3}H] GalNAc (0.2µmol) to the GlcNAc moiety of the exogenous acceptor namely GlcNAcβ1-6 (Galβ1-3) GalNAcα-(O)-Al (3µmol in the reaction mixture) as the radioactive tracer to show the hydrolysis of β1-3 linked Gal by a β- galactosidase at neutral pH. The cleavage of β1-3Gal was determined by the separation of binding and non-binding radioactive products on PNA-agarose column as well as by the separation and quantitation of the two radioactive products using TLC. The study indicated that neutral β-galactosidase activity is significantly high in human cancer cell lines. There is also a distinctive variation in the level of this activity among the cancer cell lines. All breast cancer cells and HepG2 exhibited a high level of this activity whereas this activity varied among the other cell lines. HL60 (lymphoma) and LNCaP (prostate cancer) contained lower β-galactosidase activity. Apparently, the occurrence of β1-3 galactosidase active at neutral pH for generating Tn epitope from T in cancer cell lines may have some biological significance.

Significance of β-galactosidase

The biological significance of β-galactosidase became apparent when Dimri et al. [38] described the pH6 β- galactosidase activity in human fibroblasts as a senescence-marker and designated it as SA-β-Gal. Subsequently SA- β-Gal was identified in human umbilical vein [46], fibroblasts from venous ulcers [47] and ovarian epithelial cells [48]. Field et al. [48] demonstrated that the lysosomal β-Gal at pH4.5 showed ~ 4-fold and 25-fold greater activity than at pH6 and 7, respectively. Then it was shown that β-Gal activity decreased in a similar fashion with increase in pH both in liver homogenates of different age donors and in tumor cell lysates suggesting that pH6 β-Gal activity may not be an exclusive marker of senescence [50]. Long-term cultivation of Caco2-TC7 cells showed no specific association between cultivation time (cellular aging) and pH6 β-Gal activity as pH 4.5 β-Gal exhibited the same pattern of increased activity [50]. The values for the β-Gal activity were reported as 115 and 185 nmol/mg protein at pH 4.5 for HL60 and HepG2 and at pH6 32 and 47 nmol/mg protein, respectively. It is interesting to note that the present study found the neutral β1-3 galactosidase activity as 120 and 480 nmol/mg protein for HL60 and HepG2, respectively. A recent study on human peritoneal mesothelial cells (HPMC) supports the theory that SA-β-Gal detectable at pH 6 is a reliable marker of senescence by showing that cytochemical and fluorescent methods of SA- β-Gal assessment may be informative for replication-driven cell senescence *in vitro* and time-dependent organismal aging *in vivo* respectively [51]. Another recent study reports that elevated levels of HGF (Hepatocyte growth factor) and GRO-1 (Growth-regulated oncogene) in ovarian cancer malignant ascitic fluid induced senescence in HPMC as assessed by SA-β-Gal and contribute to ovarian cancer progression [52]. A correlation between the activities of lysosomal β-galactosidase, senescence associated pH6 β-Galactosidase and the cancer cell neutral β galactosidase identified in the present study remains to be seen.

Our earlier studies have well documented that α2-3(O)ST activity is the most predominant sialyltransferase activity that accounts for 70-90% of the total sialylating activity in breast, colon and prostate cancer cell lines and also in tumor tissues of several cancers [7,8]. We found earlier that this enzyme (ST3 Gal II) has reversible sialylation activity by converting 5′ CMP and also 5′ UMP to 5′CMP- and 5′UMP-NeuAc by utilizing the donor NeuAcα2- 3Galβ1-3GalNAcα-units [53-55]. We also found highly significant level of the reversible sialylation activity in prostate cancer cells LNCaP and PC-3 [53]. We also determined that when CMPNeuAc (5mM) was incubated in 50mM Na Cacodylate pH6 containing 2% Triton CF54 and 10mg BSA/ml at 37°C for 4h, there was 30% breakdown of CMPNeuAc into CMP and NeuAc and thus 5′CMP becomes available for reverse sialylation [53].

Further it was shown that 5′UMPNeuAc is an inactive sialyl donor for the sialylation of glycans by α2-3(O)ST, α2- 3(N)ST and α2-6(N) ST [53]. 5′UMP is apparently an efficient trap for sialic acid leading to depletion of glycan sialylation. Thus, the reversible sialylation activity in conjunction with sialidase and neutral β1-3 galactosidase activity could generate Tn epitopes bearing polypeptides which apparently participate in cancer progression and metastasis. The extracellular sialylation in human plasma could then be responsible for the appearance of sialyl Tn epitope [56,57].

Significance of Type-I and Type-II LDN structures

The biological significance of Type-I and Type-II LDN structures is evident from the expression of these structures on different glycoproteins, indicating their unique functions *in vivo* [15]. Most of Type-I LDN glycoproteins are localized to intra cellular organelles, particularly to the endoplasmic reticulum whereas Type-II LDN glycoproteins are extracellular [15]. Type-I LDN structure found in O-mannose glycans on α-dystroglycan is a key structure in laminin-binding glycans [58]. Sulfated Type-II LDN plays essential roles in the regulation of circulatory half-life of pituitary glycoprotein hormones [59]. We reported earlier that the B2 ion mass spectroscopy technique could be used to differentiate Type-I and Type-II LDN structures [60]. The present study showed the separation of the isomers GalNAcβ1-3GlcNAcβ-O-Bn, GalNAcβ1-4GlcNAcβ-O-Bn and GalNAcβ1-6GlcNAcβ-O-Bn by HPLC. Further, the present study found that both Type-I and Type-II LDN structures bind to WFL-agarose whereas the weak and tight binding of Type-I and Type-II LDN products from the acceptor GlcNAcβ-O-Bn to VVL- and WGA-agarose respectively can be used for measuring β1-3 and β1-4 GalNAc-T activities in cancer cell extracts. In this context, it is important to note that the evaluation of glycosyltransferase activities is essential due to the fact that the relation between gene expression and glycosyltransferase activities is commonly not uniform and often strikingly non-linear [61,62]. The affinity chromatography on VVL- and WGA-agarose could be further developed for the isolation and identification of Type-I and Type-II LDN terminal glycans and glycoproteins.

Acknowledgement

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