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Glycosylation of the two *O*-glycosylated domains of human MUC2 mucin in patients transposed with artificial urinary bladders constructed from proximal colonic tissue

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Abstract Transposition of intestinal segments is frequently used for bladder reconstruction. Following transposition, bowel segments continue to produce mucus and a correlation between excessive mucus production and complications such as urinary tract infection or catheter blockage has been observed for a long time. However, no information is currently available on the change of mucin expression and glycosylation under these abnormal conditions. In this study, the variable number tandem repeat region and the irregular repeat domain of human MUC2 were isolated as two glycopeptide populations after reduction and trypsin digestion followed by gel chromatography from urine of patients transposed with urinary bladders. After alkaline borohydride treatment, the oligosaccharides released from the whole MUC2 mucin and the two glycosylated domains were investigated by nanoESI Q-TOF MS/MS (electrospray ionization quadrupole time-of-flight tandem mass spectrometry). More than 60 different glycans were identified, mainly based on sialylated core 3 structures. Some core 1, 2 and 4 oligosaccharides were also found. Most of the structures were acidic with NeuAc residues mainly α2–6 linked to the *N*-acetylgalactosaminitol and sulphate residues exclusively 3-linked to galactose. No expression of blood group A and B or Sda/Cad determinants was observed. Similar patterns of glycosylation were found in the tandem repeat region and the irregular repeat domain and the level of expression of the major oligosaccharides were in the same order of magnitude. The most interesting feature of this study was that sialyl-Tn antigen, which is considered as a tumour antigen, was the oligosaccharide most highly expressed. This result suggests that mucins from intestinal transposed segments are abnormally glycosylated.

Keywords MUC2 · Glycosylation · Mass spectrometry · Artificial urinary bladder

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Introduction

Mucins are large *O*-glycosylated glycoproteins with a long linear protein core and numerous carbohydrate chains along the molecule. The extreme length of these molecules, their high degree of glycosylation (more than 50% of the total weight) and their negative charge are major determinants of their ability to form gels as well as the physical properties of mucus. A distinguishing feature of mucins is that they contain *O*-linked oligosaccharides, *i.e.* the sugar chains are attached to the peptide backbone via an *O*-glycosidic linkage between a serine or threonine residue and a GalNAc residue. These *O*-glycans have many diverse functions in biological systems. They are aiding in the conformation and stability of proteins, they protect sensitive domains from proteolytic attack. They play a major role in defense of mucosae covering and protecting epithelium against various types of aggression.

As opposed to the en bloc transfer of the high-mannose oligosaccharides involved in *N*-glycosylation, *O*-glycosylation is a stepwise process including one monosaccharide at a time. The addition of GalNAc to serine and threonine residues is what governs the site-specificity, and this process is mediated by at least 14 different pp-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc Ts) [1]. Although no consensus sequence has been formulated, many studies have noted the skew in amino acid composition around mucin-type *O*-glycosylation sites ([2–5] for example) with a higher frequency of prolines, serines, threonines and alanines. A number of studies have investigated the effect of flanking residues in in vitro experiments on synthetic peptides [6–8] and especially the importance of prolines at certain positions has been confirmed. It is now well accepted that mucin-type glycosylation at multisite substrates proceeds in a hierarchical manner and is created by the successive action of several ppGalNAc Ts on a single polypeptide substrate [9–10]. Depending on the initial and subsequent substitutions on the GalNAc residue, a wide range of *O*-linked core structures is possible. Little, however, is known of the effects of peptide sequence on overall glycosylation of a particular mucin, although numerous *in vitro* and *in vivo* studies have demonstrated a strong influence of the amino acid sequence and neighbouring glycosylation on the initiation of glycosylation [11–19].

MUC2 is the main gel-forming mucin of the small and large intestine and is produced by the intestinal goblet cells [20–22]. This mucin is a major structural component of the mucus barrier covering the intestinal surface, protecting the epithelial cells from noxious agents such as micro-organisms and digestive enzymes. The MUC2 protein contains more than 5,100 amino acid residues in its most common allelic form [21, 23]. There are two central repetitive regions rich in potential *O*-glycosylation sites. The longer one is referred to as the variable number tandem repeat (VNTR) region and is composed largely of tandemly repeated 23 amino acid peptide units (consensus sequence: PTTTPITTTTTVTP TPTPTGTQT), which vary in number between alleles and are rich in threonine and proline [24–25].The shorter domain comprises a 347-amino acid-long irregular repeat rich in threonine, serine and proline, and is separated from the tandem repeat region by a 148 amino acid segment. MUC2 also contains four cysteine-rich D-domains with a high degree of sequence homology with the four D-domains of prepro-von Willebrand factor.

MUC2 is also strongly expressed in the transposed intestinal segments [26]. Transposition of intestinal segments to reconstruct or replace the bladder is commonly used in urological surgery for patients suffering from bladder cancer, but also for benign aetiology such as chronic inflammatory diseases (interstitial cystitis, tuberculosis), neurogenic bladder dysfunction and detrussor overactivity. However, the use of bowel segments is not without complications. The quality of life of these patients is affected by recurrent urinary tract infections, stone formation, excessive mucus production and an increased risk of colonic neoplasia leading to malignancy [27–29]. Indeed, many studies have demonstrated that in artificial urinary bladder, secretomotor function is maintained in the long term with mucus production and problems related to its excessive production are not decreasing with time [30–34]. Over the past 15 years, the proportion of patients receiving a neobladder has increased significantly and it becomes necessary to characterize the changes in mucin expression and glycosylation in transposed segments to better understand the relation structure/function of these proteins under abnormal conditions.

Here, we have analyzed the glycosylation of the entire MUC2 mucin comprising the two major glycosylated domains, *i.e.* the VNTR and the irregular repeat regions that have different amino acid sequences. The results reveal an important degree of glycosylation heterogeneity in the two domains which together with an overall very similar pattern and distribution of glycans suggest that oligosaccharide structure in MUC2 mucins is not strongly influenced by the peptide core. Moreover, in comparison with structures of mucin *O*-glycans found in human gut [35–39], this study demonstrates that mucins from intestinal transposed segments are abnormally glycosylated.

Materials and methods

Isolation and purification of MUC2 subunits Mucus secretions were obtained from patients having a cancer in the urinary bladder and transposed with an artificial urinary bladder constructed from proximal colonic tissue. MUC2 was collected from urine of these patients [22, 40] and purified as described previously [22]. Briefly, frozen samples were thawed and the mucus gel collected by high speed centrifugation (Beckman J2.MC centrifuge; JA rotor; 18,000 rpm; 10°C; 60 min). The mucus gel was gently dispersed with a Dounce homogenizer in 6 M guanidinium chloride, 1 mM diisopropylphosphofluoride, 5 mM *N*-ethylmaleimide, 10 mM sodium phosphate buffer pH 7.0 and left stirring at 4°C overnight. After centrifugation, as above, the soluble material was removed and the pellet re-extracted twice, as above. The final extraction residue (insoluble glycoprotein complex) was solubilized by reduction in 6 M guanidinium chloride, 10 mM dithiotreitol, 5 mM EDTA, 0,1 mM TRIS/HCl buffer pH 8.0 at 37°C overnight and alkylated with 25 mM iodoacetamide in the same buffer. After centrifugation, the supernatant was retained, dialyzed against 6 M guanidinium chloride and

subjected to isopycnic density gradient centrifugation in 4 M guanidinium chloride/CsCl (Beckman optima L70 ultracentrifuge, 15°C, 90 h in a 50.2 rotor at 36,000 rpm, starting density 1.37 g/ml). Fractions were collected from the bottom of the tube and analysed for density, absorbance at 280 nm, sialic acid, and MUC2 using anti-MUC2 antibodies.

Isolation of the tandem repeat region and the irregular repeat domain Mucin subunits obtained using density gradient centrifugation were pooled, dialysed against 0.1 M NH_4HCO_3, pH 8.0, treated with trypsin (50 μg) overnight at 37°C, and freeze-dried. Tryptic glycopeptides were dissolved in 4 M guanidinium chloride, 10 mM sodium phosphate buffer pH 7.0 and subjected to gel chromatography on a Sephacryl S-500 HR column (1.6×50 cm), eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions were collected and analysed for sialic acid which displayed two major peaks A and B corresponding to the tandem repeat region and the irregular repeat domain respectively, as described previously [22]. The two peaks were pooled, dialyzed against water and lyophilised.

Release of oligosaccharide alditols by alkaline borohydride treatment MUC2 mucin subunits and the two glycosylated domains were submitted to β-elimination under reductive conditions (0.1 M KOH, 1 M KBH_4) for 24 h at 45°C [41]. The oligosaccharide alditol mixture was purified by gel chromatography on a Bio-Gel P2 column (85×2 cm ID, 400 mesh, Bio-Rad, Richmond, CA) equilibrated and eluted with water, at 10 ml/h at room temperature. The oligosaccharide fractions, detected by UV absorption at 206 nm, were pooled for structural analysis.

Monosaccharide analysis The monosaccharide composition of the native mucins and oligosaccharide alditol fractions were determined by gas chromatography (GC) on a Shimadzu gas chromatograph equipped with a 25 m× 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ullis, France) after methanolysis (0.5 M HCl-methanol for 24 h at 80°C), *N*-reacetylation and trimethylsilylation [42–43].

Fractionation of the oligosaccharide alditols by HPLC The oligosaccharide alditols released from each glycosylated domain and from MUC2 mucin subunits were subjected to fractionation by HPLC (Dionex Chromeleon System, Sunnyvale, CA, USA) on a primary amino-bonded silica column (Supelcosyl, LC-NH_2, 4.6×250 mm, Supelco, Bellefonte, CA, USA). The column was equilibrated with the initial solvent using a mixture of acetonitrile/ H_2PO_4K 30 mM/ H_2O (80:0:20, by vol.) with a flow rate of 1 ml/min. After the injection, a linear gradient to (50: 50:0, by vol.) for 90 min was applied followed by isocratic conditions for 30 min. Oligosaccharides were detected by UV spectroscopy at 200 nm using an UVD 170U detector (Dionex, Sunnyvale, CA, USA).

MALDI-MS All mass spectra were acquired on a Voyager Elite (DE-STR) reflectron time-of-flight (TOF) mass spectrometer (Perseptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. Oligosaccharide samples were analysed in delayed extraction mode using an accelerating voltage of 20 kV, a pulse delay time of 200 ns, and a grid voltage of 66%. Detector bias gating was used to reduce the ion current for masses below 500 Da. Between 100 and 200 scans were averaged for each mass spectrum. Oligosaccharide alditols were co-crystallized with 2,5-dihydroxybenzoic acid as matrix (10 mg/ml 2,5-dihydroxybenzoic acid in methanol/water [50/50] containing 0.1% trifluoroacetic acid supplemented with 5 mM NaCl). For all measurements, the "dried droplet" preparation technique was used. Typically, 1 μl of the matrix was mixed on target with 1 μl of water-dissolved oligosaccharides and allowed to dry under an air stream. Analysis was performed in both positive and negative ion modes.

Electrospray mass spectrometry (nanoESI-MS/MS) All analyses were performed on a Q-STAR Pulsar quadrupole time-of-flight (Q-q-TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) fitted with a nanoelectrospray ion source (Protana, Odense, Denmark). Oligosaccharides dissolved in water (60 pmol/μl) were acidified by addition of an equal volume of methanol/0.1% formic acid and sprayed from a gold-coated "medium length" borosilicate capillaries (Protana). A potential of −800 V was applied to the capillary tip and the focusing potential was set at −100 V, the declustering potential varying between −60 and −110 V. For the recording of conventional mass spectra, time-of-flight data were acquired by accumulation of 10 MCA (multiple channel acquisition) scans over mass ranges of *m*/*z* 400–2,500. In the collision-induced dissociation (CID) tandem MS analyses, multiple charged ions were fragmented using nitrogen as collision gas (5.3×10^{-5} Torr), the collision energy varying between −30 and −90 eV to obtain optimal fragmentation. The CID spectra were recorded on the orthogonal TOF analyzer over a range of *m*/*z* 80–2,500. Data acquisition was optimized to supply the highest possible resolution and the best signal-to-noise ratio even in the case of low abundance signals. Typically, the full width at half maximum (FWHM) was 7,000 in the measured mass ranges. External calibration was performed prior to each measure using a 4 pmol/μl solution of taurocholic acid in acetonitrile/water (50:50) containing 2 mM of ammonium acetate.

Results

Monosaccharide composition of the MUC2 subunits (T), the tandem repeat region (A) and the irregular repeat domain (B) Mucus secretions were obtained from the urine of patients with artificial urinary bladders constructed from proximal colonic tissue [40]. After purification steps, MUC2 mucin subunits were subjected to trypsin digestion followed by gel chromatography and two major peaks (A and B) were detected with the sialic acid assay. As described in a previous paper [22], based on the amino acid compositions and the relative sizes of glycopeptides A and B, it was concluded that they strongly corresponded to the VNTR region and the irregular repeat domain respectively.

Purified MUC2 mucin subunits were analysed for their carbohydrate composition (Table 1). Little mannose (less than 5% of the total monosaccharides) was found, suggesting that only few *N*-linked oligosaccharides occur. It should be noted that *N*-linked oligosaccharides are not usually released by the alkaline conditions used here suggesting that the mannose detected most likely represents unreleased *N*-linked glycopeptides remaining after the cation-exchange step.

The recovery of saccharides after release from the protein backbone was estimated based on monosaccharide analyses of the whole mucin and of the released fraction. The yield was around 60% for each sample analysed. This is a typical yield for this procedure.

As shown in Table 1, the monosaccharide composition of the MUC2 subunits, the tandem repeat region and the irregular repeat domain are similar, except for the molar ratio of NeuAc in VNTR. This result is in keeping with a similar oligosaccharide substitution and is in agreement with previous studies [22].

Similar glycosylation patterns for MUC2 subunits, the tandem repeat region and the irregular repeat domain After the desalting step, oligosaccharide mixtures isolated from MUC2 mucin as well as the two glycosylated domains (A and B), were analysed directly by MALDI-TOF and nanoESI Q-TOF tandem mass spectrometry, without prior derivatization or fractionation step [44]. Fragment annotations applied in this study were based upon the suggested nomenclature by Domon and Costello [45] and by Karlsson *et al.* [46]. The fragment ions obtained were mainly A_i, B_i, Y_j, Z_j ions. Moreover, an α suffix was used to designate cleavages in the 6-linked branch and a β suffix for cleavage in the 3-linked branch from the GalNAcol.

Figure 1 represents the MS spectra acquired in negative ion mode for the total oligosaccharide fractions from the three mucin populations. Most of the ions could be related to $[M-H]^-$ ions of the theoretical oligosaccharide structures. The spectra illustrate a remarkable structural diversity: the true heterogeneity is likely to be even larger, since a substantial number of the molecular ions may represent oligosaccharide species with isomeric structures. To avoid confusion, the mass values used in the text, Tables and illustrated fragmentations are mainly nominal masses. As shown in Tables 2 and 3 and Fig. 1, the pattern of glycosylation for the three samples was quite similar and showed significant heterogeneity with more than 60 different structures identified by MS analysis. As expected, many of them were acidic structures, carrying mainly NeuAc residues α2–6 linked to the initial GalNAc and also NeuAc and/or sulphate residues linked to peripheral galactose residues. Small amounts of core 1, core 2 and core 4 structures were detected. However, the main core structure in all samples was the sialylated core 3 [GlcNAcβ1–3(NeuAcα2–6)GalNAcol] as found in normal intestinal mucins [35–39].

Mass spectrometry is not a quantitative analytical method, because of the different ionization of different compounds. However, comparing the same relative amount of the same compound between samples is relatively reliable. As seen in Fig. 1, the relative intensities of mass signals obtained for the oligosaccharide mixtures from the

Table 1 Monosaccharide composition

	Fuc	Gal	Man	GlcNAc	GalNAc	NeuAc	GalNAcol
Native mucin populations							
T[a]	0.3	0.6	0.1	0.5	1.0	0.9	–
A[b]	0.4	0.5	0.1	0.5	1.0	0.4	–
B[c]	0.3	0.4	0.2	0.4	1.0	0.9	–
Released oligosaccharides							
T[d]	0.5	0.7	0.1	0.7	0.4	1.4	1.0
A	0.6	1.0	0.2	1.0	0.3	0.6	1.0
B	0.5	0.7	0.3	0.7	0.7	1.4	1.0

[a] MUC2 subunits (T), [b] tandem repeat region (A) and [c] irregular repeat domain (B). The molar ratio of the different monosaccharides was calculated on the basis of one GalNAc residue for the native mucins.
[d] The molar ratio of the different monosaccharides was calculated on the basis of one GalNAcol residue for the oligosaccharides released from T, A and B.

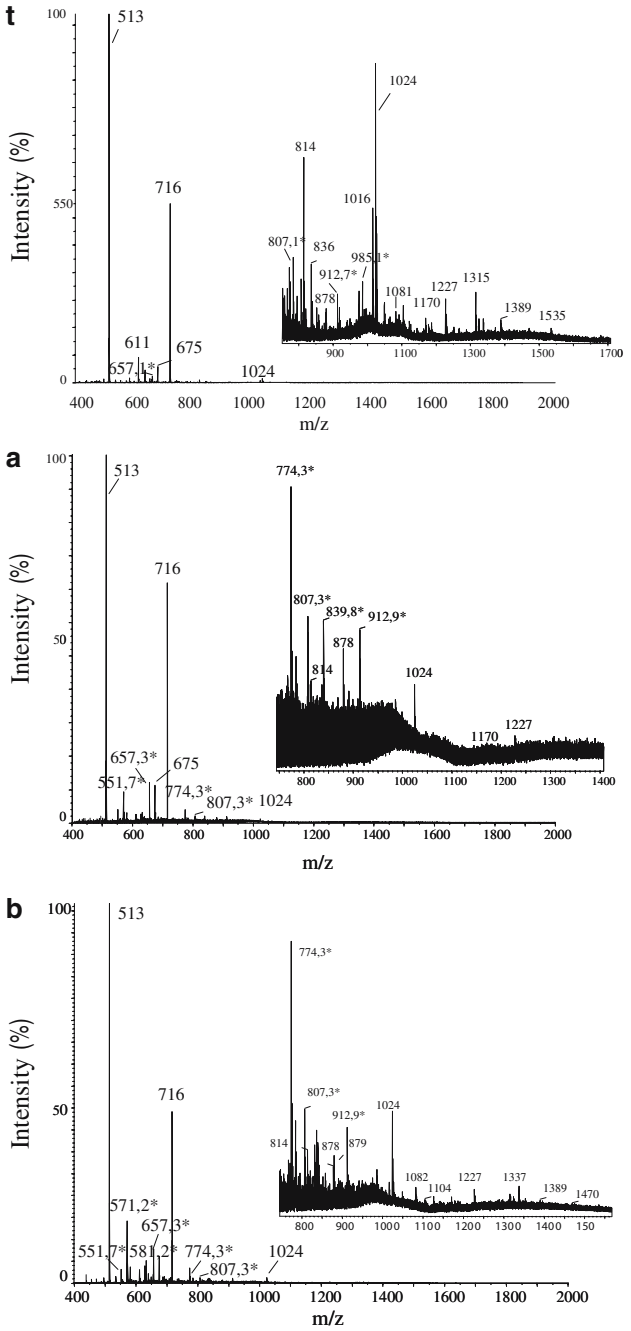

Fig. 1 Glycosylation pattern of the MUC2 mucin subunits (**t**), the tandem repeat region (**a**) and the irregular repeat domain (**b**). MS spectra of the total oligosaccharides acquired in the negative ion mode $[M-H]^-$. Signals marked with *asterisks* referred to dicharged ions $[M-2H]^{2-}$

three samples were in the same order of magnitude, suggesting that the level of expression of the major oligosaccharides is similar.

The main differences between the oligosaccharides isolated from MUC2 subunits, the tandem repeat region and the irregular repeat domain specify small structures carrying sulfoLewis X determinants (ions at *m/z* 813, 975 and 1,016 in the negative ion mode). Nevertheless, their neutral counterparts are recovered in the three spectra (ions at *m/z* 757, 919 and 960 in the positive ion mode).

HPLC fractionation of oligosaccharides released from MUC2 subunits, tandem repeat region and irregular repeat domain Oligosaccharides released from the MUC2 subunits, the tandem repeat region and the irregular repeat domain were fractionated by HPLC on a primary amino-bounded column (Fig. 2) and the fractions collected were analysed by nano-ESI Q-TOF MS (results not shown). Identification of the different fractions was also confirmed by comparing their retention times with those of standards

Table 2 Proposed neutral oligosaccharide structures identified in MUC2 by nanoESI MS/ MS in positive ion mode

Sequence /composition of oligosaccharide alditols[a]	$[M+Na]^+$	T[b]	A[c]	B[d]
Gal→3GalNAcol	408	+	+	+
GlcNAc→3GalNAcol	449	+	+	+
GalNAc→3GalNAcol	449	+	+	+
Gal→3GlcNAc→3GalNAcol	611	+	+	+
Gal→4GlcNAc→3GalNAcol	611	+	+	+
Gal→3*[GlcNAc→6]*GalNAcol	611	+	+	+
GlcNAc→3*[GlcNAc→6]*GalNAcol	652	+	+	+
Gal→3(Fuc→4)GlcNAc→3GalNAcol	757	+	+	+
GlcNAc→3(Fuc→2)Gal→3GalNAcol	757	+	+	+
GlcNAc→3*[Gal→4GlcNAc→6]* GalNAcol	814	+	−	−
Gal→4GlcNAc→3*[GlcNAc→6]* GalNAcol	814	+	−	−
HexNAc→Gal→4GlcNAc→3GalNAcol	814	+	−	−
(Fuc→2)Gal→4(Fuc→3) GlcNAc→3GalNAcol	903	+	+	+
(Fuc→2)Gal→3(Fuc→4) GlcNAc→3GalNAcol	903	+	+	+
(Fuc→)GlcNAc→3(Fuc→2) Gal→3GalNAcol	903	+	+	+
2 Gal, HexNAc, Fuc, GalNAcol	919	−	−	+
GlcNAc→3*[Gal→3(Fuc→4)GlcNAc→6]* GalNAcol	960	+	+	+
Gal→3[Fuc→4]GlcNAc→3*[GlcNAc→6]* GalNAcol	960	+	+	+
HexNAc→Gal→3[Fuc→4] GlcNAc→3GalNAcol	960	+	+	+
GlcNAc→3*[Fuc→2Gal→4(Fuc→3) GlcNAc→6]*GalNAcol	1,106	+	−	−
GlcNAc→3*[Fuc→2Gal→3(Fuc→4) GlcNAc→6]*GalNAcol	1,106	+	−	−
2 Gal, 2 HexNAc, Fuc, GalNAcol	1,122	−	+	−
2 Gal, HexNAc, 3 Fuc, GalNAcol	1,211	−	+	−
2 Gal, 2 HexNAc, 3 Fuc, GalNAcol	1,414	+	−	−

[a] The upper branch of the oligosaccharides is indicated in italic.
[b] *T* Structures recovered from MUC2 mucin subunits.
[c] *A* Structures recovered from tandem repeat region.
[d] *B* Structures recovered from irregular repeat domain.

Table 3 Proposed acidic oligosaccharide structures identified in MUC2 by nanoESI MS/MS

Sequence/composition of oligosaccharide alditols[a]	$[M+Na]^+$	$[M-H]^-$	T[b]	A[c]	B[d]
Oligosaccharides with one acidic residue					
*NeuAc→6*GalNAcol	537	513	+	+	+
Gal→3*[NeuAc→6]*GalNAcol	699	675	+	+	+
GlcNAc→3*[NeuAc→6]*GalNAcol	740	716	+	+	+
(SO_3^-)3Gal→4(Fuc→3)GlcNAc→3GalNAcol	–	813	+	–	–
Fuc→2Gal→3*[NeuAc→6]*GalNAcol	–	821	+	–	+
Gal→4GlcNAc→3*[NeuAc→6]*GalNAcol	902	878	+	+	+
Gal→3GlcNAc→3*[NeuAc→6]*GalNAcol	902	878	+	+	+
GlcNAc→3Gal→3*[NeuAc→6]*GalNAcol	902	878	+	+	+
Gal→3*[(SO_3^-)3Gal→4(Fuc→3)GlcNAc→6]*GalNAcol	–	975	+	–	+
(SO_3^-)3Gal→4(Fuc→3)GlcNAc→3Gal→3GalNAcol	–	975	+	–	+
GlcNAc→3*[(SO_3^-)3Gal→3(Fuc→4)GlcNAc→6]*GalNAcol	–	1,016	+	–	+
GlcNAc→3*[(SO_3^-)3Gal→4(Fuc→3)GlcNAc→6[*GalNAcol	–	1,016	+	–	+
GlcNAc→3*[Fuc→2Gal→4 (SO_3^-)6GlcNAc→6[*GalNAcol	–	1,016	+	–	+
Gal→4(Fuc→3)GlcNAc→3*[NeuAc→6]*GalNAcol	1,048	1,024	+	+	+
Gal→3(Fuc→4)GlcNAc→3*[NeuAc→6]*GalNAcol	1,048	1,024	+	+	+
(Fuc→)GlcNAc→3(Fuc→2)Gal→3*[NeuAc→6]*GalNAcol	1,194	1,170	+	–	+
(Fuc→2)Gal→4(Fuc→3)GlcNAc→3*[NeuAc→6]*GalNAcol	1,194	1,170	+	–	+
(Fuc→2)Gal→3(Fuc→4)GlcNAc→3*[NeuAc→6]*GalNAcol	1,194	1,170	+	–	+
HexNAc→(Fuc→2)Gal→4GlcNAc→3*[NeuAc→6]*GalNAcol	1,251	1,227	+	+	+
(SO_3^-)3Gal→4(Fuc→3)GlcNAc→3*[Fuc→2Gal→4GlcNAc→6]*GalNAcol	–	1,324	+	+	+
Gal→GlcNAc→(Fuc→2)Gal→GlcNAc→3*[NeuAc→6]*GalNAcol	–	1,389	+	+	–
2 Gal, 2 HexNAc, 3 Fuc, SO_3^-, GalNAcol	–	1,470	+	+	+
Gal→GlcNAc→(Fuc→2)Gal→(Fuc→)GlcNAc→3*[NeuAc→6]*GalNAcol	–	1,535	+	+	+
2 Gal, 2 HexNAc, 2 Fuc, NeuAc, GalNAcol	1,559	–	+	+	–
Fuc→2Gal→(Fuc→)GlcNAc→(Fuc→2)Gal→(Fuc→)GlcNAc→3*[NeuAc→6]*GalNAcol	–	1,827	+	+	+
Oligosaccharides with two or three acidic residues					
(SO_3^-)3Gal→4(Fuc→3)GlcNAc→3*[NeuAc→6]*GalNAcol	–	1,104	+	+	+
(NeuAc→3)Gal→4(Fuc→3)GlcNAc→3*[NeuAc→6]*GalNAcol	1,339	1,315	+	+	+
(SO_3^-)3Gal→(Fuc→)GlcNAc→3Gal→4(Fuc→3)GlcNAc→3*[NeuAc→6]*GalNAcol	–	1,615	+	+	+
2 Gal, 2 HexNAc, Fuc, 2 NeuAc, GalNAcol	–	1,680	+	+	+
2 Gal, 2 HexNAc, Fuc, 2 NeuAc, SO_3^-, GalNAcol	–	1,760	+	+	+
2 Gal, 2 HexNAc, Fuc, 3 NeuAc, GalNAcol	–	1,971	–	–	+

[a] The upper branch of the oligosaccharides is indicated in italic
[b] *T* Structures recovered from MUC2 mucin subunits
[c] *A* Structures recovered from tandem repeat region
[d] *B* Structures recovered from irregular repeat domain

previously characterized by NMR [39]. This was particularly important to differentiate for example between core 5 (GalNAcα1–3GalNAcol) and core 3 sialylated glycans at *m/z* 716. These studies were useful for the determination of the relative abundance of major oligosaccharides in the sample. Detection of glycans by UV at 200 nm is not as sensitive as MS and weakly expressed oligosaccharides cannot be identified. As shown in Table 4, relative proportions of the main structures detected in each sample were very similar, confirming results obtained by mass spectrometry (Fig. 1). Moreover, this work demonstrates that the two major oligosaccharides expressed on MUC2 mucin isolated from patients with artificial urinary bladders were sialyl-Tn antigen (around 50% of the recovered glycans) and the sialylated core 3 glycans: GlcNAcβ1–3 [NeuAc α2–6]GalNAcol (around 20%).

Structural characterization of oligosaccharides The structures of both neutral and acidic oligosaccharides are shown in Tables 2 and 3. Sequencing of oligosaccharides was acquired in both positive and negative ion modes to obtain complete elucidation of the structure for each mucin oligosaccharide. Positive ion mode is useful for the determination of the core-type oligosaccharide and the position of fucose, whereas negative ion mode produces characteristic cross-ring cleavages necessary for differentiation between isomers and identification of NeuAc (*N*-acetylneuraminate) and sulphate substitution. A series of diagnostic ions were used to determine the linkages between monosaccharides and between monosaccharide/sulphate residues [47]. The ions at *m/z* 306 and 513 are specific for NeuAcα2–6 linked to GalNAcol, whereas the ions at *m/z* 408 or 611 are characteristic of NeuAcα2–3 linked to a Gal residue. The

fragment ion at *m/z* 199 is a diagnostic ion for locating the sulphate group to C-4 or C-6 of GalNAc, Gal or GlcNAc but not to the C-3 of Gal or GlcNAc. A specific $^{0,2}A_i$ cleavage with concomitant loss of water from a GlcNAc substituted on C-4 allowed to differentiate between type 1 (Galβ1–3GlcNAc) and type 2 (Galβ1–4GlcNAc) chains. Some characteristic ions also allowed assigning unambiguously the presence of certain terminal structures such as the Lewis determinants and the Sda/Cad antigens. However, in complex oligosaccharides, diagnostic ions are sometimes of lower intensity, rendering the complete elucidation of the structure difficult or impossible.

Fucosylated glycans Fucose was present in a large variety of terminal linkages (Tables 2 and 3) that included blood group H_1 epitopes (Fucα1–2Galβ1–3GlcNAc), as well as Le^y: (Fucα1–2Galβ1–4[Fucα1–3]GlcNAc), Le^b (Fucα1–2Galβ1–3[Fucα1–4]GlcNAc) and Le^x (Galβ1–4)[Fucα1–3]GlcNAc) determinants.

An example of the interpretation of mass spectra is described in Fig. 3, where a mass spectrum of a tetrafucosylated decasaccharide at *m/z* 1,827 is shown. It should be noted that all the glycans with the same elongated core but with a different peripheral backbone (one fucose residue corresponding to the ion at *m/z* 1,389, two Fuc at *m/z* 1,535 and three Fuc at *m/z* 1,681) were identified in MUC2 mucin subunits. The ion at *m/z* 513 is diagnostic of a NeuAc α 2–6 linked to the GalNAcol and the ion at *m/z* 716 indicates a sialylated core 3 (GlcNAcβ1–3[NeuAc α2–6]GalNAcol). The series of Y_i ions at *m/z* 571 (Fucα1-GlcNAcβ1–3GalNAcol), 733 (Galβ1-[Fucα1-]GlcNAcβ1–3GalNAcol), 1,082 (Fucα1-GlcNAcβ1–3Galβ1-[Fucα1-]GlcNAcβ1–3GalNAcol) and 1,244 (Galβ1-[Fucα1-]GlcNAcβ1–3Galβ1-[Fucα1-]GlcNAcβ1–3GalNAcol) allow reconstruction of the sequence of the molecule, consisting of subsequent addition of fucosylated Gal and GlcNAc. This sequence is also confirmed by the series of fragment ions still carrying the NeuAc residue, at *m/z* 1,024 (Galβ1-[Fucα1-]GlcNAcβ1–3[NeuAc α2–6]GalNAcol), 1,373 (Fucα1-GlcNAcβ1–3Galβ1-[Fucα1-]GlcNAcβ1–3[NeuAc α2–6]GalNAcol) and 1,535 (Galβ1-[Fucα1-] GlcNAcβ1–3Galβ1-[Fucα1-]GlcNAcβ1–3[NeuAcα2–6]GalNAcol). No information on the type of chains (type 1 or 2) could be deduced because of the size of the molecule.

Acidic glycans Most of oligosaccharides identified in the three samples carried acidic components with NeuAc and/or sulphate residues, in agreement with previous studies published on human colonic mucins [35–39]. NeuAc residues were α2–6 linked to the GalNAcol and were easily identified in the spectra by their diagnostic ions at *m/z* 290 (B_1 fragment ion) and 513 ($Y_{1\beta}$ fragment ion). Only one structure carried a NeuAcα2–3 linked to a Gal residue. No glycans with blood group Sda/Cad antigen were recovered in these mucins.

An interesting feature of these mucins is that the oligosaccharide with the higher level of expression was the sialyl-Tn antigen (ion at *m/z* 513 in the MS spectra). This result was quite surprising since aberrant expression of sialyl Tn antigen has been widely associated with several epithelial cancers [48–50]. Moreover, such an expression of the sialyl-Tn glycan was not found in normal human mucin isolated from proximal colon [39] suggesting that MUC2 mucin in secretions from patients with artificial urinary bladders is abnormally glycosylated.

Sulphate residues were mainly found 3-linked to galactose (diagnostic ions at *m/z* 241), only one structure was recovered carrying a sulphate linked to a GlcNAc residue (diagnostic ion at *m/z* 282). Some differences occur between the MUC2 subunits, the tandem repeat region and the irregular repeat domain. These differences concern mainly oligosaccharides carrying sulfo Le^x determinants.

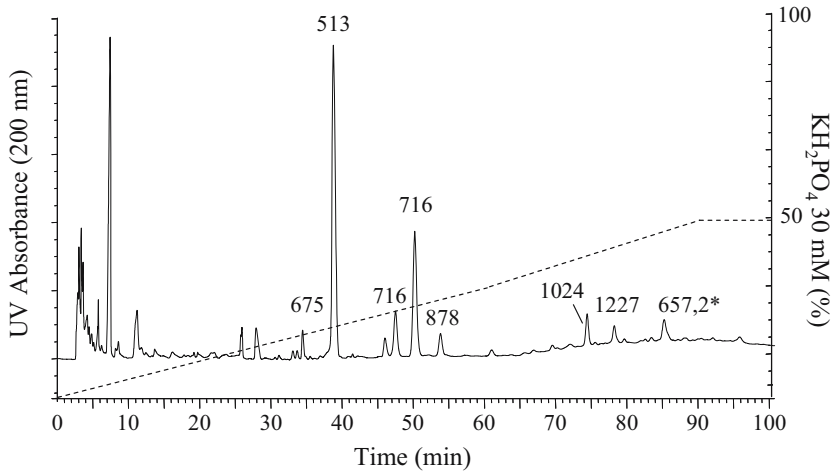

Fig. 2 HPLC profile of the MUC2 subunit oligosaccharide alditols on a primary amino-bonded column eluted with gradient described under "Materials and methods". Fractions were identified by their $[M-H]^-$ ion, fraction marked with asterisk referred to dicharged ions $[M-2H]^{2-}$

Table 4 Estimated percentage of major oligosaccharides recovered by HPLC

m/z [a]	$[M-H]^-$	$[M+Na]^+$	T[b]	A[c]	B[d]
425[e]	425	449	n.d.	1	n.d.
513	513	537	48.5	42	53
551[h]	1,104	–	1	1	1
587	587	611	1	n.d.	1
613[h]	1,227	1,251	2.5	1	2
657[h]	1,315	1,339	5	5	4.5
675	675	699	2	3.5	2
716[f]	716	740	7	7.5	4.5
716[g]	716	740	21	21.5	17.5
878	878	902	3	3	2.5
1,024	1,024	1,048	4	4	6

[a] The HPLC fractions were identified by their corresponding *m/z* ions to allow comparison with the nanoESI mass spectra
n.d. Non-determined.
[b] *T* Structures recovered from MUC2 mucin subunits
[c] *A* Structures recovered from tandem repeat region
[d] *B* Structures recovered from irregular repeat domain
[e] Ion at *m/z* 425 corresponding to both neutral core 3 and core 5 structures
[f] Ion at *m/z* 716 corresponding to a sialylated core 5
[g] Ion at m/z 716 corresponding to a sialylated core 3
[h] These ions referred to dicharged ions $[M-2H]^{2-}$

For example, Fig. 4 shows the MS/MS spectrum of the ion at *m/z* 1,016, corresponding to a sulphated pentasaccharide found only in irregular repeat domain and whole MUC2 mucin. The core 4 was easily identified on the fragmentation spectrum by the presence of the two Y_1 ions: the $Y_{1\beta}$ fragment ion at *m/z* 813 indicated that a branch consisting of Gal, GlcNAc, Fuc and a sulphate group was linked to the GalNAcol, whereas the $Y_1\alpha$ fragment ion at *m/z* 425 indicated that another branch comprised a residue of GlcNAc linked to the GalNAcol. The $^4A_0\alpha$ fragment ion at *m/z* 650 allowed the differentiation between the 6-branch and the 3-branch of the GalNAcol. Moreover, the series of $B_{i\alpha}/Y_{1\beta}$ ions was indicative of the sequence of the 6-branch of the GalNAcol, consisting of SO_3-Gal (ion at *m/z* 241) linked to a fucosylated GlcNAc residue (ions at *m/z* 444 and 590). Finally, cross-ring fragment ions $^{0.2}A_\alpha/Y_{2\alpha}$" at *m/z* 361 and 343 (loss of water) were diagnostic of a β1–4 linkage between Gal and GlcNAc residues, confirming the presence of a Le^x determinant on this glycan.

Discussion

After trypsin digestion and gel chromatography, two major glycopeptide populations (A and B) are obtained from the whole human MUC2 mucin, corresponding to the VNTR region and the irregular repeat domain. Human colonic MUC2 subunits, the tandem repeat region and the irregular repeat domain isolated from the same preparation were used in this study in order to determine the glycosylation repertoire of mucins produced following transposition of intestinal segments into the urinary tract. Using MALDI-MS and nano-ESI Q-TOF MS/MS techniques, purified oligosaccharides released from the three samples were structurally characterized and the pattern of glycosylation was compared. The oligosaccharide repertoire of each fraction comprises a complex mixture of at least 60 neutral

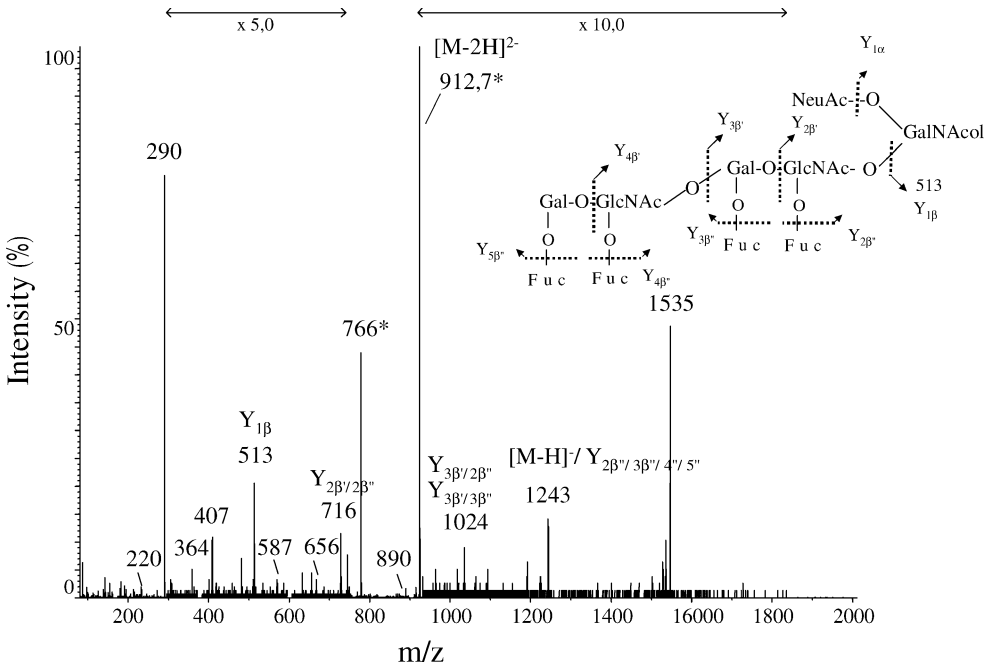

Fig. 3 MS/MS spectrum, recorded in the negative ion mode, of the dicharged ion ($[M-2H]^{2-}$ corresponding to the oligosaccharide at *m/z* 1,827

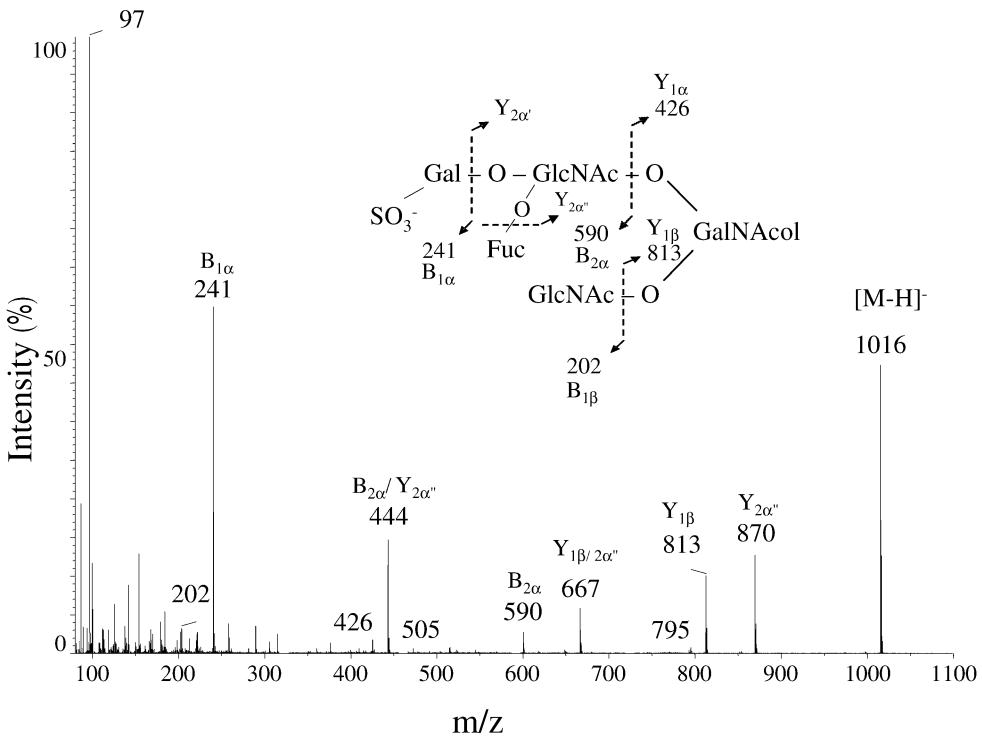

Fig. 4 MS/MS spectrum recorded in the negative ion mode ([M−H]$^-$ ions) and proposed fragmentation of the oligosaccharide at *m/z* 1,016

and acid-containing species. This analysis shows that some of the oligosaccharides are not present in all of the variable number tandem repeat region and the irregular repeat domain, however the distribution of the individual oligosaccharide species in MUC2 and in the glycopeptides are very similar. Moreover, the level of expression of major oligosaccharides is limited. We found no obvious differences in the abundance of the core structures in the three samples, indicating a similar regulation of the initial oligosaccharide biosynthesis. Altogether, our results seem to indicate that the amino acid sequence and/or neighbouring glycosylation do not affect the elongation of *O*-glycans in the two repetitive regions of MUC2 mucins. The repertoire of glycosyltransferases available in each cell or tissue, their localization in the Golgi, their relative activities in each subcompartment and their spatial arrangements in the Golgi membrane certainly seem to be important parameters regulating *O*-glycan elongation and termination. However the density and structures of each *O*-glycosylation site have not been characterized in this study and maybe that ppGalNAc Ts, which transfer a GalNAc to Ser/Thr residues, are differently regulated by the amino acid sequences of the two *O*-glycosylated domains of human MUC2 mucin.

The main differences between oligosaccharides from the tandem repeat region and the irregular repeat domain (and thus also the entire MUC2) are the sulphated oligosaccharides, particularly the sulpho-Le^x determinants (ions at *m/z* 813, 975 and 1,016 in the negative ion mode). However the neutral counterparts (ions at *m/z* 757, 919 and 960 in the MS spectra recorded in positive ion mode) are recovered in this population. Because bacteriuria, including the growth of uropathogenic strains, is favoured by the incorporation of intestinal segments into the urinary tract [30, 51–52], the absence of sulpho-Le^x epitopes in the VNTR of MUC2 may be explained by the degradation by sulphatases originating from bacterial contamination. Or the sulphotransferases are not able to efficiently transfer a sulphate group on the peripheral Gal residue in this domain.

As described previously for human colonic mucins [35–39], most of the glycans identified in this study were based on a core 3 structure, with a sialic acid α2–6 linked to the GalNAcol. Some core 1, 2, 4 and 5 oligosaccharides were also found, in agreement with previous studies. Most of the oligosaccharides presented have been described before in human intestine. Some differences occur between the present study and the other ones [37–39], concerning mainly acidic structures. Moreover, the estimated percentage of major oligosaccharides recovered after HPLC fractionation showed that more than 90% of the glycans are acidic whereas in proximal colonic mucins [39], only 70% were acidic. Two main differences were observed when comparing the studies, one concerning the neutral oligosaccharide at *m/z* 587 (1 HexNAc, 1 Hex and 1 GalNAcol): around 1% in the present work, versus 20% of total glycosylation in the caecum of adult intestinal mucin. The second glycan concerned was the sialyl-Tn antigen (NeuAc α2–6 GalNAcol) at *m/z* 513: around 50% here, versus 2% in the last study [39].

Several hypotheses may explain these contrasting results. First, the present study concerns only glycosylation of MUC2 mucin, whereas other works corresponded to glycans isolated from mucosal scrapings of the human intestine with MUC2 as the major mucin but also other MUC products such as MUC1 and MUC3, however the most likely explanation being the different sample origins. Indeed, in the present work, MUC2 has been isolated from patients with an artificial urinary bladder constructed from proximal colonic tissue. Profound changes occur in transposed segments, such as an adaptation to a different role of protection rather than absorption. Because patients lacked many of the antibacterial defenses, most of them had a urinary tract colonized with aerobic, anaerobic bacteria and carried uropathogenic strains. Considerable number of data have indicated a correlation between expression of certain glycans and bacterial colonization. This may explain the modification of expression of some oligosaccharides observed here. Moreover, the location of the intestinal mucosa in the bladder, exposed to urine, may lead to changes in glycosylation pattern of mucins. When an intestinal segment is interposed in the urinary tract, the flux of substances across the mucosa gives the urine a composition differing from that in the intact bladder and a higher level of the pH. Changes in pH may induce modification in the repertoire of oligosaccharides, as suggested by Campbell *et al.* [53]. Indeed, they have shown that intra-Golgi alkalinization affects mucin glycosylation, resulting in decreased mucin sulphation and increased expression of Thomsen–Friedenreich antigen (Galβ1–3GalNAc).

Another difference observed in the glycosylation of MUC2 compared with human colonic mucins concerned the expression of Sda/Cad blood group antigens. Such structures were not found on MUC2 mucins. As mentioned above, MUC2 mucins were collected from secretions of mucus produced by proximal colonic tissue after transposition into the urinary tract, or Sda/Cad antigens are mainly expressed in the distal part of the large intestine [37–39]. Moreover, a change in the microflora colonization [54] in such constructions may explain differences in the expression of these antigens. The GalNAc β1–4 transferase producing Sda from NeuAcα2–3Galβ1–4GlcNAc precursor may be negatively regulated as a consequence of adaptation with the bacterial strains present in these intestinal segments. Malagolini *et al.* [55] postulated for example that reduced susceptibility to Escherichia coli infection has been the selective agent responsible for the dominant expression of this glycosyltransferase.

The sialyl-Tn antigen (ion at *m*/*z* 513 in MS spectra recorded in the negative ion mode) was the major oligosaccharide expressed in MUC2 mucin. The sialyl-Tn epitope is one of the most specific tumour associated antigens described so far. It is not strictly tumor-specific since it is found in a variety of normal tissues either at an intracellular level, at cell surfaces or on secreted mucins. However, its expression is largely increased in many types of cancer including colorectal carcinomas and urinary bladder [56]. Based on these considerations, its high expression in this study may be a direct consequence of the transposition, and may reflect one of the complications observed during transposed intestinal segment surgery: the risk of developing cancer as a direct consequence of this type of surgery. This is in agreement with previous studies [26, 57]. The high expression of sialyl-Tn antigen may also be due to the new environment and the exposure to urine.

In conclusion, this study has demonstrated that mucin *O*-glycans from neobladder constructed with proximal colonic tissue are differently glycosylated than *O*-glycans found in human intestine. Moreover the two *O*-glycosylated domains of MUC2 present a similar pattern of glycosylation, suggesting that elongation of glycans is not strongly influenced by the amino acid sequence. In a future work, it would be interesting to examine both colonic and transposed samples of the same patients to determine the impact of the different environments that may induce glycosyltransferases to synthesize different structures.

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