Differential expression patterns of N-acetylglucosaminyl transferases and polylactosamines in uterine lesions

A.T.R. Clark,[1]
V.M.L. Guimarães da Costa,[1]
L. Bandeira Costa,[1]
C.L. Bezerra Cavalcanti,[1]
M.J.B. de Melo Rêgo,[1,2]
E.I.C. Beltrão[1,3]

[1]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife
[2]Núcleo para Inovação Terapêutica, Universidade Federal de Pernambuco, Recife
[3]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife-PE, Brazil

Abstract

Polylactosamine (polyLacNAc) is a fundamental structure in glycoconjugates and it is expressed in specific cells/tissues associated with the development and carcinogenesis. β1,3-*N*-acetylglucosaminyl transferases (β3GnTs) play an important role in polyLacNAc synthesis, however the roles of these glycosyltransferases and their products in cancer progression are still unclear. In this sense, this work aimed to evaluate differential expression pattern of the N-acetylglucosaminyl transferases and polylactosamines in invasive and premalignant lesions of the uterus cervix. The expression of β3GnT2 and β3GnT3 were evaluated in normal (n=10) and uterine cervix lesions (n=120), both malignant [squamous carcinoma (SC)] and premalignant [cervical intraepithelial neoplasia (CIN), grades 1, 2 and 3] using immunohistochemistry. Besides, lectin histochemistry with *Phytolacca americana* lectin (PWM) and *Wheat germ* agglutinin (WGA) was also carried out to observe the presence of polyLacNAc chains and *N*-acetylglucosamine (GlcNAc), respectively. The β3GnT3 was expressed in almost all samples (99%) and β3GnT2 was higher expressed in disease samples mainly in CIN 3, when compared with normal (P=0.002), CIN 1 (P=0.009) and CIN 2 (P=0.03). The expression of polyLacNAc was higher is SC samples, when compared with normal (P=0.03), CIN 1 (P=0.02) and CIN 3 (P=0.004), and was observed only nuclear expression in nearly 50% of the SC samples, showing a statistically significant when compared with normal (P=0.01), CIN 1 (P=0.002), CIN 2 (P=0.007) and CIN 3 (P=0.04). Deferring from transferases and polyLacNAc chains, GlcNAc (WGA ligand) reveals a gradual staining pattern decrease with the increase of the lesion degree, being more expressed in CIN 1 lesions when compared with normal (P<0.0001), CIN 2 (P<0.0001), SC (P<0.0001) and CIN 3 (P=0.0003). Our data reveal that β3GnT2 and polyLacNAc may be involved in the progression of the pre-malignant lesions of the human uterine cervix. In addition, polyLacNAc expression only in the nucleus can be associated a poor prognostic in uterine lesions.

Correspondence: Eduardo Isidoro Carneiro Beltrão, Departamento de Bioquímica and Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife-PE, 50670-901, Brazil.
Tel. +55.81.21268484 - Fax: +55.81.21268485.
E-mail: ebeltrao@hotmail.com.br

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Introduction

Squamous carcinomas (SC) of the cervix usually is one of the most common malignancies in women worldwide[1] and arises from metaplastic squamous mucosa in the region of the transformation zone;[2] it is preceded by a long phase for pre-invasive disease, called CIN (cervical intraepithelial neoplasia).[2,3] Traditionally, CIN are graded as CIN 1, CIN 2 and CIN 3, depending on the degree of differentiation.[3] An accurate diagnosis of premalignant lesions is important to clinical management, but the histological diagnosis of CIN can be complicate.[3] Studies of cell surface carbohydrates in these lesions are scarce and they can be useful for understanding the development of this type of lesion and its early diagnosis. Human cells are covered with a dense and complex array of glycoconjugates (glycoproteins, glycolipids and proteoglycans), that differs between cell types; for example, glycosylation of a single cell type significantly changes during cell development and differentiation.[4-7]

Glycosylation is one of the most common post-translational modifications in eukaryotic cells.[8-10] These glycosylated molecules are involved in a wide variety of biological events, such as cell activation,[11] differentiation,[7,12] infection,[13] cell-cell, receptor-ligand, and carbohydrate-carbohydrate interactions.[4,14,15] Furthermore, cell malignant transformations are often associated with structural alteration of carbohydrate chains in glycoconjugates,[5-7,14] and they may be directly or indirectly involved in cancer progression and malignancy.[5,6,14] The polyLacNAc structure is a linear glycan containing repeats of the *N*-acetyllactosamine (LacNAc) unit (Galβ1-4GlcNAcβ1-3)*n*. It is a fundamental. structure of the carbohydrate chains in glycoproteins and glycolipids,[4,16,17] being expressed in specific cells/tissues associated with development and cell-recognition, binding to several endogenous lectins.[16] In cancer, polyLacNAc and related structures play important roles in cell-cell and cell-matrix interaction, determining metastatic capacity.[4,17,18] Thus, the investigation of their presence may be useful for understanding the importance of polyLacNAc in cancer lesion.

PolyLacNAc and GlcNAc can be identified in tissues by *Phytolacca americana* (PWM), lectin which binds with high affinity to polyLacNAc glycans bearing three or more linear *N*-acetyllactosamine repeats, and from *Wheat germ* agglutinin (WGA), respectively. Lectins have long been used as tools to characterize cell surface glycans because of their substantial selectivity in terms of branching, linkage and terminal modifications of complex glycans.[6,19] The structure of these glycans depends on the glycosylation enzymes, glycosidases and glycosyltransferases, and the presence of appropriate sugar donors and receptors in the endoplasmic reticulum and Golgi apparatus of the eukaryotic cell,[8,10,16], and they are very complex.[5,20]. A key enzyme in this process is β1,3-*N*-acetylglucosaminyltransferases (β3GnTs) that transfers an *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a galactose on the nonreducing end of the carbohydrate chain in a β1,3-linkage.[15,21,22]. Eight β3GnTs, β3GnT1 to β3GnT8, have been isolated, and their *in vitro* activities have been characterized.[4,15-17,22-24]. However, the roles of these multiple β3GnTs on *in vivo* polylactosamine (polyLacNAc) synthesis are still unclear.

This study aimed to investigate the expression of *N*-acetylglucosaminyl transferases

(β3GnT2 and β3GnT3), the expression of polyLacNAc (one of their possible products), to evaluate the polyLacNAc structure in transformed uterine cervix tissues and visualize possible changes in the expression according to neoplasia grade.

Materials and Methods

Materials

Trypsin (T1005), streptavidin-peroxidase polymer (S2438), neuraminidase (N2133), horseradish peroxidase (P8375), lectin from *Phytolacca americana* (PWM) (L9379), *Wheat germ* agglutinin (WGA) biotin conjugated (L5141), *N*-acetyl-D-glucosamine (A8625) and antibodies included rabbit anti-B3GNT2 (HPA005897) and anti-B3GNT3 (HPA024298) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The visualization system, Advance™ HRP (K406889-2), and the chromogenic substrate (K346811-2), liquid diaminobenzidine (DAB) and substrate, were obtained from Dako (Glostrup, Denmark). Entellan® (107960) was purchased from Merck (Whitehouse Station, NJ, USA).

Samples

Malignant and premalignant lesions of uterine cervix were obtained from the Tissue Bank from Hospital das Clínicas at the Universidade Federal de Pernambuco, Northeast Brazil, after approval by the Health Science Ethical Committee from the same university (CEP/CCS/UFPE Nº 195/09). One hundred and thirty formalin-fixed and paraffin-embedded cases were collected and grouped according to their histopathological classification. In this sense, 120 samples of transformed tissues diagnosed as CIN grades 1 (n=30), 2 (n=30), 3 (n=30) and squamous carcinoma (SC, n=30). Ten samples of normal epithelium were also analyzed. Patients' age at diagnosis time ranged between 23-83 year-old (mean 48.86±14.78) to squamous-cell carcinoma; 24-89 year-old (mean 40.79±14.93) to CIN 3; 25-89 year-old (mean 38.10±15) to CIN 2 and 18-46 year-old (mean 33.08±8.75) to CIN 1. Normal tissues samples were obtained from patients with 28-68 year-old age range (mean 40.77±12.23).

Immunohistochemistry

Tissue sections (4 µm) were deparaffinized in xylene and hydrated in ethanol (100%-70%), treated with 10% (v/v) ammonium hydroxide solution for 10 min at 25ºC and with 10 mM citrate buffer pH 6.0 for 30 min at 100ºC on a steamer chamber.[25,26]. Afterwards, samples were treated with a 0.3% (v/v) H_2O_2-methanol-solution for 15 min at 25ºC, blocked with 1% (w/v) bovine serum albumin (BSA) solution in PBS (100 mM phosphate-buffered saline pH 7.2, containing 150 mM NaCl), for 1h at 25ºC. Thereafter, primary antibodies were diluted in 1% BSA-PBS (B3GnT2 1:50 and B3GnT3 1:100) and incubated for 2h at 37ºC. Incubation with Advance™ HRP link (Dako) was performed for 45 min, followed by Advance™ HRP Enzime (Dako) for 45 min both at 25°C, as a biotin-free polymer method. Peroxidase reaction was revealed with 3,3'-diaminobenzidine (Dako) and sections were counterstained with Harry's hematoxylin, dehydrated and mounted with Entellan® (Merck). Between each step, samples were washed twice (5 min each) with PBS. Positive staining control was developed with gastrointestinal tract samples[22] and negative ones by replacing the primary antibodies for blocking solution.

Lectin histochemistry

After deparaffinination in xylene and hydration in ethanol (100%-70%), four micrometer thick tissues were treated with a 0.1% (w/v) trypsin solution for 2 min at 37ºC, followed by 100 mU/mL neuraminidase solution for 1 h at 37ºC and a 0.3% (v/v) H_2O_2-methanol solution for 15 min at 25ºC. PWM and WGA lectins incubations (20 µg/mL) were carried out for 2 h at 4ºC. Biotynilated WGA lectin was visualized using streptavidin-peroxidase polymer (1:1000) for 45 min at 25ºC. PWM, a non-conjugated lectin, after tissue incubation were also incubated with a 5% (w/v) peroxidase solution for 1h at 25ºC. Peroxidase was revealed with DAB according to de manufacturer's instructions. Sections were then counterstained with Harry's hematoxylin, dehydrated in ethanol and mounted with Entellan® (Merck). Between each step, samples were washed twice (5 min each) with 100 mM PBS solution pH 7.2, containing 150 mM NaCl. Inhibition of lectin-carbohydrate recognition (staining control) was developed by incubating tissues sections with lecting inhibited with their specific sugar (300 mM of *N*-acetyl-D-glucosamine for 30 min to WGA) prior to tissue incubation and negative staining control was developed by replacing lectins for PBS.

Staining analysis and image capture

Image capture and analysis of tissues sections were carried out using a Nikon Eclipse 50i light microscope (USA) with a NIS-Elements F software (version 2.30). Random areas (µm²) were analyzed taking into account the number of stained cells per area. Staining intensity was measured according to Dornelas[27] and Ferreira,[28] as: 0, negative staining; 1+, low staining for up to 1/3 of cells stained; 2+, moderate staining for up to 2/3 of cells stained; and 3+, intense staining for more than 2/3 of cells stained. For statistical analysis we used a method where the results were dichotomized as high (3+ and 2+) or low (1+ and 0) expression.[29,30].

Statistical analysis

The Fischer's test was used to evaluate the relationship between positive staining and lesions grades. When $P<0.05$ differences were considered significant. GraphPad Prism version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used for data analysis.

Results

β3GnT3 showed to be expressed (Figure 1-1a) in the cytoplasm of 99% samples (Table 1). However, this expression was not different in among analyzed groups (Figure 2). β3GnT2

Table 1. Expression of β3GnT3, β3GnT2, GalNAc, PolyLacNAc and nuclear PolyLacNAc in normal, premalignant and malignant uterine lesions.

	β3GnT3		β3GnT2		GalNAc		PolyLacNAc		Nuclear PolyLacNAc	
	Lower	Higher	Lower	Higher	Lower	Higher	Lower	Higher	Lower	Higher
Normal	10	0	9	1	9	1	9	1	10	0
CIN 1	30	0	21	9	3	27	24	6	28	2
CIN 2	27	3	19	11	18	12	26	4	27	3
CIN 3	30	0	10	20	17	13	22	8	25	5
SC	30	0	16	14	23	7	15	15	17	13

SC, squamous carcinoma; CIN, cervical intraepithelial neoplasia.

expression was observed in the cytoplasm (mainly in CIN 3 lesions) and it was higher than β3GnT3 (Figure 1-2a and Table 1) when compared with normal (P=0.002), CIN 1 (P=0.009) and CIN 2 (P=0.03) samples (Figure 2). The presence of GlcNAc terminal residues was also most commonly found in the cytoplasm and cell surface of CIN 1 lesions (Figure 1-3a and Table 1) when compared with normal (P<0.0001), CIN 2 (P<0.0001), SC (P<0.0001) and CIN 3 (P=0.0003) samples (Figure 2). It was also observed a gradual expression decrease of this saccharide with the increasing degree of lesions (CIN 1, CIN 2, CIN 3, and SC), opposing to what was observed with the expression of both glycosyltransferases, β3GnT2 and β3GnT3 (Figure 2).

PolyLacNAc chains, like GlcNAc terminal residues, were more expressed in cytoplasm and cell surface of lesion samples (Figure 1-4a). It was highly expressed in SC samples (Table 1), especially when compared with normal (P=0.03), CIN 1 (P=0.02) and CIN 2 (P=0.004) samples (Figure 2). In this sense, an increased expression of these saccharide chains was observed with an increased histologic degree of the lesion (Figure 2). For SC lesions nearly 50% of samples (Table 1) showed a polyLacNAc expression only in nucleus (Figure 3), whereas in premalignant lesions and normal samples this staining pattern was observed only in few cases (Figure 3) showing a statistically significant difference when compared with normal (P=0.01), CIN 1 (P=0.002), CIN 2 (P=0.007) and CIN 3 (P=0.04).

Discussion

In 2001, Shiraishi *et al.*[22] demonstrated the expression of β3GnT2 in significant levels in many tissues, including uterus, while β3GnT3 was found but at lower levels. Togayachi and co-workers[21] showed that β3GnT2 and β3GnT3 are considerably expressed in human cell lines of colon adenocarcinoma, lung squamous cell carcinoma, lung adenocarcinoma, stomach cancer, hepatoblastoma, prostatic cancer and pancreas cancer. Additionally, β3GnT2 was also significantly express in human leukemia and promyelocytic leukemia cell lines. Using immunohistochemistry our data also showed higher levels of β3GnT2 when compared with β3GnT3 in the normal uterine cervix. We also

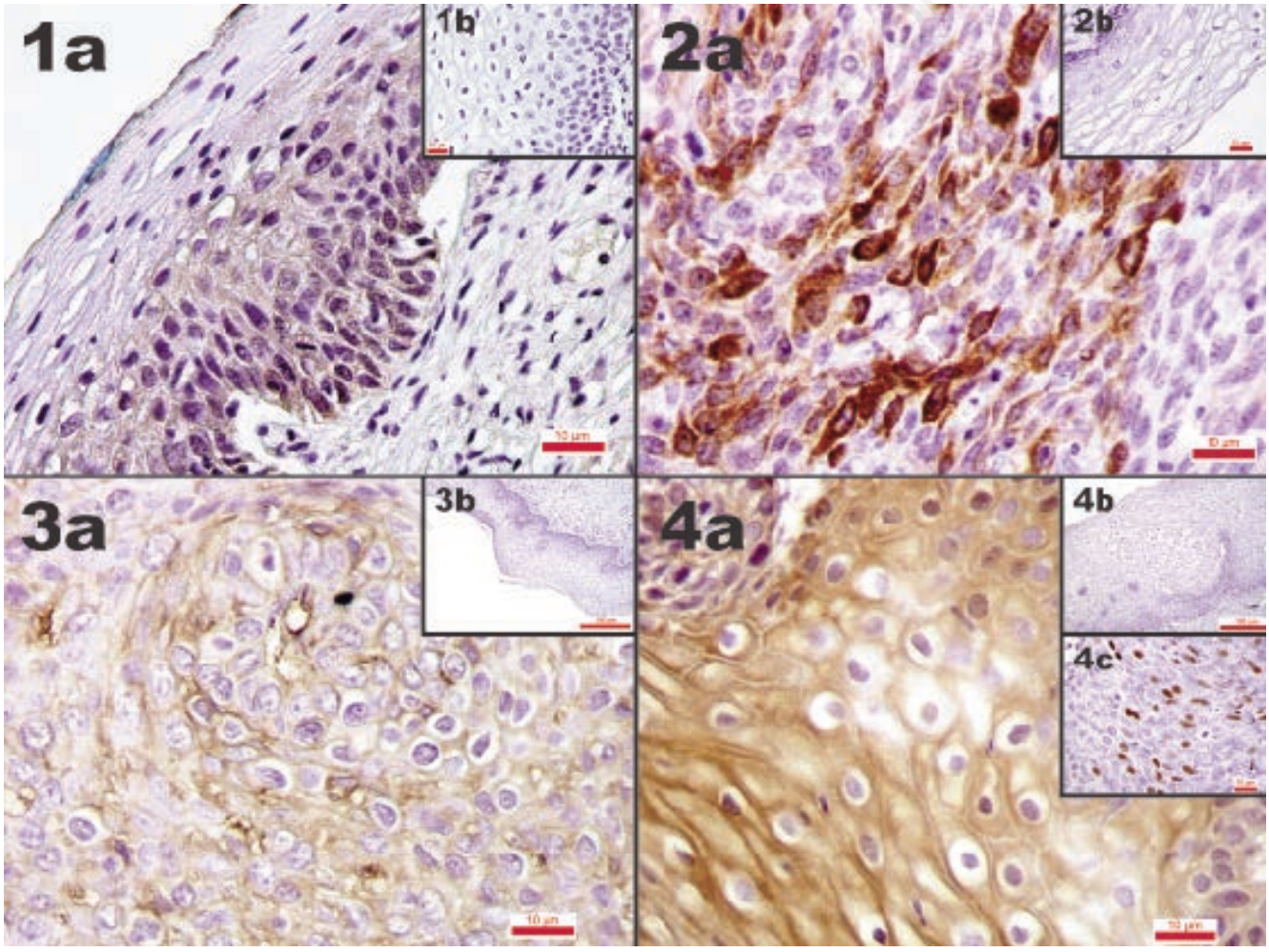

Figure 1. Immunohistochemistry and lectin histochemistry of premalignant and malignant lesions of human uterine cervix. 1a) Anti-β3GnT3 staining of cervical intraepithelial neoplasia grade 2, showing moderate dysplasia confined to the basal 2/3 of epithelium. 2a) Anti-β3GnT2 staining of invasive squamous carcinoma, showing staining of the invasive cells. 3a) *Wheat germ* agglutinin (WGA) staining squamous epithelium with cervical intraepithelial neoplasia grade 2. 4a) *Phytolacca americana* lectin (PWM) staining of cervical intraepithelial neoplasia grade 1. 1b, 2b, 3b, 4b) Negative controls for respective assay. 4c) *Phytolacca americana* lectin (PWM) staining of nucleus in invasive cells of squamous carcinoma.

showed an expression of both glycosyltransferases in transformed human uterine cervix tissues, there are, however, higher levels of β3GnT2. Furthermore, our data demonstrate a progressive upregulation of β3GnT2 expression during the transformation progression of uterine cervix, maybe involved in essentials events in the establishing of the pre-malignant lesions. In transitional cell carcinoma of bladder, Gromova *et al.*[14] observed lower levels of β3GnT2 transcript in the invasive tumors, compared with their noninvasive counterparts, establishing a downregulation of β3GnT2 during bladder cancer progression. These findings show that glycosyltranferases play different roles according to the cell or tissue type involved. Tanaka *et al.*,[31] in nude mice, administered four cell lines in the abdominal cavities of the animals, where MKN45 (a human gastric cell line) was used to overexpress β-1,6-*N*-acetylglucosaminyltransferase, which led to an increased number of cell surface *N*-glycans containing polyLacNAc. They observed that the presence of larger polyLacNAc structures enhanced the metastatic potential of this tumor cell, when compared to the control cell lines.

Several other studies have demonstrated the importance of polyLacNAc expression in the development and progression of many cancer types, as involved during differentiation,[32] hyperproliferation,[33] immune response[18] and especially cancer growth and metastasis.[34-37] In our results the polyLacNAc expression also demonstrated an involvement of this structure with the cancer process progression. We can also assign that the decreased expression of terminals residues of GlcNAc during the increasing of the lesion degree can been explained by the appearance of other carbohydrate antigens, such as Sialyl-Lewis A, Sialyl-Lewis X, Lewis Y and Thomsen-Friedenreich antigens, which may imply advantages for these cells and to the lesion progression process.[38] Recently, Togayachi and co-workers[4] have suggested that polyLacNAc chains on glycoproteins are synthesized mostly by β3GnT2 after observing a dramatically reduction in their expression in thymus, spleen, lymphocytes and macrophages of β3GnT2 knockout mice. They suggest that β3GnT2 is the major

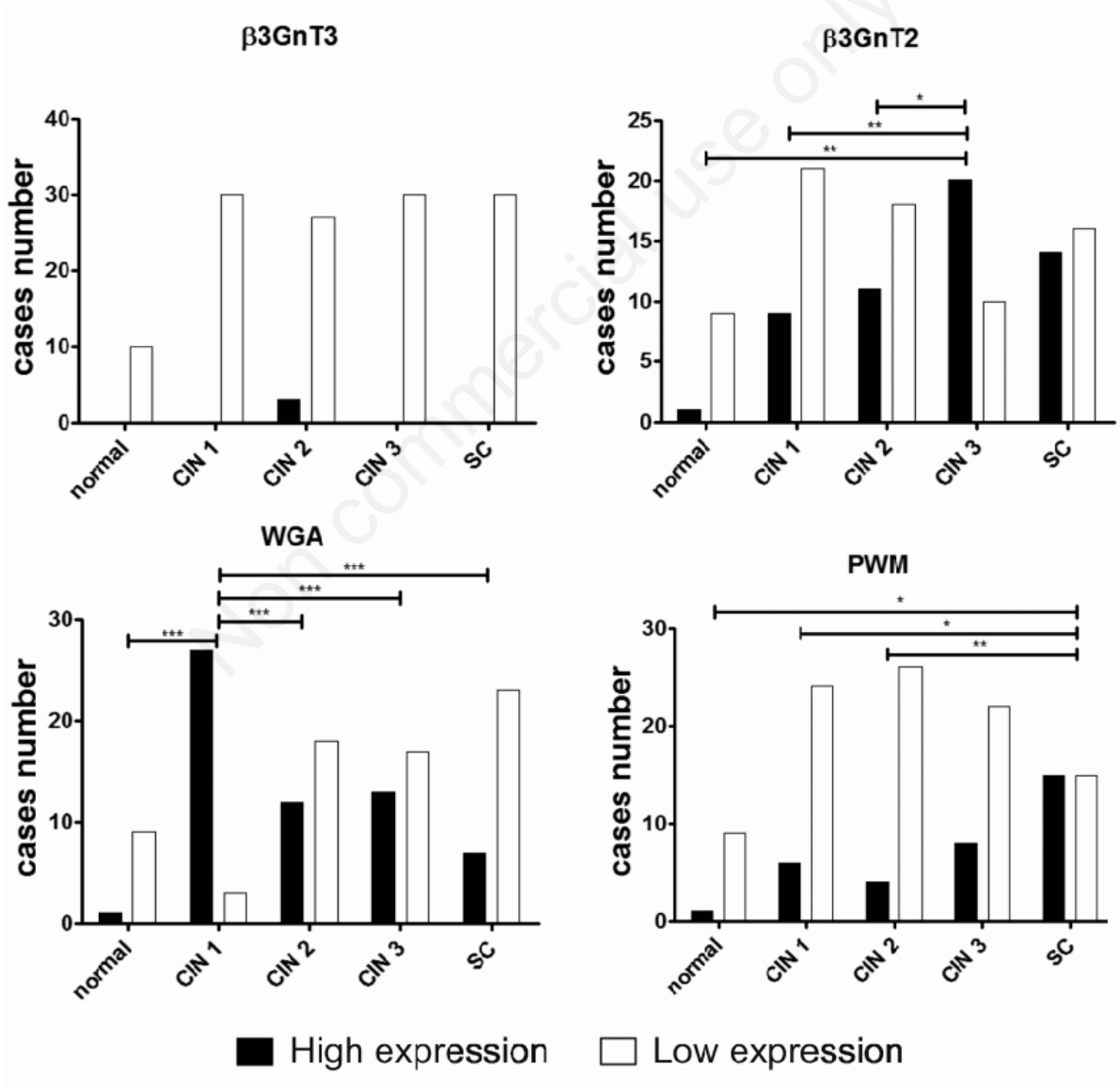

Figure 2. Statistical analysis of anti-β3GnT2, anti-β3GnT3, *Phytolacca americana* lectin (PWM) and *Wheat germ* agglutinin (WGA) staining. *P<0.05; **P<0.005; ***P<0.0005; CIN, cervical intraepithelial neoplasia; SC, squamous carcinoma.

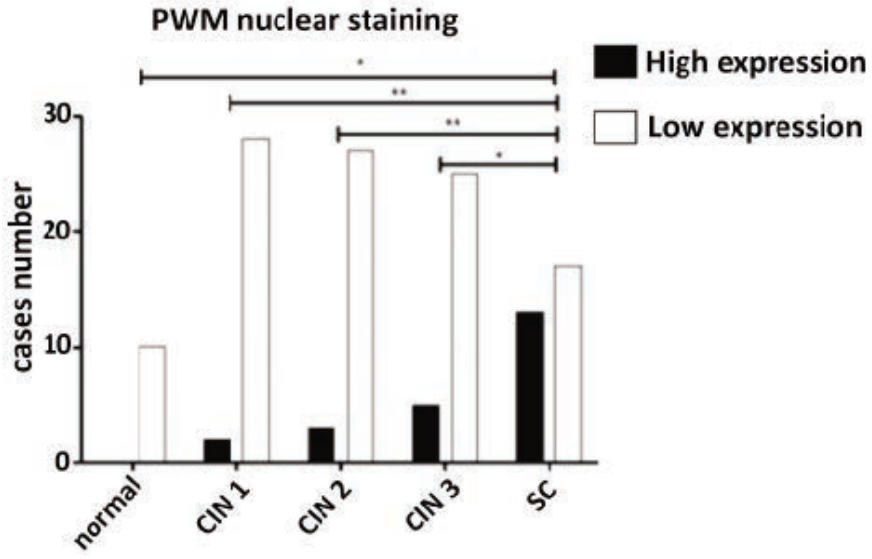

Figure 3. Statistical analysis of nuclear staining of *Phytolacca americana* lectin (PWM). *P<0.05; **P<0.005; ***P<0.0005; CIN, cervical intraepithelial neoplasia; SC, squamous carcinoma.

enzyme responsible for the synthesis of polyLacNAc chains on *N*-glycans *in vivo*. Another study[16] also suggests that an increase in the activity of β3GnT2 increases polyLacNac chains in HL-60 cells, a human promyelocytic leukemia cells. This work showed a significant difference in the expression pattern of β3GnT2, GlcNAc and polyLacNAc chains among uterine lesions. β3GnT2 seems to be involved with the progression of the pre-malignant lesions, increasing their expression levels with the increasing of the grade of lesion and decreasing with the establishment of the malignant lesion. The opposite occurred with the GlcNAc expression; the expression decreased with the progression of pre-malignant lesions. PolyLacNAc chains expression showed to be progressive with the increasing of lesions grade but differently to β3GnT2 expression the former continues to increase until to the malignant lesion, being highly expressed in SC. When polyLacNAc is present only in the nucleus this feature was an indicative of poor prognosis. Its expression seems to be associated with the increase of the β3GnT2 during the progression of the pre-malignant lesions and may provide some advantages to tumor development such as differentiation, hyperproliferation, growth or metastasis.[5,6,14] β3GnT2 and polyLacNAc expression can mediate pathophysiological key events during various stages of uterine lesions progression, including differentiation, hyperproliferation, growth and metastasis.[5,6,14]

Our study was the first in the literature which aimed to analyze the expression of β3GnTs in uterine cervix and results showed the potential involvement of β3GnT2 and polyLacNAc chains in uterine lesions progression and to show that the nucleus expression of polyLacNAc chains can be associated to a poor prognostic in uterine lesions.

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