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# Enhanced sialyltransferases transcription in cervical intraepithelial neoplasia.

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**Key words:** Sialyltransferase, cervical intraepithelial neoplasia, cervix, cervical cancer, mRNA expression.

**Abstract.** Altered sialylation observed during oncogenic transformation, tumor metastases and invasion, has been associated with enhanced sialyltransferases (STs) transcription. Increased mRNA expression of STs (ST6Gal I, ST3Gal III) has been detected in invasive cervical squamous cell carcinoma. A study of the sialic acid concentration in local tissue of cervix and in serum showed a slight elevation in benign inflammatory lesions and a moderate elevation in severe neoplasia, but to date, altered expression of STs in cervical intraepithelial neoplasia has not yet been evaluated. This study investigates the changes in mRNA expression of three STs (ST6Gal I, ST3Gal III, and ST3Gal IV) in cervical intraepithelial lesions (CIN). Alterations of these STs mRNA expression were examined in 35 cervix specimens classified as normal, CIN 1, CIN 2 and CIN 3, by semiquantitative reverse transcription-polymerase chain reaction. mRNA expression of the three STs was enhanced in CIN 1, CIN 2 and CIN 3 with respect to normal tissue, with a significant difference of $p < 0.001$ (Mann-Whitney U test) for all the enzymes. Our results suggest that altered expression of ST3Gal III, ST3Gal IV and ST6Gal I in CIN could play an important role during malignant transformation and could be related with the enhanced sialic acid expression detected in neoplasic tissues.

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**Incremento de la transcripción de sialiltransferasas en muestras de cérvix con neoplasia intraepitelial cervical.**
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**Palabras clave:** Sialiltransferasa, neoplasia intraepitelial cervical, cérvix, cáncer cervical, expresión de ARNm.

**Resumen.** La sialilación alterada que se ha detectado durante la transformación maligna, en los tumores con invasión y metástasis ha sido asociada con un incremento en la transcripción de sialiltransferasas (STs). En carcinoma escamoso cervical invasor ha sido detectado un incremento en la expresión del ARNm de STs (ST3Gal III y ST6Gal I). Un estudio realizado en muestras de cérvix mostró un ligero incremento en la expresión de ácido siálico en lesiones inflamatorias benignas y un incremento moderado en neoplasia severa, con respecto al tejido normal, sin embargo, a la fecha la expresión alterada de STs en la neoplasia intraepitelial cervical no ha sido evaluada. Este estudio tuvo como finalidad investigar los cambios en el nivel de transcripción de tres STs (ST3Gal III, ST3Gal IV y ST6Gal I) en la neoplasia intraepitelial cervical (NIC). Para ello se analizaron 35 biopsias de cérvix clasificadas como: normal, NIC 1, NIC 2 y NIC 3, mediante ensayos semicuantitativos de RT-PCR. El nivel de transcripción de las tres STs se incrementó en las muestras con diagnóstico de neoplasia intraepitelial cervical con respecto al tejido normal, con una diferencia significativa de $p < 0.001$ (Mann-Whitney U test) para todas las enzimas. Nuestros resultados sugieren que la expresión alterada de las STs: ST3Gal III, ST3Gal IV y ST6Gal I, en la neoplasia intraepitelial cervical puede tener un papel importante durante la transformación maligna y estar relacionada con los incrementos en la expresión de ácido siálico detectado en tejido con neoplasia cervical.

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## INTRODUCTION

Malignant transformation is frequently accompanied by a marked alteration of the cell surface oligosaccharide expression. Carbohydrate determinants expressed on cancer cells contain predominantly an increase in sialylated structures (1-6) catalyzed by a family of enzymes named sialyltransferases (STs) (7). Levels of ST expression differ in various human tissues and changes in their expression have been observed in several cancerous tissues and cells (8-10). ST expression is regulated in a cell type-specific manner and is mainly achieved at the transcriptional level. Increased ST mRNA expression has been correlated with poor outcome in breast cancers (11-13) and colon carcinoma (14-15). In human cervical cancer, enhanced expression of ST6Gal I and ST3Gal III was associated with invasiveness such as lymph node metastasis. Overexpression of ST6Gal I was relevant for poor prognostic factors such as deep stromal invasion and lymph or vascular space involvement (16, 17). Roy et al (18) determined the sialic acid concentration of cervical tissue and serum of women with

healthy and unhealthy cervix, they found slight elevation in benign inflammatory lesions, moderate elevation in severe dysplasia and preinvasive carcinoma and marked elevation in invasive carcinoma cervix. These results suggested that the sialylation is altered in stages previous to cancer, and it could be the result of altered transcription of sialyltransferases.

In the present study we analyzed the transcription level of STs (ST3Gal III, ST3Gal IV and ST6Gal I) in specimens with different stages of CIN in order to determine its putative association with neoplastic transformation.

## MATERIALS AND METHODS

### Tissues

A retrospective study was carried out using material from the tissue collection at the Clinics of Dysplasia, Hospital General Regional No. 36 and Hospital General de Zona No. 5, Metepec, Mexican Institute of Social Security.

Pathological specimens were obtained from cervical cones with CIN. Each sample had 80% of abnormal epithelium taken from transformation zone. Samples of normal cervix were obtained from uterus of patients who had undergone total hysterectomy due to uterine myoma. All samples were obtained according to the guidelines of the Human Ethics Committee of our Institution. This study involved 35 specimens classified according to the Bethesda System: 11 cases of CIN 1, 17 cases of CIN 2 and CIN 3 and seven samples of normal epithelium.

### RNA extraction

Tissues were powdered by freezing with liquid nitrogen. RNA was extracted using the RNA/DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RNA was dissolved in RNAase-free water and stored at –70°C until use. RNA yield and quality were determined spectrophotometrically.

### Reverse transcription-polymerase chain reaction (RT-PCR)

ST mRNA was analyzed and amplified according to reported primers described by Recchi et al. (11); for a 371-bp fragment of the human ST6Gal I 5´-TATCGTAAGCTG CACCCCAATC-3´ (forward primer), and 3´-GAAGGCCTGGTAAGTGACGATT-5´ (reverse primer), for a 300-bp fragment of the human ST3Gal III 5´-CGGATGGCTTCT GGAAATCTGT-3´ (forward primer), and 3´-AGTTTCTCAGGACCTGCGTGTT-5´ (reverse primer), for a 458-bp fragment of the human ST3Gal IV 5´-CCCAAGAACATCCA GAGCCTCA-3´ (forward primer), 3´-CTA ATTCGTCTTCGGGTGGTGC-5´ (reverse primer), for a 100-bp fragment of the human cyclophilin 5´-ATGGTCAACCCCA CCGTGTT-3´ (forward primer) and 3´-CCC ACCACTGAAGTGTGC-5´ (reverse primer). In order to quantify the expression of ST mRNA, the housekeeping gene cyclophilin was used as an internal control. One-$\mu$g samples of RNA were subjected to reverse transcription and polymerase chain reaction using the SuperScript One Step RT-PCR Kit (Invitrogen, Carlsbad, CA). The RT-PCR mixture consisted of 0.5 $\mu$L RT/Platinum Taq mix, 12.5 $\mu$L 2X Reaction mix, 1 $\mu$g RNA template, and 0.2 $\mu$M of each primer and distilled water added to a final volume of 25 $\mu$L. Samples were overlaid with two drops of mineral oil (Sigma Chemical Corp., St Louis, MO). Reactions were run in a PTC-100TM thermal cycler (MJ Reseach, Watertown, MA) using the following conditions: 55°C for 30 min, 30 cycles for the ST3Gal III and ST3Gal IV, and 35 cycles for ST6Gal I (previously standardized), of 1 min at 94°C, 1 min at 58°C and 1.5 min at 72°C.

Negative control reactions were done by replacing total RNA template with sterile water. Ten-$\mu$L aliquots of RT-PCR reaction were size-separated in 1% agarose gel equilibrated in Tris-borate-EDTA (TBE). Sizes of the generated fragments were estimated according to the migration of a 1-kb DNA ladder. Gels were stained with ethidium bromide (1 $\mu$g/mL), observed on a UV transilluminator, and photographed using a Kodak DC290 Zoom Digital Camera.

**Semiquantitative analysis of mRNA expression**

RT-PCR products were quantified by using the image analysis software Quantity One (Bio-Rad Laboratories, Hercules, CA). The density of each ST band was compared with the density of cyclophilin band and, the ratio (ST band density unit/cyclophilin density units) was calculated. The statistical analysis was done for normal samples, CIN 1 samples, and CIN 2 and CIN 3 that were analyzed as one group (severe neoplasia).

**Statistical analysis**

Statistical analysis was done using the statistical software SPSS, version 10.0 (SPSS Inc., Chicago, IL). Mann-Whitney U test was used to test differences between expression of a given ST in the CIN 1, CIN 2-CIN 3 and normal tissue ($P < 0,05$). Spearman analysis was used to determine the association of the mRNA expression level between the STs analyzed and different types of samples (normal, CIN 1 and CIN 2-CIN 3).

## RESULTS

All analyzed STs were detected in normal cervical tissues, ST3Gal III showed the higher expression. RT-PCR results of STs in normal and pathological tissues are shown at Fig. 1.

Expression of STs increased in CIN 1 and CIN 2-CIN 3 with respect to normal tissue (Fig. 2). The transcription mean values in normal samples were ST6Gal I (0.73), ST3Gal III (1.5), and ST3Gal IV (0.90); the

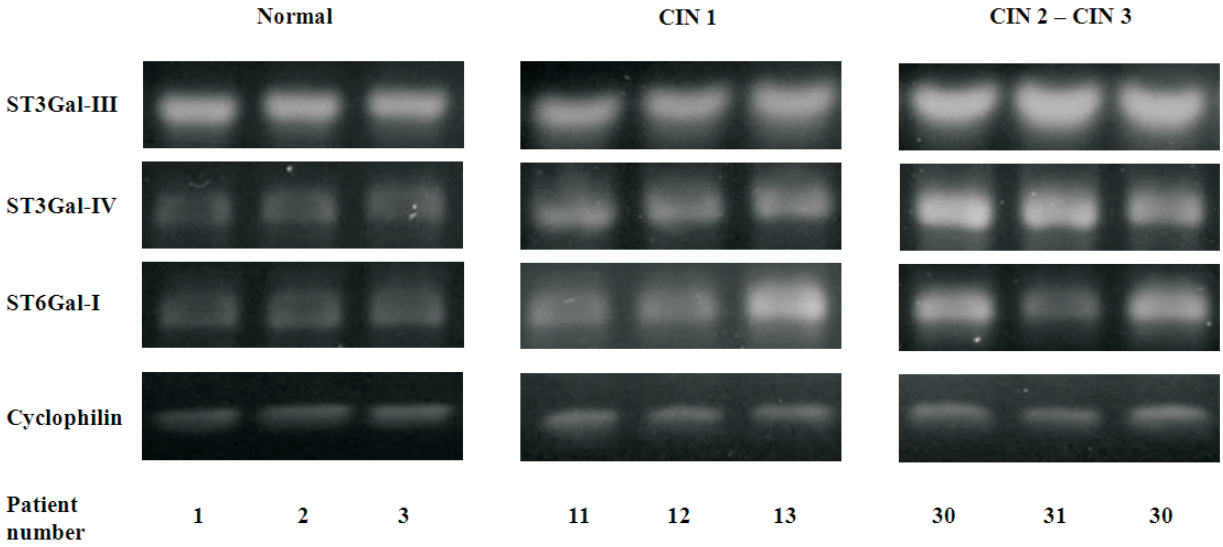


Fig. 1. RT-PCR analysis of sialyltransferases ST3Gal III, ST3Gal IV, ST6Gal I from normal (Patient No. 1, 2, 3) CIN 1 (Patient No. 11, 12, 13) and CIN 2-CIN 3 (Patient No. 30, 31, 32) of cervical samples. Cyclophilin was used as internal control. Each sample was subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

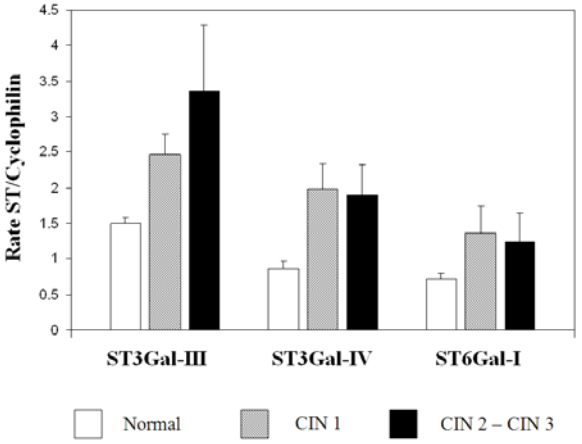


Fig. 2. Amount of STs mRNA normalized for the cyclophilin mRNA in the cervical samples. Data are the mean (±) SD of each group (normal, CIN 1 and CIN 2-CIN 3) with the different enzymes.

mean values in CIN 1 samples were ST6Gal I (1.36), ST3Gal III (2.47), and ST3Gal IV (1.98); and in CIN 2-CIN 3 were ST6Gal I (1.24), ST3Gal III (3.36), and ST3Gal IV (1.89). The increase in the mRNA expression of the three analyzed enzymes showed statistical significance ($p < 0.001$) when either CIN 1 or CIN 2-CIN 3 were compared with normal samples, but only the ST3Gal III showed a significant difference between CIN 1 and CIN 2-CIN 3 ($p = 0.008$).

Spearman analysis showed an association, high levels of ST3Gal IV mRNA were associated with high levels of ST6Gal I in both CIN 1 and CIN 2-CIN 3 ($p < 0.001$). There was not association between the ST3Gal III and ST3Gal IV mRNA expression nor ST3Gal III and ST6Gal I in CIN and normal samples.

It is important to note that distribution of the ST/cyclophilin ratio values is different between normal and pathological samples. The three analyzed enzymes in normal samples showed low dispersion values, but in pathological samples we observed broad dispersion values. The highest dispersion values were detected for the ST3Gal III in CIN 2-CIN 3 samples (Fig. 3).

The results show that the transcriptional regulation of STs is affected at early stages of cell transformation before the cancer development.

## DISCUSSION

There are extensive reports in the literature describing the variations in ST expression related to cancer and invasive properties of cancer cells (11-17). Expression of STs in carcinoma tissue has been used as a prognostic factor and potential target for therapeutic approaches (19). Although the mechanisms of control of expression have not yet been characterized, there is evidence that it is mainly regulated at the transcriptional level (8). Most stud-

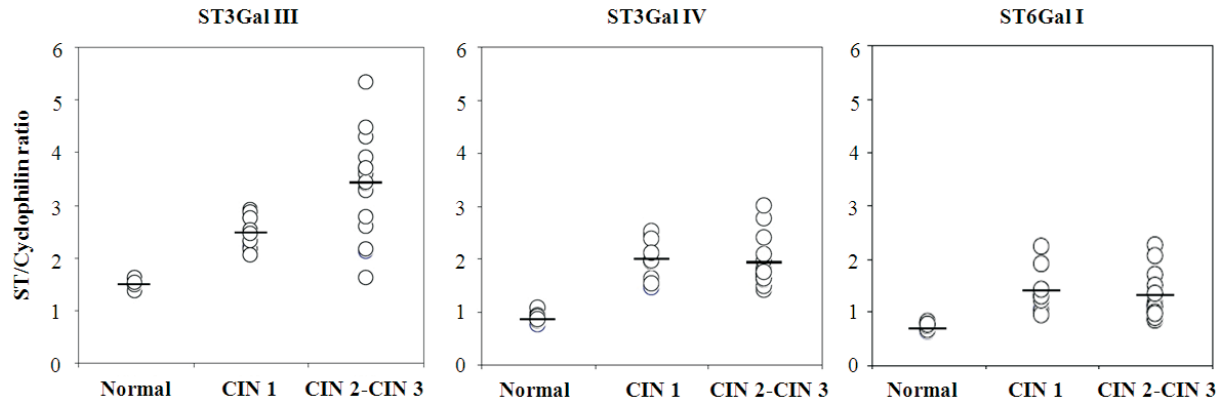


Fig. 3. Dispersion of values of ST/cyclophilin among normal, CIN 1 and CIN 2-CIN 3 samples.

ies of glycosylation have been done in malignant tissue of different tumor cancers but there are few reports in premalignant tissue. The sialic acid concentration in cervical tissue was found to be increased with the pathological process (18). This change of expression could be related with altered expression of sialyltransferases. Our aim was to analyze transcription of three STs in normal samples and with diagnosis of CIN 1, CIN 2-CIN 3. We found an enhanced transcriptional level in all of the enzymes in samples with CIN. Upregulated expression of ST6Gal I has been shown in colorectal cancer (14), breast cancer (11) and cervical cancer (17). Expression of ST6Gal I may have different effects in different cancer types. Elevated alpha-2,6 sialylation inhibited formation of glioma *in vivo* (20). Reports have shown that expression of ST6Gal I and ST3Gal III mRNA increased in cervical cancer tissues with lymph node metastases compared to those without metastases (17). High ST6Gal I expression was also associated with other invasive properties of cervical cancer such as deep stromal invasion and presence of lymph-vascular space involvement. Those findings suggest that these enzymes could play a role in metastases (16, 17). Our study found increased expression of ST6Gal I, ST3Gal III, and ST3Gal IV genes in non malignant tissue, indicating their change in mRNA expression is also relevant at early stages in neoplastic transformation. Gretschel et al (21) found increased transcription of ST3Gal III and ST6Gal I in tumor tissue of gastric cancer, interestly, also the nonmalignant and uninvolved mucosa of tumor patients in some cases showed enhanced sialyltransferases levels indicative of the alteration of glycosylation very early during tumorigenesis. The ST6Gal I catalyzes the transfer of sialic acid in α2,6-linkage to the acceptor molecule Galβ1,4GlcNAc (*N*-acetyl-lactosamine), a sequence commonly found in *N*- and *O*-linked chains of glycoproteins (7). The broad tissue distribution of its acceptor *N*-acetyl-lactosamine is the likely reason for the association of ST6Gal I with several types of cancer (1). In this regard, changes in tumor cell surfaces due to increased sialylation and ST6Gal I expression have been associated with loss of cellular adherence, larger tumor size, invasiveness and metastases in several carcinomas (8, 22-25). This change of mRNA expression in non malignant tissue could be related with the alteration in the tissue layers organization observed in CIN.

Wang et al (17) proposed that increased expression of ST3Gal III might be a late event in cancer development; in our work, ST3Gal III increased early in neoplastic transformation. This enzyme is involved in the sialylation of the Lewis X and A antigens, tumor-associated carbohydrate structures that mediate the adhesion of malignant cells to the vascular endothelium (23, 26). The change of mRNA expression could be related to the change of alpha 2,3 sialic acid expression in carbohydrates structures that could play a role in the cellular adherence. In ovarian cancer the increased expression of ST3Gal I is detected in the samples with increased alpha2,3-linked sialylation (27).

We found that the level of ST3Gal IV mRNA was also increased in CIN 1 and CIN 2-CIN 3 compared with normal tissue. Wang et al (17) described a decreased ST3Gal IV expression in cervical cancer. This apparent contradiction could be explained by changes of expression by ST3Gal IV during the neoplastic transformation because it can be up- or downregulated, whereas mRNA of other STs decreases or increases. This may be due to an internal gene control or to expression of other cellular genes or transcription factors (28).

ST3Gal IV mRNA expression reports are contradictory, the level of mRNA ex-

pression of this enzyme was significantly enhanced in gastric carcinoma (29) but it was down regulated in renal cell carcinoma (30). Zangh et al (31) reported a decreased expression of the ST3Gal IV mRNA in colorectal carcinoma. Kudo et al (32) reported a significant up-regulation of ST3Gal IV in poorly differentiated colorectal carcinoma. Down regulation of the ST3Gal IV seems to be restricted to certain subpopulations of colorectal carcinomas (14). Transcriptional regulation of this enzyme could be more complex than we suppose, and it is necessary to investigate the mechanism of transcriptional regulation to explain the different pattern of expression. We found a statistical correlation between the expression levels of the ST3Gal IV and ST6Gal I. This association could be the result of common mechanisms of transcriptional regulation, but it is necessary realize further studies to clarify this.

The manipulation of cell-surface carbohydrate expression by the sialyltransferase gene may provide a new therapeutic approach for treatment of malignant tumor cells (20). In conclusion, we found enhanced transcription of the ST3Gal III, ST3Gal IV and ST6Gal I genes in cervical intraepithelial neoplasia. RT-PCR assays of specific STs may be helpful for earlier detection of cervical neoplastic transformation. These results may be important and helpful to analyze the role of the sialylation in early neoplastic transformation before the presence of cancer.

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