RESEARCH ARTICLE

Promoter Polymorphisms of *ST3GAL4* and *ST6GAL1* Genes and Associations with Risk of Premalignant and Malignant Lesions of the Cervix

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Abstract

Sialyltransferase gene expression is altered in several cancers, including examples in the cervix. Transcriptional regulation of the responsible genes depends on different promoters. We aimed to determine the association of single-nucleotide polymorphisms in the B3 promoter of the *ST3GAL4* gene and the P1 promoter of the *ST6GAL1* gene with cervical premalignant lesions or cervical cancer. A blood sample and/or cervical scrapes were obtained from 104 women with normal cytology, 154 with premalignant lesions and 100 with cervical cancer. We also included 119 blood samples of random donors. The polymorphisms were identified by sequencing from PCR products. For the B3 promoter, a fragment of 506 bp (from nucleotide -408 to +98) was analyzed, and for the P1 promoter a 490 bp (-326 to +164) fragment. The polymorphism analysis showed that at SNP rs10893506, genotypes CC and CT of the *ST3GAL4* B3 promoter were associated with the presence of premalignant lesions (OR=2.89; 95%CI 1.72-4.85) and cervical cancer (OR=2.23; 95%CI 1.27-3.91). We detected only one allele of each polymorphism in the *ST6GAL1* P1 promoter. We did not detect any genetic variability in the P1 promoter region in our study population. Our results suggest that the rs10893506 polymorphism -22C/T may increase susceptibility to premalignant and malignant lesions of the cervix

Keywords: SNPs, single-nucleotide polymorphism - sialyltransferase - cervical cancer - cervical neoplasia

Introduction

Sialyltransferases are a family of enzymes that transfer sialic acid to glycoconjugates (Harduin-Lepers et al., 2001). In cervical cancer, increased mRNA levels of the sialyltransferase genes *ST6GAL1* and *ST3GAL3* have been reported. Higher levels of *ST6GAL1* mRNA are associated with invasion to the stroma and lymphatic nodule (Wang et al., 2002). In premalignant lesions, higher levels of *ST6GAL1*, *ST3GAL3* and *ST3GAL4* mRNAs have been reported (López-Morales et al., 2009). Increased

expression of sialic acid and also sialylated antigens, such as sLewis(x), have been reported in premalignant and malignant lesions of the cervix (Roy and Chakraborty, 2005; López-Morales et al., 2010; Velázquez-Márquez et al., 2012).

The gene *ST6GAL1* codes for different mRNA isoforms, three of which are better characterized than the others. Isoform 1, also called isoform H, is increased in cervical cancer tissue (Aasheim et al., 1993; Wang et al., 1993; Wang et al., 2003). This isoform results from the activity of the P1 promoter, which has been characterized

in the cervical cancer cell line C33A (Milflores-Flores et al., 2012). Six mRNA isoforms have been reported for *ST3GAL4*: A1, A2, B1, B2, B3 and BX (Kitagawa et al., 1996; Taniguchi and Matsumoto, 1998). The B3 promoter shows greater activity in the cervical cancer cell line HeLa (Taniguchi and Matsumoto, 1999).

Single-nucleotide polymorphisms (SNPs) of different genes have been associated with the risk of cervical cancer (Stanczuk et al., 2001; Zhang et al., 2007; Yu et al., 2009; Jiang et al., 2010). No association studies of SNPs in sialyltransferase gene promoters and cervical cancer have been reported. Only for a few diseases, such as schizophrenia and IgG nephropathy, have promoter SNPs been associated with the disease and with change in the gene expression (Arai et al., 2006; Li et al., 2007). One polymorphism in the promoter of GALNT1 has been associated with decreased risk of ovarian cancer (Sellers et al., 2008). SNPs have excellent potential to be used as biomarkers for diagnosing genetic diseases, including cancer.

The objective of this work was to analyze the polymorphisms in the P1 promoter of *ST6GAL1* and B3 promoter of *ST3GAL4* and their associations with the presence of premalignant and malignant lesions of the cervix.

Materials and Methods

Study population
The study was carried out using cervical scrapes and peripheral blood of patients assigned to different clinics and hospitals of the Instituto Mexicano del Seguro Social. This study included cervical scrapes and/or peripheral blood of 104 specimens of from healthy women, 154 patients with a diagnosis of cervical lesions and 100 patients with cervical cancer. Additionally, we included 119 blood samples of donors in order to have a reference for the allelic distribution in the population. All samples were obtained according to the guidelines of the Human Ethics Committee of our Institute. The purpose and procedures of the study were explained to patients, and informed consent was obtained from all participants.

Collection of specimens
Two cervical samples were collected from the endo- and exocervix with a cytobrush and an Ayre spatula, respectively. The first sample was used for cytological diagnosis. Papanicolaou smears were evaluated by a pathologist according to the Bethesda diagnosis criteria. The second sample was used for the polymorphism analysis and was placed in a tube containing 3 mL of ice-cold phosphate-buffered saline (PBS). Blood samples were collected using a needle and placed in tubes containing 0.5 mL of EDTA buffer. All samples were stored at 4°C and processed within 24 h of their arrival at the laboratory. Samples of blood donors were obtained from the buffy coats from the blood bank of the UMAE No. 80, Instituto Mexicano del Seguro Social.

DNA extraction
Blood samples and cervical scrapes were centrifuged at 4000 rpm for 10 min at 4°C. The leukocytes and cervical cell pellets were used for DNA extraction. DNA was extracted according the protocol for purification of total DNA from animal blood or cells QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA was dissolved in 100 μL DNase-free water and stored at -20°C until use. All samples were tested for the integrity of DNA by 1% agarose gel electrophoresis. DNA yield was determined spectrophotometrically.

PCR assays
The region -326 to +164 of the P1 promoter was amplified using the primers 5'-AGT AGA GAT GGA GTT TCA CC-3' (forward primer) and 5'-CCT TAC CTG CTT CTG GCT AAT CC-3' (reverse primer). All the amplifications were performed using 40 cycles of 1 min at 95°C, 45 s at 58°C and 1 min at 72°C, and this was followed by a single 7 min extension at 72ºC. The region -408 to +98 of the B3 promoter was amplified with the primers 5'-GAGGACTCAAGCCCTCACAC-3' (forward primer) and 5'-GGATGACACTCAC GGGACTT-3' (reverse primer). Amplifications were performed using 40 cycles of 1 min at 95°C, 45 s at 57°C and 1 min at 72°C, followed by a final extension of 7 min at 72ºC. The PCR mixture consisted of 25 µL PCR Master Mix 2X (Promega, Madison, WI), 40-50 ng DNA template, and 0.2 µM of each primer, with distilled water added to a final volume of 50 µL. Reactions were run in a PTC-200 thermocycler (MJ Research, Watertown, MA). PCR products were electrophoresed, and the band was purified according the protocol of the Wizard SV gel and PCR clean-up system, DNA kit (Promega, Madison, WI).

SNP selection and genotyping
The SNPs analyzed in this study are found in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects / SNP/). For the P1 promoter of the *ST6GAL1* gene, the SNPS selected were rs141894607 (-306G/T), rs150796963 (-275C/T), rs116918425 (-259A/G), rs187589817 (-237G/T), rs116705383 (-216C/T), rs139268780 (-208C/T), rs149979727 (-202A/G), and rs73071448 (-123C/T). For the B3 promoter of the *ST3GAL4* gene, the SNPs were rs148809885 (-278T/C), rs2186716 (-267A/G), rs7933887 (-218A/C), rs141571632 (-215T/C), rs11603211 (-152A/G), rs115057909 (-57T/C), rs10893506 (-22C/T), rs190884962 (+24T/C) and rs184073997 (+25A/G).

We analyzed whether the alleles of the reported SNPs were part of the consensus binding sequence of any transcription factor using the Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess/tess). Genotyping was performed by direct sequencing of the PCR products of all samples using the Sanger method in the Laboratory of Biodiversity and Genomics, CINVESTAV, Irapuato, México.

Statistics
Hardy-Weinberg equilibrium (HWE) was tested with the χ^2 goodness-of-fit test. Before statistical analysis, we determined each SNP's allelic and genotype frequencies according to the formula $F = PAA/PA + Paa/Pa - 1$, where PAA and Paa are the respective genotype frequencies

for homozygotes and pA and pa the respective allele frequencies. The χ^2 test (2-tailed probability) was used to analyze the difference between genotypes. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression analysis. IBM SPSS 20 was used for statistical analysis (SPSS Inc., Chicago, IL); p<0.05 was considered statistically significant.

Results

SNP sequence analysis of promoter P1 of the ST6GAL1 gene

The database of SNPs (dbSNP, http://www.ncbi.nlm.nih.gov/snp) reported 8 SNPs for the P1 promoter fragment analyzed in this study. To determine if the presence of certain alleles of the SNPs could generate transcription factor binding sites, we used the Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess/tess) and found that only some of the alleles of the SNPs rs141894607, rs150796963, rs187589817 and rs116705383 could generate consensus binding sequences for transcription factors (Table 1).

Analysis of SNPs of the P1 promoter in normal and cervical cancer groups

The P1 promoter fragment (-326 bp to +164 bp) was sequenced to identify the SNPs present in the P1 promoter of the normal and cervical cancer groups. We analyzed 100 samples of women with normal cytology and 100 with cervical cancer. The SNP sequence analysis showed that all the samples were homozygous for every SNP in the P1 promoter, and the allele identified for all the SNPs was the major allele reported in dbSNP, which was the ancestral allele (Table 1).

Analysis of SNPs of the B3 promoter of ST3GAL4

In the B3 promoter region of the *ST3GAL4* gene, dbSNP reported 9 SNPS, rs148809885, rs2186716, rs7933887, rs141571632, rs11603211, rs115057909, rs10893506, rs190884962 and rs184073997. The analyses with the Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess/tess), showed that several consensus sequences for transcription factors could be generated depending of the allele present (Table 2).

Association of SNPs from the B3 promoter with cervical premalignant lesions and cervical cancer

The sequence of the B3 promoter of the *ST3GAL4* gene was analyzed in 104 samples of women with normal

Table 1. Information about SNPs in the P1 Promoter of the ST6GAL1 Gene According to dbSNP*

ID dbSNP	Position	Alleles	Ancestor allele	Putative transcription factor binding site	Genotype identified in normal and CC groups
rs141894607	-306	G	G	c-Myc	GG
		T		------	
rs150796963	-275	C	C	PR	CC
		T		------	
rs116918425	-259	A	G	------	GG
		G		------	
rs187589817	-237	G	G	------	GG
		T		PR	
rs116705383	-216	C	C	------	CC
		T		AP1	
rs139268780	-208	C	C	------	CC
		T		------	
rs149979727	-202	A	A	------	AA
		G		------	
rs73071448	-123	C	C	------	CC
		T		------	

*The position is given relative to the transcription start site. Shown are the alleles of each polymorphism, the ancestral (major) allele, the putative transcription factor binding site depending on the allele present and the only genotype identified in the normal and cervical cancer groups

Table 2. Minor Allelic Frequencies of SNPs in the B3 Promoter of ST3GAL4 in the Different Study Groups*

ID dbSNP	Position	Alleles	Putative transcription factor binding site	Ancestor allele	Genotype identified	MAF dbSNP	MAF Control lesions	MAF Pre malignant cancer	MAF Cervical
rs148809885	-278	T	------	C	CC	0.0018	------	------	------
		C	BRCA1						
rs2186716	-267	A	FOXC1	G	GG	0.1056	0.02	0.03	0.04
		G	FOXC1		AG				
					AA				
rs7933887	-218	A	------	C	CC	0.1791	0.03	0.04	0.06
		C	------		AC				
					AA				
rs141571632	-215	T	REL	C	CC	0.0014	------	------	------
		C	REL						
rs11603211	-152	A	SP1	C	GG	0.0955	0.02	0.03	0.04
		G	GATA2		AG				
					AA				
rs115057909	-57	T	ETS1	C	CC	0.0087	------	------	------
		C	ETS1						
rs10893506	-22	C	AP2	T	TT	0.4853	0.23	0.42	0.37
		T	------		TC				
					CC				
rs190884962	24	T	Myc	C	CC	0.0023	------	0.009	0.005
		C	BCRA1		TC				
rs184073997	25	A	AP2	G	GG	0.0005	------	------	------
		G	BCRA1						

*The position is given relative to the transcription start site. Shown are the alleles of each polymorphism, the ancestral (major) allele, the putative transcription factor binding site depending on the allele present and the genotypes identified in the study population

Table 3. Magnitudes of Associations (Odds Ratio; OR) of SNP rs10893506 (-22C/T) with Pre-Malignant Cervical Lesions and Cervical Cancer

ID dbSNP	Genotype	Normal group n=104 n (%)	Premalignant group n=154 n (%)	χ^2(95% CI) p value	Normal group n=104 n (%)	Cervical Cancer group n=100 n (%)	χ^2 (95% CI) p value
rs2186716	GG	100 (96.2)	144 (93.5)	0.41	100 (96.2)	91 (91)	1.48
-267	GA	4 (3.8)	10 (6.5)	p=0.521	4 (3.8)	9 (9)	p=0.222
	AA	0 (0)	0 (0)		0 (0)	0 (0)	
rs7933887	CC	98 (94.2)	142 (92.2)	0.142	98 (94.2)	86 (86)	3.03
-218	AC	5 (4.8)	11 (7.1)	p=706	5 (4.8)	13 (13)	p=0.081
	AA	1(1)	1 (0.65)		1(1)	1 (1)	
rs11603211	GG	100 (96.2)	144 (93.5)	0.41	100 (96.2)	91 (91)	1.48
-152	GA	4 (3.8)	10 (6.5)	p=0.521	4 (3.8)	9 (9)	p=0.222
	AA	0 (0)	0 (0)		0 (0)	0 (0)	
rs10893506	TT	59 (56.7)	48 (31.2)	15.67	59 (56.7)	37 (37)	7.19
-22	TC	36 (34.6)	82 (53.2)	p<0.001	36 (34.6)	51 (51)	p=0.007
	CC	9 (8.6)	24 (15.6)		9 (8.6)	12 (12)	

CI: confidence interval

Table 4. Associations of SNPs Identified in the B3 Promoter of *ST3GAL4*

ID dbSNP	Genotype	Normal group n=104 n (%)	Premalignant group n=154 n (%)	OR(95% CI) p value	Normal group n=104 n (%)	Cervical Cancer group n=100 n (%)	OR(95% CI) p value
rs10893506	CC	45 (43.2)	106 (68.8)	2.89 (1.72-4.85)	45 (43.2)	63 (63)	2.23 (1.27-3.91)
-22	CT			p=0.0004			p=0.003
	TT	59 (56.7)	48 (31.1)		59 (56.7)	37 (37)	

*The analysis compared the results of premalignant cervical cancer groups to the normal group. All comparisons were made using the χ^2 test (2-way probability)

cytology, 154 samples with a diagnosis of premalignant lesions and 100 samples of cervical cancer. In addition, 119 DNA samples from female blood donors were sequenced.

We observed a homozygous genotype for the SNPs rs148809885 (CC), rs141571632 (CC), rs115057909 (CC) and rs184073997 (GG). For the other SNPs, we identified both SNP alleles: rs2186716 A/G, rs7933887 A/C, rs11603211 A/G, rs10893506 C/T and rs190884962 T/C. The MAFs for these SNPs were determined in the normal, premalignant and malignant groups. The T allele from SNP rs190884962 (+24 bp) was not identified in the control group; in the premalignant and malignant groups the MAFs obtained for this SNP were 0.009 and 0.005, respectively (Table 2).

The Hardy Weinberg (HWE) equilibrium was calculated only for the SNPs -218 and -22 because these SPNs presented the genotype heterozygous and both genotypes homozygous.

The genotype proportions observed for the SNPs -218 in the study groups: normal cytology (χ^2=6.919 p=0.031), premalignant lesions (χ^2=2.146 p=0.341), and cervical cancer (χ^2=0.403 p=0.817), are in agreement with the expected proportions for the HWE in Mexican population (χ^2=0.092 p=0.954), there's no statistical differences (χ^2=2.784 p=0.248). The genotype proportions observed for the SNPs -22 in the study groups: normal cytology (χ^2=1.030 p=0.597), premalignant lesions (χ^2=1.289 p=0.524), and cervical cancer (χ^2=0.772 p=0.679) are in agreements with the expected proportions in the HWE in Mexican population (χ^2=1.922 p=0.382), there´s no statistical differences between them (χ^2=0.582 p=0.968).

The association of SNPs rs2186716, rs7933887,

rs11603211 and rs10893506 with the presence of premalignant lesions and cervical cancer was further determined (Table 3). No association was obtained for the SNP rs2186716, rs7933887, and rs11603211 in the premalignant and malignant groups, but the C allele and CC genotype of rs10893506 were associated with the presence of premalignant lesions (OR=2.67, p<0.001; χ^2= 7.19 p=0.007) respectively. Genotype frequencies are shown for each group in Table 3.

Association between the -22C>T genotype and the risk of premalignant lesions and cervical cancer

We determined the ORs for the individual alleles and genotypes of SNP rs10893506 (-22C>T) to determine the risk of premalignant and malignant lesions using a logistic regression model. This SNP was associated with the risk of premalignant cervical lesions (OR= 2.89; 95%CI 1.72-4.85) and cervical cancer (OR= 2.23; 95%CI 1.27-3.91) (Table 4)

Discussion

High levels of the mRNAs of the sialyltransferase genes *ST6GAL1* and *ST3GAL4* have been reported in cervical cancer and in premalignant lesions (Wang et al., 2003; López-Morales et al., 2009). The upregulation of sialyltransferase genes has been correlated with increased sialylation of membrane glycoconjugates. Hypersialylation contributes to an increased cell invasion capacity and tumor metastasis (Chirtie et al., 2008; Büll, et al., 2013; Fukusawa et al., 2013). Transcriptional regulation of sialyltransferases genes is complex because some sialyltransferases genes, including *ST3GAL4* and

ST6GAL1, have distinct promoters that confer different tissue expression and also different levels of expression (Appenheimer et al., 2003; Taniguchi et al., 2003).

The presence of certain promoter activity-modifying SNPs in promoters of sialyltransferase genes has been associated with schizophrenia and IgG nephropathy. One SNP in the *ST6GALNT* gene has been associated with decreased risk of ovarian cancer (Seller et al., 2008).

The aim of the present study was to determine whether SNPs in the P1 promoter of *ST6GAL1* and B3 promoter of *ST3GAL4* are associated with premalignant lesions and cervical cancer. Database of dbSNP reports 8 SNPs for the P1 promoter region. Our analysis of these SNPs in both normal and cervical cancer groups showed the presence of only one of the two reported alleles for each of these SNPs, the major allele, which in each case was also the ancestral allele. The analysis of the SNPs of this promoter suggest that the Mexican population is monomorphic, but to confirm this, is necessary to increase the sample size.

For the B3 promoter of the *ST3GAL4* gene, we analyzed a fragment of 506 bp (-408 to +98). dbSNP reports 9 SNPs in this promoter, only five of which included both alleles in our population (rs2186716 A/G, rs7933887 A/C, rs11603211 A/G, rs10893506 C/T and rs190884962 T/C). For these SNPs, the minor allele in our population was the minor allele reported in dbSNP. SNP rs10893506 (-22C/T) was significantly associated with premalignant lesions of the cervix and cervical cancer. The presence of allele C of SNP 10893506 was associated with the risk to developing premalignant lesions and cervical cancer.

The *in silico* analysis of the rs10893506 promoter sequence and the detection of the different SNP alleles using the Transcription Element Search System showed that the presence of the -22C allele of the B3 promoter generates a consensus binding sequence for the transcriptional factor AP2. By contrast, when the T allele is present, no transcription factor binding sequence is generated. The presence of a binding site for AP2 could modify the promoter activity. AP2 activates the transcription of cellular and viral genes (Williams and Tjian, 1991; Hilger-Eversheim et al., 2000; Zhao et al., 2001). Therefore, the presence of the C allele could be related to increased expression of *ST3GAL4*. But further studies of this promoter activity and the resultant mRNA levels should be performed to determine the effect of the C allele.

Genotype CC of SNP rs190884962 (+24) was only identified in the premalignant and cervical cancer groups. The presence of this allele generates a BCRA1 binding site. BRCA1 can modulate the transcription of several genes through its association with sequence-specific transcription factors, BRCA1 helps to repair damaged DNA, therefore, play a role in ensuring the stability of the cell's genetic material (Mullan et al., 2006). The analysis of this SNP in a larger sample size could be of interest to determine its possible association with premalignant and malignant cervical lesions.

One limitation of our study is the sample size. To confirm whether the Mexican population is monomorphic for the P1 promoter, is necessary to analyze a larger sample. The second limitation is that *ST3GAL4* expression data are lacking for the study population. Such expression data could confirm that the presence of the C allele in SNP rs10893506 modifies the expression of the gene.

In conclusion, the present study has reported the first association between a sialyltransferase gene promoter SNP and the presence of premalignant and cervical cancer. SNP -22C/T of the B3 promoter of the *ST3GAL4* gene was associated with the presence of premalignant lesions and cervical cancer.

Acknowledgements

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