



***BORIS* and *CTCF* are overexpressed in squamous intraepithelial lesions and cervical cancer**

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ABSTRACT. We investigated the expression of Brother of Regulator of Imprinted Sites (*BORIS*) and CCCTC-binding factor (*CTCF*) in squamous intraepithelial lesions and cervical cancer. To analyze *BORIS* and *CTCF* expression, an endocervical cytobrush sample was taken for total RNA isolation. *CTCF* and *BORIS* mRNA was quantified from total RNA using quantitative reverse transcription-polymerase chain reaction. A total of 71 samples were collected and classified according to the Bethesda Classification of squamous intraepithelial lesions. *BORIS*

expression was observed in 9 (12.7%) samples; of these, 5.3, 5.9, 14.8, and 37.5% in the groups that were cytology negative for intraepithelial lesion or malignancy, low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and invasive cervical carcinoma, respectively. The expression level of *BORIS* was significantly higher in the group with invasive cervical carcinoma as compared with the groups negative for intraepithelial lesion or malignancy, LSIL, and HSIL ($P < 0.0005$). *CTCF* mRNA was expressed in all samples. *CTCF* expression was significantly higher in carcinoma groups compared with LSIL, HSIL, and negative for intraepithelial lesion or malignancy groups. We found that *BORIS* and *CTCF* expressions in the LSIL and invasive cervical carcinoma groups were higher than expression in cytological normal samples. Additional studies should be conducted to examine the function of transcription factors during different stages of the transformation of cervical cancer cells.

Key words: *BORIS*; Cervical cancer; *CTCF*; Squamous intraepithelial lesions

INTRODUCTION

Despite the implementation of screening health programs, cervical cancer is one of the major causes of death among women worldwide; according to a recent estimate, it is the third most commonly diagnosed cancer, accounting for 9% (529,800) of new cancer cases (Jemal et al., 2011; Jin et al., 2011).

Although the infection by human papillomavirus (HPV) (Bosch et al., 2002) plays an important role in the pathophysiology of cervical cancer, most women infected with HPV do not develop cancer, indicating that other factors contribute to its progression. In addition, given that HPV molecular tests do not discriminate between transient and persistent infection, it is necessary to investigate more sensitive and specific markers related to the risk of developing cervical cancer (Pinto et al., 2012). In this regard, genetic and epigenetic alterations such as global DNA hypomethylation, hypermethylation of tumor suppressor genes, and histone modifications, which are involved in cervical carcinogenesis, may be targets in the development of new markers (Reuschenbach et al., 2011; Mazumder et al., 2011).

The CCCTC-binding factor (CTCF) and its paralog Brother of the Regulator of Imprinted Sites (BORIS) are implicated in epigenetic reprogramming events. Typically, CTCF and BORIS are expressed in a mutually exclusive pattern that correlates with the resetting of methylation marks during male germ cell differentiation (Loukinov et al., 2002; Pugacheva et al., 2010; Campbell et al., 2010).

BORIS is considered to be a new oncogene, as abnormally high levels of *BORIS* transcripts have been observed in a wide range of human tumors and cancer-derived cell lines, and its expression coincides with CpG hypomethylation in cancer cells (Klenova et al., 2002; Woloszynska-Read et al., 2011; Kleiner, 2012).

BORIS has been studied in patients with gynecological cancers and its expression in endometrial and uterine mixed mesodermal tumors has been frequently observed (Risinger et al., 2007). The ratio of *BORIS/CTCF* mRNA expression has been associated with DNA hypo-

methylation and poor prognosis in epithelial ovarian cancer (Kleiner, 2012).

However, the expression of *BORIS* and *CTCF* has not been studied in cervical cancer; thus, we investigated the expression of *BORIS* and *CTCF* in squamous intraepithelial lesions and cervical cancer.

MATERIAL AND METHODS

Study population

The study was approved by the Institutional Ethics Committee of the Ministry of Health of Durango State (002943), and informed consent was obtained from participants before conducting this cross-sectional study.

Women aged 18-63 years were recruited from the Family Care Clinic at the Scientific Research Institute of the Juarez University of Durango State, and the Clinic of Dysplasia at the General Hospital of the Ministry of Health of Durango State.

The participants underwent sampling for Papanicolaou (Pap) smear, colposcopic examination, and biopsy for allocation into the study groups, which were stratified according the Bethesda Classification of squamous intraepithelial lesions (Solomon et al., 2002): 1) cytology negative for intraepithelial lesion or malignancy (NILM), 2) low-grade squamous intraepithelial lesions (LSIL), 3) high-grade squamous intraepithelial lesions (HSIL), and 4) invasive cervical carcinoma (ICC).

Patients with a history of cryotherapy, cone therapy, chemotherapy, or radiotherapy were excluded from the study.

Sociodemographic data and risk factors for cervical cancer such as smoking status, age at menarche, educational status, occupation, number of pregnancies, number of vaginal deliveries, use of oral contraceptives, and age at first sexual intercourse were collected.

Definitions

According to the Bethesda Classification of squamous intraepithelial lesions (Solomon et al., 2002), the NILM indicates that there is no cellular evidence of neoplasia. LSIL typically appears as mild dysplasia [cervical intraepithelial neoplasia (CIN)] (Jemal et al., 2011), likely caused by HPV infection and typically diagnosed following a Pap smear. HSIL refers to cytologically detected lesions reported as CIN2, CIN3, or carcinoma *in situ*. ICC refers to squamous cell carcinoma/unspecified histology and adeno/adenosquamous carcinoma.

Assays

Cervical specimens were collected using a standard cytobrush technique; 1 smear sample was prepared and stained using the Papanicolaou method. All patients were examined colposcopically after application of 5% acetic acid to the cervix. Colposcopic findings were recorded as follows: normal; abnormal but inconsistent with CIN; CIN 1, 2, or 3; or cancer (test-positive, CIN 1 or worse). Directed biopsy and endocervical curettage was performed and processed in the General Hospital Pathology Department using standard methods.

To analyze *BORIS* and *CTCF* expression, a second smear sample was obtained using a cytobrush, which was immersed and stored in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and immediately frozen at -80°C for total RNA isolation.

The concentration and purity of RNA were assessed by absorbance at UV_{260} and $UV_{260/280}$, respectively. RNA integrity was verified by 2% agarose gel electrophoresis.

According to manufacturer instructions, mRNA quantitation of *CTCF* and *BORIS* was conducted from total RNA using quantitative reverse transcription-polymerase chain reaction with the QuantiTect SYBR Green RT-PCR Kit and QuantiTect Primer Assay primers (Qiagen, Hilden, Germany). Relative gene expression levels were assessed and normalized using the gene for glyceraldehyde 3-phosphate dehydrogenase as an internal control.

Statistical analysis

Differences between groups were estimated using the unpaired Student *t*-test (Mann-Whitney U-test for skewed data) for numerical variables, and the Fisher exact test for categorical variables.

One-way analysis of variance with *post hoc* Bonferroni's correction was used to estimate statistical differences between more than 2 groups.

Multivariate logistic regression analysis was performed to determine the relationship between the expression of *BORIS* and *CTCF* (independent variables) with squamous intraepithelial lesions and cervical cancer (dependent variables). The model was adjusted by variables that in bivariate analysis showed differences between groups.

The statistical software package used was SPSS for Windows, version 15.0 (SPSS, Inc., Chicago, IL, USA). Expression analysis and statistical evaluation was conducted using the pairwise fixed re-allocation randomization test in the Qiagen REST 2009 Software, V2.0.13.33.

RESULTS

Patient characteristics

A total of 71 women were enrolled, including 19 (26.8%), 17 (23.9%), 27(38%), and 8 (11.3%) women in the groups with NILM, LSIL, HSIL, and ICC, respectively.

The number of vaginal deliveries was significantly higher in women with ICC compared with other groups, whereas education status was significantly higher in women with NILM. There were no other significant differences between groups (Table 1).

Expression analysis of *BORIS* and *CTCF*

BORIS expression was observed in 9 (12.7%) samples; of these, 1 (5.3%), 1 (5.9%), 4 (14.8%), and 3 (37.5%) were in the groups with NILM, LSIL, HSIL, and ICC, respectively. Relative *BORIS* expression was significantly higher in the ICC group ($106.6 \times 10^{-3} \pm 168.8 \times 10^{-3}$) compared with in the NILM ($2.5 \times 10^{-3} \pm 11.0 \times 10^{-3}$), LSIL ($1.2 \times 10^{-3} \pm 5.0 \times 10^{-3}$), and HSIL ($5.6 \times 10^{-3} \pm 25.9 \times 10^{-3}$) groups ($P < 0.0005$).

CTCF mRNA was expressed in all samples. The expression level of *CTCF*, relative to the gene for glyceraldehyde 3-phosphate dehydrogenase, was significantly higher in the carcinoma group (1620 ± 2342) compared with in the LSIL (69 ± 140), HSIL (227 ± 661), and NILM (264 ± 848) groups ($P < 0.0005$).

Interestingly, although no statistical significant differences were observed, *CTCF* expression was significantly down-regulated in the LSIL group compared with in the NILM group.

Table 1. Patient characteristics according to cytological diagnosis.

	NILM	LSIL	HSIL	ICC	F	P value
N	19	17	27	8		
Age (years)	38.6 ± 7.4	34.2 ± 10.9	38.4 ± 12.4	42.7 ± 8.2	1.3	0.27
Menarche (years)	12.4 ± 0.8	12.5 ± 1.4	13.0 ± 1.8	13.0 ± 1.6	0.9	0.45
Education status (years)	13.3 ± 4.1	7.3 ± 3.6	7.3 ± 3.3	8.6 ± 4.1	11.5	<0.0005**
Age at first sexual intercourse	19.8 ± 3.4	16.9 ± 3.1	18.4 ± 3.0	18.4 ± 4.4	2.4	0.07
Number of sexual partners*	1 (1-2.8)	2 (1-3)	2 (1-2.5)	2 (1-5.5)	-	0.36
Pregnancies*	2.5 (1.2-3)	3 (2-5)	4 (2-6)	4 (2.5-7.5)	-	0.06
Vaginal deliveries (N*)	2 (0.5-3)	3 (2-4)	3 (2-5)	4 (2.5-6)	-	0.007 [‡]

NILM, negative for intraepithelial lesion or malignancy; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; ICC, invasive cervical cancer. Data are reported as means ± SD, or otherwise indicated. *Data are median (25th-75th percentile). **P < 0.0005 between NILM and HSIL, estimated using one-way ANOVA with *post hoc* Bonferroni's test. [‡]P < 0.05 between NILM group as compared with LSIL, HSIL, and ICC groups, estimated using the Kruskal Wallis test.

DISCUSSION

The causal association between HPV infection and cervical cancer is well-known; however, HPV molecular testing methods lack specificity because they do not discriminate between transient and persistent infection. Therefore, other markers that play a role in deregulation of the cell cycle that occurs in cervical neoplasia, such as p16 (INK4A), MIB-1, and BD-ProEx CTM, have been examined. These markers show a high correlation with the presence of HSIL (Pinto et al., 2012). In this regard, BORIS is aberrantly expressed in different cancers and is absent or low in normal human tissues or cell lines; therefore, it has been proposed to be a potential biomarker in cancer (D'Arcy et al., 2006, 2008; Chen et al., 2013).

BORIS expression has been observed in gynecological neoplasias, such as ovarian and uterine cancers (Risinger et al., 2007; Woloszynska-Read et al., 2011; Renaud et al., 2011). BORIS expression has also been examined in the normal cervix and the HeLa cervical adenocarcinoma cell line (Renaud et al., 2011; Jones et al., 2011) but not in LSIL, HSIL, or ICC. In our study, expression of BORIS mRNA was observed in 12.7% of the samples analyzed, with a higher proportion of positive samples in the ICC group than in the LSIL and HSIL groups (P < 0.0005).

BORIS is considered to be an oncogene because its aberrant expression has been detected in ~70% of all primary tumors and cancer cell lines analyzed (de Necochea-Campion et al., 2011); however, the direct role of BORIS in the pathophysiology of neoplasia is not completely understood.

A recent study found that induction of BORIS expression resulted in decreased proliferation and clonogenic capacity of normal and transformed cells; therefore, BORIS may act as a tumor suppressor gene rather than an oncogene, similar to its paralog CTCF. In addition, it has been hypothesized that aberrant expression of BORIS in cancer may be a normal cellular response to protect against abnormal proliferation (Tiffen et al., 2013).

With regards to CTCF, we observed significantly higher levels of CTCF in the ICC group than in the LSIL, HSIL, and NILM groups. CTCF overexpression has been associated with growth suppression in different cellular systems; therefore, it is considered to be a tumor suppressor (Rasko et al., 2001; Rakha et al., 2004; Torrano et al., 2005; Docquier et al., 2005; Recillas-Targa et al., 2006; Ramli et al., 2012; Ouboussad et al., 2013). However, the opposite effect has been observed in other cell types (Heath et al., 2008); thus, it has been hypothesized that these differences in gene transcription and regulation of cell growth mediated by CTCF

depend on the chromatin environment, transcriptional machinery, and metabolic state of different cell types (Huang et al., 2013).

The expression of *CTCF* in LSIL or ICC has not been studied previously. Recently, Huang et al. (2013) reported that ectopically expressed *CTCF* promotes cell proliferation in a cell line of cervical cancer cells (HeLa-S3), suggesting that *CTCF* is an oncogene rather than a tumor suppressor in cervical cancer cells.

Worldwide, ICC is one of the most commonly diagnosed cancers in women. Cytological screening programs using conventional Pap tests have significantly reduced the incidence and mortality of cervical cancer; however, biomarkers that improve the standardization and quality control in the diagnosis and prognosis of ICC are needed.

In conclusion, we found that *BORIS* and *CTCF* expression in LSIL and ICC is higher than expression in cytologically normal samples. The possible utility of *BORIS* and *CTCF* as biomarkers in cervical neoplasias requires further analysis to determine its regulation at different stages in the transformation of the cervical cancer cells.

Conflicts of interest

The authors declare no conflict of interest.

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