Immunohistochemical WWOX Expression and Association with Angiogenesis, p53 Expression, Cell Proliferation and Clinicopathological Parameters in Cervical Cancer

Avaliação da expressão do gene WWOX por avaliação imunohistoquímica, sua associação com marcador de angiogênese, expressão do p53, proliferação celular e parâmetros clinicopatológicos no câncer de colo uterino

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Abstract

Objective The current study evaluated the expression of WW domain-containing oxidoreductase (WWOX), its association with clinicopathological features and with p53, Ki-67 (cell proliferation) and CD31 (angiogenesis) expression in patients with invasive cervical squamous cell carcinoma (ICSCC). To the best of our knowledge, no other study has evaluated this association.

Methods Women with IB stage-ICSCC ($n = 20$) and women with uterine leiomyoma ($n = 20$) were prospectively evaluated. Patients with ICSCC were submitted to type B-C1 radical hysterectomy and pelvic lymphadenectomy. Patients in the control group underwent vaginal hysterectomy. Tissue samples were stained with hematoxylin and eosin for histological evaluation and protein expression was detected by immunohistochemistry studies.

Results The WWOX expression was significantly lower in the tumor compared with the expression in the benign cervix ($p = 0.019$). The WWOX expression was inversely associated with the CD31 expression in the tumor samples ($p = 0.018$). There was no association between the WWOX expression with the p53 expression ($p = 0.464$) or the Ki-67 expression ($p = 0.360$) in the samples of invasive carcinoma of the cervix. There was no association between the WWOX expression and tumor size ($p = 0.156$), grade of differentiation ($p = 0.914$), presence of lymphatic vascular invasion ($p = 0.155$), parametrium involvement ($p = 0.421$) or pelvic lymph node metastasis ($p = 0.310$) in ICSCC tissue samples.

Conclusion The results suggested that WWOX may be involved in ICSCC carcinogenesis, and this marker was associated with tumor angiogenesis.

Keywords

- cervical neoplasia
- immunohistochemistry
- tumor suppressor gene
- WWOX

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Resumo

Objetivo O presente estudo avaliou a expressão do WWOX, sua associação com características clinicopatológicas e com a expressão do p53, ki-67 (proliferação celular) e CD31 (angiogênese) em pacientes com carcinoma invasivo de células escamosas do colo uterino, ou simplesmente câncer do colo uterino (CCE).

Métodos Foram avaliadas prospectivamente pacientes com CCE no estágio IB ($n = 20$) e mulheres com mioma uterino, no grupo controle ($n = 20$). As pacientes com CCE foram submetidas à histerectomia radical e à linfadenectomia pélvica do tipo B-C1. As mulheres no grupo-controle foram submetidas à histerectomia vaginal. As amostras de tecido foram coradas com hematoxilina e eosina para avaliação histológica e a expressão das proteínas foi detectada por imuno-histoquímico.

Resultados A expressão do WWOX foi significativamente menor no tumor quando comparada com sua expressão no colo do útero benigno ($p = 0,019$). A expressão tumoral de CD31 foi inversamente associada à expressão de WWOX ($p = 0,018$). Sua expressão não foi associada à expressão tumoral de p53 e Ki-67 em pacientes com CCE ($p = 0,464$ e $p = 0,360$, respectivamente). Não houve associação entre a expressão de WWOX e o tamanho do tumor ($p = 0,156$), grau de diferenciação ($p = 0,914$), presença de invasão vascular linfática ($p = 0,155$), comprometimento do paramétrio ($p = 0,421$) ou metástase dos linfonodos pélvicos ($p = 0,310$) em pacientes com CCE.

Conclusão Os resultados sugeriram que o WWOX pode estar envolvido na carcinogênese do CICECU e esse marcador foi associado à angiogênese tumoral.

Palavras-Chave

- câncer de colo uterino
- imunohistoquímica
- gene supressor tumoral
- WWOX

Introduction

Cervical cancer is the most common gynecological neoplasia in the developing world.[1] Developing countries account for two-thirds of the cases and for more than 85% of all deaths due to cervical cancer.[2,3] In 2016, 16,630 new cases are expected in Brazil alone, representing the third most common malignancy and the fourth leading cause of death among women.[3]

Studies have shown that persistent infection with human papillomavirus (HPV) plays a critical role in cervical carcinogenesis.[4,5] However, HPV infection alone is not sufficient to induce malignant transformation, and additional genetic or epigenetic changes in tumor cells are required for tumorigenesis.[6–8] The development and progression of cervical cancer are likely to be associated with the loss of growth suppression, increased cell growth rates and angiogenesis.[9,10] These combinations of genetic abnormalities generate cells that divide more rapidly or evade cell death, liberating them from growth control and cell cycle checkpoints.[11]

The analysis of tumor suppressor genes expression in human cancer is very important to gain a better insight in the process of tumorigenesis and for the early diagnosis of malignant transformation.[12] The WW domain-containing oxidoreductase (WWOX) gene is located at the site of chromosome 16 (16q23.3–24.1), specifically the FRA16D site.[13,14] This region displays profound chromosomal instability and is the second most active fragile site in the human genome.[15,16] The WWOX expression is altered in several types of tumor, including breast cancer, prostate and esophageal cancer, and also seems to be involved in the progression and prognosis of these cancers.[17–24]

Despite the potential relevance of the WWOX gene in carcinogenesis, surprisingly, little research has focused on its role in the development of cervical cancer.[8] Given the high incidence of this cancer, the current study evaluated the immunohistochemical expression of WWOX in women with invasive cervical squamous cell carcinoma (ICSCC) and its association with the expression of genes p53, CD31 and Ki-67, which are involved in important stages of carcinogenesis, such as angiogenesis and cell proliferation. In addition, we also investigated the potential association between the WWOX expression and clinicopathological parameters.

Methods

The study protocol was approved by the local Research and Ethics Committee, and all patients signed an informed consent form before being included in this study.

The study group consisted of 20 women with stage IB ICSCC and the control group was composed of 20 women with uterine myoma. The mean age of the patients was prospectively evaluated at 49.1 ± 1.7 years (mean ± standard errorof the mean [SEM], range 27–78 years).

The patients with cervical cancer were submitted to Piver-Rutledge class III radical hysterectomy and pelvic lymphadenectomy.[25] This was the primary treatment for all patients because none had previously been submitted to radiotherapy and/or chemotherapy. The clinical stage was defined preoperatively by pelvic examination under general anesthesia, according to the recommendations of the International Federation of Gynecology and Obstetrics (FIGO) Vaginal hysterectomy was performed for the uterine myomas, according to the modified Heaney technique.[26,27]

The cervix tissue samples were fixed in 10% neutral-buffered formalin immediately after the surgery. Then they were embedded in paraffin and stained with hematoxylin and eosin for histological evaluation. The pathological specimens were analyzed by two pathologists, according to the recommendations of the American Society of Pathologists.[28] The clinicopathological characteristics, such as tumor size, differentiation grade, lymphatic vascular invasion, parametrial involvement and status of pelvic lymph nodes, were recorded.

Immunohistochemistry

Tissue sections from ICSCC and normal cervices samples were stained with WWOX (Upstate, NY, USA), p53 (clone DO7, DAKO), Ki-67 (clone MIB-1, DAKO) and CD31 (clone JC/70A, DAKO) antiserum. Briefly, 4-µm paraffin-embedded sections were dewaxed in xylene and hydrated with graded ethanol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in water for 10 minutes. Heat-induced antigen retrieval was performed with 1 mM EDTA buffer at pH 8.0 for 30 minutes in a steamer at 96°C.

Primary polyclonal rabbit antiserum was used at 1:100, 1:100, 1:100 and 1:40 dilutions for WWOX, p53, Ki-67 and CD31, respectively, for 18 hours at 4°C. This was followed by incubation with the labeled streptavidin-biotin NovoLink Max Polymer Detection System (Leica Biosystems, Nussloch, Germany). The peroxidase activity was developed with DAB (Sigma, St Louis, MI, USA) with timed monitoring using a positive control sample. The sections were then counterstained with hematoxylin, dehydrated and mounted.

Analysis of WWOX, p53, Ki-67 and CD31 staining

All slides were examined under light microscopy. The staining for WWOX, p53, Ki-67 and CD31 was evaluated according to the number of positive stained cells by two pathologists blinded to the clinical information of each patient. The WWOX protein is expressed in the cytoplasm (►**Fig. 1**). The p53 and Ki-67 proteins demonstrated only nuclear reactivity, while the CD31 showed both nuclear and cytoplasmic staining in the cells. For each protein, epithelium cells for WWOX, p53 and Ki-67 and endothelium cells for CD31 presenting any expression were considered positive and counted, regardless of the staining intensity.

The immunostaining was analyzed semiquantitatively. At least 1,000 epithelium cells were analyzed in 10 fields, at 200x magnification. In other words, for each field, at least 100 epithelium cells were checked. With the data obtained from all the analyzed fields, the positive index was calculated using the following formula:

$$\textbf{Positive Index} = \frac{\text{Sum of all positive cells per field x } 100}{\text{Total cells per field}}$$

Therefore, the positive index indicated the percentage of positive cells over the number of total epithelial cells analyzed. For the WWOX, p53, Ki-67 and CD31 proteins, the following grades were considered:

- Grade 1: 0 to 25% of immunopositive cells;
- Grade 2: 26 to 50% of immunopositive cells;
- Grade 3: 51 to 75% of immunopositive cells;
- Grade 4: 76 to 100% of immunopositive cells.

Statistical Analysis

The statistical analysis was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The data were analyzed using the chi-squared test to evaluate significant differences between the groups. The level of significance was set at $p < 0.05$. Power calculations showed that the sample size ($n = 20$) provided a minimal detectable difference of 35% between the two prevalence rates, with a power of 80% and a type I error of 5%.

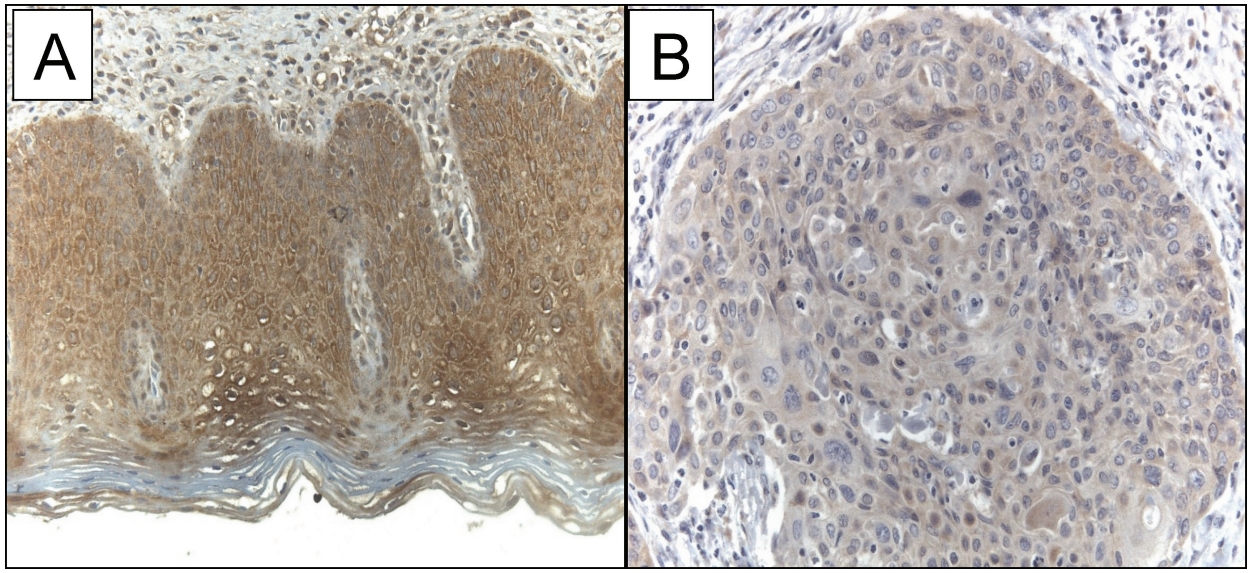

Fig. 1 WWOX cytoplasmic immunostaining. Normal cervical epithelium (A) and invasive squamous cell carcinoma (ISCC) (B). Less intense expression of WWOX can be observed in the ISCC compared with the benign tissue. (original magnification x200).

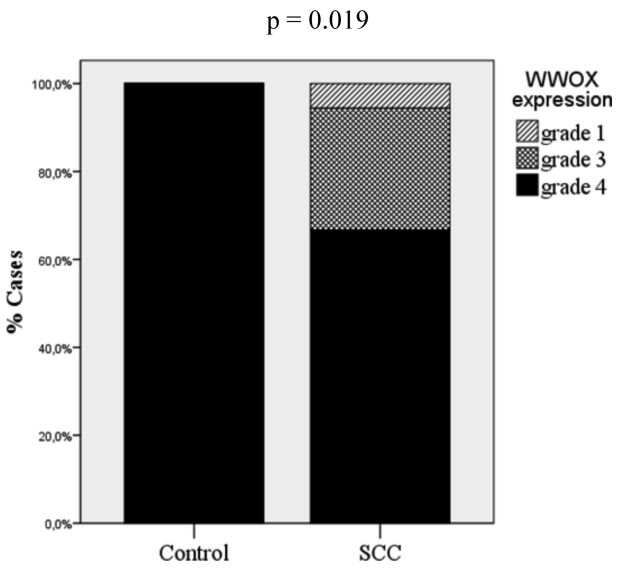

Fig. 2 Expression of WWOX in the benign cervix samples (control group = 20 samples) and in the squamous cell carcinoma samples (study group = 20 samples). The higher WWOX expression was observed in all control group samples. **Note:** Differences between groups were assessed by χ2 test (two groups). Grade 1: less than 25% of cells showed positivity; grade 2: 26–50% expression; grade 3: 51–75% expression; grade 4: greater than 75% expression.

Results

The clinical stage (FIGO) was IB1 in 14 patients (70%) and IB2 in 6 patients (30%). The average tumoral volume was 18.4 ± 19.1 cm^3 (0.3–140.0 cm^3). The tumor was well-differentiated (G1) in 1 case (5%), moderately differentiated (G2) in 15 cases (75%) and poorly differentiated in 4 (20%) cases. Lymphatic vascular invasion (LVSI) was present in four patients (20%). The average number of dissected lymph nodes was 17.9 ± 5.1 (range 10–28). Parametrium involvement was noted in 8 patients (40%). Pelvic lymph node metastasis was observed in 9 patients (45%) and the average number of pelvic lymph nodes affected by the tumor at the time of the pathological examination was 1.3 ± 2.3 (range 0–9 nodes).

The WWOX immunostaining was lower in the tumor compared with the benign cervix ($p = 0.019$). In 100% of the controls ($n = 20$) the WWOX expression was grade 4. This high expression was observed in 65% ($n = 13$) of the study group (►**Fig. 2**). However, WWOX lower grades expression, grade 3 ($n = 6$) and grade 1 ($n = 1$), occurred only in the cervical squamous cell carcinoma samples.

There was no association between tumor WWOX expression and tumor size ($p = 0.156$), differentiation grade ($p = 0.914$), presence of lymphatic vascular invasion ($p = 0.155$), parametrium involvement ($p = 0.421$) and pelvic lymph node metastasis ($p = 0.310$) in patients with invasive carcinoma of the cervix (study group) (►**Table 1**).

The WWOX expression was not associated with tumor expression of p53 and Ki-67 in patients with ICSCC ($p = 0.464$ and $p = 0.360$, respectively) (►**Fig. 3A** and **B**). The tumor expression of CD31 was associated inversely with the WWOX expression ($p = 0.018$). Tumor samples with higher grades of WWOX expression presented lower grades of CD31 expression (►**Fig. 3C**).

Discussion

The WWOX is a tumor suppressor gene and its genomic location in a common fragile site makes this gene attractive for several research groups.[13,22] The altered expression of WWOX is usually caused by previous carcinogenic exposure; in cervical cancer, the HPV infection could play this role. However, studies investigating WWOX expression involved in the development of invasive primary cervical cancer remain incipient.[29]

This study aimed to evaluate the WWOX expression in the squamous cervical carcinoma. The association between the WWOX expression with clinicopathological features, p53 expression, Ki-67 expression (cell proliferation biomarker)

Table 1 Association of tumor size, grade of differentiation, presence of lymphatic vascular invasion, parametrium involvement and pelvic lymph node metastasis with tumor WWOX expression in patients with invasive squamous cell carcinoma of the cervix

Variables	WWOX expression			*p*
	Grade 1	Grade 3	Grade 4	
Tumor size				0.156
< 4 cm	1 (7.7%)	2 (15.4%)	11 (76.9%)	
≥ 4 cm	0	4 (66.7%)	2 (33.3%)	
Grade of differentiation				0.914
G1	0	0	1 (100%)	
G2	1 (7.7%)	5 (30.8%)	9 (61.5%)	
G3	0	1 (25%)	3 (75%)	
Lymphatic vascular invasion	1 (25%)	1 (25%)	2 (50%)	0.155
Parametrial invasion	1 (12.5%)	2 (25%)	5 (62.5%)	0.421
Lymph node metastasis	1 (12.5%)	3 (37.5%)	4 (50%)	0.310

Note: Differences between groups were assessed by χ2 test (two groups). Grade 1: when less than 25% of cells showed positivity; grade 2: with 26–50% expression; grade 3: with 51–75% expression; grade 4: with greater than 75% expression.

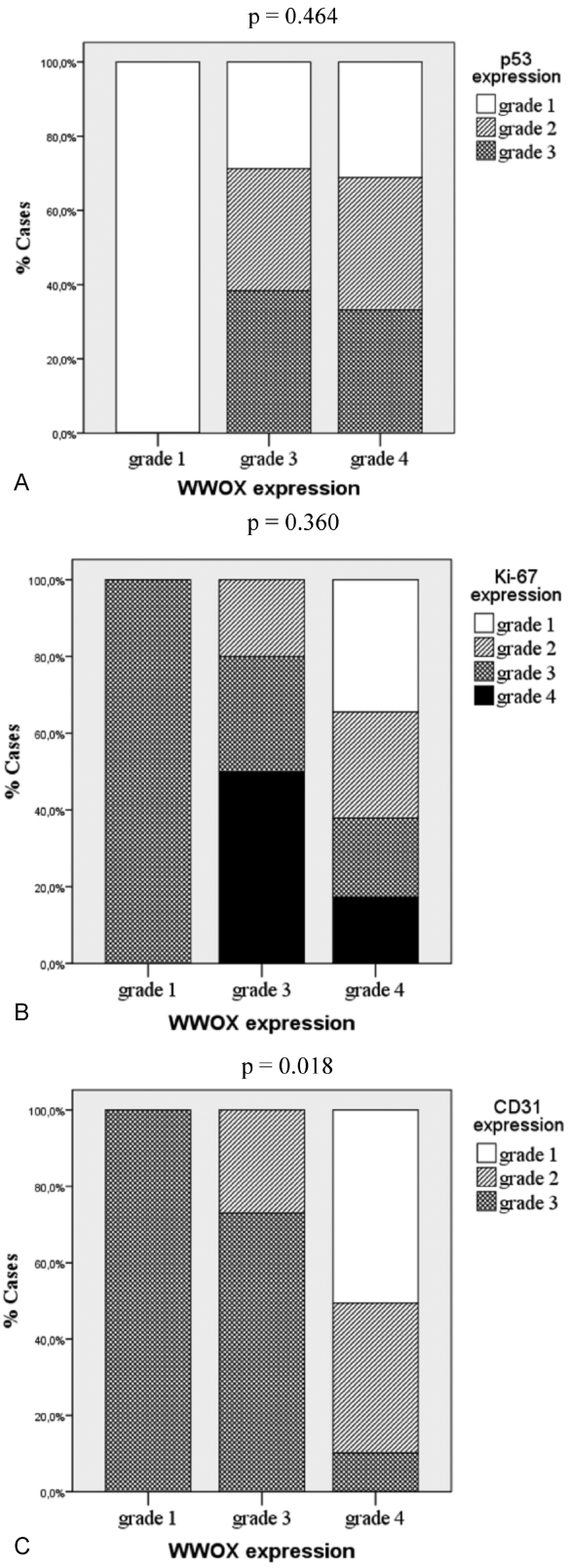

Fig. 3 Graphs demonstrate the association between the grades of WWOX immunostaining and the p53 (A), Ki-67 (B) and CD31 (C) grades of expression in patients with invasive squamous cell carcinoma (ISCC) ($n = 20$). In (C), the higher CD31 expression was significantly associated with a reduced WWOX expression ($p = 0.018$). **Note:** Differences between groups were assessed by the $\chi 2$ test (two groups). Grade 1: less than 25% of cells showed positivity; grade 2: 26–50% expression; grade 3: 51–75% expression; grade 4: greater than 75% expression.

and CD31 expression (angiogenesis biomarker) in patients ICSCC was also investigated. To the best of our knowledge, no other study has evaluated this association, and this is the strength of our study.

The WWOX expression was decreased in samples of invasive cervical squamous cell carcinoma compared with the benign cervix. These results are consistent with the only previous study that evaluated WWOX expression in cervical cancer, which identified a reduction or absence of WWOX protein expression in 69% of the patients with invasive cervical cancer. In preinvasive lesions, they observed that the expression was low or undetectable in 43.1% of cervical intraepithelial neoplasia (CIN) grade 1 and in 50% of CIN grades 2/3. The authors also detected a greater loss of WWOX expression from CIN2/3 to ICSCC than from CIN 1 to CIN2/3, suggesting that the WWOX protein is more important during cervical cancer progression than in the initial process of carcinogenesis.[8] Our study did not investigate preinvasive lesions and cannot confirm these results for CIN.

The current study also showed that the WWOX expression is inversely associated with the CD31 expression. Angiogenesis is a prerequisite for tumor growth and is also correlated with the potential for solid tumor metastasis.[30] The CD31 expression is related to neovascularization and it seems to be associated with the clinical course of cervical cancer.[31] The results suggest that decreased WWOX expression in ICSCC can allow CD31 overexpression. Since the WWOX is a tumor suppressor gene, it may suppress tumor angiogenesis,[32] inhibiting the expression of CD31; so, it has potential to be a prognostic marker in ICSCC. The inverse association observed between the expressions of WWOX and CD31 strengthens this hypothesis. However, further research will be required to validate these findings and establish this link. Different pathways implicated in cervical cancer angiogenesis should be evaluated.

No significant association was found between the WWOX expression and the p53 and Ki-67 expressions in ICSCC, which may suggest that the loss of WWOX expression occurs earlier than the alteration in the p53 and Ki-67 expression. This result may be explained by the fact that the samples used in this study were obtained from women with ICSCC classified as IB1 and IB2 stages, according to the FIGO classification. The p53 gene has been extensively studied to explain the oncogenicity of high-risk HPV types in cervical cancer and mutations occur very rarely in early stages of the tumor.[33,34] In more advanced stages of cervical carcinoma, the p53 expression may be greater either due to increased abnormalities in the control of its expression or degradation, or due to an increased incidence of p53 mutations.[35,36] If the study had included women with advanced stages of cervical cancer, an association between the WWOX and p53 expressions would possibly have been established.

Interestingly, our results did not show any statistically significant association between the clinicopathological features and the WWOX expression in ICSCC. Previous studies have shown that carcinogenesis markers are related to clinicopathological features. For example, in studies with advanced tumor stages, the p53 expression was reported to correlate

with stage, tumor size and grade.[33,37] On the other hand, another study did not show an association between the p53 expression and other prognostic histological variables (tumor grade, depth, lymphovascular space invasion) in early-stage cervical carcinomas.[38,39] The same behavior can be observed when evaluating the WWOX expression and the clinicopathological parameters. These unexpected findings could be attributed to the evaluated cervical cancer stages and to our limited sample size. To better evaluate this association, further studies are warranted, with a greater number of patients and including ICSCC samples of other stages.

Studies have shown that multiple mechanisms may be responsible for reducing the WWOX expression in carcinomas. The most common mechanism for the decreased WWOX expression is hemizygous deletions.[40] Another mechanism that reduces WWOX transcriptional level is hypermethylation of its promoter and coding regions. This mechanism may play a role in the downregulation of WWOX expression in several cancer cell lines by silencing the gene.[41,42] The mechanism involved in the inactivation of WWOX expression in cervical tumors was not evaluated in this study. For this reason, future studies should focus on the methylation status of the promoter region of WWOX or other epigenetic alterations which may influence the WWOX expression.

Conclusion

In conclusion, the present study suggested that the WWOX gene may be involved in ICSCC carcinogenesis and it is associated with tumor angiogenesis. A better characterization of the WWOX expression is necessary in normal, preneoplastic and ICSCC to more fully understand how the loss of the WWOX gene expression contributes to the carcinogenesis of cervical cancer. A detailed definition of WWOX functions may lead to the identification of new targets for intervention in tumor development and progression.

Contributors

Seabra M. A. L., Cândido E. B., Vidigal P. V. T., Lamaita R. M., Rodrigues A. N. and Silva Filho A. L. contributed with the project and the interpretation of data, writing of the article, critical review of the intellectual content and final approval of the version to be published.

Conflict of Interests

The authors declare that there was no conflict of interests.

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