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Loss of ZNF516 protein expression is related with HR-HPV infection and cervical preneoplastic lesions

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Abstract

Purpose Cervical cancer is an important health issue among women worldwide. Cervical smear and human papillomavirus detection are the most used screening methods to detect preneoplastic and neoplastic lesions. However, as neither can predict cervical development, new markers are needed for this disease. *ZNF516*, a potential tumor suppressor gene, has been found altered in cervical cancer. The objective of this study was to determine ZNF516 immunohistochemistry frequency in cervical biopsies and its association with clinicopathological parameters, to evaluate its potential as marker in cervical lesions.

Methods A retrospective series of 452 formalin-fixed, paraffin-embedded (FFPE) cervical biopsies, obtained between 2002 and 2007, were selected for immunohistochemistry of ZNF516, p16 and Ki-67 markers. Human papillomavirus genotyping was performed on 272 of these samples through reverse line blot assay.

Results An inverse relation between ZNF516 expression and cervical lesions grade ($P < 0.001$) was observed, given this protein was found mainly expressed in normal tissues, while was decreased in cervical lesions. As expected, the proliferation markers p16 and Ki-67 were found highly expressed in cervical cancer compared to normal tissues, and inversely correlated to ZNF516 expression ($P < 0.01$). High oncogenic risk-Human papillomavirus presence also was related to the lack of ZNF516 expression in cervical lesions ($P < 0.05$), and the detection of these two parameters showed a high sensitivity (70.9%) for preneoplastic lesions detection.

Conclusions The loss of ZNF516 expression was found in cervical lesions, and its detection potentially could be used as a complementary marker of early diagnosis in cervical lesions.

Keywords ZNF516 · Cervical cancer · Immunohistochemistry · p16 · Ki-67 · Human papillomavirus

Introduction

Cervical cancer is the fourth most common cancer in women around the world with an estimated of 570,000 new cases and 310,000 deaths in 2018 [1]. High incidence rates

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of cervical cancer and its precursor lesions can be found mainly in transitioning countries [1]. Particularly in Chile, incidence rate of cervical cancer was estimated at 12.2 per 100,000 women and mortality rate in 5.0 per 100,000 women, according to GLOBOCAN data, thus becoming the eighth cancer-related cause of death in female Chilean population [1].

Human papillomavirus (HPV) infection is the main cause of cervical precursor lesions and subsequent cervical cancer [2]. The preneoplastic lesions could be classified into two main groups: low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) according to the probability of progression to cancer. According to this classification, cervical epithelia withsigns of HPV infection and cervical intraepithelial neoplasia grade 1 (CIN 1) are considered as LSIL; meanwhile CIN 2, CIN 3 and Carcinoma in situ are included in HSIL [3]. Invasive cervical cancer occurs when tumorigenic cells exceed basal membrane of the epithelia. Histologically, cervical cancer presents as either squamous cell carcinoma (SCC) or adenocarcinoma (AC), where SCC is the most common type of cervical cancer [4].

Cytology-based cervical screening (Pap smear) has enabled to decrease the incidence and mortality of cervical cancer around the world, but its sensitivity is approximately 55% [5]. HPV detection has also contributed to the early recognition of preneoplastic lesions. However, despite cervical carcinogenesis is a consequence of high-risk HPV (HR-HPV) infection, only a small fraction of infected women will develop cervical cancer, implying there are others factors involved in cervical carcinogenesis [6].

Several proteins have been described as overexpressed in cervical cancer and its precursors [7]. Two of the most important are p16INK4a (p16) and Ki-67, whose expression is routinely tested by immunohistochemistry for cervical preneoplastic lesions severity classification [7, 8]. However, p16 and Ki-67 detection has limitations such as interpretation, use of non-standardized antibodies and protocols, among others [9, 10]. Therefore, novel biomarkers are required to complement the efforts in diagnosis and prognosis traceability in cervical cancer.

ZNF516 (zinc finger protein 516) is a gene located on chromosome 18q23, which has been identified as a tumor suppressor gene implied in chromosome instability in colorectal cancer. The loss of its expression leads to DNA replication stress, structural chromosome abnormalities and chromosome missegregation [11].

Recent genome-wide profiling studies have shown that *ZNF516* is hypermethylated in cervical cancer but not in normal cervical epithelia [12], suggesting *ZNF516* as a potential tumor suppressor gene and a likely methylation biomarker for early detection of cervical cancer [12, 13]. However, the expression of ZNF516 and its clinical relevance in cervical tissue remain undefined. The aim of this study was to evaluate the immunohistochemical expression of ZNF516 in cervical tissues and its association with clinicopathologic features, to evaluate its potential as marker in cervical lesions.

Materials and methods

Patients and tissue samples

To detect ZNF516 protein expression, an immunohistochemical staining was performed on a retrospective series of 452 formalin-fixed, paraffin-embedded (FFPE) biopsy samples. These samples included 45 normal cervical tissues, 169 cervical intraepithelial neoplasia grade 1 (CIN 1), 83 cervical intraepithelial neoplasia grade 2 (CIN 2), 100 cervical intraepithelial neoplasia grade 3 (CIN 3) and 55 squamous cervical carcinomas (SCC), collected between 2002 and 2007, and the analysis was performed in 2014. Normal cervical tissue samples were obtained from patients who underwent hysterectomy for benign conditions (myoma). Preneoplastic lesions were extirpated through loop electrosurgical excision procedure (LEEP) and SCC cases were obtained by surgery resection before treatment with radio or chemotherapy. The FFPE tissues were retrieved from the surgical pathology archives at Dr. Hernán Henríquez Aravena Hospital, Temuco, Chile. The study was approved by the ethical scientific committee of School of Medicine (Document no. 17/012), Universidad de La Frontera, Temuco, Chile.

Tissue microarrays (TMA)

Tissue microarrays were constructed with 2 mm cores of a representative area of each case, as indicated by an expert pathologist. Unstained 3 µm-thick sections were cut from each TMA for Histo-Clear (National Diagnostics, Atlanta, GA, USA) de-waxed and re-hydrated through graded concentrations of ethanol.

ZNF516 immunohistochemistry

Slides containing tissue cores were washed with 1 × phosphate-buffered saline (PBS) and then quenched with 5% hydrogen peroxide for 10 min before they were placed in an antigen retrieval solution (0.1 M citrate buffer, pH 6.0) for 20 min at 96 °C. After cooling for 30 min, tissues were incubated with a 1:50 dilution of rabbit polyclonal Anti-ZNF516 antibody (ab121486, Abcam, Cambridge, UK) at 4 °C,

overnight. After washing three times with 1 × PBS, slides were incubated with mouse/rabbit polydetector HRP Label (Bio SB, Santa Barbara, CA, USA). Labeling was detected with the DAB-Chromogen system (Dako North America Inc, Carpinteria, CA, USA) according to the manufacturer's protocol. All sections were counterstained with Harris's hematoxylin, dehydrated, and mounted. Normal prostate tissues were included as positive control and negative controls were prepared substituting primary antibody for PBS buffer.

Ki-67 and p16 immunohistochemistry

Ki-67 and p16 immunohistochemistry were performed in an automatized stainer (Leica BOND MAX, Leyca Biosystems, Melbourne, Australia). Briefly, slides were dewaxed at 72 °C for 30 min with dewax Solution (Leica Biosystems Newcastle Ltd. Newcastle, UK). After an ethanol rinse and wash, tissues were enzymatically pretreated before epitope retrieval at 100 °C. Peroxidase was blocked with 3% hydrogen peroxide previous to 15 min of incubation with primary antibody at room temperature. A polymer enhancer was applied followed by poly-HRP anti-mouse/anti-rabbit IgG before mixed DAB refined labeling and hematoxylin contrast. The antibodies Bond™ Ready-to-Use Primary Antibody Ki-67 (K2) (PA0230, Leica Biosystems, Newcastle Ltd, Newcastle, UK) and CINtec® p16 Histology (725-4713, Ventana Medical Systems, Inc., Tucson, AZ, USA) were used for the study.

Evaluation of immunohistochemistry

For semiquantitative evaluation of ZNF516, Ki-67 and p16, the immunohistochemically stained slides were digitalized using the digital scanner Aperio ScanScope CS2 (Leica Biosystems Imaging, Inc., Vista, CA, USA).

ZNF516 was found expressed in nucleus and cytoplasmic compartment in cervical epithelia. However, only nucleus staining was considered for the analysis, according to antibody datasheet. First, a semi-quantitative scale was used to score the reactivity of the samples based on the labeling intensity (0 = absent; 1 = weak; 2 = moderate; 3 = strong) and the percentage of positive cells (1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%). Subsequently, after data analysis, we concluded that a final score, which classified samples as negative (absence of staining) or positive (any percentage or intensity of positivity), was the better approach for ZNF516 scoring. Ki-67 scoring was based in a semiquantitative scale to describe the percentage of positively stained tumor cells as follows: Negative (below 5%), weak positive (1 + = 5–25%), moderate positive (2 + = 26–50%) and intense positive (3 + = greater than 50%) [14]. In the case of p16 scoring, strong and diffuse block staining for p16 were considered as positive; meanwhile, all other patterns were considered negatives [8]. All scores were assigned by an expert pathologist.

HPV genotyping

Due to amount of tissue needed for DNA extraction we genotype a total of 272 biopsies: normal tissues (44), CIN 1 (86), CIN 2 (44), CIN 3 (53) and SCC (45). Each case was manually dissected from FFPE samples for DNA extraction. DNA quality was evaluated through an integrity control, based on the amplification of beta globin gene (268 bp). Amplification of viral L1 gene for HPV detection was performed using primers GP5+ and biotin labeled GP6+ followed by reverse line blot analysis previously described [15, 16]. Briefly, modified oligoprobes complementary to 36 HPV genotypes were bound covalently to a membrane (Biodyne C; Pall Bio-Support West Chester, OH) using a Miniblotter system (MN45; Immunetics, Boston, MA). The GP5+/bioGP6+ PCR products were added to each Miniblotter channel, perpendicularly to oligoprobes lines for hybridization. Afterwards, membranes were incubated with a streptavidin–peroxidase conjugate (Roche, Basel, Switzerland) before chemiluminescent detection with ECL reagent (Amersham Biosciences, Piscataway, NJ). Membranes were then exposed to a film (Hyperfilm; Amersham Biosciences) for final detection.

Statistical analysis

The analyzes were performed using the statistical package SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The background data were compared between negative ZNF516 and positive ZNF516 groups. The correlation of ZNF516 expression with the clinical and pathological variables was assessed using Chi-square test or Fisher's exact probability test (two-sided). Kaplan–Meier survival curves were plotted for patients with negative versus positiveZNF516 expression and compared using a stratified log-rank test. Only the carcinoma cases were used for survival analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC) were calculated to analyze the potential use of ZNF516 as early diagnosis marker in comparison with HR-HPV. To establish the association between lesion grade, p16, Ki-67 and ZNF516 expression multinomial or binomial logistic regression were used, considering normal epithelia tissues as reference group. In multinomial and binomial logistic regression, the statistical significance, odd ratio and confidence interval was determined. P values < 0.05 were considered significant, with 95% of confidence.

Results

Association between ZNF516 expression and sociodemographic characteristics

The mean age of patients was 37.5 years (standard deviation 14.1; range 17–86 years). Two groups of patients were established according to age range: less or equal to 35 and over 35 years old, due to the major incidence of cervical cancer occurs over 35 years of age. Also, patients were grouped as White/Hispanic ($n=314$; 69%) or Mapuche ($n=138$; 31%) according their ethnicity.

Concerning to the relationship between sociodemographic data and ZNF516 expression, no significant association was found between age and immunostaining of ZNF516 ($P=0.851$). However, a significant association was observed between ethnicity and ZNF516 expression, where Mapuche participants showed a higher expression of ZNF516 compared to White/Hispanic participants ($P<0.05$) (Table 1).

Loss of ZNF516 expression was frequently found in preneoplastic and neoplastic lesions

Results showed a marked decrease of ZNF516 nuclear immunostaining in preneoplastic and neoplastic lesions compared with cervical normal epithelia (Fig. 1). The 80% of normal tissue samples were positives for nuclear ZNF516 immunostaining, while only 49.1% of SCC tissues were positive for ZNF516. The preneoplastic lesions also showed a decrease of ZNF516 nuclear staining with percentages of positivity of 57.4%, 49.4% and 42.0% for CIN 1, 2 and 3, respectively (Table 1; $P<0.001$). Significant statistical differences were found between ZNF516 expression in normal tissue compared to CIN 1 ($P<0.01$), CIN 2 ($P<0.01$), CIN 3 ($P<0.001$) and SCC ($P<0.01$), and between CIN 1 and CIN 3 ($P<0.05$) (Fig. 2). Multinomial logistic regression showed that patients with loss of ZNF516 expression showed an increased risk to develop a preneoplastic or neoplastic lesion of cervix (Table 2).

Table 1 ZNF516 immunohistochemical expression and its relationship with clinicopathological and sociodemographic features

	Cases	ZNF516 Negative		ZNF516 Positive		*P*
	n	*n*	%	*n*	%	
Total cases	452	209	46.2	243	53.8	–
Age						0.851[a]
< 35 years-old	229	107	46.7	122	53.3	
≥ 35 years-old	223	102	45.7	121	54.3	
Ethnicity						**0.031**[a]
Hispanic/White	314	156	49.7	158	50.3	
Mapuche	138	53	38.4	85	61.6	
Histological diagnosis						**0.000**[b]
Normal	45	9	20.0	36	80.0	
CIN 1	169	72	42.6	97	57.4	
CIN 2	83	42	50.6	41	49.4	
CIN 3	100	58	58.0	42	42.0	
SCC	55	28	50.9	27	49.1	
p16						**0.000**[a]
Negative	194	68	35.1	126	64.9	
Positive	258	141	57.7	117	45.3	
Ki-67						
Negative	90	29	32.2	61	67.8	**0.002**[b]
1 +	143	62	43.4	81	56.6	
2 +	106	52	49.1	54	50.9	
3 +	113	66	58.4	47	41.6	

Normal normal cervical tissues, *CIN* cervical intraepithelial neoplasia, *SCC* squamous cervical carcinoma

P values < 0.05 were considered significant, with 95% of confidence

[a]Fisher exact test

[b]Pearson Chi-square test

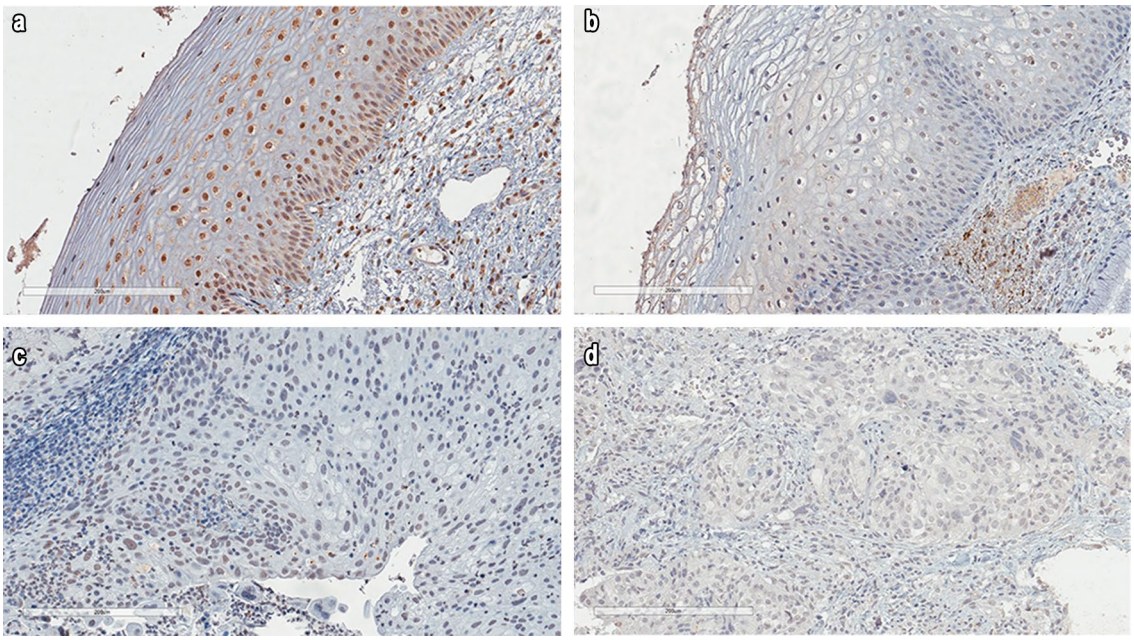

Fig. 1 ZNF516 expression in different cervical tissues detected by immunohistochemistry. **a** Normal cervical squamous epithelium showing a positive expression of ZNF516. **b** Low-grade squamous intraepithelial lesions (LSIL), showing a moderate positive staining. **c** High-grade squamous intraepithelial lesions (HSIL) showing a weak immunohistochemical staining. **d** Invasive squamous cell carcinoma expressing negative immunohistochemical staining in small nests of tumor (400 ×)

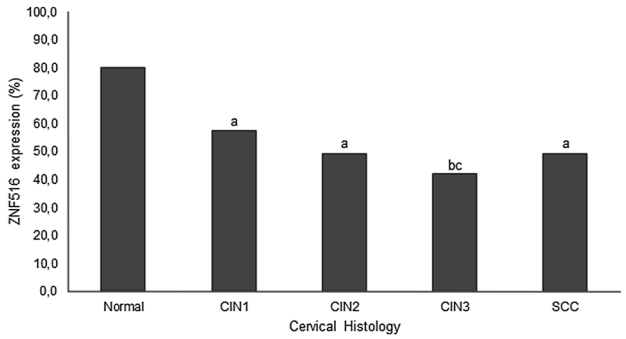

Fig. 2 Frequency of ZNF516 expression distribution in normal tissue, preneoplastic and neoplastic lesions of the cervix. *Normal* normal cervical tissues, *CIN* cervical intraepithelial neoplasia, *SCC* squamous cervical carcinoma. (a: $P < 0.01$ compared to normal; b: $P < 0.001$ compared to normal; c: $P < 0.05$ compared to CIN 1)

Correlation between ZNF516, p16 and Ki-67 in cervical tissues

To correlate ZNF516 expression with frequently used biomarkers in different histological diagnosis of cervical tissue, we performed an immunohistochemical analysis of p16 and proliferation marker Ki-67, which are the most used markers in diagnosis of cervical lesions. A direct relationship between p16 and Ki-67 expression with cervical lesions severity degree was found ($P < 0.001$) (Table 3). In normal tissues, Ki-67 and p16 were poorly expressed and most samples were considered with negative or low expression (Ki-67: 44/45; p16: 44/45) for these markers. In preneoplastic lesions, the intensity of Ki-67 and p16 immunostaining was gradually increasing, while

Table 2 ZNF516 expression association with histological cervical lesions degree and p16 and Ki-67 immunohistochemical expression

	P	OR	95% CI
Histology			
Normal	Reference[a]	Reference	Reference
CIN 1	0.007	2.969	1.345–6.552
CIN 2	0.001	4.098	1.755–9.565
CIN 3	0.000	5.524	2.405–12.686
SCC	0.002	4.148	1.684–10.220
p16 expression			
Negative	Reference[b]	Reference	Reference
Positive	0.000	2.233	1.522–3.277
Ki-67 expression			
Negative	Reference[a]	Reference	Reference
1 +	0.091	1.610	0.927–2.797
2 +	0.018	2.026	1.130–3.630
3 +	0.000	2.954	1.655–5.271

CIN cervical intraepithelial neoplasia, *SCC* squamous cervical carcinoma, *OR* odd ratio, *CI* confidence Interval

[a]Multinomial logistic regression

[b]Binomial logistic regression

Table 3 Expression of ZNF516, p16 and Ki-67 in relation to grade of cervical lesions

Histology	ZNF516			p16			Ki-67				
	Negative	Positive	*P**	Negative	Positive	*P**	Negative	1 +	2 +	3 +	*P**
	n (%)	*n* (%)		*n* (%)	*n* (%)		*n* (%)	*n* (%)	*n* (%)	*n* (%)	
Normal	9 (20.0)	36 (80.0)	0.000	44 (97.8)	1 (2.2)	0.000	28 (62.2)	16 (35.6)	1 (2.2)	0 (0.0)	0.000
CIN 1	72 (42.6)	97 (57.4)		121 (71.6)	48 (28.4)		54 (32.0)	87 (51.5)	27 (16.0)	1 (0.6)	
CIN 2	42 (50.6)	41 (49.4)		20 (24.1)	63 (75.9)		7 (8.4)	23 (27.7)	39 (47.0)	14 (16.9)	
CIN 3	58 (58.0)	42 (42.0)		8 (8.0)	92 (92.0)		1 (1.0)	15 (15.0)	30 (30.0)	54 (54.0)	
SCC	28 (50.9)	27 (49.1)		1 (1.8)	54 (98.2)		0 (0.0)	2 (3.6)	9 (16.4)	44 (80.0)	
Total	209 (46.2)	243 (53.8)		194 (42.9)	258 (57.1)		90 (19.9)	143 (31.6)	106 (23.5)	113 (25.0)	

Normal normal cervical tissues, *CIN* cervical intraepithelial neoplasia, *SCC* squamous cervical carcinoma

*Chi-square test

negative cases were almost null. Finally, over a 98% of SCC cases studied (Ki-67: 55/55; p16: 54/55) were positive for Ki-67 and p16 with an intense immunostaining (Figs. 3, 4).

Given that ZNF516 expression was high in normal cervical tissue and low in SCC, an inverse association between ZNF516 expression and p16 ($P < 0.001$) and Ki-67 ($P < 0.01$) expression was found (Table 1). In fact, the odds ratio for p16 positive expression and Ki-67 with more than 50% of positive cells increase by 2.233 and 2.954, respectively, when the expression of ZNF516 is lost (Table 2).

ZNF516 and HPV status

To establish the relationship between HPV infection and ZNF516 expression the genotyping of 60.2% (272/452) of samples included in the study was performed. Analysis showed that 64.0% (174/272) of the studied samples were positives for HPV: 18.2% of normal tissues, 60.5% of CIN 1, 79.5% of CIN 2, 81.1% of CIN 3 and 80% of SCC. The most frequent HPV genotype found was HPV16, which was present in 51.7% of positive samples (Table S1). Among HPV positive samples, 95.4% of them had DNA from an oncogenic or high-risk type of HPV (HR-HPV). Regarding the

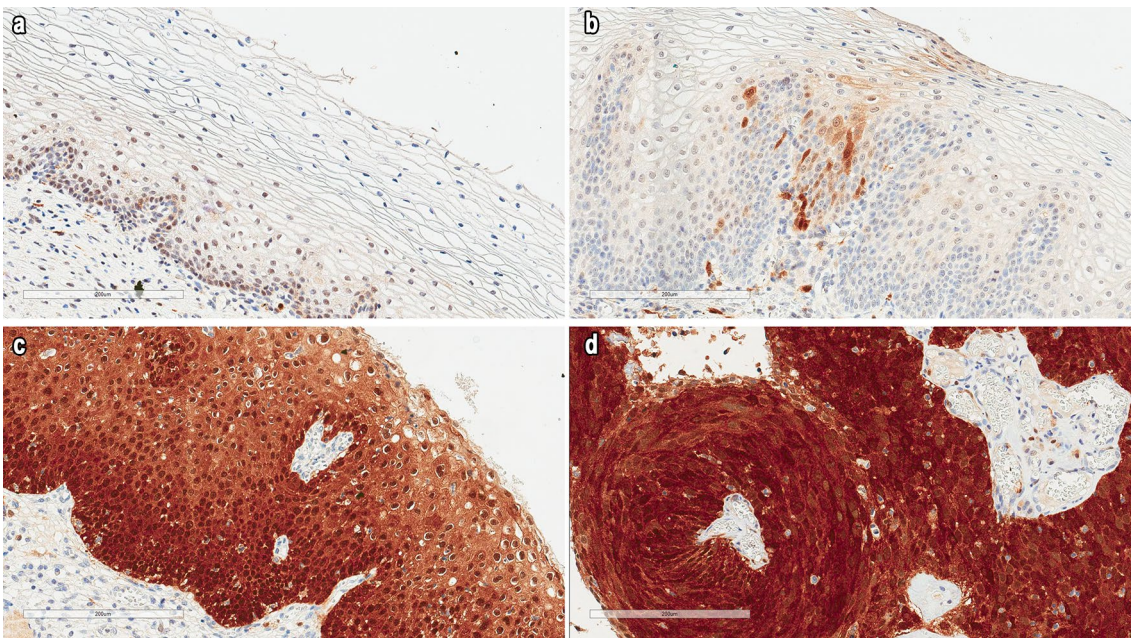

Fig. 3 p16 expression in different cervical tissues detected by immunohistochemistry. **a** Normal cervical squamous epithelium and **b** low-grade squamous intraepithelial lesions (LSIL), showing a negative expression of p16. **c** High-grade squamous intraepithelial lesions (HSIL) and **d** invasive squamous cell carcinoma showing a positive expression of p16 (400 ×)

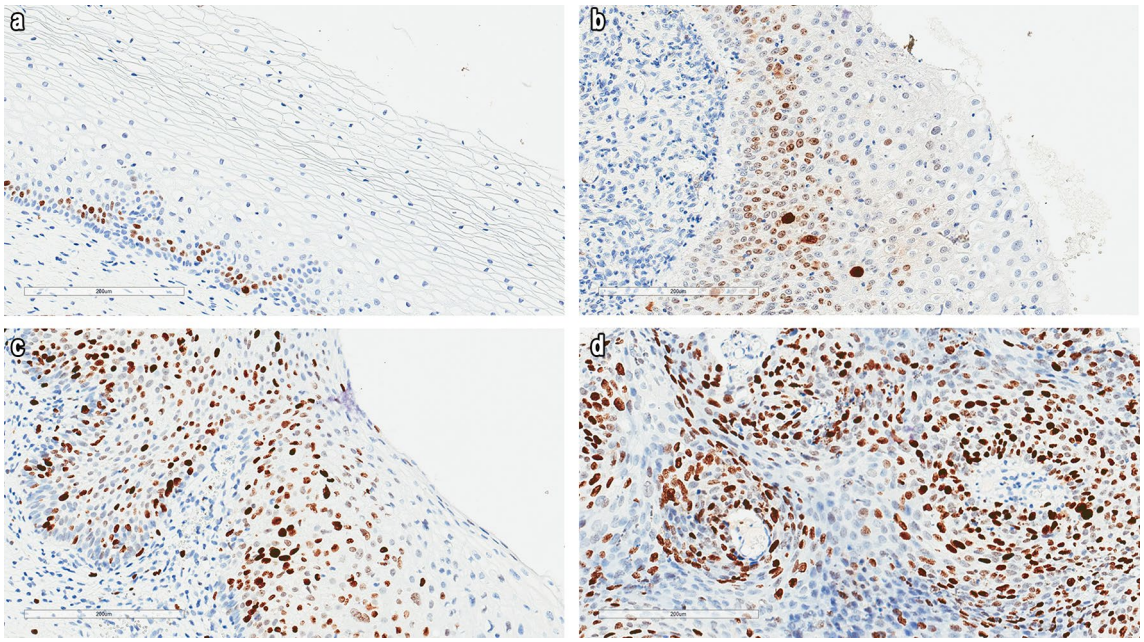

Fig. 4 Ki-67 expression in different cervical tissues detected by immunohistochemistry. **a** Normal cervical squamous epithelium showing nuclear expression of Ki-67 only in basal membrane. **b** Low-grade squamous intraepithelial lesions (LSIL), showing a mild positive staining in cells ubicated in the third part near to basal membrane of the stratified epithelia. **c** High-grade squamous intraepithelial lesions (HSIL) showing a strong immunohistochemical staining in almost all cells of the cervical epithelia. **d** Invasive squamous cell carcinoma expressing a strong immunohistochemical staining in small nests of tumor (400 ×)

relationship between HPV status and ZNF516 expression, in cervical tissues it was possible to observe that HPV negative samples or those infected with a low-risk HPV (LR-HPV), frequently had a positive expression of ZNF516 compared to HR-HPV positive samples ($P < 0.05$) (Table 4). Sensitivity, specificity, PPV, NPV and AUC were calculated for ZNF516 immunochemical expression and HR-HPV detection in relation to their capacity of discriminate CIN 1 from normal tissue (Table 5). Both markers used alone showed a relative low sensitivity (42.6% and 52.3% for ZNF516 and HR-HPV, respectively) and high specificity (80.0% and 84.1% for ZNF516 and HR-HPV, respectively) to detect CIN 1. However, when both parameters were analyzed together, an AUC higher than 0.7 was reached and the sensitivity to detect CIN1 was increased (70.9%).

ZNF516 expression and clinical outcome

Clinical outcome was analyzed in 55 patients with SCC. Patients who died from other causes not related to the disease were discarded from the study (5/55). The follow-up time ranged from 2 to 168 months, with a median time of 38 months. The entire group had an estimated 5-year survival rate of 11.5% with a median survival of 18 months.

Table 4 ZNF516 immunohistochemical expression and its relationship with HPV infection

	Total cases	ZNF516 Negative		ZNF516 Positive		*P**
	n	*n*	%	*n*	%	
HPV infection						0.030
Negative	98	34	34.7	64	65.3	
Positive	174	85	48.9	89	51.1	
HPV genotype						0.012
Negative/LR-HPV	106	36	34.0	70	66.0	
HR-HPV	166	83	50.0	83	50.0	

HPV human papillomavirus, *LR-HPV* low oncogenic risk-HPV, HR-*HPV* High oncogenic risk-HPV

*Fisher exact test

Table 5 Sensibility, specificity, positive and negative predictive values and area under curve of ZNF516 immunohistochemistry, HR-HPV genotyping and the combination of both methodologies, in relation to their capacity of discriminate CIN 1 from normal tissue

	Sensibility (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
ZNF516	42.6	80.0	88.9	27.1	0.613
HR-HPV	52.3	84.1	86.5	47.4	0.682
ZNF516/HR-HPV	70.9	70.5	82.4	55.4	0.707

HR-HPV high oncogenic risk Human papillomavirus, *PPV* positive predictive value, *NPV* negative predictive value, *AUC* area under curve

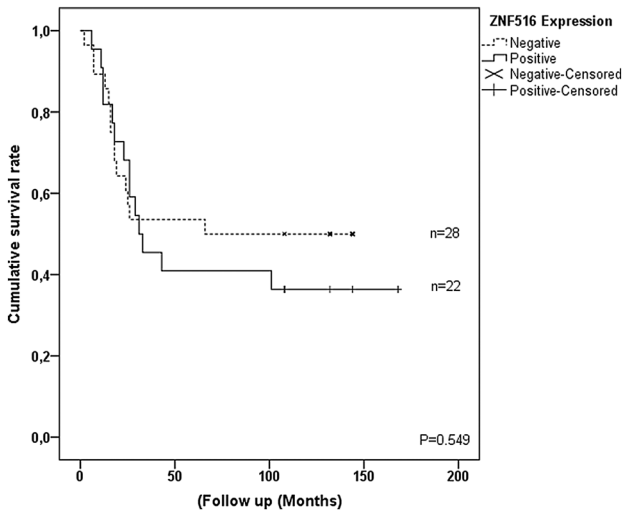

Fig. 5 Five-year survival curves (Kaplan–Meier) for patients with SCC. The solid line indicates patients whose tumors express high levels of ZN516 and the dotted line indicates patients with low expression of ZNF516 ($P=0.549$; stratified log-rank test)

Log-rank test and Kaplan–Meier plot showed that patients negative for ZNF516 had a 5-year survival rate of 54% (15/28) with a median survival of 16 months, whereas patients with positive ZNF516 expression had a 5-year survival rate of 41% ($n=9/22$) with a median survival of 23 months. Moreover, log-rank test showed no association between ZNF516 expression and overall survival in SCC patients with advanced carcinoma ($P=0.549$) (Fig. 5).

Discussion

Cervical cancer remains an important public health problem and the high rates of incidence and mortality persist [17]. Early detection of preneoplastic and neoplastic lesions, through PAP smear and more recently HPV detection, has been one of the most important advances in cervical cancer prevention [5, 6]. However, there are some deficiencies in cervical cancer screening tests that include low sensitivity of PAP and the fact that not all HPV infected women will develop a neoplasia [5, 6]. Therefore, early detection of biomarkers in cervical carcinogenesis is a pivotal topic for the screening and follow-up of this malignancy.

ZNF516 is a potential tumor suppressor protein that seems to be a strong candidate for diagnosis and marker in cervical cancer. This protein is found in the cell nucleus and apparently to have a role as transcription regulator with potential DNA-binding functions [18]. In this study, we determined the immunohistochemical expression patterns of ZNF516 in normal tissue and preneoplastic and neoplastic lesions of the cervix. In normal tissues, the expression of ZNF516 was found positive in most cases and differed significantly from preneoplastic lesions and SCC, which would suggest that ZNF516 potentially may be used as early diagnostic marker of cervical lesions. In addition, we found ZNF516 expression decreased in preneoplastic lesions and cervical cancer. In fact, the loss of ZNF516 protein expression was associated with a higher risk of suffering a cervical lesion. In 2016, differences in ZNF516 immunohistochemical expression were observed in another study involving cervical cancer, but a detailed description of the results was not shown [13]. Futhermore, our results reaffirm the idea that ZNF516 could act as a tumor suppressor protein probably repressed in carcinogenesis. Indeed, previous studies have shown that *ZNF516* gene is highly methylated in cancer but not in normal tissues [12], which agrees with the well-known fact that CpG islands promoter methylation is one of the most important transcription repressor of tumor suppressor genes in cancer development [19].

Two of the most used biomarkers for clinical diagnosis of cervical abnormalities are p16 and Ki-67 [20]. p16 is a cyclin-dependent kinase inhibitor implied in cell cycle arrest. The inactivation of RB (retinoblastoma) protein by HPV E7 oncoprotein triggers a high phosphorylation of cyclin-dependent kinases, leading to a high expression of p16, as negative feedback cell response to the lack of RB protein [10]. The proliferation marker Ki-67 is regularly expressed in nucleus of cell cycle active cells (phases G1, S, G2 and mitosis). Ki-67 is also affected by RB inactivation caused by HPV infection, due to increment of cell proliferation [21]. p16 and Ki-67 are used for preneoplastic lesion classification, especially to improve specificity and positive predictive value of ambiguous atypical squamous cells of undetermined significance (ASCUS) or LSIL Pap

test results [7, 22]. Specifically, p16 immunostaining is used in specific cases such as differentiation of HSIL from benign lesions, helping in CIN 2 or CIN3 diagnosis [8]. In this study, both markers were found highly expressed in cervical cancer and HSIL and poorly expressed or absent in LSIL and normal epithelia. These results confirm previous evidence that describe p16 and Ki-67 as good progression markers of cervical lesions, allowing the classification of cervical preneoplastic lesions [10, 21, 22]. The immunohistochemical expression of p16 and Ki-67 in cervical tissues was inversely proportional to ZNF516 expression, because p16 and Ki-67 expression increased according to the cervical lesion severity, while ZNF516 levels decreased. In addition, the probability to find a tissue with a strong expression of p16 or Ki-67 was higher in ZNF516 negative tissues indicating that ZNF516 absence is related somehow to cervical cells proliferation.

Regarding sociodemographic features, a previous research showed a slight association between ZNF516 promoter methylation and ethnicity, indicating that ZNF516 methylation was less frequently observed in Mapuche than in White/Hispanic participants ($P=0.05$) [12]. In this study, the majority of Mapuche subjects had a higher expression of ZNF516 than non-mapuche participants, which is correlated with methylation data from previous reports. Further experiments involving a larger amount of Mapuche individuals should be conducted to clarify the implications of ethnicity association with ZNF516 expression and cervical cancer.

On the other hand, HPV was genotyped to establish a potential relationship between ZNF516 expression and the presence of this virus in cervical tissue. HPV was found in 80% of analyzed cervical cancer cases, which is correlated to others studies performed in FFPE tissues [23]. Interestingly, the loss of ZNF516 expression in pathologic cervical tissues seems to be related to HR-HPV infection. In this point, a previous report showed a relationship between HPV status and ZNF516 methylation [12]. HPV E7 oncoprotein induces an increased activity of DNA methyltransferase 1 (Dnmt1) leading to methylation and silencing of several genes [24]. This phenomenon could be related to the loss of immunohistochemical expression of ZNF516 found in our study; however, further experiments are needed to prove this hypothesis. In addition, we evaluated the ability of HR-HPV detection and ZNF516 immunohistochemistry to differentiate CIN 1 from normaltissue. In this regard, both tests showed a relative low sensitivity and NPV, but a high specificity and PPV. Other reports have shown similar values of sensitivity of HPV detection (approximately 50%) to discriminate CIN 1 from normal cytology [25]. Nevertheless, testing both, HR-HPV and ZNF516, increased the probability of early detection of incipient preneoplastic lesions as CIN 1, with a sensibility of 70.9% and a AUC > 0.7, which could be considered as good accuracy for diagnosis [26]. To date, PAP smear and HPV genotyping, the recommended screening methods for preneoplastic and neoplastic cervical lesions, cannot properly distinguish between CIN 1 and normal tissue [5, 6]. In response to this problem, it is necessary novel complementary markers, which alone or in combination can diagnose CIN 1 cases or evaluate the risk of cervical carcinogenesis. The combination of ZNF516 immunohistochemistry and HR-HPV detection could be used as a potential biomarker of early diagnosis of cervical preneoplastic lesions, especially in cases with lack of certainty in the diagnosis.

In conclusion, ZNF516 expression decreased in cervical lesions in comparison with normal tissue. In fact, the loss of ZNF516 protein expression in preneoplastic and neoplastic lesions was found associated with a high expression of markers of HPV infection and proliferation in cervical carcinogenesis. Also, the absence of ZNF516 seems to be related to HR-HPV infection and the detection of both parameters can accurately discriminate CIN 1 from normal tissue. Therefore, immunohistochemistry staining of ZNF516 potentially could be used as a complementary tool of early diagnosis of cervical preneoplastic lesions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research is a retrospective study including biopsy tissue samples of archive. Therefore, for this type of study formal consent is not required. The use of these samples was approved by the ethical scientific committee of School of Medicine (Document no. 17/012), Universidad de La Frontera, Temuco, Chile.

Informed consent This research is a retrospective study including biopsy tissue samples of archive. Therefore, for this study informed consent was not obtained. The use of these samples was approved by

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