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Original Research

Expression and Analysis of Rsf-1 and P16 in Cervical Intraepithelial Neoplasia

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Abstract

Objective: The molecular mechanism in the occurrence and progression of cervical intraepithelial neoplasia (CIN) is of great theoretical significance to improve the early diagnosis, treatment and prognostic judgement of CIN in clinical practice. The current study aimed to detect the expression of remodel and space factor-1 (Rsf-1) and P16 in CIN, and to analyze the expression of Rsf-1 and P16 in different stages of CIN and to investigate the possible correlation. **Methods**: A total of 110 samples of CIN were collected from the Department of Pathology, the First Affiliated Hospital of Bengbu Medical College in 2018. Rsf-1 and P16 was detected by immunohistochemical staining in pathological tissue sections. The correlation between Rsf-1 expression and P16 expression in CIN was further analyzed. **Results**: The immunohistochemical results of 110 cases of CIN showed that the positive rate of P16 and Rsf-1 gradually increases with the grade of CIN increases. The expression of P16 was significantly different between different CIN grade ($p = 0.011$). The expression of Rsf-1 was also significantly different in different CIN grade ($p = 0.015$). **Conclusions**: Rsf-1 and P16 was highly expressed or over-expressed in high-grade CIN, and the positive rate of their expression increased with the increase in CIN grade.

Keywords: Rsf-1; P16; cervical intraepithelial neoplasia; immunohistochemistry

1. Introduction

Cervical cancer has become one of the most common female malignant tumors worldwide, with the highest mortality following breast cancer [1,2]. At present, it is conceived that the pathogenesis of cervical cancer is related to multiple factors, such as early marriage, early childbirth, prolificacy and sexual life disorder. Sexually transmitted high-risk human papillomavirus (hr-HPV) infection may be the main pathogenic factor for cervical intraepithelial neoplasia (CIN). It is clinically conceived that it generally takes 5–10 years for CIN to progress into cervical cancer. Early detection of precancerous lesions or cervical cancer can greatly improve the curative rate of patients with cervical cancer. The main clinical screening methods of cervical cancer include cytological and virological detections [3–5], however, with missed diagnosis in part of the patients. Therefore, new biomarkers are likely to increase the diagnostic rate of cervical lesions.

CIN is theoretically defined as the continuous epithelial squamous epithelial dysplasia with atypical nuclei of the cervix epithelium and the possible progression into invasive carcinoma [6]. Persistent infection with high-risk human papillomavirus is an important factor in promoting the occurrence of CIN [7–10]. Studies have found immune escape in patients with persistent HPV infection, making it impossible to be cleared by immunity, and the local immune change of the genital tract after HPV infection plays an important role in the transformation of infection in cervical squamous lesions, the loss of function is caused by HPV E7 [11,12].

Remodeling and spacing factor-1 (*Rsf-1*), also known as Hepatitis B X-antigen associated protein (*HBXAP*), located at 11q13.5, was originally discovered in the nucleus of Hela cells [13]. Chromosome region 11q13.5 is one of the most common amplification regions in human tumors, and is closely correlated with the occurrence and progression of tumors. The over-expression of *Rsf-1* gene is involved in stimulating cell proliferation and malignant transformation, which are considered as an oncogene. It can bind with human sucrose non-fermenting protein 2 homologue (*hSNF2H*) to form the chromatin remodeling complex *Rsf* in the nucleus, further promoting the repair and remodeling of tumor cells, and preventing tumor cells from apoptosis [2,14], which is also significantly associated with the degree of malignancy, proliferation ability, cell cycle, chromosomal stability, tumor invasion, drug resistance of tumor cells. *Rsf-1* has been confirmed to be over-expressed in many tumors, and it is related to poor prognosis, but there is no relevant report on cervical cancer and precancerous lesions [15,16]. Therefore, we explored whether the expression of Rsf-1 has changed in the precancerous stage, and whether it can be used to assist in the diagnosis of precancerous lesions.

The cell cycle-dependent protein kinase inhibitor *P16*, a basic gene in the cell cycle, is a tumor suppressor gene located at human chromosome 9p21 and consisting of three exons and two introns with approximately 8.5 kb in length [17]. *P16* is involved in cell cycle regulation, negatively regulating cell proliferation and division. Methylation of *P16* gene and decreased protein expression of P16 can lead to the binding of CDK4 to cyclinD to further promote cell growth and division, leading to uncontrolled cell growth and massive proliferation of malignant tumor cells [18]. *P16* gene is involved in tumorigenesis by gene deletion and widespread mutation. P16 is a protein that can inhibit cells from entering S phase from G1 and play a negative regulatory role [19]. Tsiaambas *et al*. [20] have reported that the overexpression of P16 protein is a marker that reflects the persistent infection of cervical cells by hr-HPV virus and causes the proliferation state, and has high clinical value in the early screening of CIN. It is of great significance to detect whether *P16* gene is altered in judging the easiness of tumorigenesis and patient prognosis in clinical practice [21]. Experimental studies have confirmed that the expression of *P16* is correlated in the diagnosis of high-grade CIN and cervical cancer, and P16 is an important indicator for judging the degree of cervical cancer lesions. Therefore, the expression of P16 is of great significance in the diagnosis of cervical lesions and cervical cancer [22].

Among gynecological tumors, cervical cancer has a high degree of malignancy and poor prognosis. Cervical cancer is one of the most susceptible tumors in women globally, and the number of patients is also increasing year by year in China [23–26]. The developmental course of cervical cancer is from normal cervical tissue to CIN, further progressing into cervical invasive carcinoma. Thin-layer liquid-based exfoliated cell examination (TCT) combined with human papillomavirus (HPV) is a common clinical screening method for CIN. It has high specificity and sensitivity diagnostic method, or in a second step before colposcopy. Subjective factors of the tested physician, HPV (high/low-risk type) probe cross-reaction influence, possibly missed diagnosis. Moreover, the characteristics of exfoliated cells are not exactly the same as those of living tissue cells. At the same time, they are affected by the techniques of obtaining materials and making (reading) slices, and it is impossible to determine the specific lesion location and extent. The emergence and rapid development of molecular biotechnology has swift the study focus of the occurrence and development of cervical cancer to the molecular level [27]. Therefore, the early detection of CIN is an important way to prevent cervical cancer, and the exact identification of CIN classification is also of great significance in selecting clinical therapeutic methods [28]. In the present study, we aimed to investigate the expression and correlation of Rsf-1 and P16 in cervical lesions, and to examine the related molecular mechanisms, which might have certain clinical significance for the early diagnosis, treatment and prognostic judgments of cervical lesions.

2. Materials and Methods

2.1 Clinical Samples

In total, 110 cases of CIN were collected from the Department of Pathology, the First Affiliated Hospital of Bengbu Medical College in 2018, and reviewed by three senior pathologists. There were 35 cases of CIN grade I, 33 cases of CIN grade II, and 42 cases of CIN grade III. The median age was 47 years, ranging from 24 to 66 years old.

2.2 Immunohistochemistry (IHC) Assay

The paraffin-embedded samples were cut into 4 μm-thick slices, treated with L-poly-polylysine for the sake of anti-dislodging, baked, dewaxed in xylene and hydrated in gradient ethanol solution. The SP (streptavidin-perosidase) method was used to label Rsf-1 (1:1000, GC-E1448; Gene Copoeia, Rockville, MD, USA) and P16 (1:100, orb228200; Biorbyt, Cambridge, UK) protein according to the manufacturers' instructions. The known positive slice was used as a positive control, and PBS replacing primary antibodies was used as a blank control. DAB was utilized for visualization.

2.3 Fluorescence Quantitative PCR Detection

Took 1 mL of the mixed sample, centrifuged at 12000 r/min for 5 min (centrifugation radius was 16 cm), discard the supernatant. Added 1 mL of sterilized saline, shook to mix, and centrifuged again to discard the supernatant. Added 100 μL of nucleic acid extraction solution, mixed well, and placed in a metal bath at 100 °C for 10 min. After centrifugation, took the supernatant, which is the DNA template. The PCR reaction solution was mixed with Tap enzyme and centrifuged for a while, and 4 μL of DNA template, negative and positive control were added to the reaction reagent in 36 μL/tuber respectively. Centrifuged briefly and tested on the computer. Amplification parameters: 94 °C-2 min, 93 °C for 10 s-62 °C for 31 s, 40 cycles; single-point fluorescence detection at 62 °C. Judged the results according to the instructions, tested 14 high-risk HPV–DNA such as 16-type, 18-type, 33-type, 52-type, 58-type, etc.

2.4 Judgment Criteria

The positive staining was yellow or brown for IHC results. The Rsf-1 was expressed in the nucleus, and P16 was expressed in the nucleus/cytoplasm. Positive cells are yellow or brown: the cells are single. If the percentage of positive cells is less than 5%, the test result would be negative. If the stained cells are scattered or in small cell clusters, with $\geq$5% but $\leq$24% stained cells, it is considered weak positive. If the cell staining is in sheets or clusters of cells, with $\geq$25% but $\leq$50% of the cells staining, is considered positive. When the cell staining is diffuse and the proportion of positive cells is $>$50%, it is considered strongly

positive. Used Image-Pro Plus software 6.0 (Media Cybernetics, Rockville, Maryland, USA) for image analysis, and the integrated optical density (IOD)/area value was used to judge the expression of Rsf-1 and P16 protein.

The presence of koilocytes in the squamous epithelium of the cervix is helpful in diagnosing HPV. Koilocytes are larger than normal cells, with enlarged nuclei in the center, round, oval or irregular, with dark staining, dual or multinucleated cells, and a hollow halo in the perinuclear cytoplasm. Dilated capillaries and lymphatic vessels were seen in the dermis, and a large number of chronic inflammatory cells were infiltrated.

2.5 Statistical Analysis

SPSS23.0 (SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Chi-square test was used for comparison between groups. $p < 0.05$ was considered as statistical significance. Spearman rank correlation was employed for correlation analysis.

3. Results

3.1 Protein Expression of RSF-1 in Different Grades of CIN

IHC results showed that the positive expression of Rsf-1 was yellow or brown, located in the nucleus. We found that the expression of Rsf-1 was increased with the increase of CIN grade in the 110 cases (Fig. 1). The positive rate of Rsf-1 protein expression was 62.86% out of the 35 cases of CIN grade I (22/35); the positive rate of Rsf-1 protein expression was 75.76% out of the 33 cases of CIN grade II (25/33); and positive rate of Rsf-1 protein expression was 90.48% out of the 42 cases of CIN grade III (38/42). The different expression of Rsf-1 in CIN grade I, CIN grade II and CIN grade III was considered statistical significance with HPV infection. ($p = 0.015$) (r = 0.82) (Fig. 2, Table 1).

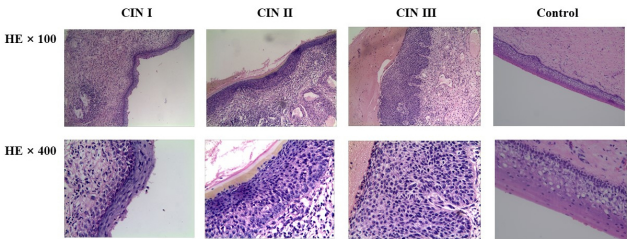

Fig. 1. Representative hematoxylin and eosin (HE) staining image of each group.

3.2 Protein Expression of P16 in Different Grades of CIN

IHC results revealed that the positive expression of P16 was yellow or brown, located in the nucleus/cytoplasm. We found that the expression of P16 increased with the increase of CIN grade in 110 cases CIN (Fig. 1). The positive rate of P16 protein expression was 65.71% out of the 35 cases of CIN grade I (23/35); the positive rate of P16 protein expression was 72.73% out of the 33 cases of CIN grade II (24/33); and positive rate of P16 protein expression was 92.86% out of the 42 cases of CIN grade III (39/42). The expression of P16 in different CIN grade was considered statistical significance with HPV infection ($p = 0.011$) (r = 0.78) (Fig. 2, Table 2).

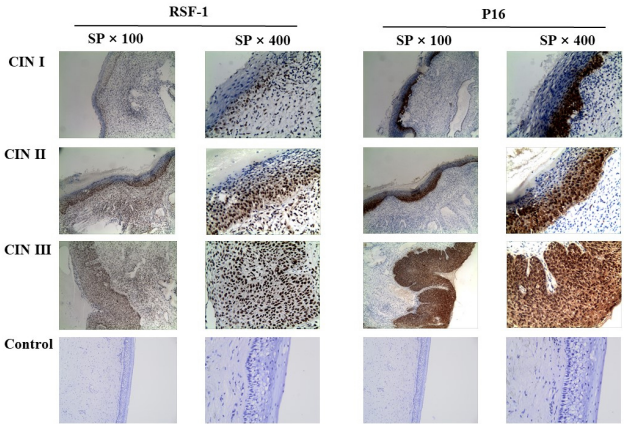

Fig. 2. Representative immunohistochemical staining showed the expression changes of RSF-1 and P16 in each group.

4. Discussion

CIN is a precancerous lesion of the cervix, and reasonable intervention in cervical intraepithelial lesions can effectively prevent the occurrence and development of cervical cancer. At this stage, the main methods of cervical cancer screening include cervical exfoliative cell examination, human HPV detection, colposcopy, etc., but the CIN classification is not specific, and it is difficult to rely on only one examination as a treatment certificate for patients. The diagnosis of CIN is mainly based on pathological examination, but HE staining cannot fully identify it, so it is necessary to choose and explore new diagnostic criteria to differentiate and diagnose different grades of CIN [29]. Therefore, this study analyzed the clinical value of the combination of P16 and Rsf-1 in HPV-positive cervical intraepithelial lesions.

The *Rsf-1/HBXAP* gene functions by combining with human sucrose nonfermenting protein-2 homologue (*hSNF2H*) in the nucleus to form a chromatin remodeling complex. The *Rsf-1/HBXAP* gene acts as a histone chaperone to adjust the binding activity of the complex to DNA, while *hSNF2H* has ATPase activity and helicase activity, and participate in the movement of nucleosomes and the unwinding of DNA, thereby changing the structure of chromosomes or nucleosome position, affects gene transcription, replication and other processes [30]. Rsf-1 protein plays an important role in the life course of cells, including

Table 1. The RSF-1 and HPV expression in CIN I, CIN II and CIN III.

Grade	Cases	RSF-1			HPV			*p* value
		Positive	Negative	Positive rate	Positive	Negative	Positive rate	
CIN I	35	22	13	62.86%	30	5	85.71%	
CIN II	33	25	8	75.76%	29	4	87.88%	0.015
CIN III	42	38	4	90.48%	40	2	95.24%	

r = 0.82.

Table 2. The P16 and HPV expression in CIN I, CIN II and CIN III.

Grade	Cases	P16			HPV			*p* value
		Positive	Negative	Positive rate	Positive	Negative	Positive rate	
CIN I	35	23	12	65.71%	29	6	82.86%	
CIN II	33	24	9	72.73%	28	5	84.85%	0.011
CIN III	42	39	3	92.86%	39	3	92.86%	

r = 0.78.

maintaining normal cellular morphology, cell senescence, cell death and apoptosis [31,32]. Studies have confirmed that Rsf-1 promotes tumor cell growth and reproduction mainly through various proteins involved in cell growth, and Rsf-1 may promote tumor cell proliferation and metastasis by cyclinD1/ERK-related pathways [2,33]. Rsf-1 has been confirmed to be highly expressed in tumors, and it is related to malignant biological behaviors such as hyperproliferation, invasion, metastasis, and drug resistance. The possibility mechanisms have been verified in gastric cancer [34], non-small cell lung cancer [35], and ovarian cancer [36]. Abnormal expression of *Rsf-1* gene is part of the manifestations of HPV infection, which probably result in host cell mutation. Rsf-1 acts through the NF-κB signaling pathway [31]. In the case of persistent HPV infection, HPV is expected to result in cervical cancer through three stages such as infection, integration, and cancerization. The occurrence and development of pre-lesion causes the maturation of vaginal epithelial cells to become impaired, and the dysplasia of vaginal epithelial cells is formed. The abnormal expression of Rsf-1 is correlated with high-risk HPV infection. It has been found that the expression of Rsf-1 is elevated in cervical cancer [37] and CIN and is related to poor prognosis. However, the mechanism of Rsf-1 in cervical cancer needs to be further investigated.

A number of studies have reported that the *Rsf-1* gene was involved in the regulation of nuclear factor κB (NF-κB) and ERK signal transduction pathway related gene expression regulation, thereby promoting the evolution of tumor cells. *Rsf-1* gene specific acts as activator or repressor of transcription, depending on cell type and/or interaction with transcriptional co-activators/mediators such as HBX proteins [38]. Therefore, we speculated that *Rsf-1* gene might interact with other transcriptional co-activators to stimulate the transcription of NF-κB pathway in tumor cells. NF-κB-dependent gene expression involves a protein family of transcriptional co-activators that most likely act to promote or bridge sequence-specific activators of basal transcriptional machinery and alter chromatin structure [39]. Combined with the above research results, it is indicated that the *Rsf-1* gene may play a role in the development and evolution of cervical lesions, thereby affecting the prognosis.

In this study, different cervical tissues were stained by immunohistochemistry, and the results showed that the expression of Rsf-1 in CIN tissue and normal cervical tissue was significantly different. The content of protein expression product was significantly higher than that in normal tissue, and the expression difference of Rsf-1 protein in different cervical intraepithelial lesion tissues was statistically significant. The above results showed that the content of Rsf-1 protein in cervical tissue increases with the degree of cervical intraepithelial lesions, which probably suggested the dynamics of histone acetylation and deacetylation caused by Rsf-1 protein overexpression in normal cervical tissue cells. The balance was destroyed, and the nucleosome structure was abnormal, so that the proliferation and differentiation process of cervical cells were abnormal, and then they were transformed into infinitely proliferating malignant tumor cells. It was suggested that this factor may play a key role in the process of cervical normal tissue malignant transformation into cervical cancer.

The P16 protein is the first tumor suppressor gene found at human chromosome 9p21, which is directly involved in the regulation of cell cycle [40]. P16 is an important molecular marker for HPV infection into cervical cells, and could accumulate in cells, which could be clearly detected by IHC [41–43]. Previous studies have shown that the tumor suppressor gene *P16* was overexpressed in 99% of hr-HPV positive cervical cancer and precancerous lesions, therefore, P16 protein can be used as a biomarker for early screening of cervical cancer and precancerous lesions [44].

HPV infection is a necessary factor for the occurrence of cervical squamous cell carcinoma and epithelial neopla-

sia. HPV infection is asymptomatic and relatively common in most countries. There are currently more than 100 types of HPV viruses, most of which are low-risk viruses and do not cause cervical cancer. In addition, there are 13 types of HPV that can be continuously infected. The common HPV types that cause cervical cancer are type 16 and type 18. They multiply in a warm and humid environment. The external genitalia and perianal parts of the human body are the most prone to infection. Cervical cancer is easily induced after hr-HPV infection. After being infected by hr-HPV, the DNA integrated into the host cell, triggering the expression of oncogenes *E6* and *E7* [45]. Through the combination of E6 and p53 protein and E7 and pBb protein, the two inhibitory links of the cell cycle would be blocked, and the host cells would undergo malignant proliferation. The tumor suppressor gene *P16* and its products can inhibit the phosphorylation of retinoblastoma protein, which can prevent cells from G1 phase to S phase, thereby inhibiting the malignant proliferation of cells. The E7 protein in HPV-infected cervical tissue can cause the inactivation of pBb function, and finally relieve the inhibition of pBb on the expression of P16 protein, resulting in the overexpression of *P16* [46]. The results of this study showed that with the aggravation of cervical neoplasia, the expression level of P16 also increased, indicating that the detection of P16 protein level probably utilized to distinguish cervical neoplasia and cervical cancer. This also suggested that HPV infection could cause cervical neoplasia to progress to cervical phosphorus cancer. This process was accompanied by the interaction between cyclins and oncogenes. Joint monitoring of HPV infection and the expression of P16 maybe result in the diagnosis rate of cervical cancer greatly increase [47].

In this study, the IHC outcomes showed that the expression levels and expression rates of Rsf-1 protein and P16 protein increased with in the increasing grade of CIN. Rsf-1 protein and P16 protein were highly expressed or overexpressed in abnormal epithelial cells of CIN, but not in cervical epithelium of normal or benign lesions. The expression levels of Rsf-1 and P16 were significantly different in different grades of CIN. To be specific, the expression level and expression rate of Rsf-1 and P16 protein were significantly higher in CIN grade III tissues than those in CIN grade I and II tissues. However, the IHC results are relatively subjective in judgment, and certain degree of bias could be caused due to different judgment criteria because of immunohistochemistry can detect the protein expression by using in situ hybridization. In addition, the specific molecular mechanism and whether there are more related markers would be further validated in subsequent experiments. P16 and Rsf-1 can be used to assist in the diagnosis of CIN and can reflect the prognosis, which may play a prompting role for clinical treatment.

5. Conclusions

We investigated the significance of *Rsf-1* and *P16* proteins in the occurrence and progression of CIN, aiming at providing a theoretical basis for early clinical diagnosis, targeted therapy and prognostic judgement. *Rsf-1* and *P16* are closely associated with the occurrence and evolution of CIN. Results of this study were consistent with previous researches that *P16* is a valuable molecule to evaluate the cervical squamous lesion. Meanwhile, *Rsf-1* was highly expressed in high-grade CIN, and the positive rates of *Rsf-1* and *P16* increased with the increasing grade of CIN. *Rsf-1* could be used to assist in the diagnosis of CIN and reflect the prognosis, which may play a prompting role for clinical treatment. However, the specific molecular mechanism and whether there are more related target genes would be further validated in subsequent experiments. The progress of related gene research, various genetic testing and intervention methods are expected to play a role in the diagnosis and treatment of cervical lesions.

Abbreviations

CIN, cervical intraepithelial neoplasia; *Rsf-1*, remodel and space factor-1; hr-HPV, high-risk human papillomavirus; *HBXAP*, Hepatitis B X-antigen associated protein; *hSNF2H*, human sucrose non-fermenting protein 2 homologue; IHC, Immunohistochemistry.

Author Contributions

LW designed and conducted the study. QZ collected and analyzed the data, and wrote the manuscript. GZ collected and analyzed the data. MT collected and analyzed the data. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The current study followed the requirements of Declaration of Helsinki and was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College (Approval No: BBMEC-2020-08), and written informed consent forms were obtained from all patients.

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Conflict of Interest

The authors declare no conflict of interest.

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