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Decreased expression of GRIM-19 and its association with high-risk HPV infection in cervical squamous intraepithelial neoplasias and cancer

Abstract

Purpose: GRIM-19 has been shown to be down-regulated in cervical cancers. This study investigated the expression of GRIM-19 in cervical intra-epithelial neoplasias and its association with high-risk human papillomavirus (HR-HPV) infection.

Methods: The expression of GRIM-19 was assessed in cervical exfoliated cells and cervical intra-epithelial neoplasia tissues by immunohistochemistry, and the level of GRIM-19 was also evaluated by Western blotting using cervical exfoliated cells. HR-HPV infection of cervical exfoliated cells was detected by HC II.

Results: GRIM-19 is predominantly expressed in the cytoplasm of the middle layer of normal cervical epithelial cells, whereas the surface layer cells of the normal cervix showed no GRIM-19 expression. The expression of GRIM-19 gradually decreased from atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) to squamous cell carcinoma (SCC); a pattern which was also observed in cervical intra-epithelial neoplasias tissues. The reduced expression of GRIM-19 was correlated with HR-HPV infection.

Conclusion: GRIM-19 may regulate the differentiation of normal cervical tissue, and a decrease in GRIM-19 may be the result of HR-HPV infection, which in turn leads to the malignant transformation of the cells.

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Cervical precancer is graded histologically as cervical intraepithelial neoplasia I (CIN I), CIN II or CIN III. The latter two grades are typically grouped together as high-grade CIN, which tends to develop into cancer without treatment [1]. The molecular basis of development of normal cervical squamous epithelia into cervical intraepithelial neoplasia and invasive carcinoma has not been completely elucidated.

Persistent infection by high-risk human papillomaviruses (HR-HPV), such as HPV18 and HPV16, is vital for the oncogenicity of cervical cells [2,3]. E6 and E7 oncogenes of HR-HPV play critical roles in the development of the malignant phenotype of cervical cancer by interfering with the expression of tumor suppressor protein p53 [4-6] and retinoblastoma protein (pRb) [7-9]. The most common type-specific HR-HPVs in Chinese women are HPV16, HPV52, HPV58, HPV33 and HPV18 [10]. In addition to persistent infection with HR-HPV, other host genetic changes and the dysregulated expression of various oncogenes and tumor suppressor genes also contribute to the development of cervical cancer.

GRIM-19 is associated with apoptosis [11], growth inhibition [12-16], migration, invasion and epithelial-mesenchymal transition (EMT) [17-19]. Low GRIM-19 expression occurs in some human malignant tumors, such as those of the kidney, cervix, breast, liver and lung [14-15, 20-23]. GRIM-19 can suppress tumor growth by down-regulating STAT3-induced gene expression and inducing p53 accumulation through a disruption of the E6/E6AP complex and an induction of E6AP auto-ubiquitination in cervical cancerous cells [14, 21]. In the present study, the expression of GRIM-19, and its association with HR-HPV infection in malignant cervical exfoliated cells, was investigated further. GRIM-19 was found to gradually decrease from atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) to squamous cell carcinoma (SCC), and the reduced expression of GRIM-19 was positively correlated with HR-HPV infection.

Materials and Methods

Ethics Statement

This study was approved by the ethics review board of Anhui Provincial Hospital. Written consent was obtained from each patient.

Tissue specimen and exfoliated cells

The patients were selected from the gynecologic cytology practice at Anhui Provincial Hospital, which is affiliated with An-

hui Medical University in Hefei, China. The samples considered for this study included 40 slides of normal squamous cells, 40 slides of ASCUS, 40 slides of LSIL, 40 slides of HSIL and 20 slides of SCC from patients with cervical cancer. Cases were excluded if the patients were younger than 21 years, if the samples were obtained from women with previous hysterectomies, or if there was insufficient residual fluid to extract cellular proteins. All Pap tests were collected in PreservCyte solution and processed using ThinPrep methodology (Hologic, Marlborough, MA, USA). All of the slides were screened by at least two certified cytotechnologists.

A total of 20 cases of normal cervical tissues, 35 cases of CIN I, 30 cases of CIN II, 30 cases of CIN III and 20 cases of SCC were used for the study. Colposcopy-directed biopsies were fixed in 10% buffered formalin and stained with hematoxylin and eosin using routine methods. The biopsies were included if they were obtained within three months of the abnormal Pap smear. All Pap smear slides and histological biopsy slides were reviewed by at least one experienced gynecologic pathologist who was also certified in cytopathology and were interpreted using established criteria. The Bethesda System was used for the cytological diagnosis, and the biopsies were reported using CIN terminology.

Immunohistochemistry and evaluation of immunoreactivity

The tissue sections were fixed with 4% formaldehyde, dehydrated, embedded and cut into 2 μ m serial sections. All Pap slides were used for the detection of GRIM-19 staining after diagnosis by the experienced gynecologic pathologist.

The slides were deparaffinized in xylene for 10 min, fixed using 100% ethanol for 5 min and then dehydrated with graded ethanol. The endogenous peroxide activity was quenched by incubation with 3% hydrogen peroxide in methanol for 10 min. The sections were washed twice with phosphate-buffered saline (PBS) and incubated with the primary antibody for 2 hours at routine temperature. The samples were then rinsed twice with PBS and incubated with the corresponding secondary antibody for 30 min at 37°C. The sections were then washed with PBS and incubated for 1 min with a high-sensitivity substrate (DAB). Lastly, the slides were counterstained with 10% hematoxylin, and photographs were captured using a microscope with a digital camera.

Immunoreactivity in the tissue was evaluated independently by two pathologists who were blinded to the clinical data and other immunohistochemical results. Normal cervical tissues were used as a positive control. Negative controls were included in each slide assay (the omission of primary and secondary antibodies), and all controls yielded the appropriate

results. Monoclonal antibodies against GRIM-19 (1:100) were purchased from Santa Cruz Biotechnology, and the secondary antibodies were obtained from Santa Cruz Biotechnology. The positive cells displayed brownish granules in the cytoplasm.

Immunoreactivity was semi-quantitatively evaluated based on the staining intensity and the distribution using the immunoreactive score [24]: immunoreactive score = intensity score $\times$ proportion score. The intensity score was defined as follows: 0, negative; 1, weak; 2, moderate; or 3, strong. The proportion score was defined as follows: 0, negative; 1, <10%; 2, 11-50%; 3, 51-80%; or 4, >80% positive cells. The total score ranged from 0 to 12. The immunoreactivity was divided into three levels on the basis of the final score, such that negative immunoreactivity was defined as a total score of 0, low immunoreactivity was defined as a total score of 1-4, and high immunoreactivity was defined as a total score >4. The stained tumor tissues were scored by two researchers blinded to the clinical patient data.

Immunoblotting

Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with monoclonal antibodies against GRIM-19 (1:1000). The membranes were then incubated with a 1:5000 dilution of a peroxidase-conjugated corresponding secondary antibody (Sigma, St. Louis, MO, USA). The blots were developed using an enhanced chemiluminescence kit (Pierce, Thermo Scientific, Northumberland, UK).

HPV DNA testing

DNA from the cervical tissues or cervical exfoliated cells was tested for the presence of HR-HPV DNA by the HC2 method according to the manufacturer's instructions (Digene Inc., Gaithersburg, MD, USA). The probe cocktail is capable of testing HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, all of which are important in the initiation of cervical

cancers. A positive cut-off value of 1.00 relative light units per positive control (RLU/pc) was used to score the presence of HR-HPV DNA in the samples. Each sample was tested in duplicate. Although it can detect HPV DNA in the sample, the HC2 test does not distinguish among the HPV genotype(s) present. The results are reported as positive or negative for HR-HPV.

Statistical analysis

All statistical analyses were performed using the statistical package SPSS (Chicago, IL, USA), version 13. A P-value <0.05 was considered statistically significant.

Results

The expression of GRIM-19 in cervical exfoliated cells by immunohistochemistry

Cervical squamous epithelial tissue is generally composed of three layers: the surface layer (cornified cells), middle layer and basal layer. The cervical exfoliated cells used for a Pap smear are mainly cells from the middle and surface layers. In the exfoliated cells, GRIM-19 was highly expressed in $94.2 \pm 2.8\%$ ($188.4 \pm 5.5/200$) of the normal middle layer squamous cells (Fig. 1 a and a') and in $0 \pm 0.0\%$ ($0 \pm 0.00/200$) of the normal surface layer squamous cells (Fig. 1 b and b'). Two hundred ASCUS cells, 200 LSIL cells, 200 HSIL cells and 200 SCCs in the corresponding ASCUS, LSIL, HSIL, and SCC Pap slides were viewed to detect the expression of GRIM-19. The expression of GRIM-19 was decreased in the ASCUS, LSIL, HSIL and SCC cells (Fig 2) and was negative in 16.5% of the ASCUS cells, 32.0% of the LSIL cells, 60.5% of the HSIL cells, and 97.5% of the SCCs (Table 1). These results suggest that the expression of GRIM-19 was gradually down-regulated in the progression of malignant cell transformation. There was a

TABLE 1. Correlation between expression of GRIM-19 and cytological diagnoses

Cytological diagnoses	GRIM-19 expression, n (%)			Total
	Negative	Low	High	
ASCUS	33 (16.5)	67 (33.5)	100 (50.0)	200
LSIL	64 (32.0)	100 (50.0)	36 (18.0)	200
HSIL	121 (60.5)	59 (29.5)	20 (10.0)	200
SCC	195 (97.5)	5 (2.5)	0 (0.0)	200
Total	413	231	156	800

NOTE: There was a highly significant correlation between expression of GRIM-19 and cytological diagnoses ($\chi^2 = 371.799$, $r = -0.616$, $P < 0.001$).

FIGURE 1. Fig. 1 The expression of GRIM-19 in the exfoliated Pap smears ($\times 400$). Left panel: Pap smear of cervical exfoliated cells. Right panel: immunohistochemistry staining of GRIM-19 of the corresponding figures in the left panel. a and a', normal middle layer squamous cells; b and b', normal surface layer squamous cells.

highly significant correlation between GRIM-19 expression and the cytological diagnosis ($P < 0.001$).

The expression of GRIM-19 in cervical exfoliated cells by Western blotting

GRIM-19 levels were further explored in cervical exfoliated cells by Western blotting. Because GRIM-19 was not expressed in the surface layer exfoliated cells, these cells were counted (as a percentage) on each slide. After the GRIM-19/GAPDH proteins were quantified, the final relative expression level of GRIM-19 was multiplied by the middle layer exfoliated cells/(middle layer exfoliated cells+surface layer exfoliated cells) $\times 100\%$, and GRIM-19/GAPDH in the normal middle layer exfoliated cells was normalized as 1. Decreased GRIM-19 expression was found in the LSIL, HSIL and SCC cells. Compared to the normal middle layer squamous cells, the expression of GRIM-19 was dramatically reduced in the LSIL cells ($P < 0.05$), HSIL cells ($P < 0.05$), and SCCs ($P < 0.001$) (Fig. 3A-C).

The expression of GRIM-19 and its association with HR-HPV infection in cervical precancers

As consistent HR-HPV infection is closely associated with SCC, the correlation of HR-HPV infection with the expression of GRIM-19 was explored in exfoliated cells. There was a highly significant correlation between the expression of

FIGURE 2. The expression of GRIM-19 in ASCUS, LSIL, HSIL and SCC Pap smears ($\times 400$). The typical abnormal cells were shown with black arrows.

GRIM-19 and HR-HPV infection ($\chi^2 = 57.583$, $r = -0.268$, $P < 0.001$) (Table 2).

GRIM-19 expression in cervical intraepithelial neoplasias and SCCs by immunohistochemistry

To confirm the results found in exfoliated cells, GRIM-19 expression was investigated in CIN I, CIN II, CIN III and SCC (Fig. 4 A-E). High cytoplasmic expression of GRIM-19 was found in 100% (20/20) of the middle and basal layer cells of the normal cervical epithelium. The negative expression of GRIM-19 was found in 20.0% (7/35) of CIN I, 23.3% (7/30) of CIN II, 56.7% (17/30) of CIN III and 95% (19/20) of SCC. The staining scores showed that, compared to the normal tissue, the expression of GRIM-19 was significantly decreased in CIN II ($P < 0.05$), CIN III ($P < 0.05$) and SCC ($P < 0.001$) (Fig. 4F).

Discussion

The basal layer of the normal cervix is considered to be composed of stem-like cells that can differentiate into keratinocytes in the upper stratum of the epithelium. Once cell cycle arrest occurs, most basal cells will enter into terminal differentiation and die by apoptosis [25]. In epidermal keratinocytes, terminal differentiation and cornification is a specialized form of apoptosis [26-27]; thus, the disturbance of normal cornification through the loss of the ability to differentiate can lead to ma-

TABLE 2. Correlation between expression of GRIM-19 and HR-HPV infection

HR-HPV infection	GRIM-19 expression, n (%)			Total
	Negative	Low	High	
Negative	78 (32.8)	82 (34.4)	78 (32.8)	238
Positive	335(59.6)	149(26.5)	78 (13.9)	562
Total	413	231	156	800

NOTE: There was a highly significant correlation between expression of GRIM-19 and HR-HPV infection ($\chi^2 = 57.583$, $r = -0.268$, $P < 0.001$).

lignant squamous cell transformation [28-29]. In this study, GRIM-19 was found to be predominantly expressed in the cytoplasm of the middle layer of the cervix and also in the basal layer, although it was completely absent in the surface layer of the cervix. These findings imply that the expression of GRIM-19 might contribute to the differentiation of normal middle and basal layer cervical epithelia. Once the expression of GRIM-19 is reduced, the cells of these layers will advance toward abnormal differentiation and enter into the cell cycle for proliferation.

CINs are now formally is classified as LSIL (CIN I) and HSIL (CIN II-III). The large majority of CIN I will spontaneously regress to normal or ASCUS within 6 to 12 months [30-

31], and approximately 13% of women with a diagnosis of LSIL will develop an HSIL in 3 years [32-33]. In our study, expression levels and percentages of GRIM-19 were found to gradually decrease from ASCUS, LSIL, HSIL to SCC. These data suggest that CINs might regress after the restoration of GRIM-19 expression using interferon and retinoids, which are also used for anti-viral infection in cervical pre-cancers [35-35].

The positive correlation between decreased GRIM-19 expression and HR-HPV infection suggests that GRIM-19 might be an important protein, preventing cervical cells from malignant transformation, and that the decrease in GRIM-19 might be the result of HR-HPV infection. The exact molecular events that mediate the reduction of GRIM-19 expression in

FIGURE 3. The expression of GRIM-19 in cervical exfoliated cells was detected by Western blotting. A, typical slides of the exfoliated cells used for detecting GRIM-19 expression ($\times 200$), typical abnormal cells were shown with black arrows; B, the expression of GRIM-19 protein in cervical exfoliated cells by Western blotting; C, the relative expression of GRIM-19 in cervical exfoliated cells. NS, normal surface layer exfoliated cells; NM, normal middle layer exfoliated cells; A, ASCUS cells; L, LSIL cells; H, HSIL cells; S, SCC cells.

TABLE 3. Correlation between expression of GRIM-19 and biopsy diagnoses

Biopsy diagnoses	GRIM-19 expression, n (%)			Total
	Negative	Low	High	
Normal tissue	0(0)	0(0)	20(100.0)	20
CIN I	7(20.0)	8(22.9)	20(57.1)	35
CIN II	7(23.3)	18(60.0)	5(16.7)	30
CIN III	17(56.7)	9(30.0)	4(13.3)	30
SCC	19(95.0)	1(5.0)	0(0)	20
Total	50	36	49	135

NOTE: There was a highly significant correlation between expression of GRIM-19 and biopsy diagnoses ($\chi^2 = 95.550$, $r = -0.646$, $P < 0.001$).

HR-HPV-infected cervical cells are unclear. Research is underway to explore whether the binding of E6/E6AP to GRIM-19 can reduce the expression of GRIM-19.

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FIGURE 4. The immunoreactivity of GRIM-19 in the normal cervix, CIN I, CIN II, CIN III and SCC ($\times 200$). A, normal cervix – the circled inserts show a magnified view ($\times 800$); B, CIN I; C, CIN II; D, CIN III; E, SCC; F, summary of the GRIM-19 score results in specimens representing different histological groups.

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