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Original Article

EGFR protein expression and gene amplification in squamous intraepithelial lesions and squamous cell carcinomas of the cervix

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Abstract: The purpose of this study was to evaluate the protein expression and gene amplification of epithelial growth factor receptor (EGFR) in intraepithelial neoplasias and squamous cell carcinoma of the cervix and to determine the value of EGFR in carcinogenesis, progression, and prognosis of cervical cancer. EGFR protein expression and gene amplification involved gene copy number in 75 cases of cervical various lesions were evaluated using immunohistochemistry and by fluorescence in situ hybridization (FISH) techniques. Expression of EGFR was observed in 76.00% of the high-grade CIN and 79.17% of the invasive carcinomas. In contrast, there were low levels of EGFR expression in chronic cervicitis (1/10) and low-grade CIN (7/16). There were statistically significant differences among them ($P<0.05$). Gene amplification was detected in 20.51% high-grade CIN and invasive carcinoma, but there only 4.35% EGFR gene amplification was observed in chronic cervicitis and low grade CIN. Among the 42 patients with negative or low levels of EGFR expression, 26 patients (61.90%) were found to have diploidy and 11 patients (26.20%) to have balanced triploidy. However, among the 20 patients with an intermediate and high levels of EGFR protein expression, 13 (65.00%) were found to have balanced polyploidy or gene amplification. All cases of EGFR gene amplification involved intermediate and high levels of protein expression. EGFR may be involved in the carcinogenesis of the cervix and may be an early event during the carcinogenesis. Overexpression of EGFR protein may result from gene amplification and increases in gene copy number.

Keywords: Cervix neoplasms, EGFR, protein expression, gene amplification, gene copy number, FISH

Introduction

Although the morbidity and mortality from cervical cancer have decreased considerably in developed countries, cervical cancer is still one of the most common malignant tumors suffered by women in other parts of the world, including China. Statistics show that of the 500,000 or so new cases of cervical cancer are reported each year, 80% are in developing countries [1]. In China, cervical cancer is one of the top three malignant disorders diagnosed in women. Much work is still needed to control cervical cancer. One of focus of cervical cancer research has always been the mechanism of the initiation and development of cervical squamous intraepithelial lesions and squamous cell carcinoma and on factors that influence prognosis.

Previous studies have shown that the epidermal growth factor receptor (EGFR) gene is overexpressed in various human tumors, and the invasiveness of tumors with EGFR overexpression is higher than that of tumors with normal or low EGFR expression. So far, research into the role of EGFR in the carcinogenesis and progression of cervical cancer has been plenty, however the findings are quite controversial. The role of EGFR precancerous and cancerous cervical lesions and the value of its detection in the clinical management of these diseases have not been well accepted. The present study attempts to evaluate the protein expression and gene amplification of epithelial growth factor receptor (EGFR) in intraepithelial neoplasias and squamous cell carcinoma of the cervix and to explore the value of EGFR in the carcinogenesis, progression, and prognosis of cervical

squamous cell carcinoma. Immunohistochemical and fluorescence in situ hybridization (FISH) techniques were used to detect EGFR protein expression and gene amplification involved gene copy number in intraepithelial neoplasias and squamous cell carcinoma of the cervix.

Materials and methods

Case selection

Formalin-fixed paraffin-embedded (FFPE) specimens from seventy-five patients who had cervical pathological examination were collected at the Nanjing Maternity and Child Health Hospital during a 6 month period lasting from June 2010 to June 2011. The patients were from 22 to 70 years old with a median age of 46. CIN diagnosis and classification were made according the *WHO Tumor Pathology and Genetics Classification - Female Reproductive Organs Tumor Histological Classification Standard* was used for. Of the 75 cases, 10 patients had chronic cervicitis, 16 had low-grade cervical intraepithelial neoplasias (CIN), 25 had high-grade CIN, and 24 had cervical cancer. All of these patients with squamous cell carcinoma had not been treated with preoperative radiotherapy and chemotherapy before surgery. This study was reviewed and approved by our local Ethics Review Committee at Nanjing Medical University. Biopsy samples and surgical specimens were routinely processed using 4% neutral buffered formaldehyde for fixation, graded alcohol for dehydration, xylene for clearing and finally, paraffin infiltration, embedding and sectioning. Three sections were made from each of the paraffin blocks for routine H&E staining, immunohistochemistry, and FISH detection respectively.

Immunohistochemistry analysis

The two-step EnVision immunohistochemical method was used to detect EGFR protein in FFPE materials. After dewaxing and re-hydration, a 1% hydrogen peroxide solution in PBS was used to block the activity of endogenous peroxidase in the sections. The rest of staining procedure was carried out according to the kit instruction. Lastly hematoxylin counterstaining and neutral resin sealing were performed before microscopic observation. The rabbit anti-human EGFR is a polyclonal antibody (working concentration 1:100), produced by the Santa Cruz Company (Santa Cruz, CA). The DAB color reaction kit and adhesive poly-l-lysine used in slide preparation were purchased from Fujian Wallace New Biotechnology Company. During the experiment, the known EGFR positive breast cancer sections from Boster (Wuhan, China) were used as positive controls, and for negative control, PBS instead of primary antibody were used in the procedure.

Microscopic evaluation

Evaluation standard for EGFR immunohistochemical staining is as following. The specific EGFR staining on cell membrane and cytoplasm were both considered. Each section was evaluated by two pathologists who were doubly blind to the purpose of the experiment. Representative high-power fields on each of the five immunohistochemistry sections were examined using light microscope. From each section, a total of 200 cells to 1000 cells were counted. The sections were scored according to the relative number of stained cells and the intensity of the staining. Each section with 0% stained cells received 0 points; sections in which between 1% and 50% of the cells were stained received 1 point; sections in which between 51% and 80% of the cells were stained received 2 points; and sections in which more than 80% of the cells were stained received 3 points. Staining intensity was judged in reference to the color of majority of cells in the section. No coloring was scored with 0 points; slight brown, 1 point; brown, 2 points, and dark brown, 3 points. Using these two indicators of the score, the sections were then divided into four levels: Sections with 0 points were considered negative (-). Section with 2 points were considered weakly positive (+). Sections with 3-4 points were considered moderately positive (++). Sections with 5-6 points were considered strongly positive (+++).

Fluorescence in situ hybridization (FISH) analysis

The procedure was performed as suggested in the kit instruction. Overall, the FISH protocol for paraffin sections was divided into two stages: slide pretreatment and denaturation hybridization. The former included xylene elution, ethanol hydration, protease digestion, gradient alcohol dehydration, and acetone fixation, fol-

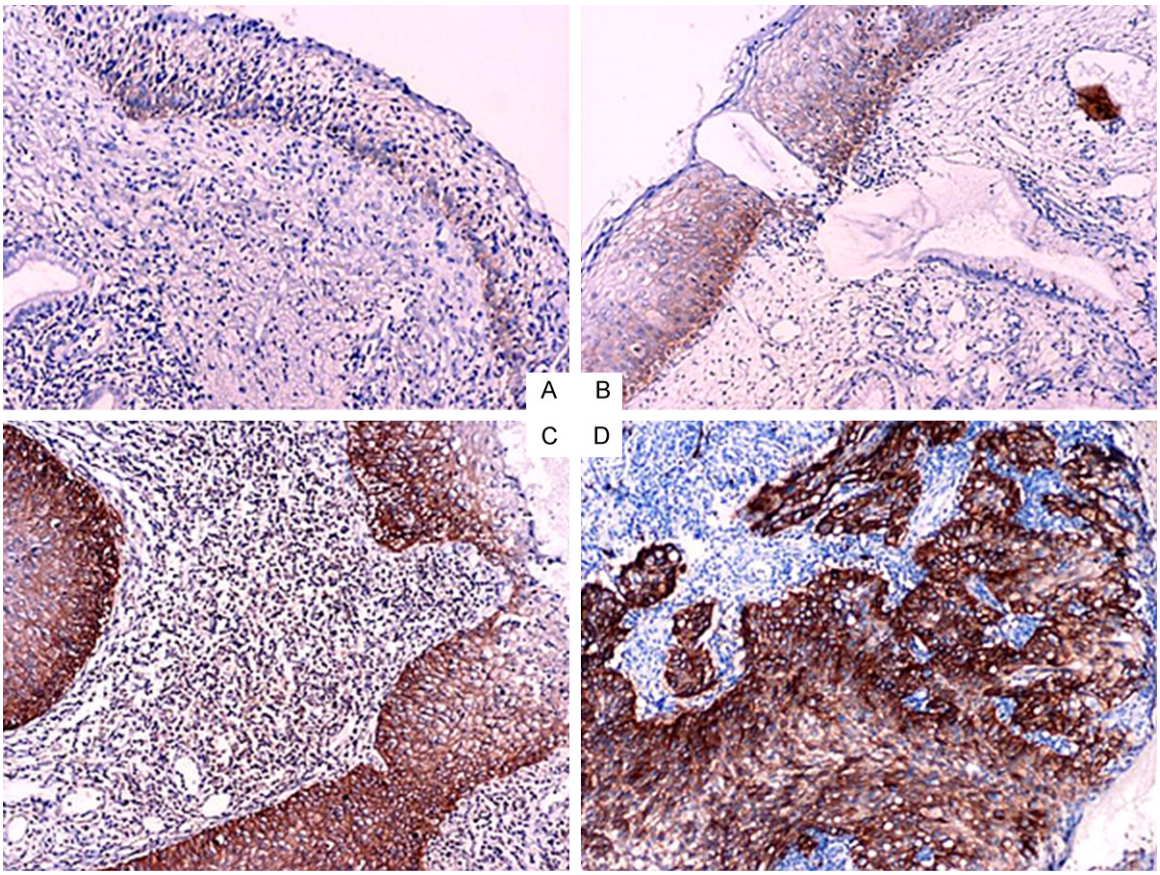

Figure 1. EGFR protein expression was observed in the plasma membrane and cytoplasm in all cervical lesions by EnVision immunohistochemistry. In (A) chronic cervicitis, (B) low-grade CIN, (C) high-grade CIN and involved glands, and (D) squamous cell carcinomas, positive expression appears gradually from the basal cells, spinous cells to all the layers and tumor nests, and its intensity grows (Magnification, ×200).

lowed by incubation overnight at 56°C. The latter mainly included specimen preparation and denaturation, probe mixture preparation (2 μl hybridization buffer, 1 μl deionized water, and 2 μl probe), probe denaturation, overnight probe and sample hybridization, and washing of the slides. Finally the slides were left in the dark to air dry, and 15 μl DAPI counterstaining agent drops were added to the hybridization zone. The slide was immediately mounted with proper medium and coverslip. After being put in the dark for 10 to 20 min, the slide was ready for the fluorescence microscopy observation, with a suitable filter group.

Fluorescence microscope evaluation

The turquoise FISH probe, LSI EGFR/CEP 7, was purchased from Beijing Gold Pujia Medical Technology Co., Ltd. For the EGFR gene, it showed red, and for the chromosome 7 kinetochore sequence it gave the green signal. In the experiment, the cell nuclei were counterstained with DAPI. Hybridization results were examined with the fluorescent microscope Olympus BX60 from Olympus. (Lake Success, NY, U.S.) for signal analysis. The analysis software used was FISH2.0 (Imstar, France). Two pathologists randomly counted cells for the observation, and the results were considered good only if both pathologists agreed. After a random counting of 100 cells in the experiment, the ratio value (ratio value = total number of red signals per 100 nuclei/total number of green signals per 100 nuclei) was calculated. When the ratio value ≥2 and the amplified cells appeared in lumps or clusters, the sample was considered positive for EGFR gene amplification.

Statistical analysis

The SPSS10.0 statistical package was used for data analysis. The Chi-square test with exact probability method was applied in comparing the difference in rates. A *p*-value of less than 0.05 was considered statistically significant.

Table 1. EGFR protein expression in cervical lesions

Group	Cases	EGFR immune staining				
		-	+	++	+++	Rate (%)
Chronic cervicitis	10	9	1	0	0	10
Low-grade CIN	16	9	5	2	0	43.75
High-grade CIN	25	6	13	4	2	76
Cervical squamous carcinoma	24	5	5	9	5	79.17
Total	75	29	24	15	7	

Table 2. Relationship between EGFR protein expression and clinicopathologic features of cervical cancer

Clinical-pathologic factors		Cases	EGFR immune staining				
			-	+	++	+++	Rate (%)
Degree of differentiation	High	9	2	2	4	1	77.78
	Low	15	3	3	5	4	80
Lymph node metastasis					0	0	
	Metastatic	19	5	4	7	3	73.68
	Non-metastatic	5	0	1	2	2	100

Results

Expression of EGFR in cervical lesions

By immunohistochemistry, as shown in **Figure 1**, EGFR showed only low expression in non-malignant cervical (chronic cervicitis) squamous epithelial cells. All these cells were present in the basal layer (such cells were used for inherent controls). Small amounts of basal layer cells that showed positive staining were regarded as negative. From CIN level I, expression of EGFR in the cells in the spinous layers began to appear, and staining of cells in the mid-surface gradually manifested in CIN II/III. Cells on the edge of the cancer nest were more strongly stained than those in the middle. In the development of the lesion at all levels from chronic cervicitis, low-grade CIN and high-grade CIN to squamous carcinoma, the rate of expression increased gradually, and the intensity also increased gradually. The rate of EGFR expression in squamous carcinoma cells was higher than high-grade CIN cells, but not statistically significant (P=0.791). However, the differences between the squamous carcinoma cells, the low-grade CIN group, and chronic cervicitis group were significant (P=0.021; P=0.000). The rate of EGFR expression in the high-grade CIN group was higher than in the low-grade or chronic cervicitis groups, and the difference was significant (P=0.036; P=0.001). The rate of EGFR expression in the low-grade CIN group and chronic cervicitis group showed no significant differences (P=0.099), as shown in **Table 1**.

EGFR expression and its correlation with pathological features of cervical squamous cell carcinoma

As shown in **Table 2**, of the 24 cases of cervical squamous carcinoma, 19 showed at least some EGFR expression (79.17%), 5 of them scored +, 9 scored ++, and the other 5 scored +++. High differentiation and low differentiation groups showed no significant differences in expression (P=1.000). The rate of expression differed between the lymph node metastasis group and the lymph node negative group, but the difference was not statistically significant (P=0.544).

EGFR gene amplification and gene copy number in cervical lesions

62 cases out of the 75 cases showed valid signals in FISH detection while the remaining 13 cases showed insufficient hybridization and incalculable cell packing. **Figure 2** shows the four main hybridization signal patterns. The variations in the patterns of gene copy number detected in the cervical lesions were consistent with those reported in previous studies [2]. The corresponding ratio value was calculated by two pathologists using the number of red and green signal points. An average value was recorded. Changes in red and green signals reflected variations in the EGFR gene number and copy number of chromosome 7. They also reflect changes in ploidy among aneuploids. **Table 3** indicates that in most triploid and polyploid cases EGFR gene amplified via balanced ploidy increase, that is to say, EGFR gene and chromosome 7 had balanced amplification. Among the 9 cases where EGFR gene amplification was detected, 5 of them were low-level amplification and 4 high-level. The gene amplification ratio of high-grade CIN groups to squamous carcinoma group was 20.51% (8/39), but for the chronic cervicitis group and low-grade

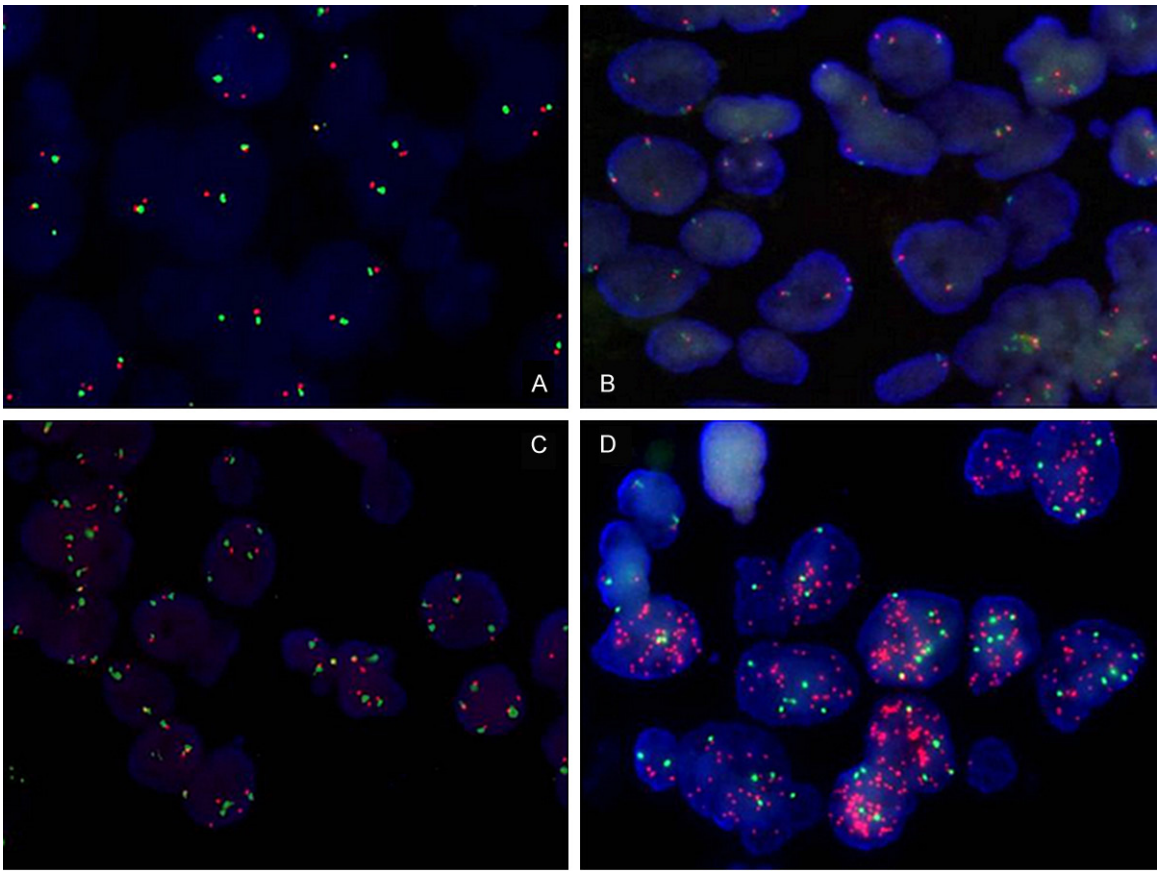

Figure 2. Patterns of EGFR gene copy number variations detected using the FISH technique. In the picture, red signals represent the EGFR gene and green signals represent the centromeric sequence in chromosome 7, which served as a control. A: Balanced disomy (each with 2 red and green signals, ratio=1). B: Balanced triploidy (each with 3 red and green signals, Ratio=1). C: Balanced polysomy (each with more than 4 red and green signals, Ratio<2). D: Gene amplification (with the red signals to green signals Ratio>2, manifesting as clustered amplification) (Magnification, ×1000).

CIN group, the ratio was 4.35% (1/23). The difference between the two ratios was relatively large, but it was not statistically significant (P>0.05). EGFR gene diploidy was prominent in the chronic cervicitis group and low-grade CIN group, accounting for 69.57% (16/23); but only 30.77% of the cases of high-grade CIN and squamous carcinoma groups exhibited diploidy (12/39). The copy number of the EGFR gene was higher than average in 11 cases (57.89%) in the high-grade CIN group and 16 (80.00%) in the squamous carcinoma group.

Relationship between EGFR protein expression, gene amplification, and gene copy number

Table 4 shows the relationship among protein expression, gene amplification, and gene copy number. In the negative and low EGFR protein expression groups, 61.90% cases were found to be diploid and 26.20% were found to be triploid as indicated by FISH detection patterns, but only 11.90% were found to be polyploid, and no EGFR gene amplification was detected. However, in moderate and strong EGFR protein expression groups, only 10.00% were diploid, 25.00% were triploid, and 20.00% were polyploid. Nine cases showed EGFR gene amplification. EGFR protein expression levels were found to be closely related to the increase in gene copy number (P<0.05). All the cases of EGFR gene amplification showed moderate to strong levels of protein expression. The data suggest that low levels of EGFR gene copy number (including diploid and triploid patterns) did not have much impact on protein expression, but high levels of EGFR gene copy number showed significant influence on protein expression

Table 3. Changes in EGFR gene copy number in cervical lesions

FISH signal pattern		Cases	Ratio	Histological type			
				Chronic cervicitis	Low-grade CIN	High-grade CIN	Invasive squamous carcinoma
Diploid		28	0.8~1.2	8	8	8	4
Triploid		16	0.9~1.4	1	4	4	7
Polyploid		9	0.8~1.5	0	1	4	4
Gene amplification	Low level	5	2.0~2.7	0	1	2	2
	High level	4	>3.0	0	0	1	3
Total		62		9	14	19	20

Table 4. Relationship between EGFR protein expression and gene copy number

Protein expression	FISH signal mode							
	Diploid		Triploid		Polyploid		Gene amplification	
	n	%	n	%	n	%	n	%
-	16	80.00	3	15.00	1	5.00	0	0
+	10	45.46	8	36.36	4	18.18	0	0
++/+++	2	10.00	5	25.00	4	20.00	9	45.00
Total	28	45.16	16	25.80	9	14.52	9	14.52

(including polyploidy and EGFR gene amplification).

Discussion

As early as in 1914, Boveri claimed that chromosome abnormalities were the fundamental cause of tumorigenesis. Modern cytogenetics and molecular biology studies show that most human tumor cells, especially solid tumor cells, often present chromosome instability. Research on the instability of cervical cancer chromosome suggests that aberrations of chromosomes 3, 8, 5, 7, X, and 18 may be early events in the carcinogenesis of cervical cancer [3, 4]. EGFR is the expression product of proto-oncogene c-erbB-1 (HER-1), which is located on human chromosome 7 p13-7 p12, and it is a type I transmembrane growth factor receptor tyrosine kinase. Its ligand binding with EGF can increase the proliferation and differentiation of cells and accelerate the transformation of malignant cells.

It has been reported that high or abnormal EGFR expression were found in cancerous tissues of many solid tumors such as brain glioma, esophageal cancer, gastric cancer, breast cancer, lung cancer and ovarian cancer [5-10]. Research shows that EGFR also has high levels of expression in cervical cancer, and this expression may be related to cervical cancer prognosis [11]. Longatto-Filho et al. reported that EGFR showed negative expression in normal cervical tissue epithelia and low expression in the epithelia of low-grade CIN patients, but the ratio and intensity of expression in high-grade CIN and cervical cancer increased gradually [12]. This study also found that detection of EGFR expression can be used for the early diagnosis and prognosis of cervical cancer. Other studies have suggested that the detection of EGFR expression may have important clinical significance for the prognosis of cervical cancer [13].

The present study examined EGFR protein expression in 75 cases with various levels of cervical CIN and cervical cancer using immunohistochemistry. The rate of expression of EGFR protein increased gradually in invasive squamous carcinoma lesions from chronic cervicitis patients and in both low-grade and high-grade CIN patients with invasive squamous carcinoma. The rate of expression of high grade CIN group and squamous carcinoma was markedly higher among samples from patients with the low-grade CIN or chronic cervicitis. This suggests that high levels of EGFR protein expression may be involved in the progression of cervical cancer. The present study also explored the relationship between EGFR protein expression and clinicopathological factors for cervical cancer. Though no statistically significant differences were observed, the present study showed the rate of EGFR protein expression for those with lymph node metastasis to be higher than for those without lymph node metastasis.

This suggests that EGFR protein expression may be related to the invasive biological behaviors of cervical cancer.

Most previous studies of the relationship between EGFR and cervical lesions have been limited to assessment of protein expression levels. Our study evaluated not only EGFR protein expression, but also changes in gene copy number in various cervical lesions using the FISH technique. Four FISH expression patterns were identified using changes in the number of EGFR genes and kinetochore sequences in chromosome 7. Results showed that, in high-grade CIN and squamous carcinoma, both the EGFR gene copy number and gene amplification were higher than in other groups. All cases with gene amplification showed overexpression of EGFR protein, which suggests that EGFR gene amplification may be one of the molecular mechanisms underlying EGFR overexpression. The relationship between overexpression of EGFR protein and increases in gene copy number further indicate that the superimposed effects of gene copy number may also be one of the important mechanisms underlying EGFR protein overexpression. The increase in the gene copy number includes two factors: specific gene amplification with its corresponding increase in chromosome copy number, and superimposed effect of gene dosage resulted in the increase of protein expression. The findings of the present study differ slightly from that of the a study performed by Marzano et al. [14]. They found no EGFR gene amplification but considerable polyploidy in cervical lesions, and they argued that this may be attributed to the heterogeneity of tumor cells. However, findings reported by Hirsch et al. on non-small cell lung cancer were similar to those of this study [2]. These studies suggest that changes in chromosome 7 may be early events in the carcinogenesis of cervical cancer. EGFR gene amplification and increases in gene copy number on chromosome 7 showed a close relationship with the carcinogenesis and progression of cervical cancer. For this reason, the EGFR gene amplification and the instability (aneuploidy) of chromosome 7 can be considered genetic markers of high-risk progression of cervical intraepithelial lesions.

The expression of EGFR protein is caused by a combination of factors which influence protein expression, including gene amplification, abnormal transcription, gene mutations, and gene polymorphism. According to previous reports, EGFR protein expression is mainly related to mRNA expression, and CA-SSR1 polymorphism has been found to influence EGFR expression to an extent [15]. Due to the complexity and diversity of EGFR expression and the limits of experimental techniques, it is currently not practical to analyze the regulation of EGFR expression in specific individuals or specific tumors. Although a correlation between EGFR protein expression and variation in gene amplification and gene copy number was observed in the present study, no correlation was observed between the two factors. The rate of protein expression was found to be considerably different from that of EGFR gene amplification. There may also be other molecular mechanisms that can cause overexpression of EGFR protein, so further study is needed.

Many preclinical and clinical studies show that blocking EGFR can inhibit tumor growth. Because it is a critical component for signal transduction, EGFR tyrosine kinase has become an important therapeutic target in cancer therapy [16]. One of the hottest topics in recent research is the combined use of anti-EGFR agents and chemotherapy with radiation as a therapeutic strategy for clinical treatment of cancers. According to reports from recent literature, gefitinib, an EGFR inhibitor, has some effect on cases of recurrent cervical cancer that are resistant to standard treatment [17]. However, large-scale clinical trials must be performed before it can be formally used in clinical settings.

The present study not only confirmed the overexpression of EGFR in high-grade cervical CIN and squamous carcinoma but also showed that a greater EGFR gene copy number and gene amplification may significantly affect protein expression levels. The increases in the EGFR gene copy number and gene amplification are part of the molecular mechanism underlying protein overexpression. The detection of EGFR protein at all levels of cervical intraepithelial neoplasias, squamous carcinoma expression, gene amplification, and variations in gene copy number can be used not only in the differential diagnosis of high-grade and low-grade CIN but also in the early diagnosis of cervical cancer.

They can also be used as biological indicators of the preliminary prognosis of cervical cancer. The present study also reinforces the new train of thought for the treatment of cervical cancer with anti-EGFR agents.

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Disclosure of conflict of interest

None.

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