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Original Article

Gene amplification of EGFR and its clinical significance in various cervical (lesions) lesions using cytology and FISH

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Abstract: The purpose of this study was to investigate the gene amplification and clinical significance of the epithelial growth factor receptor (EGFR) gene in cervical lesions. This study was designed to detect the EGFR gene amplification by liquid-based cytology and fluorescence in situ hybridization (FISH) techniques in 78 cases of cervical various lesions [28 cases of normal control cervix, 26 low grade squamous intraepithelial lesion (LSIL) and 25 high grade squamous intraepithelial lesion (HSIL)]. Positive gene amplification rates of the EGFR gene in normal cervix, LSIL and HSIL were 7.14%, 23.08% and 62.50%, respectively. There was a significantly difference between HSIL and normal control cervix or LSIL ($P<0.01$). At the same time, the gene amplification rate of EGFR was also significantly different between the cases of LSIL with positive follow-up and negative follow-up ($P<0.01$). In addition, the correlation between EGFR gene amplification and HPV viral load in the same sample was evaluated in some cases. Then the increase of EGFR gene amplification in HSILs and LSILs with positive follow-up suggests that EGFR gene amplification is associated with the severity of cytologic findings. Therefore, detection of EGFR gene may provide an objective genetic test for the assessment of cells in Pap smears and serves as a screening marker for HSILs or LSILs, which may help determine the progressive potential of individual lesions.

Keywords: Cervical intraepithelial lesion, EGFR, FISH, cytogenetics, gene amplification

Introduction

Invasive cervical carcinoma (ICC) is one of the major malignant tumors seriously threating women's health. Screening out the precancerous lesions and prognosis monitoring are critical to decrease the morbidity and mortality of the ICC. At present, the cervical cytology and HPV detection are methods often used for cervical cancer screening. The aim of cervical cytology screening is to identify the real precancerous lesions (high level intraepithelial neoplasia, HSIL), which requires to strictly differentiate HSIL from low grade squamous intraepithelial lesions (LSIL) on the one hand, and to predict correctly prognosis of LSIL on the other. Though most LSILs will normally go away on its own, a few cases can further progress into HSIL or ICC [1]. So far no method, however, has been found effective at LSIL prediction and HSIL prognosis evaluation.

Epidermal growth factor receptor (epithelial growth factor receptor, EGFR) is the expression of the c-erbB-1 (HER-1) proto-oncogene. Previous studies have proved that the protein expression of EGFR and gene amplification increase gradually during the progression from chronic cervicitis to low grade CIN, high grade CIN and squamous cell cancer at all levels [2]. Using liquid-based cytology and fluorescent in situ hybridization (FISH) technique to detect EGFR gene amplification in specimens (ThinPrep, Hologic, Marlborough, MA) of cervical lesions at different levels, the present study aims to find valid methods to predict LSIL and evaluate prognosis for HSIL. In addition, the correlation between EGFR gene amplification and viral load of the high-risk human papilloma virus (hHPV) in the same specimens will be evaluated in some cases.

Materials and methods

Case selection

Cervical cytology specimens of 78 cases were collected from June 2008 to December 2008

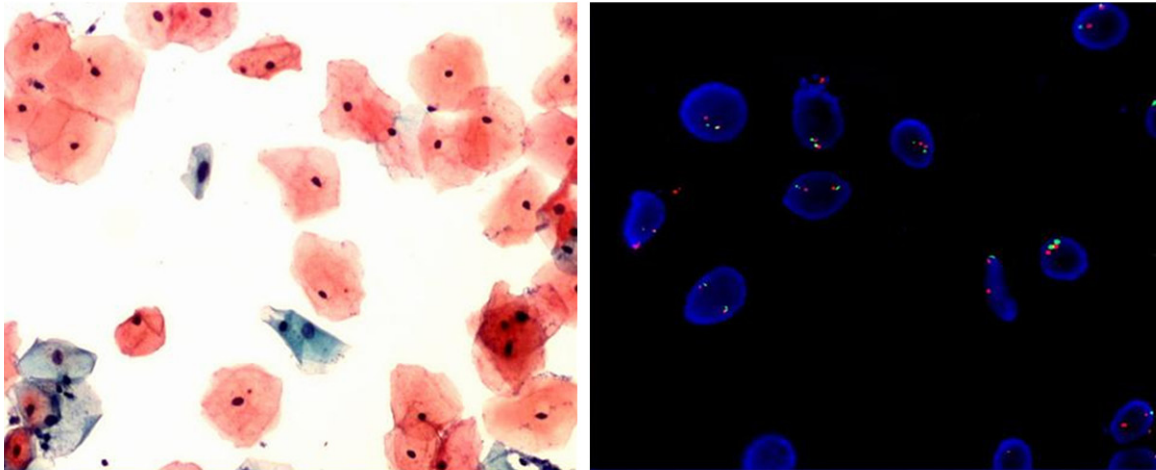

Figure 1. Normal cervical squamous epithelium cells. EGFR shows disomy.

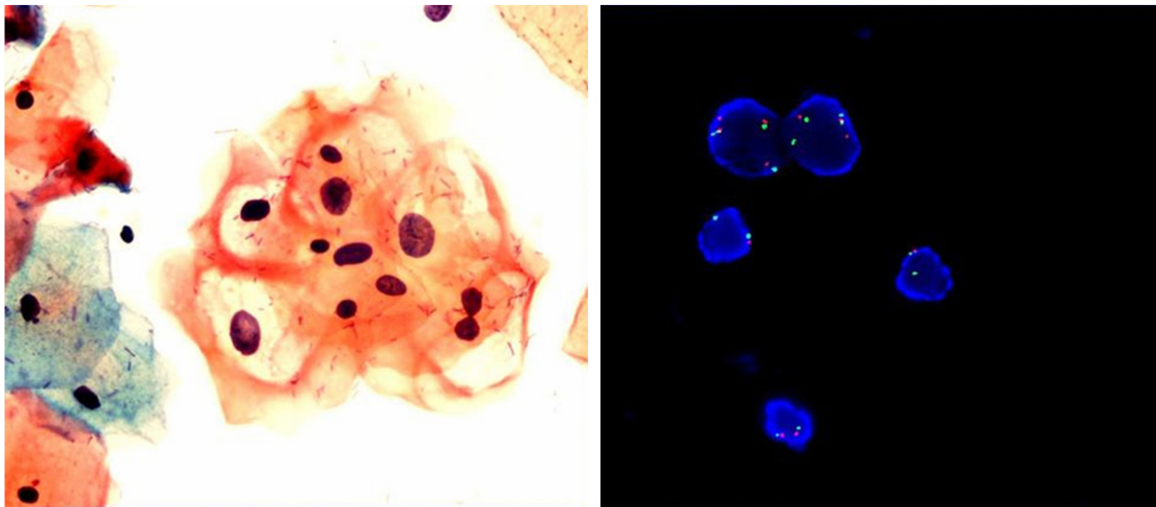

Figure 2. Low-grade squamous intraepithelial lesions show EGFR gene amplification, manifesting balanced trisomy and tetrasomy.

from Nanjing Maternity and Child Health Hospital affiliated to Nanjing medical university, including 26 cases with low-grade squamous intraepithelial lesions (LSIL), 24 with highly squamous intraepithelial lesion (HSIL), and 28 relatively normal controls (of the 28 controls, 20 were used to establish threshold for EGFR gene amplification). The standard for cytological diagnosis classification was the 2001 TBS system [3].

All the diagnosis were jointly made by two pathologists, one was responsible for preliminary cell screening and the other for the screening review. LSIL and HSIL were confirmed histologically to correspond to low level CIN and high level CIN respectively. All specimens were satisfactory archived liquid-based cytology smears. All LSILs underwent cytological follow-up 6 months later, and half of which had follow-up 12 months later.

FISH detection

FISH analysis was made on liquid-based smears. (1) probe: double-colored FISH probes (LSIEGFR/CEP7) were bought from Beijing Golden Pu Jia Medical Company. Fluorescence microscope (Olympus Bx60, Lake Success, NY) with bright vision was used for the analysis. Analysis software was FISH 2.0 (Imstar, France). (2) steps for smear pretreatment: Kerstin Heselmeyer-Haddad, et al reported in 2003 that FISH can be applied to cytological smears

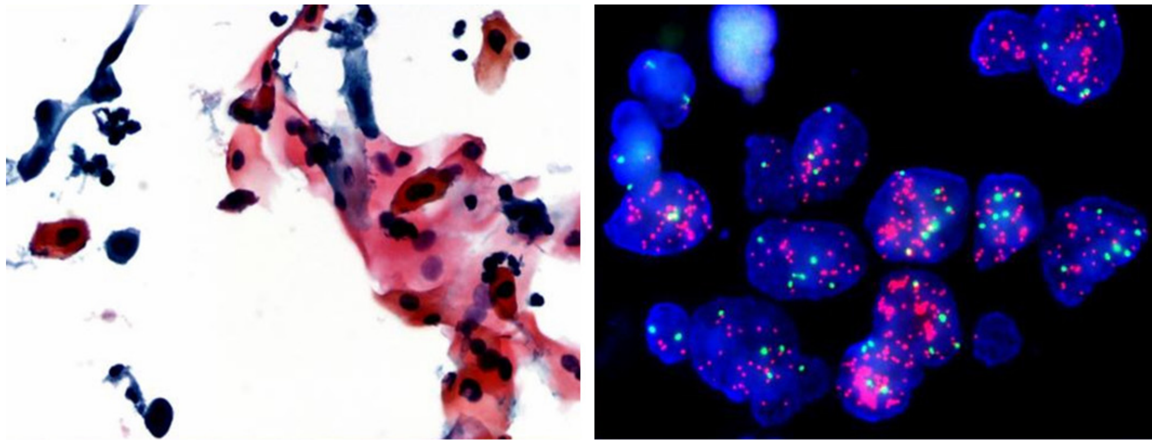

Figure 3. Squamous intraepithelial lesions show positive EGFR gene amplification, manifesting cluster amplification.

with steps similar to histological ones, only slightly different in terms of sample pretreatment [4]. The archived smears (coverless slides) were first placed in xylene for 2 days, and then gradiently decolored by ethanol, and then rinsed, digested by pepsin respectively, and heated to 56°C. (3) steps for FISH: in dark light, the slides and probe mixture (7 μl of hybrid buffer solution, 1 μl of deionized water and 2 μl probe) were degenerated in 70% denaturation solution (formamide/2 x SSC) at 73 ± 1°C for 5 minutes, then dehydrated gradiently for 3 minutes by 70%, 85% and 100% ethanol precooled at -20°C. After the slides dried naturally, the mixture was preheated in the baking machine at 46°C and hybridized with a probe. The hybridized area were dripped with 10 μl hybridized droplets, then covered by a cover slip and sealed with rubber glue, then kept at 42°C incubator overnight for 16 hours' hybridization. Slides elution in the next day: at the temperature of 46°C, the cover removed slides were placed in the 50% solution (formamide/2 x SSC) for 10 min x 3 times (the elution time-keeping started when the last slide was put into the Kaupthing bottle), then placed in 2 x SSC solution for 10 mins, and finally to the 2 x SSC/0.1% NP240 solution for 5 mins. After the slide dried naturally, 10 μl DAPI counterstain solution was dripped on it, sealed with a cover slide, put into the cassette for counterstain for 20 mins. The slide was then observed through suitable filter glass group under the fluorescence microscope.

HPV detection

Of the 26 LSIL cases, 15 were done hHPV test at the same time (materials obtained from the archived data). Hybrid Capture II test (HC II) was done to detect hHPV. The HCII-HPV-DNA was detected by hybrid Capture method, and the instrument was gene hybridization signal amplification System in Hybrid CaptureR System (Model DML 2000) produced by Digene Company, America. The HPV testing kit was bought from Shanghai Wright Company, and the operation procedures were in strict accordance with the instruction manual on the reagent box.

Threshold value for EGFR judgment

The threshold was set up by detecting the expression of EGFR gene by FISH in nuclei off the normal cervix in 20 cases. Threshold = mean + 3 x standard deviation. Through calculation, threshold value = 5.32%. When cells are ≥6, red signals >2 and only when green signals are ≥2 too, the expression can be judged as positive.

EGFR FISH detection

The probes were LSI EGFR/CEP7 (CEP7 was control probe). The marker color was double red/green, red signal standing for EGFR gene (TRITC channel image), and green for 7 centromeric sequence of chromosome (TRITC channel image). Nucleus was identified by DAPI filter. Two pathologists observed the randomly chosen counting cells, only when both agreed on the observation, can the result be confirmed. Cells differentiable by each channel signal were chosen for counting, while those which may affect signal judgment were excluded, such as those from hybrid heterogeneous region, with

Table 1. EGFR gene amplification in different cervical lesions

Group	Cases	EGFR gene amplification		
		negative	positive	positive rate (%)
normal	28	26	2	7.14
LSILs	26	20	6	23.08
HSILs	24	9	15	62.50
Total	78	54	24	30.77

Table 2. LSILs follow-up

	Follow-up 6 months	Follow-up 12 months
LSIL (-)	12	15
EGFR gene amplification	1 (8.33%)	1 (6.67%)
LSIL (+)	14	7
EGFR gene amplification	5 (35.71%)	3 (42.86%)
Missed follow-up	0	4
P	<0.05	<0.01

unclear cell nucleus contour or region in dark background. 200 cells randomly were counted. If more than 10% of the cells were abnormal, it meant that EGFR gene amplification happened; and when the ratio is less than 10% but abnormal EGFR gene cells gathered (cluster amplification), it also meant EGFR gene amplification.

HPV detection

HC II can detect simultaneously 13 types of high-risk HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The judgment standard was, when the ratio of relative light units and cutoff (RLU/CO) was >1.0, the result was regarded as positive.

Statistical analysis

SPSS 10.0 statistical software package was used for statistical data analysis, chi-square test and exact probability method were also used. *P*<0.05 was defined as significant.

Results

Correlation between EGFR gene amplification and the cervical lesions at all levels

Gene amplification of different degree of cervical lesions, as shown in **Figures 1-3**, most of the negative cytology cases showed balanced disomy, only 2 cases appeared balanced trisomy. Of the 26 LSILs cases, none of them showed monosomy, 20 were balanced disomy, and 6 (accounting for 23.08%) were positive. Of the 6 positive cases, 4 were balanced trisomy, the other 2 polysomy and cluster amplification. For the 24 patients with HSILs, the positive rate of EGFR gene amplification was 62.50% (15/24), and most of them showed polysomy and cluster amplification (see **Table 1**). With the development of cervix lesion, the positive rate of EGFR gene amplification increased, there was significant difference in the positive rate between HSILs and LSILs, and HSILs and the normal control group (*P*<0.01). Using EGFR gene amplification to differentiate low-grade lesions and high grade lesions, the sensitivity, specificity, positive prediction value and negative prediction value for HSILs were 71.4%, 69.0%, 62.5% and 76.9% respectively.

LSILs follow-up

After cytological diagnosis, patients with LSILs were followed up for 6~12 months. During the follow-up, if HSILs appeared, they were referred for colposcopy and biopsy screening. All the 26 patients with LSILs took part in the first round review at the end of 6 months, the results showed that 12 patients have regressed to normal, accounting for 46.15% (12/26); The other 14 patients had different degrees of pathological changes (1 case was ASC-US, 10 remain LSILs, 3 developed into HSILs). 3 HSILs cases were referred for colposcopy and biopsy, one case showed no pathological changes, the biopsy of other two proved to be high level intraepithelial neoplasia (CIN 2/3), then the two were operated on cervix conization (the follow-up were made separately). For the second round follow-up at the end of the 12 months, 22 cases were obtained, 4 cases were lost to follow-up (2 cases with HSILs, 1 with LSILs, 1 was negative at the first round follow-up). Of the 22 followed-up cases, 7 showed different degree of lesions (2 with ASC-US, 3 with LSILs, and 2 with HSILs), the other 15 cases were negative cytologically. For 3 cases with HSILs, colposcopy and biopsy showed progressive lesions, confirmed as severe dysplasia, and these 3 cases were operated on the conization of cervix (the follow-up were made separately). Of the 12 cases with LSILs who were identified negative at the first follow-up, 2 cases (16.67%) showed EGFR gene amplification with 1 trisomy and 1 polysomy. Of the 14 cases with LSILs

Table 3. Relationship between EGFR gene amplification and hHPV virus load

Follow-up	positive/ negative	cases	EGFR gene amplification	hHPV virus Load value
6 months	positive	9	4	703
	negative	6	1	487
12 months	positive	6	2	934
	negative	5	1	359

who were found positive at the first follow-up, 5 cases (35.71%) showed EGFR gene amplification, including 2 case with trisomy, 2 polysomy, and 1 cluster amplification. The positive rates of the two had difference ($P<0.05$). In addition, 2 cases confirmed as HSILs at the follow-up showed EGFR gene amplification (1 case with polysomy, the other with cluster amplification). Of the 15 patients found negative at the follow-up 12 months later, 1 case was positive. And of the 7 cases found positive at the same follow-up, 3 cases (42.86%) were positive EGFR gene amplification. The positive rate of the two had significant difference ($P<0.01$, see **Table 2**).

HPV test

As shown in **Table 2**, of the 26 cases with LSILs, 15 cases got hHPV detection and their file could be checked. Of these 15 cases, 12 were positive, 3 negative (positive rate was 80%), and 6 recovered, 9 showed progressive lesions. The follow-up six months later found that the 3 hHPV negative cases were back to normal. Viral load of the hHPV positive cases found at the follow-up increased more than that of the negative cases, but there was no significant difference ($P>0.05$, see **Table 3**).

Discussions

Application of FISH technology in cervical exfoliated cells

Using fluorescently labeled DNA probe to detect chromosome changes of the cell, FISH is a rapid and effective molecular cytogenetic technique, which can use specific nucleic acid sequence probe to locate precisely the metaphase chromosomes, and can also measure the target interphase nucleus. FISH can detect various genetic changes in cells, including heteroploid, reproduction, missing, amplification and translocation. FISH can also make up for the drawbacks of traditional banding technique. It has been proved that FISH has important role in detecting chromosome aberration or determining its fracture point. FISH has been applied widely at present in areas such as prenatal and pre-implantation genetic diagnosis, cancerous tissue detection including ovarian epithelial tumors, breast cancer and nasopharyngeal carcinoma, and so on. At the same time, FISH can also be applied in the cytological field; there have been reports on detecting urine exfoliated cells for the diagnosis of bladder cancer, detecting pleural effusion in the lung malignancy diagnosis and detecting telomerase in cervical exfoliated cells to predict cervical cancer [5-7]. The technology of FISH to detect the cervical exfoliated cells has been relatively mature [8, 9]. With the help of the fluorescent signal interpretation the judgment of the result can be more objective, hence the subjectivity of the operators can be minimized to ensure the accuracy of the results.

Using FISH with double-color probe, the present research successfully detected EGFR amplification in various cervical exfoliated cells with lesions. The signal was stable and results reliable. In this study, archived liquid -based smears were used with the advantage of having satisfying cells distribution and abundant abnormal cells. And using FISH, Papanicolaou staining pictures can be taken before hybridization, and abnormal cells can be marked on the back of the smear for probe dripping and effective hybridization.

Relationship between EGFR and cervical cancer lesions

EGFR is the expression of c-erbB-1 (HER-1) proto-oncogene, its gene locates on 7p13-7p12, combination of which with ligand EGF can promote cell proliferation differentiation thus accelerate cell malignant transformation. A number of studies conducted in China and other countries showed that EGFR has high expression in cervical cancer tissues, and may have correlation with cervical cancer prognosis [10].

The present study showed that EGFR gene amplification in the cervical exfoliated cells increased from normal to LSIL and HSILs. Compared with LSILs and the normal, the posi-

tive rate of amplification for HSIL is significantly different. And positive and negative rate of EGFR gene amplification for follow-up cases with LSILs also had difference. EGFR gene amplification was associated with the severity of pathological changes, which showed that the EGFR gene amplification may encourage the occurrence and development of cervical squamous intraepithelial lesions. Abnormal activation of EGFR may occur in the early stages of cervical cancer, suggesting that EGFR can serve as one of the biological markers of cervical cancer, and can be used in cervical cancer screening, early diagnosis and disease progression predicting.

Significance of EGFR gene amplification detection in the cervical exfoliated cells

The popularity of the cervical cytologic screening has greatly reduced the morbidity and mortality of cervical cancer, but as cytologic screening is a kind of morphological screening influenced by factors such as material selection, smear quality and subjectivity of the observer, there are deficiencies such as low sensitivity, poor repeatability and unsatisfactory smear, and so on. Although test for HPV DNA, the high risk factor of cervical cancer, has now been a test item, the positive prediction value of HPV DNA is low, because most HPV infection (regardless of cytologically abnormal) is temporary, especially for sexually active young women, only a few long-term persistent infections have a cancer risk. In fact, mildly abnormal cells are common in young women, but the rate of precancerous lesions (high level intraepithelial neoplasia) is not high. This study found that hHPV infection rate for LSILs was as high as 80%, the negative cases in the subsequent follow-up all went back to the normal, which suggests that negative prediction value of hHPV detection is valid. Though the HPV virus load of positive follow-up cases with LSILs was higher than that of the negative follow-up cases, the quantitative difference has no statistical significance. Therefore, other monitoring indicators are needed to make up for the inadequacy of the previous ones.

FISH with specific diagnostic probe is used to detect EGFR gene amplification and centromere copy number of chromosome 7 in cervical cells collected from cytological examination. Chromosomal aneuploidy can be made visible experimentally, thus the method is feasible and valid. Specific applications of FISH include: (1) diagnosing intractable cases especially with LSIL and HSIL. It is easier to identify morphologically low and high grade lesions, but it is rather difficult to accurately distinguish atypical lesions in some cases. Especially for subsequent progression for young female patients, EGFR gene amplification in cervical exfoliated cells detected by FISH can be used as an auxiliary diagnosis indicator. This study showed that there were significant difference in the positive EGFR gene amplification rates between normal cervix, LSIL and HSIL. Using liquid-based cytology directly, without collecting additional materials, the technique is feasible and convenient. (2) It can be used as the clinical marker for low-grade lesions follow-up There are complete difference in follow-up treatment of low-grade lesions (biopsy shows low level intra epithelial neoplasia) and high grade lesions (biopsy shows high level intraepithelial neoplasia), the former only needs follow-up observation while the latter needs the cervical conization. Although most low-grade lesions can go back to normal, still a few lesions have different degree of progression. This study showed that the positive rate of EGFR gene amplification was much higher than that of the negative follow-up cases. Therefore, for some low-grade lesion patients with positive EGFR gene amplification, especially those middle-aged and old women with polysomy and cluster amplification, the cervical conization is recommended rather than a follow-up. Close follow-up should be given to cases with positive EGFR gene amplification. (3) assist postoperative follow-up and prognosis evaluation on conization of high-grade intraepithelial lesions. Cases with negative EGFR gene amplification have good prognosis.

To conclude, FISH can be used to detect EGFR gene amplification in the cervical exfoliated cells on liquid-based cytology from regular cervical cytology specimens. Therefore, detection of EGFR gene may provide an objective genetic test for the assessment of cells in Pap smears and serves as a screening marker for HSILs or LSILs, which may help determine the progressive potential of individual lesions. But due to the technical difficulties and high cost of FISH detection, its clinical applications need further research.

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Disclosure of conflict of interest

None.

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