RESEARCH ARTICLE

A Piezoelectric Immunosensor for Early Cervical Cancer Detection

Li Yang[1,3]*, Xianhe Huang[1], Liang Sun[2]

Abstract

Background: A piezoelectric immunosensor for early cervical cancer detection was developed involving short analyis time and less invasive technique for $p16^{INK4a}$, a protein that has been linked to cervical cancer. Materials and Methods: 5μL of 5.0 mg/mL $p16^{INK4a}$ antibody and then supernatant from different clinical samples from West China Second University Hospital (Sichuan, China) were dripped on the center of the AT-cut crystal through a micro-injector. Absorption of the $p16^{INK4a}$ by antibody caused a shift in the resonant frequency of the immunosensor, and the resonant frequency was correlated to the amount of the $p16^{INK4a}$ in the supernatant. Results: The greater severity of lesion grading, the greater the expression level of $p16^{INK4}$. Conclusion: Degree of cervical cancer lesion development could be determined by detected amount of p16INK4a in different clinical samples.

Keywords: Quartz crystal microbalance - immunosensor - early cervical cancer - detection

Asian Pac J Cancer Prev, **15 (21)**, 9375-9378

Introduction

The cervical cancer ranks the second for its incidence against women in the global context. (Ferlay et al., 2008) It is also one of the most common cancers in developing Asian countries. Human papilloma virus (HPV) infection is a main pathogeny of cervical cancer (Sahebali et al., 2006; Darragh et al., 2012; Jing et al., 2013; Zhang et al., 2014).

Study shows that, amid most of the cancers, $p16^{INK4a}$ protein is in under-expression while amid the cervical cancer, the $p16^{INK4a}$ is over-expressed, and closely related to HPV infection and cervical cancer development (Ishikawa et al., 2006) In the cases of cervical intraepithelial neoplasia grade I, II, III and cervix squamous carcinoma, Ishikawa found that high risk-HPV (HR-HPV) had infection rates of 69.8%, 97.5%, 91.7% and 100%, respectively, and in the HR-HPV positive cases $p16^{INK4a}$ positive expression rates reached 32.4%, 82.1%, 93.2% and 100%, respectively, and all the HR-HPV specimens showed protein expression, which increased with the lesion progression. Ekalaksananan studied 241 cases of cervical liquid based cytology smears with papanicolaou stain grading, detected HR-HPV with in-situ hybridization (ISH) and $p16^{INK4a}$ with immunohistochemistry, and in all high-grade squamous intraepithelial lesions, the HR-HPV was found positive, the $p16^{INK4a}$ was over-expressed; so it is believed that the $p16^{INK4a}$ and HR-HPV detection is appropriate for cervix smear screening (Ekalaksananan et al., 2006). Eleuterioet detected 13 cases of high-grade squamous intraepithelial lesions, 26 cases of low-grade squamous intraepithelial lesions and 57 cases of $p16^{INK4a}$ expression in normal cervical tissues with immunohistochemical method, finding that the positive expression rates reached 92.3%, 15.4% and 0%, respectively; as to high-grade squamous intraepithelial lesions, the diagnostic sensitivity, specificity, positive predictive value and negative predictive value of $p16^{INK4a}$ expression was 92.3%, 100%, 100% and 98.3%, respectively, the correlation coefficient of $p16^{INK4a}$ and HR-HPV with high risk intraepithelial neoplasia was 0.95 and 0.47, respectively. They believed the $p16^{INK4a}$ is more sensitive than HR-HPV for cervical intraepithelial neoplasia diagnosis (Eleuterioet et al., 2012)

Previous studies suggested that $p16^{INK4a}$ could be a biomarker to predict the outcomes of cervical lesions, Therefore, we examined $p16^{INK4a}$ expression in cervical cytology specimen.

The quartz crystal microbalance (QCM) has been widely used as highly sensitive sensor which commonly configure with electrodes on both sides of thin disk AT-cut quartz. The crystal can be electrically excited into resonance because of the piezoelectric properties. In the late 1950s , Sauerbrey found the relationship between resonant frequency and mass deposit on surface of quartz in gas phase (Sauerbrey et al., 1956)

$$\Delta f=-[(2f_0^2)\div A(\mu_q\rho_q)^{1/2}]\Delta m \quad (1)$$

In which Δf is the observed frequency change (in

[1]*Frequency Control Laboratory, School of Automation and Engineering, University of Electronic Science and Technology of China,* [2]*Department of Pathology, West China Second University Hospital, Sichuan University,* [3]*College of Electrical & Information Engineering, Southwest University for Nationalities, Chengdu, Sichuan, China *For correspondence: swun_yangli@163.com*

Hz) , μq and ϱq are the shear modulus and density of the crystal respectively, A is the active electrode area, and Δm is the mass change on the surface of the crystal (in ng). in this study, AT-cut quartz crystal was choose, ϱq and μq are equal to 2.65 g/cm^3 and 2.95N/m^2 respectively. Since the work of Sauerbrey, the quartz crystal microbalance (QCM)has been widely used to monitor slight mass change (nanogram) in many research area such as biology, physics, medicine and so on.

In the current study, A piezoelectric (PZ) sensor for early cervical cancer detection was developed. PZ crystals were coated with mouse polyclonal antibody induced by p16^{INK4a}. the mass on the crystal changed when antibody absorbed p16^{INK4a}, at the same time, the resonant frequency of QCM shifted. The frequency shift had a linearity relation with the concentration of the antigen. Schematic illustration PZ immunoser for the detection of p16^{INK4a} is shown in figure1.using this method, we succeeding in measuring the p16^{INK4a} expression level in different clinical sample, and experiment data indicated that this method is capable of nanogram detection of p16^{INK4a} protein at room temperature. Because of the relationship between p16^{INK4a} concentration and cervical cancer, this method can be used in early cervical cancer detection.

Materials and Methods

Sample colletion

Clinical supernatant were obtained from West China Second University Hospital for cervical cancer screening. Study materials consisted of cervical ovaginal cytology specimens that had been selected on the basis of ThinPrep slides. Only specimens that had 2 mL of residual fluid with visible, floating, tissue-like fragments after preparation of ThinPrep slides were included. Cervicovaginal cytology specimens were collected in PreservCyt solution, and ThinPrep slides were prepared, screened, and interpreted using the 2001 Bethesda reporting system. For the purposes of the current study, all slides were reviewed, and diagnoses were confirmed or reassigned based on a consensus reached by two pathologists. ThinPrep Pap test cytologic diagnoses were reported as squamous cancer in 2 specimens, high-grade squamous intraepithelial lesions (HSIL) in 2 specimens, low-grade squamous intraepithelial lesions (LSIL) in 2 specimens, Diagnoses of negativity for intraepithelial lesions or malignancy (NILM) were reported in 2 specimens.

Equipment and reagents

All the PZ crystals were AT-cut which had nearly zero frequency drift with temperature around room temperature. The resonance frequency of crystal is 10MHz. The crystal (diameter: 8 mm)was placed between two gold electrodes, mounted in metal holder. An symmetric electrode pattern was used so that the upper electrode and the lower electrode had same radius (2.5mm). They were purchased from Tongfang Guoxin Electronics co., Ltd (Beijng, China). The quartz crystal was driven at its resonant frequency with a homemade oscillator circuit and frequency measurement was performed with Agilent 531323a , with a precision of 0.1 Hz at a gate time of 0.1 s. Because of the symmetric electrode pattern, the device had high uniform mass sensitivity distribution. (Hillier et al., 1991; Michael et al., 1992; Fabien et al., 1998)

Measurements Micro-injector (range from 0.2ul to 10ul) was obtained from KeXiao co., Ltd (Hang Zhou, China).

Human p16^{INK4a} full length protein (ab84075) was purchased from Abcam Co. (U.S.A).

Antibodies to p16^{INK4a} (mouse monoclonal antibody, clone 6H12) were purchased from Maixin Co. (Huzhou, China).

Phosphate-buffered saline (PBS) was composed of 137 mM NaOH, 2.7 mM KCL, 8.0 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.2).

Measurements

The PZ crystal was dipped in 1.2 M NaOH and 1.2M HCl for 10 minutes respectively, then washed with dual-distilled water and ethanol twice. The contaminants on the electrode were removed by this procedure, and a hydrophilic gold surface that could guarantee antibody Immobilize on the surface of electrode would be obtained. Then, make the crystal drying at the room temperature, the basic frequency F0 of the QCM was measured by frequency counter. The cleaned PZ crystal would prepared for antibody immobilization. The 5μl (5mg/ml) antibody was dropped on the surface of PZ crystal by a 5-μL syringe, Then incubated over silica gel blue at 4°C for 6 hours, washed subsequently with PBS (PH7.2) and distilled water, the cleaning procedure were repeated twice, then make the crystal air drying and the resonance frequency in gas phase (F1) was measured. At last, 5μl clinical cytology samples with different concentrations of p16 INK4 was applied on the surface of crystal, the crystal were incubated over silica gel blue at 4°C for 2 hours, then washed by PBS (PH7.2) and distilled water. The resonance frequency in gas phase (F2) was taken after crystal air drying.

Results

Stability of the system:

The system was composed of quartz crystal microbalance, home-made oscillator and frequency counter. Stability of this system direct determined whether the system can be used in clinical examination. The stability of the crystal is the most critical factor for system stability. A 10MHz, 3rd overtone AT-cut crystal was choose in this study, and the resonant frequency of one crystal was measured for 5 times with the same method at room temperature . Table 1 showed the result.

Above result indicated that the deviation of the each frequency within 2Hz, so the crystal had high stability in the process of measurement.

The form of the antibody layer

The immobilization of antibody is very important for the cervical cancer detection. In this study, the hydrophilic gold surface was formed, it would help the immobilization of antibody on the surface of crystal. After 6hours incubated, the resonant frequency (F1)was measured

.the frequency shift caused by absorb of antibody was calculated as equation1,

$$\Delta f_1 = F_0 - F_1 \qquad (2)$$

The average frequency shift of 10 crystals was 303Hz and the relative standard deviation was 0.137.we cloud find that the amount of antibody for all crystals was similar and all crystals obtained the stability antibody layer.

The response for different clinical sample

The supernatant was obtained from different clinical samples, and these clinical samples had different cervical lesion degree (2negative, 2LSIL, 2HSIL, 2cancer). After immobilization of the antibody, the supernatant containing various concentration of $p16^{INK4a}$ was evenly applied on the surface of crystal , then the resonant frequency (F2) were measured .the resonant frequency shift caused by antibody-antigen reaction was calculated with equation2,

$$\Delta f_2 = F_1 - F_2 \qquad (3)$$

Using the equation (1) and equation (3) , the amount of $p16^{INK4a}$ in different sample could calculated. Comparison between the resonant shift and amount of $p16^{INK4a}$ to the biopsy result was showed in Table 3

Table 1. The Measurement of Basic Frequency of the Crystal (25°C)

Times	1	2	3	4	5
F0(Hz)	10.009373	10.009372	10.009372	10.009371	10.009372

Table 2. The Frequency Shift Caused by Absorb of Antibody

Crystalnumber	1	2	3	4	5	6	7	8	9
Δf1(Hz)	300	296	308	302	302	319	298	305	300

Table 3. Comparison between the Resonant Shift to the Biopsy Result

Crystal number		Δf1(Hz)	Δm(ng)
Negative	1	15	33
	2	20	43
LSIL	3	108	239
	4	117	258
HSIL	5	232	513
	6	259	573
Cancer	7	398	881
	8	406	898

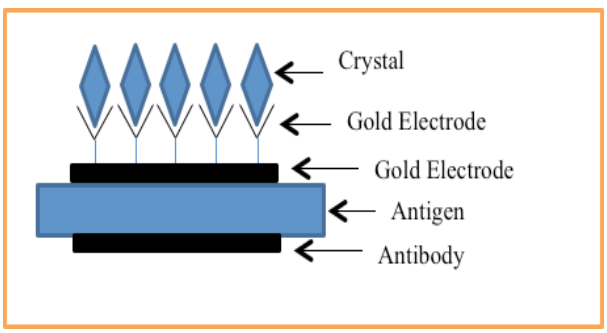

Figure 1. Schematic Illustration PZ Immunosensor for the Detection of $P16^{INK4a}$

The resonant frequency of all crystals changed after clinical supernatant covered on the surface of crystals. Two patients which diagnosed by histologically as negative had lower amount of $p16^{INK4a}$ range from 33ng to 43ng. Two patients which diagnosed by histologically as low-grade squamous intraepithelial lesions (LSIL) had amount of $p16^{INK4a}$ range from 239ng to 258ng. The amount of $p16^{INK4a}$ in two patients which diagnosed by histologically as HSIL ranged from 513ng to 573ng. Two patients which diagnosed by histologically as cancer had higher amount of $p16^{INK4a}$ range from 881ng to 898ng. So, it is obviously to see that this detection system is able to detect $p16^{INK4a}$ at the level of nanogram. The amount of $p16^{INK4a}$ was related to the cervical lesion degree. The greater severity of lesion grading, the greater the expression level of p16INK4. furthermore, the amount of p16INK4 in different lesion degree had no overlapping. So, the detection system which proposed in this paper could make judgment of cervical cancer degree from detected amount of p16INK4.

Discussion

The 5-years survival rate for cervical cancer patients in 2002-2007 was reported to be 95.1% in the screened group and 83.4% in the non-screened in Korea (Eun et al., 2013). It is obviously that the feasible inspection means could increase cervical cancer survival rates. The other feasible inspection method for early cervical cancer detection is Pap smear, this method only could determine whether the analyte presence or absence and interpreting slides is a labor-intensive, time consuming, and subjective process. Compare with other methods, the method proposed in this study is more objective, faster, and less reliant on technical expertise.

The result of this experiment is very important. It proved the feasibility and simplicity of the quartz crystal microbalance in detecting p16INK4 which associated with cervical lesion degree in the gas phase. From detection the amount of p16INK4 in different clinical samples, we could better separated those women who had no risk develop into cervical cancer and those women who had risk develop into cervical cancer. Cervical cancer incidence and mortality could reduce by this method. But it is necessary investigate further to increase the stability and precision.

Acknowledgements

This work was supported by the central college basic business expenses special funds of Southwest university for nationalities in 2013 (Young teachers fund projects) , project number: 13NZYQN06

References

Darragh TM, Colgan TJ, Cox JT (2012). The lower anogenital squamous terminology standardization project for hpv-associated lesions: background and consensus recommendations from the college of american pathologists and the American society for colposcopy and cervical pathology. *Arch Pathol Lab Med*, **10**, 1266-97

Ekalaksananan T, Pientong C, Sriamporn S, et al (2006). Usefulness of combining testing for p16 protein and human papillomavirus (HPV) in cervical carcinoma screening, *Gynecol Oncol*, **103**, 62-6.

Eleuterioet J, Giraldo PC, Cavalcante DL (2009). Papillary squamous cell carcinoma of the uterine cervix, high-risk human papilloma virus infection and p16 (INK4a) expression: a case report, *Acta Cytol*, **53**, 188-90.

Ferlay J, Shin HR, Bray F (2008). Estimates of worldwide burden of cancer in 2008: GLOBOCAN. *Int J Cancer*, **127**, 2893-917.

Hillier AC, Ward MD (1992). Scanning electrochemical mass sensitivity mapping of the quartz crystal microbalance in liquid media. *Anal Chem*, **64**, 2539-54.

Ishikawa M, Fujii T, Saito M, et al (2006). Overexpression of $p16^{INK4a}$ as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia, *Gynecol Cancer*, **16**, 347-53.

Josse F, Lee Y (1998). Analysis of the radial dependence of mass sensitivity for modified-electrode quartz crystal. *Anal Chem*, **70**, 237-47.

Jing L, Rong H, Johannes E, You L (2013). Epidemiological features of human papillomavirus (HPV) infection among women living in mainland China, *Asian Pac J Cancer Prev*, **14**, 4015-24

Michael D, Edward J (1991). Radial mass sensitivity of the quartz crystal microbalance in liquid media anal. *Chem*, **63**, 886-90.

Sahebali S, Depuydt CE, Boulet GAV (2006). Immunocytochemistry in liquid-based cervical cytology: analysis of clinical use following a cross-sectional study. *Int J Cancer*, **118**, 1254-60.

Sauerbrey G (1958). Use of quartz vibrator for weighting thin film on a microbalance. *Z.phys*, **155**, 206-12.

Zhang L, Lin Y, Li JK (2014). Concordance in cervical HPV detection between Hybrid Capture 2 and HPV GenoArray tests. *Asian Pac J Cancer Prev*, **15**, 4465-6.