

RESEARCH ARTICLE

A Piezoelectric Immunosensor for Early Cervical Cancer Detection

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Abstract

Background: A piezoelectric immunosensor for early cervical cancer detection was developed involving short analysis time and less invasive technique for p16^{INK4a}, a protein that has been linked to cervical cancer. **Materials and Methods:** 5 μ L 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^{INK4a}. **Conclusion:** Degree of cervical cancer lesion development could be determined by detected amount of p16^{INK4a} in different clinical samples.

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

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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)

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

In which Δf is the observed frequency change (in

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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 chosen, ρq and μq are equal to 2.65 g/cm^3 and 2.95 N/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 areas 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 of PZ immunosensor for the detection of p16^{INK4a} is shown in Figure 1. Using this method, we succeeded in measuring the p16^{INK4a} expression level in different clinical samples, and experimental 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 collection

Clinical supernatant were obtained from West China Second University Hospital for cervical cancer screening. Study materials consisted of cervical vaginal 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 10 MHz. The crystal (diameter: 8 mm) was placed between two gold electrodes, mounted in metal holder. A symmetric electrode pattern was used so that the upper electrode and the lower electrode had same radius (2.5 mm). They were purchased from Tongfang Guoxin Electronics Co., Ltd (Beijing, 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 0.2 μl to 10 μl) 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 Na₂HPO₄, and 1.5 mM KH₂PO₄ (pH 7.2).

Measurements

The PZ crystal was dipped in 1.2 M NaOH and 1.2 M 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 immobilization on the surface of electrode would be obtained. Then, make the crystal drying at the room temperature, the basic frequency F_0 of the QCM was measured by frequency counter. The cleaned PZ crystal would be prepared for antibody immobilization. The 5 μl (5 mg/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 (pH 7.2) and distilled water, the cleaning procedure were repeated twice, then make the crystal air drying and the resonance frequency in gas phase (F_1) was measured. At last, 5 μl clinical cytology samples with different concentrations of p16^{INK4} were applied on the surface of crystal, the crystal were incubated over silica gel blue at 4°C for 2 hours, then washed by PBS (pH 7.2) and distilled water. The resonance frequency in gas phase (F_2) 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 directly determined whether the system can be used in clinical examination. The stability of the crystal is the most critical factor for system stability. A 10 MHz, 3rd overtone AT-cut crystal was chosen 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 each frequency within 2 Hz, 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 6 hours incubation, the resonant frequency (F_1) was measured

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

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

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,

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

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

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

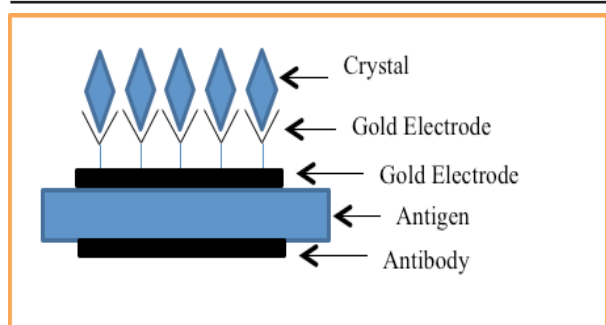


Figure 1. Schematic Illustration PZ Immunosensor for the Detection of P16^{INK4a}

biopsy result was showed in Table 3

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 p16^{INK4}. furthermore, the amount of p16^{INK4} 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 p16^{INK4}.

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 p16^{INK4} which associated with cervical lesion degree in the gas phase. From detection the amount of p16^{INK4} 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.

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