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 p16INK4a, a protein that has been linked to cervical cancer. Materials and Methods: 5μL of 5.0 mg/mL p16INK4a 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 p16INK4a by antibody caused a shift in the resonant frequency of the immunosensor, and the resonant frequency was correlated to the amount of the p16INK4a in the supernatant. Results: The greater severity of lesion grading, the greater the expression level of p16INK4a. 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

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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, p16INK4a protein is in under-expression while amid the cervical cancer, the p16INK4a 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 p16INK4a 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 p16INK4a with immunohistochemistry, and in all high-grade squamous intraepithelial lesions, the HR-HPV was found positive, the p16INK4a was over-expressed; so it is believed that the p16INK4a 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 p16INK4a 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 p16INK4a expression was 92.3%, 100%, 100% and 98.3%, respectively, the correlation coefficient of p16INK4a and HR-HPV with high risk intraepithelial neoplasia was 0.95 and 0.47, respectively. They believed the p16INK4a is more sensitive than HR-HPV for cervical intraepithelial neoplasia diagnosis (Eleuterioet al., 2012).

Previous studies suggested that p16INK4a could be a biomarker to predict the outcomes of cervical lesions, Therefore, we examined p16INK4a 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)

\[ \Delta f = - \left( \frac{2f_0}{\rho A} \right) \Delta m \] (1)

In which \( \Delta f \) is the observed frequency change (in
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Ink4a

and frequency measurement was performed with Agilent resonant frequency with a homemade oscillator circuit were purchased from Tongfang Guoxin Electronics co., Ltd (Hang Zhou, China).

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 immunosor for the detection of p16\(^{INK4a}\) is shown is figure 1 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 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 slides. Only specimens that had been selected on the basis of ThinPrep were included. Cervicovaginal cytology specimens were collected in PreservCyt solution, and ThinPrep slides were included. Cervicovaginal cytology samples 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 (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 0.2ul 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\(_2\)HPO\(_4\), and 1.5 mM KH\(_2\)PO\(_4\) (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 immobilization 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 equation 1,

$$\Delta f = F_0 - F_1$$

(2)

The average frequency shift of 10 crystals was 303Hz and the relative standard deviation was 0.137. We could 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 p16INK4a 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 equation 2,

$$\Delta f = F_0 - F_1$$

(3)

Using the equation (1) and equation (3), the amount of p16INK4a in different sample could be calculated. Comparison between the resonant shift and amount of p16INK4a to the 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 p16INK4a range from 33ng to 43ng. Two patients which diagnosed by histologically as low-grade squamous intraepithelial lesions (LSIL) had amount of p16INK4a range from 239ng to 258ng. The amount of p16INK4a 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 p16INK4a, range from 881ng to 898ng. So, it is obviously to see that this detection system is able to detect p16INK4a at the level of nanogram. The amount of p16INK4a 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-year 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

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References


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

<table>
<thead>
<tr>
<th>Times</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>FO(Hz)</td>
<td>10.009373</td>
<td>10.009372</td>
<td>10.009372</td>
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Table 2. The Frequency Shift Caused by Absorb of Antibody

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
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<tbody>
<tr>
<td>Δf1(Hz)</td>
<td>300</td>
<td>296</td>
<td>308</td>
<td>302</td>
<td>319</td>
<td>298</td>
<td>305</td>
<td>300</td>
<td>300</td>
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</table>

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

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<th>Crystal number</th>
<th>Δf1(Hz)</th>
<th>Δm(ng)</th>
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<tbody>
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<td>Negative</td>
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<td>15</td>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td>LSIL</td>
<td>3</td>
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Figure 1. Schematic Illustration PZ Immunosensor for the Detection of P16INK4a
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