Rapid detection of high-risk HPV16 and HPV18 based on microchip electrophoresis

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A B S T R A C T
Researches on detection of human papillomavirus (HPV) high-risk samples were carried out by polymerase chain reaction (PCR) coupled with microchip electrophoresis (MCE). Herein, we introduced a simple, rapid, automated method for detecting high-risk samples HPV16 and HPV18. In this research, general primers were initially selected to obtain sufficient detectable yield by PCR to verify feasibility of MCM method for HPV detection, then type-specific primers were further used to evaluate the specificity of MCE method. The results indicated MCE method was capable of specifically detecting high-risk HPV16 and HPV18, and also enabled simultaneous detection of multiplex samples. This MCE method described here has been successfully applied to HPV detection and displayed excellent reliability demonstrating by sequencing results. The inherent capability of MCE facilitated HPV detection conducted in a small chip with automated, high throughput, massive parallelized analysis. We envision that MCE method will definitely pave a way for clinical diagnosis, and even on-site screening of cervical cancer.

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1. Introduction

Epidemiological studies have revealed persistent infection with high-risk human papillomavirus (HPV) is the leading etiological cause of cervical cancer. Typically HPV16 and HPV18 are the subtypes with the highest infection rate worldwide and classified as “high-risk” type. It is well known that cytological examination is the gold standard for diagnosing HPV infection and is routinely used in hospitals. However, traditional cytological examination is difficult to distinguish viral infection at an early stage, as a result of high possibilities of false negative. Moreover, the observation scope of Papanicolaou staining is limited, consequently hitting the roof of the sensitivity [1–6]. Therefore, there has been a sustained effort to develop new strategies for HPV detection.

DNA investigation plays a prominent role in cervical cancer screening, because direct molecular analysis can achieve higher sensitivity and accuracy to some extent. Highly sensitive HPV DNA investigation strategies have been expeditiously developed that rely on molecular biology techniques such as PCR, sequence, and Southern Blot [7]. In general, a series of PCR based methods require instruments equipped with corresponding analytical apparatus; their products are usually analyzed by agarose gel electrophoresis or capillary electrophoresis, which is time-consuming and requires numerous manual operations [8]. Sequence is of importance in DNA profiling, but it is quite expensive and requires a high degree of proficiency or professionalism of the operators [9]. Given these limitations, microchip electrophoresis (MCE) has attracted great interest due to its advantages of automation, miniaturization, and high-throughput [10]. Similar to conventional electrophoresis, microchip electrophoresis employs high voltage direct current electric field (HVDC) as the driving force for sample injection, separation and detection. Miniaturizing the amplification reaction and combining it with sample detection on one chip significantly reduce consumption of expensive reagents and avoid cross contamination.
All extracted DNA were stored at buffer (Sangon Biotech, Shanghai, China) before quality determination. The extracted DNA was diluted with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) using U-3900s spectrophotometer (Hitachi, Japan). The extracted DNA was estimated from A260/A280 absorption ratio for further use.

DNA and HPV18 DNA were extracted from CaSki cells and Hela cells microfuge 5417C (Hamburg, Germany) for DNA extraction. HPV16 2.0 cell counter (Millipore, U.S.), and then centrifuged by Eppendorf and BW indicate sample, sample waste, buffer and buffer waste, respectively.

2. Experimental

2.1. Cell culture and DNA extraction

Two cervical cancer cell lines, CaSki cells and Hela cells, were supplied from Cancer Institute and Hospital of Chinese Academy of Medical Science (Beijing, China). CaSki cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (V/V) fetal bovine serum (FBS) and 0.1% (V/V) penicillin-streptomycin, while Hela cells were cultured in RPMI 1640 supplemented with 10% (V/V) FBS and 0.1% (V/V) penicillin-streptomycin. DMEM, RPMI 1640, FBS, and penicillin-streptomycin were purchased from Corning Corporation (Grand Island, U.S.). All cells were cultured in an incubator with a humid air atmosphere of 5% CO2 at 37 °C. Cells were counted by ScepterTM 2.0 cell counter (Millipore, U.S.), and then centrifuged by Eppendorf Microfuge 5417C (Hamburg, Germany) for DNA extraction. HPV16 DNA and HPV18 DNA were extracted from CaSki cells and Hela cells respectively by using TIANamp Micro DNA kit (Tiangen, Beijing, China) according to the manufacturer’s protocol. The quality of extracted DNA was estimated from A260/A280 absorption ratio using U-3900s spectrophotometer (Hitachi, Japan). The extracted DNA was diluted with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer (Sangon Biotech, Shanghai, China) before quality determination. All extracted DNA were stored at −20 °C until required for further use.

2.2. PCR primers

HPV general PCR primers were obtained according to L1 open reading frame (ORF). The general primer sequence is detailed in Table S1. HPV type-specific PCR primers were designed by the DNAMAN software (Lynnon Biosoft, USA) according to E7 ORF. The primer sequence is detailed in Table S2. The L1 and E7 information of HPV16 and HPV18 was obtained through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and was aligned by the DNAMAN software. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

2.3. PCR assay

Briefly, 20 μl general primer PCR system contained 10 ng DNA, 10 μM PCR buffer (Promega, Shanghai, China), 3 mM MgCl2 (Promega, Shanghai, China), 0.2 mM deoxyribonucleoside triphosphate (dNTP) solution (Sangon Biotech, Shanghai, China), 1.5 unit of Taq DNA polymerase (Promega, Shanghai, China), 10 pM each of primer, and double distilled water (ddH2O) was used to make up a deficiency. 20 μl type-specific primer PCR system is similar to general primer system, except the concentration of MgCl2 is 2.5 mM. PCR reactions were performed in Eppendorf Master cycler Gradient (Eppendorf, Hamburg, Germany). The optimal cycling program was as follows: denaturation at 95 °C for 9 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension step at 72 °C for 5 min. The PCR products were detected by microchip electrophoresis system (MCE 202MultiNA, Shimadzu, Japan).

2.4. Microchip electrophoresis

The extracted HPV DNA was labeled online with SYBR Gold SYBR Gold (Invitrogen, Germany). The DNA ladder (Invitrogen, Germany) consisting of 19 DNA fragments was used as internal standard to identify the size of DNA fragments with the smallest fragment being 25 bp and the largest fragment being 500 bp; the size interval between the two fragments was 25 bp (excluding 475 bp), and diluted with 1 x TE buffer when using. The DNA 500 Marker (Shimadzu, Kyoto, Japan) included a low maker (LM) and an upper marker (UM). MCE can only detect DNA fragments between LM and UM. A blue light emitting diode (LED) at 470 nm and the fluorescence detection at 525 nm was equipped in MCE.

In order to obtain reliable electrophoresis results, appropriate pre-treatment of the microchip was necessary. Before analysis, the chip channel was cleaned with ultrapure water to ensure the smoothness, followed by drying with air. Afterwards, the separation buffer, the DNA ladder, the marker, and the sample were placed on the corresponding positions of microchip, and the on-chip online mixing mode was selected to set the corresponding parameters. Sample analysis can start after confirming steps stated above. For HPV detection, the parameters are as follows, sample loading: V5 = 280 V, VSW = 510 V, VB = 320 V, and VBW = 0 V; sample analysis: V5 = 250 V, VSW = 250 V, VB = 0 V, and VBW = 1000 V. HPV analysis based on MCE can easily achieve sample consumption of 2 μl and analysis time less than 3 min.

3. Results and discussion

3.1. Quality of extracted DNA

The quality of extracted DNA from CaSki cells and Hela cells was investigated, indicating the DNA extraction was highly purified (Fig. S1). PCR reaction was performed using the extracted HPV DNA as templates to enrich target content. The PCR reaction condition was constructed to 39 identical PCR cycles. The procedure for each cycle typically consisted of (1) denaturing, (2) annealing, and (3) extension.
enables simultaneous amplification of multiple HPV subtypes, the optimized primers significantly increased the signal intensity of the HPV amplification product. However, due to the PCR amplification product fragments of various HPV subtypes are close in size, it is impossible to distinguish subtypes from the electropherogram; therefore, the positive results can only indicate the presence of HPV infection, but it is hard to distinguish subtypes.

3.3. HPV detection by type-specific primer PCR-MCE

Type-specific detection of HPV16 and HPV 18 were performed using type-specific primers corresponding to genotype to amplify HPV DNA, and then analyzed by MCE. Type-specific primers for HPV16 and HPV18 were designed according to the HPV E7 conserved region to further assess the specificity of HPV detection by MCE method [24,25]. Part of PCR amplified products were sent to sequence. The detection results are shown in Fig. 3. The signals corresponding to HPV16 and HPV18 were clearly visible, indicating the type-specific primers were effective and highly specific. To comprehensively evaluate the specificity of HPV detection by MCE method, HPV16 and HPV18 type-specific primers were utilized to amplify HPV18 DNA and HPV16 DNA, respectively. As shown in Fig. 3, the target signal intensity was stronger when using corresponding type-specific primers, indicating the type-specific primers designed in this study had good specificity and there was no cross-amplification between two subtypes. The sequencing results are shown in Table S4, which suggested that the length of the amplified product fragment of HPV16 is 203 bp, and the length of the amplified product fragment of HPV18 is 244 bp. After comparison, it was consistent with the existing sequences in GeneBank, which further demonstrated the reliability of MCE method.

Furthermore, the sensitivity of HPV detection by MCE method was evaluated, and different HPV DNA concentrations were obtained by controlling the cell density of the extracted DNA. To study the detection limitation, the density of CaSki cells and Hela cells used for HPV16 and HPV18 extraction was controlled to a series of 10 fold dilutions (from $10^6$ cells/mL to $10^2$ cells/mL). Corresponding experiments were conducted for various cell densities. The detection results of various cell densities after MCE analysis are shown in Fig. 4. It can be seen from the results that the intensity of target signal was increased along with the increase of cell density. For HPV16 analysis, the MCE system could detect a CaSki cell density as low as 10$^2$ cells/mL. And for HPV18 analysis, a Hela cell density down...
to $10^2$ cells/mL could be well detected and analyzed by MCE method. Therefore, it can be concluded that the sensitivity of both HPV16 and HPV18 detection is $10^2$ cells/mL.

In addition, it is well accepted that cervical cancer may be caused by simultaneous infection with certain kinds of HPV such as HPV16 and HPV18. Therefore, the MCE method was further evaluated by simultaneous detection of HPV16 and HPV 18. We added HPV16 type-specific primers, HPV18 type-specific primers, HPV16 DNA and HPV18 DNA for multiplex PCR in the same reaction and finally successfully achieved the simultaneous detection of two subtypes. As shown in Fig. 5, the results indicated the MCE method was target specific for HPV analysis. Although the amplification signal of HPV18 is obviously stronger while that of HPV16 is a little weaker, this may be due to a competition existing between the two amplifications in the same system, resulting in an obvious target peak of one amplification and a weaker amplification of the other. Overall, the results suggested that the MCE method described here enabled simultaneous detection of HPV16 and HPV18, as well as distinguishing two high-risk HPV subtypes.

4. Conclusion

In summary, a rapid and sensitive method for high-risk HPV16 and HPV18 detection was established, and sequencing results confirmed the reliability of this method. This method for HPV detection exhibited several superiorities to other methods. First of all, the inherent factor of integration feasibility rendered HPV analysis more practical, which is previously difficult for conventional methods, the integrated microchip detection improves the efficiency of separating DNA fragments and reduces cross contamination. Secondly, automated analysis significantly overcame multistep sample manipulation and reduced execution time. Finally, this method remarkably reduced reagent consumption and enabled high-throughput analysis with high sensitivity, specificity, and reproducibility. The method proposed here may not be as versatile as a dreamful “golden method” that is expected to solve all the chemical and biological problems we meet, but we hold the promise that the establishment of this assay is conductive to the screening of cervical cancer, and has good development prospects in life science, clinical testing and on-site cancer monitoring.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.04.003.

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