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Laser Capture Microdissection and PCR for Analysis of Human Papilloma Virus Infection

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Summary

Human papilloma virus (HPV) infection is considered one of the main factors involved in the pathogenesis of endocervical adenocarcinoma. However, the cellular location of HPV in this type of tumor is controversial. We have developed a method to determine the presence of HPV type 16 in endocervical cancer cells using laser capture microdissection followed by DNA extraction and qualitative polymerase chain reaction. Our results show that HPV type 16 is present in endocervical adenocarcinoma cells.

Key Words: Human papilloma virus; endocervical adenocarcinoma; laser capture microdissection.

1. Introduction

Human papilloma virus (HPV) infection is one of the main factors implicated in the pathogenesis of adenocarcinoma of the cervix ***(1–3)***. HPV infection is often found in the tumor. The antecedent cervical cytology and the HPV subtypes most commonly associated with cervical adenocarcinoma are types 16 and 18 ***(4)***. The reported prevalence of HPV infection in cervical adenocarcinoma ranges from 25 to 48%. This variation can be explained by the specific patient cohort sampled and variations in the method employed to detect individual HPV subtypes.

The incidence of concomitant squamous cervical intraepithelial neoplasia in endocervical adenocarcinoma is also well recognized and has been quoted to be as high as 40% ***(5)***. High-grade squamous cervical intraepithelial neoplasia is strongly correlated with HPV infection ***(6)***. However, it appears that cervical

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screening may be a less-sensitive tool for detection of glandular as opposed to squamous intraepithelial neoplasia.

To date, most studies into the relationship between HPV infection and endocervical adenocarcinoma have been based on polymerase chain reaction (PCR) analysis of DNA extracted from whole tissue sections *(7)*. These sections will often contain squamous epithelium, which will frequently be infected with HPV. To establish the true prevalence of HPV infection in endocervical adenocarcinoma it is necessary to specifically analyze endocervical adenocarcinoma tumor cells.

The laser capture microdissection system developed at the National Institutes of Health and Arcturus Engineering enables the selective sampling of cells of interest from both archival and fresh-frozen histological sections under direct microscopic control *(8,9)*. The LCM system has the dual advantages of (1) allowing the analysis of specific cell types and (2) reducing the dilution of the target DNA by ensuring that DNA is extracted only from target cells. In this chapter we describe a method to determine the presence of HPV-16 in endocervical adenocarcinoma using laser microdissected cells.

2. Materials

1. Formalin-fixed wax-embedded sections of endocervical adenocarcinoma.
2. Xylene.
3. Ethanol.
4. PixCell II Laser Capture Microdissecting System (Arcturus Engineering, Mountain View CA).
5. Toluidine blue.
6. Digestion buffer (2 mg/mL proteinase K, 0.05 *M* Tris-EDTA buffer, pH 8.5, containing 0.5% Tween-20).
7. β-globin forward primer: 5' ACA CAA CTG TGT TCA CTA GC 3' (*see* **Note 1**).
8. β-globin reverse primer: 5' CAA CTT CAT CCA CGT TCA CC 3' (*see* **Note 1**).
9. HPV-16 forward primer: 5' TCA AAA GCC ACT GTG TCC TG 3' (*see* **Note 2**).
10. HPV-16 reverse primer: 5' CGT GTT CTT GAT GAT CTG CA 3' (*see* **Note 2**).
11. Magnesium chloride.
12. dNTPs (Applied Biosystems).
13. AmpliTaq Gold DNA Polymerase (Applied Biosystems).
14. Sterile water.
15. Hybaid PCR Express thermocycler (Hybaid).
16. Agarose gel.
17. Ethidium bromide.
18. Gel loading buffer: (6X; 10% Ficoll 400, 025% bromophenol blue, 0.25% xylene cyanol FF, 0.4% orange G, 10 m*M* Tris-HCl, pH 7.9, and 50 m*M* EDTA).
19. Horizontal gel electrophoresis apparatus for agarose gels.
20. Ultraviolet light transilluminator.
21. Polaroid type 665 instant film.

3. Methods

3.1. DNA Extraction From Whole Tumor Sections

1. For wax-embedded tumor biopsies, five 5-μm sections of each biopsy were used (approximate surface area = 10 mm × 10 mm). The sections were placed in a 1.5-mL Eppendorf tube.
2. The sections were dewaxed in xylene for three 10-min periods.
3. The sections were then rehydrated in 100% ethanol for three 10-min periods to remove the xylene (*see* **Note 3**).
4. The specimens were then left to dry, to allow the ethanol to evaporate, a heating block set at 56°C for 10 min.
5. Sections were then digested in 50 μL of digestion buffer for 4 h at 55°C in a shaking heating block.
6. The proteinase K was inactivated by incubation at 98°C for 8 min.
7. Samples were centrifuged in a benchtop microfuge at 13,000*g* for 15 s and the supernatants carefully transferred to fresh sterile 0.5-mL microfuge tubes, ready for subsequent PCR analysis.

3.2. DNA Extraction From Laser Microdissected Tumor Cells

1. Sections of 5-μm thickness were cut from the wax-embedded tissue blocks onto uncoated glass slides (*see* **Note 4**).
2. The sections were dewaxed in xylene for three 10-min periods.
3. The sections were then rehydrated in 100% ethanol for three 10-min periods to remove the xylene (*see* **Note 3**).
4. The sections were then stained by immersing the sections in 0.25% toluidine blue (pH 4.5) for 5 s at room temperature. Excess staining solution was removed by washing the sections briefly in 100% ethanol, and then the sections were dehydrated sequentially in 100% ethanol and xylene (*see* **Note 5**).
5. Endocervical adenocarcinoma cells were microdissected from the stained tumor sections using the PixCell II laser microdissection system. The following settings of the laser were used: laser spot diameter15 μm, laser power 100 mW, and the duration of laser pulse was 10 ms (*see* **Note 6**). Under direct microscopic control the tumor cells of interest were aligned with the laser beam and the laser was fired at the targeted cells, causing the polymer film mounted on a disposable cap (CapSure HS™) directly above the targeted cells to be focally melted and the targeted cells captured onto the polymer film.
6. Approximately 500 laser pulses per tumor sample were used to obtain sufficient cells (corresponding to approx 1000 cells) for analysis.
7. The polymer film with the microdissected cells was carefully removed from the plastic cap, placed in an 1.5-mL Eppendorf tube, and digested in 50 μL of digestion buffer for 4 h at 55°C in a shaking hot block. This was followed by an 8-min incubation at 98°C to inactivate the proteinase K. PCR amplification for β-globin was then performed to confirm an adequate amount of PCR-amplifiable DNA.

3.3. β-Globin PCR

1. The amount of each primer was 150 pmol in a total reaction volume of 50 μL containing 2 m*M* $MgCl_2$, 200 m*M* of each dNTP, 0.25 units of AmpliTaq Gold DNA polymerase, and 1 μL of template DNA (*see* **Note 7**). The reaction volume was made up to 50 μL with sterile water.
2. DNA extracted from a fresh unstained cervical smear and distilled water were used as positive and negative controls, respectively.
3. PCR was carried out using a PCR Express thermocycler with the following cycling conditions: Initial 15-min period at 94°C followed by 40 two-step cycles at 72°C for 45 s and 55°C for 45 s.
4. The PCR products were then analysed by agarose gel electrophoresis (*see* **Subheading 3.5.**)

3.4. HPV-16 PCR

1. Only those samples showing amplifiable DNA as assessed by qualitative β-globin PCR were analyzed for HPV-16.
2. The amount of each primer was 150 pmol in a total reaction volume of 50 μL containing 200 m*M* of each dNTP, 0.25 units of AmpliTaq Gold DNA polymerase, and 1 μL of template DNA. The $MgCl_2$ concentration was 3.5 m*M* (*see* **Note 7**). The reaction volume was made up to 50 μL with sterile water.
3. The positive control was 1 μL of DNA extracted (as described in **Subheading 3.1.**) from five 10-μm sections of a paraffin-embedded block of SiHa cells (*see* **Note 8**).
4. The negative control consisted of 1 μL of sterile water.
5. PCR was carried out using a Hybaid thermocycler with the following conditions: initial 15-min period at 94°C followed by 40 two-step cycles of 72°C for 45 s and 55°C for 45 s.
6. When the PCR was complete the PCR products were then analyzed by agarose gel electrophoresis (*see* **Subheading 3.5.**).

3.5. Agarose Gel Electrophoresis

1. Gel electrophoresis using 2% agarose gels containing ethidium bromide (0.5 mg/mL) was used to analyze all PCR products. Ten μL of PCR product and 5 μL of loading buffer were added to each well.
2. Electrophoresis conditions were set at 80 V for 2 h. When the electrophoresis was complete, gels were placed on an ultraviolet light transilluminator to visualize the PCR products, and the gels were photographed using Polaroid type 665 film with a 12-s exposure time to obtain a permanent record of each gel.
3. Positive PCR results were regarded as being distinct bands of the predicted size for each PCR product (*see* **Notes 1** and **2**).
4. With DNA extracted from whole tumor sections, 33 cases were positive by PCR for β-globin. PCR was then carried out for HPV-16 in the 33 samples that were positive for β-globin; eight of those samples were positive for the HPV-16.

5. Using DNA extracted from laser microdissected cells, 52 of the 55 cases were positive for β-globin. The 52 samples that were β-globin-positive were analyzed for the presence of HPV-16 and 21 of the samples were HPV-16 positive.

4. Notes

1. The presence of amplifiable DNA in each sample is assessed using β-globin as an internal control ***(10)***. The β-globin primers produce an amplicon of 110 base pairs. Formalin fixation and wax embedding result in marked DNA fragmentation; thus, only short fragments (up to 200–300 bp) of DNA are usually amplifiable. Therefore it is important to use primers designed to amplify a short segment of DNA.
2. The primer sequences for HPV-16 were derived from the L1 region of the HPV-16 genome, as sequence variations in this region characterize individual HPV subtypes. The HPV primers produce a 119-bp amplicon.
3. It is essential to completely remove all traces of xylene from the samples prior to proteolytic digestion, as xylene prevents the action of proteinase K.
4. It is important to use noncoated slides to ensure satisfactory microdissection. Slides coated with adhesive, e.g., poly-L-lysine or aminoproprylethoxy silane, prevent the satisfactory transfer of tissue from the glass slide to the plastic cap.
5. The stained tissue sections require complete dehydration prior to laser microdissection. Even a trace of moisture appears to inhibit the successful capture of microdissected cells.
6. The optimum settings of the laser have to be determined by the individual user and for their particular application.
7. The optimum magnesium chloride concentration for each PCR must be determined by the individual investigator.
8. The SiHa cell line is derived from a squamous carcinoma of the cervix and contains two copies of HPV-16 per cell.

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