Development of Human-Derived Cell Culture Lines for Recurrent Respiratory Papillomatosis

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Abstract

Recurrent respiratory papillomatosis (RRP) is mainly caused by human papillomavirus (HPV) 6 and 11. While various adjuvant therapies have been reported, no effective therapy has been documented to universally "cure" this disease. In the era of precision medicine, it would be valuable to identify effective intervention based on drug sensitivity testing and/or molecular analysis. It is essential to be able to successfully carry out in vitro culture and expand tumor cells directly from patients to accomplish this goal. Here we report the result of successful culture of HPV-infected cell lines (success rate 70%, 9/13) that express the E6/E7 RNA transcript, using pathologic tissue biopsies from patients treated at our institution. The availability of such a system would enable ex vivo therapeutic testing and disease modeling.

Keywords

recurrent respiratory papillomatosis, human papillomavirus, cell culture

Methods

Tissues and Cell Culture

Informed consent was obtained per institutional human subject protections requirements (UCSD IRB#151676). Fresh papilloma specimens were collected from patients with RRP. Multiple exophytic wart-like lesions within the respiratory tract were biopsied from patients at our institution. The presence of HPV infection was confirmed by HPV DNA sequencing. Fresh papilloma specimens were collected from patients with RRP and immediately processed for cell culture and expansion. The availability of such a system would enable ex vivo therapeutic testing and disease modeling.

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Recurrence of respiratory papillomatosis (RRP) is a rare disease with an estimated incidence of 4.3 and 1.8 cases per 100,000 in those ≤14 and ≥15 years, respectively. Approximately, 1500 to 2000 new pediatric cases occur in the United States each year. RRP is characterized by multiple exophytic wart-like lesions within the respiratory tract, most commonly involving the larynx, especially the vocal cords.1 Low-risk human papillomavirus (HPV) types 6 and 11 account for more than 90% of all RRP cases.1 This disease poses a challenge to the patient, family, and treating physicians.2,3 According to the National Registry of Children with RRP, patients undergo an average of 4.4 (0.2-19.3) procedures per year.4 Many patients require operative intervention for years and may undergo 80 to 100 procedures. No universally effective drug has been documented.5 It would be valuable to identify individualized therapy based on drug sensitivity test and/or molecular analysis, especially for refractory RRP. It is essential to have an in vitro model for culturing tumor cells directly from patients to accomplish this goal.

The generation of human-derived cell culture lines for RRP is a nuanced laboratory technique and has been previously described in clinicopathologic and genetic profiling of recalcitrant and aggressive disease in a single patient.6 However, it is not clear whether a similar approach could work for more typical and less aggressive tumors and whether the HPV genomes would be lost if cell lines were established. Here we report successful development of a disease model with 9 robust HPV-infected cell lines using pathologic tissue biopsies from patients treated at our institution.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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active papilloma (Table 1) undergoing surgical debridement, and control specimens were obtained from children undergoing routine tonsillectomy.

Tissue samples were minced and digested with Dispase II (5 mg/mL; Roche Life Science, Indianapolis, Indiana) and then trypsin. Cells were cultured in EpiLife Medium (Gibco, Attra et al 639

**Table 1. Specimen Details.**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>HPV Type</th>
<th>Cell Growth</th>
<th>No. of Surgeries</th>
<th>Sex</th>
<th>Age, y</th>
<th>Diagnosis</th>
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<td>6</td>
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<td>F</td>
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<tr>
<td>2</td>
<td>6</td>
<td>Y</td>
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</tr>
<tr>
<td>3</td>
<td>11</td>
<td>N</td>
<td>68</td>
<td>M</td>
<td>12.5</td>
<td>Papillomatosis of trachea</td>
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<td>4</td>
<td>6</td>
<td>N</td>
<td>29</td>
<td>F</td>
<td>25.5</td>
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<tr>
<td>5</td>
<td>11</td>
<td>Y</td>
<td>92</td>
<td>F</td>
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<tr>
<td>6</td>
<td>6</td>
<td>Y</td>
<td>21</td>
<td>F</td>
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</tr>
<tr>
<td>7</td>
<td>6</td>
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<td>7</td>
<td>F</td>
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<tr>
<td>8</td>
<td>11</td>
<td>Y</td>
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<td>M</td>
<td>2.3</td>
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</tr>
<tr>
<td>9</td>
<td>11</td>
<td>Y</td>
<td>3</td>
<td>M</td>
<td>16.4</td>
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<tr>
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<td>6</td>
<td>N</td>
<td>21</td>
<td>F</td>
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</tr>
<tr>
<td>11</td>
<td>11</td>
<td>N</td>
<td>21</td>
<td>F</td>
<td>11.0</td>
<td>Recurrent glottic respiratory papillomatosis</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
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<td>29</td>
<td>F</td>
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<td>6</td>
<td>Y</td>
<td>47</td>
<td>M</td>
<td>22.2</td>
<td>Papillomatosis of larynx</td>
</tr>
</tbody>
</table>

Figure 1. Recurrent respiratory papillomatosis (RRP) and tonsil cells in culture. (A) Pathological diagnosis. (B) Cultured cell morphology (phase contrast). (C) Cytokeratin staining of cultured RRP cells. (D) Cytokeratin staining of tonsil cells. DIC, Differential Interference Contrast; IF, immunofluorescence.
ThermoFisher, Massachusetts) supplemented with human keratinocyte growth supplement (Gibco), bovine pituitary extract (Gibco), 10 to 40 ng/mL recombinant human EGF protein (R&D Systems, Minneapolis, Minnesota), 10 to 30 ng/mL recombinant human FGF basic protein (R&D systems), and addition of 5 to 10 µmol/L Y-27632 (Enzo Life Sciences, Farmingdale, New York) without feeder cells to facilitate downstream analyses.

Immunofluorescence

Cells grown on coverslips were fixed with 4% formaldehyde; blocked with 3% bovine serum albumin; incubated in mouse anti–cytokeratin 5 (ab17130; Abcam, Cambridge, Massachusetts), rabbit anti–cytokeratin 8+18 (ab53280; Abcam), mouse immunoglobulin (SC-2025; Santa Cruz Biotechnology, Santa Cruz, California), or rabbit immunoglobulin (#2729; Cell Signaling Technology, Beverly, Massachusetts); detected with Alexa Fluor 488 anti–mouse IgG (Life Technologies, Carlsbad, California) or Alexa Fluor 568 anti–rabbit antibody (Life Technologies); and mounted with ProLong Gold Antifade Mountant with DAPI (Life Technologies). To demonstrate the specificity of the immunofluorescent staining, cells were also stained with secondary antibodies without primary antibodies. Images were captured on a Nikon A1R confocal microscope with lasers at 405-, 488-, and 561-nm excitation wavelengths using a 60×1.4 NA objective at a resolution of 0.2 microns per pixel (Nikon, Melville, New York).

Nucleic Acid Analysis

All of the clinical samples were subject to HPV typing (13 high-risk and 24 low/intermediate-risk genotypes) by polymerase chain reaction (PCR) L1 gene for reverse blot hybridization with a linear array (Quest Diagnostics, San Juan Capistrano, California).

For in-house E6/E7-based typing, total RNA and DNA were isolated from the samples using the RNeasy Mini Kit (Qiagen, Valencia, California) and Quick-DNA Universal
Kit (Zymo Research, Irvine, California), respectively. Purified RNA was reverse-transcribed into complementary DNA (cDNA) using SuperScript V1LO cDNA Synthesis System (Invitrogen, Carlsbad, California) with a random primer. The PCR reactions for the detection of the genomic DNA and E6 messenger RNA (mRNA) of HPV contained 2 μL DNA/cDNA, 200 μM dNTP, 1.5 mM MgCl2, 1 U Taq DNA polymerase, and 200 nM of each primers. For the multiplex PCR to genotype HPV 6 and 11, 1 HPV6/11 universal forward primer and 2 genotype-specific reverse primers (HPV 6: 5’-TTA TGA ACC GTG CCT TGG TTA G-3’; HPV 11: 5’-CAA CGA CCC TTC CAC TGG TTA-3’) were used. Two sets of primers were used to genotype HPV 16 and HPV 18. The PCR products were resolved in 2% agarose gels and stained with ethidium bromide.

Results
All of the clinical diagnoses of RRP were validated by pathological examination of biopsied tumor samples (Figure 1A). Thirteen patients (Table 1) were recruited and 9 samples were successfully cultured and expanded. Three lines of evidence suggest that these cell lines were derived from RRP tumor cells. First, the cell morphology showed typical cobblestone-like epithelial cells (Figure 1B). Second, these cells were positive for epithelial cell markers, cytokeratins (Figure 1B). Third, these cell lines were positive for either HPV 6 or HPV 11 (see below).

In addition, 19 of 23 tonsil cell lines were established from patients with benign diseases.

While all of the clinical specimens from RRP debulking were positive for either HPV 6 or 11 (Table 1) using a genotyping array for 37 HPV types, it was not clear whether cultured cell lines would lose HPV episomes. E6-specific primers were used for HPV 6/11 typing and for detecting viral RNA expression in cells. Representative results of 2 tonsil (negative control) and 3 RRP cell lines are presented in Figure 2A. Our analyses reveal that HPV DNA (lanes 3, 6, 7, and 10) was present and the E6/E7 mRNA transcript (lanes 5, 8, and 12) was expressed in cultured cells. No HPV DNA was detectable in the cultured medium (lanes 5, 9, and 13), suggesting no viral particle was produced from cell culture. No HPV 16 and 18 were detected in all cell lines (Figure 2B,C), which is consistent with clinical genotyping result.

Discussion
In this short communication, we report a high success rate (69.2%, 9/13) of primary tumor cell culture directly from patient biopsy specimens by applying a modified protocol. The same protocol also works efficiently for culturing tonsil epithelial cells, which are used for control experiments. Characterizing some of these patient-derived cells revealed persistence of E6/E7 expression. However, no viral particle was detectable in the culture medium. This is consistent with the fact that HPV viral cycle is tightly regulated by host cell differentiation and the Rho kinase inhibitor-treated cells were conditionally reprogrammed to a less differentiated state. Nevertheless, cell differentiation can be restored when a proper medium is used without this inhibitor. Therefore, specialized protocols can be developed to accommodate individual needs. Further development is ongoing; having been successful at growing these cell cultures, we have begun to test them with various chemotherapeutic/antimicrobial compounds, beginning with the antiviral agent cidofovir. It would also enable the development of potentially curative adoptive cell immunotherapy for RRP, given the promising report of applying HPV-targeted tumor-infiltrating lymphocytes to treat patients with refractory cervical cancers.

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Author Contributions
James Attra, clinical data acquisition, drafting manuscript; Li-En Hsieh, experimental design, HPV analyses, drafting manuscript; Linda Luo, cell line development, clinical data acquisition, drafting manuscript; Jun Qin Mo, clinical data acquisition, drafting manuscript; Matthew Brigger, clinical data acquisition, drafting manuscript; Yu-Tsung Liu, overall experimental design and conception, data analysis, drafting manuscript; Seth Pransky, overall clinical research design and conception, data analysis, drafting manuscript.

Disclosures
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References


