APPENDIX
APPENDIX A

Solutions

PBS pH 7.2

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 1.52 \text{ gm} \\
\text{KH}_2\text{PO}_4 & \quad 0.08 \text{ gm} \\
\text{NaCl} & \quad 8.5 \text{ gm} \\
\text{ddH}_2\text{O} & \quad 1000 \text{ ml}
\end{align*}
\]

pH of solution was adjusted to 7.2 and solution filter sterilized.

Proteinase K (20 mg/ml)

\[
\begin{align*}
\text{Proteinase K} & \quad 100 \text{ mg} \\
\text{Sterile ddH}_2\text{O} & \quad 5 \text{ ml}
\end{align*}
\]

Solution was filter sterilized with a 0.2 μ milipore filter, aliquoted and stored at -20°C.

RNase (10 mg/ml)

\[
\begin{align*}
\text{RNase} & \quad 10 \text{ mg} \\
10 \text{ mM Tris Cl pH 7.5} & \quad 10 \text{ ml}
\end{align*}
\]

Solution was filter sterilized with a 0.2 μ milipore filter, aliquoted, heat denatured at 92°C for 5 minutes and stored at -20°C.

20 x SSC pH7.0

\[
\begin{align*}
\text{NaCl} & \quad 175.3 \text{ gm} \\
\text{Sodium Citrate} & \quad 8.2 \text{ gm} \\
\text{ddH}_2\text{O} & \quad 1000 \text{ ml}
\end{align*}
\]

pH of solution was adjusted to 7.0 with 10 N NaOH.
Cell lysis solution (10 mM Tris pH 8.0, 0.05 M EDTA pH 8.0, 100 μg/ml proteinase K, 1% SDS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 8.0</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.5 M EDTA pH 8.0</td>
<td>10 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5 ml</td>
</tr>
<tr>
<td>Sterile ddH₂O</td>
<td>84 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100 ml</strong></td>
</tr>
</tbody>
</table>

To 1 ml of the above, 10 μl of a 20 mg/ml stock of proteinase K solution was added prior to DNA extraction.

Digestion buffer (1 mM Tris HCl, 1.5 mM MgCl₂, 0.45% Tween 20 and 60 μg/ml proteinase K)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris HCl</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Sterile ddH₂O</td>
<td>99.30 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100 ml</strong></td>
</tr>
</tbody>
</table>

To 1 ml of above, 3.3 μl of a 20 μg/ml stock of proteinase K solution was added prior to DNA extraction.
Solutions for culture of Probe

**LB broth**

- Trypton 1.0 gm
- NaCl 1.0 gm
- Yeast extract 0.5 gm
- ddH$_2$O 100 ml

**LB agar**

- Trypton 1.0 gm
- NaCl 1.0 gm
- Yeast extract 0.5 gm
- Agar 1.8 gm
- ddH$_2$O 100 ml

**Ampicillin (20 mg/ml)**

- Ampicillin 100 mg
- Sterile ddH$_2$O 5 ml

Solution was filter sterilized with a 0.2 μm Milipore filter, aliquoted and stored at -20°C.
Solutions for Plasmid Isolation (Qiagen Kit)

Buffer P1 pH 8.0
- RNase A: 100 µg/ml
- Tris-HCl: 50 mM
- EDTA: 10 mM

Buffer P2
- NaOH: 200 mM
- SDS: 1%

Buffer P3 pH 4.8
- KAc: 2.55 M

Buffer QBT pH 7.0
- NaCl: 750 mM
- MOPS: 50 mM
- Ethanol: 15%
- Triton X-100: 0.15%

Buffer QC pH 7.0
- NaCl: 1.0 M
- MOPS: 50 mM
- Ethanol: 15%

Buffer QF pH 8.2
- NaCl: 1.25 M
- MOPS: 50 mM
- Ethanol: 15%
Solutions for Agarose gel electrophoresis

**TBE (5x)**

- Trisma base 54 gm
- Boric acid 27.5 gm
- 0.05 M EDTA pH 8.0 20 ml
- distilled water to 1 L

**Gel loading buffer 6x**

- Bromophenol blue 0.25% w/v
- Glycerol 30% w/v
- Solution was stored at 4°C

**Ethidium bromide (10 mg/ml)**

- Ethidium bromide 100 mg
- Sterile ddH$_2$O 10 ml
- Solution was stored in the dark.
Solutions for Southern hybridization (Boehringer Mannheim, 1989)

Hybridization solution (5xSSC, 0.1% w/v N-lauroylsarcosine, Na Salt (0.02% w/v SDS, 1% w/v Blocking reagent (vial II))/

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20xSSC</td>
<td>25 ml</td>
</tr>
<tr>
<td>N-lauroylsarcosine, Na Salt (Sigma)</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>SDS</td>
<td>0.02 gm</td>
</tr>
<tr>
<td>Blocking reagent (vial II)</td>
<td>1 gm</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>75 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution was dissolved for at least 1 hour at 68°C.

Note: Solution does not become clear.

Wash Solution 1 (2xSSC, 0.1% w/v SDS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20xSSC</td>
<td>10 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>90 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Wash Solution (0.1xSSC, 0.1% w/v SDS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20xSSC</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>99.5 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Solution for detection of hybridized products (Boehringer Mannheim (1989))

Buffer 1 pH 7.5 (100 mM, Tris HCl, 150 mM NaCl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris HCl pH 7.5</td>
<td>100 ml</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>150 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>750 ml</td>
</tr>
<tr>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Buffer 2 (prepared fresh)

0.5% w/v blocking reagent (vial II) in buffer 1.
Solution was dissolved at 68°C for at least 1 hour before use.

Buffer 3 pH 9.5 (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 9.5</td>
<td>100 ml</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>100 ml</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>50 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>750 ml</td>
</tr>
<tr>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Buffer 4 pH 8.0 (10 mM Tris, 1 mM EDTA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 8.0</td>
<td>10 ml</td>
</tr>
<tr>
<td>0.05 M EDTA</td>
<td>200 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>790 ml</td>
</tr>
<tr>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Colour solution

4-Nitroblue tetrazolium chloride (NBT) 45 µl
5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) 35 µl
Buffer 3 10 ml

Antibody-conjugate (150 mU/ml) diluted to 1:5000 (prepared fresh)

Anti-digoxigenin-alkaline phosphatase 4 µl
Buffer 1 20 ml

Solutions for cell culture

RPMI 1640

RPMI 1640 1 sachet
NaH₂CO₃ 2 gm
HEPES 1 gm
ddH₂O upto 1000 ml

Solution was filter sterilized with a 0.2 µ milipore filter. Media was stable for 3 months at 4°C.

Reviving media

Fetal Calf Serum (Gibco) 20 ml
Penicillin and Streptomycin 2 ml
Fungizone 1 ml
L-glutamine 0.012 gm
RPMI 1640 77 ml

Solution was filter sterilized with a 0.2 µ milipore filter and stored for a maximum of 2 weeks at 4°C.
10 % Culture Medium

Fetal Calf Serum 10 ml
Penicillin and Streptomycin 2 ml
Fungizone 1 ml
L-glutamine 0.012 gm
RPMI 1640 87 ml

Solution was filter sterilized with a 0.2 μ milipore filter and stored for a maximum of 2 weeks at 4°C.

Solutions for immunohistochemistry

Denhardt's solution (50x)

Ficoll 5 gm
Polyvinylpyrrolidone 5 gm
Bovine Serum Albumin 5 gm
ddH₂O to 500 ml

Solution was filter sterilized with a 0.2 μ millipore filter, aliquoted and stored at -20°C.

Organosilane solution (1% v/v)

3-amino-propyl-trithoxysilane (Sigma) 1 ml
ddH₂O 99 ml

100 ml
**Glutardialdehyde (10% v/v)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutardialdehyde (Sigma)</td>
<td>10 ml</td>
</tr>
<tr>
<td>PBS</td>
<td>90 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**0.1 M Sodium-m-periodate**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium-m-periodate</td>
<td>2.14 gm</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Sufficient Detection of Human Herpesvirus 6 in Oral Carcinoma

Yadav, A. Chandrashekar, M. Vasudevan, D. V. Ablashi*

Human herpesvirus 6 (HHV-6) was recently isolated from patients with lymphoproliferative disorders and acquired immunodeficiency syndrome (AIDS) (1). It was later shown to be highly prevalent in various human populations worldwide (2). The virus is the etiologic agent of exanthem subitum (3), of some cases of febrile illness in young children (4), and of some cases of heterophil-body-negative infectious mononucleosis (2). HHV-6 has also been reported to be associated with several diseases, including lymphomas (5,8), but it has not previously been reported to be associated with epithelial cell tumors of the oral cavity.

Oral carcinoma ranks as the eighth most common tumor in the developed world. In Asia and the Pacific regions, however, it is a major tumor of the head and neck, particularly in populations among whom chewing of betel quid and tobacco is the cultural norm (9). Carcinogens have been suspected in the pathogenesis of oral carcinoma (10), but the role of viral agents in oral carcinogenesis remains unclear.

In a preliminary study (11), we found significantly elevated levels of immunoglobulin G (IgG) antibody to HHV-6 in sera from oral carcinoma patients from South India (geometric mean titer [GMT] = 2042) compared with sera from healthy donors from the same population (GMT = 47). In contrast, Yadav and Ablashi (12) found normal levels of HHV-6 antibody in sera from patients with nasopharyngeal carcinoma, which is the most common tumor in the Chinese populations of Southeast Asia (GMT = 14) and which has been linked to Epstein-Barr virus (EBV) (13).

In the present study, we extend these observations to detection of HHV-6 DNA and viral antigens in tissues of oral tumors.

Fresh tumor tissue samples of histologically diagnosed squamous cell carcinoma of the buccal mucosa (one sample each from nine patients) were obtained from the Medical College of Trichur, India. Fifty nanograms of genomic DNA from each sample was used as the template for polymerase chain reaction (PCR) amplification, using HHV-6-specific nested primers to a conserved region of the virus, and amplified for 30 cycles (14). We found that six (67%) of the nine oral carcinoma tissue samples were positive for HHV-6 and the PCR amplicons specifically hybridized to a digoxigenin (Boehringer Mannheim, Mannheim, Federal Republic of Germany)-labeled oligonucleotide probe derived from the plasmid pZVH-14 containing an 8.9-kilobase (kb) insert of HHV-6 DNA (Fig. 1, A and B).

Further studies were performed on archival formalin-fixed and paraffin-embedded sections of biopsy tissue from seven oral mucosal tumors. The sections were incubated at a dilution of 1:50 with mouse monoclonal antibody (MAb) (GASG3) to the HHV-6 glycoprotein (gp) 116K/64K/54K component (15), which is a late protein in the viral replicative cycle. The tissue-bound GASG3 MAb was visualized by the immunoperoxidase reaction (16), and all seven (100%) of the biopsy specimens were seen to express HHV-6–associated antigen in the transformed cells (Fig. 2, A and B). The antigen was localized in the cytoplasm and sometimes also strongly localized in the membrane and nucleus of squamous cells, which had a typical epithelial morphology. The squamous cells also stained specifically to cytokeratin MAb (BioGenex Laboratories, San Ramon, Calif.), confirming their epithelial nature. It is interesting that paraffin-embedded sections from three biopsy specimens of nasopharyngeal carcinomas were negative with the GASG3 MAb, showing that the HHV-6 antigen was not expressed in the nasopharyngeal carcinoma tissues. Control tissue sections from normal oral mucosa were also negative for the GASG3 MAb. Moreover, the GASG3 MAb failed to react with oral carcinoma tissue sections following adsorption of the antibody with heat-inactivated HHV-6 particles, thus confirming its specificity.

PCR for HHV-6 was also conducted on DNA extracted from the paraffin-embedded sections that were positive for GASG3 MAb; of seven samples analyzed, five (71%) were positive. Thus, the immunohistochemical staining was more sensitive in the overall detection

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Correspondence to: Professor M. Yadav, Ph.D., Department of Genetics and Cellular Biology, University of Malaya, 59100 Kuala Lumpur, Malaysia.

See "Notes" section following "References."
Fig. 1. Detection by nested PCR of HHV-6 sequence in genomic DNA extracted from fresh oral carcinoma tissue. A) The PCR-amplified nested fragment (8 µL) was electrophoresed in 2% agarose gel and stained with ethidium bromide. Lanes 1 and 13: molecular weight markers, 100-base-pair (bp) ladder. Lanes 2-10: oral tumor DNA amplified by PCR. The 186-bp fragment is visible in lanes 2-5, 8, and 10. Lane 11: positive control DNA derived from plasmid pZVH-14 with an 8.9-kb HHV-6 insert. Lane 12: negative control. B) DNA from the gel was transferred to a Hybond (Amer sham International, Buckinghamshire, England), positively charged, nylon filter and then hybridized to a digoxigenin-labeled oligonucleotide HHV-6 probe. The 186-bp PCR products amplified by inner primers are noted in lanes 2-5, 8, and 10, and these amplified products and the positive control in lane 11 hybridized to the probe. In lanes 8, 10, and 11, hybridization signals can also be seen to the 435-bp PCR fragment that was amplified by the outer primers.

Fig. 2. Immunohistochemical detection of HHV-6-associated protein in formalin-fixed and paraffin-embedded sections of the buccal mucosal tumor. Following deparaffinization, the tissues were successively treated with MAb GA5G3, anti-mouse immunoglobulin labeled with biotin, streptavidin, hydrogen peroxide, and the substrate 3-amin-9-ethylcarbazole (counterstained with Mayer's hematoxylin, original magnification x1000). A) Section shows intense reactivity (reddish brown) of the membrane, cytoplasm, and nucleus with GA5G3 MAb to HHV-6 (gp 116K/64K/54K) protein. Nonreactive cells show only blue hematoxylin counterstain. B) Negative controls were achieved by omitting the primary antibody (GA5G3 MAb).
noted in precancerous lesions. These viruses probably act by a “hit-and-run” mechanism (10). HHV-6 has not as yet been suspected as a possible cofactor in the development of oral tumors, however. Recent observations of HHV-6 transactivation of human papillomavirus in cervical carcinoma cell lines (24) and HHV-6 transformation of simian virus 40- and adenovirus 7-immortalized cells (25,26) lend support to the hypothesis that HHV-6 in combination with other cofactors, e.g., carcinogens, could play a role in the oncogenesis of oral carcinoma.

References


Notes

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