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Frequent presence of incomplete HPV16 E7 ORFs in lung carcinomas: Memories of viral infection

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ABSTRACT

Background: HPV16 E6/E7 oncoproteins are critical for cervical carcinogenesis. The corresponding oncogenes are also detected in head and neck cancer, but in lung cancer their presence is strongly debated. PCR-based detection protocols amplify different target sequences.
Objectives: To examine the frequency of different length HPV16 E7 segments in lung carcinomas.
Study design: We designed four different amplification schemes for the detection of overlapping segments of the HPV16 E7 ORF, all suitable for specific HPV detection in cervical carcinoma. In two schemes, the entire E7 ORF was targeted while in the remaining schemes internal, smaller sequences were targeted. In total, 76 specimens were used; 29 lung carcinoma specimens, 16 non-cancerous lung tissue specimens from the same patients and 31 bronchial washings from different lung cancer patients.
Results: Amplification of the entire HPV16 E7 ORF, using two protocols, demonstrated the absence of the specific HPV16 E7 sequences (74 samples either tested negative by the first PCR protocol or false positive by the second, based on sequencing or AvaII or PvuII digestion). However, both schemes targeting smaller E7 segments revealed the frequent presence of HPV16 E7 sequences in lung carcinoma specimens (14/23 positive by either scheme).
Conclusions: HPV16 E7 sequences are frequently observed in lung carcinomas. Decreasing the size of PCR-target sequences increases the detection frequency, possibly indicating the presence of incomplete viral ORFs. Restriction endonuclease analysis is critical for verifying the reliability of the detection of these sequences.

1. Background

Intensive research over several decades has established the role of high risk HPVs in the pathogenesis of cervical cancer, as well as in other sites such as oral cavity, esophagus and bronchi.[1,2] Syrjanen et al. first reported morphological resemblance of papillomatous bronchial lesions with cervical cancer tissue, implying the possible role of HPV in lung carcinogenesis.[3] HPV exerts its activity at the squamous-columnar junction (uterine to cervical, anal to rectal and laryngeal to tracheal transition).[4] The respiratory epithelium of smokers, all along the bronchial tree, frequently presents multifocal metaplastic areas from which squamous bronchogenic carcinomas may arise.[5] Therefore, this locus represents an area for potential HPV infection.

To date there are several studies that correlate HPV infection and lung carcinogenesis.[6–12] Results of these studies are conflicting as the frequency of detection of HPV varies from 0% to 79%.[13] In addition to epidemiologic or environmental reasons, variability in these frequencies could depend on different HPV detection methods.[13]

In cervical carcinoma specimens, integrated HPVs can be detected easily using polymerase chain reaction (PCR) methods targeting the HPV16 E7 sequence. However, the epigenetic state of DNA differs in smokers and lung cancer patients compared to non-smokers and healthy individuals.[14] These differences, and the possibility that incomplete open reading frames (ORFs) of the corresponding oncogenes might only be incorporated, suggest that experimental conditions and target sequences used in PCR amplification protocols for HPV detection in lung cancer could vary from those used in cervix.

2. Objectives

In the present study we designed novel protocols for the detection of overlapping segments of decreasing size of the HPV16 E7 ORF. Both these novel and other common protocols were used for HPV16 E7 detection in lung carcinoma specimens, non-cancerous

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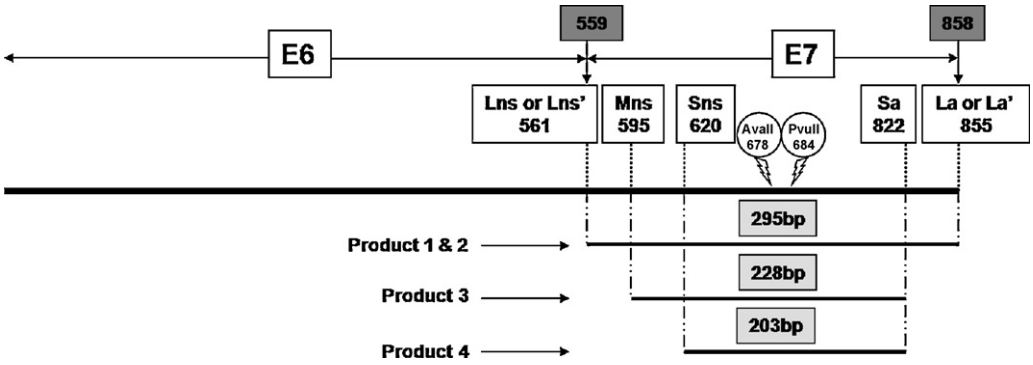

Fig. 1. Diagrammatic presentation of the HPV16 E7 detection schemes. The termini of E6 and E7 coding sequences are indicated in dark boxes. Dashes indicate the first nucleotides of forward primers Lns, Lns′, Mns and Sns and the last nucleotides for reverse primers Sa, La and La′. In circles the restriction sites of AvaII and PvuII endonucleases are shown. In light grey boxes the sizes of amplicons using the corresponding primers are indicated. The expected amplified sequences for schemes 1 or 2, 3 and 4 are 295, 228 and 295 bp, respectively.

lung tissue from the same patients and bronchial washings from different lung cancer patients.

3. Study design

3.1. Clinical samples

In this study, a total of 76 specimens were analyzed. The sample distribution was as follows: 29 tissue specimens of non-small cell lung cancer (16 adenocarcinomas, 10 squamous carcinomas and 3 large cell carcinomas), 16 corresponding macroscopically non-cancerous tissues from the same lung cancer patients (8 adenocarcinomas, 7 squamous carcinomas and 1 large cell carcinoma) and 31 bronchial washings from different lung cancer patients (7 adenocarcinomas and 23 squamous carcinomas). Ten HPV-positive cytological samplings from cervical carcinoma patients were used as controls for the presence of HPV16. Tissue samples were frozen in liquid nitrogen immediately after excision and stored at −70 °C until further processing. The bronchial washings were stored in PreservCyt®, CYTYC.

3.2. DNA preparation from tissue specimens

DNA isolation was performed using proteinase K treatment and phenol/chloroform extraction. Only high quality DNA specimens were used for the PCR reactions. Measures taken to avoid PCR contamination included a laminar flow hood equipped with a UV light for periodic UV irradiation, pre- and post-PCR areas in separate rooms and dedicated instrumentation and consumables (filtered tips).

3.3. PCR amplification

Two-step PCR protocols were used. The sequences of the oligonucleotide primers used and their location in the viral genome are shown in Table 1. The corresponding amplicon sizes are schematically represented in Fig. 1. The DNA, $MgCl_2$ and primer concentrations, as well as the two-step PCR amplification conditions for each amplification scheme (1–4) are analytically presented in Table 2. Products were analyzed by agarose gel electrophoresis (2% or 3% NuSieve).

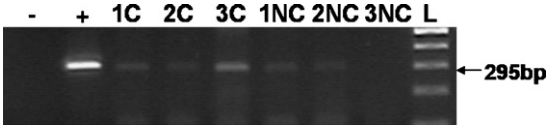

Fig. 2. Amplification of HPV16 E7 DNA from lung carcinoma specimens and macroscopically non-cancerous tissues from the same patients according to amplification scheme 2 (295 bp). The amplified products correspond to a sequence from human Chromosome 1. C, cancerous specimens; NC, non-cancerous specimens; +, cervical carcinoma specimen as positive control; −, negative control; L, 100 bp DNA ladder.

3.4. Restriction enzyme analysis

PCR products were analyzed by PvuII and AvaII restriction endonuclease digestion. The corresponding digestion sites are shown in Fig. 1. In each case, 10 μl of PCR product were digested either with PvuII (2 U) or AvaII (3 U) at 37 °C for 1 h, and analyzed by agarose gel electrophoresis as described above.

3.5. Sequencing of the PCR products

To determine the nucleotide sequence of selected PCR amplicons, the products were gel extracted and purified using the QIAquick gel extraction kit (Qiagen) and sequenced (Lark Technologies).

4. Results

4.1. Amplification of the complete reading frame in lung carcinoma specimens and non-cancerous tissue from the same patients using different amplification schemes

The E7 ORF was first amplified using primers La and Lns/La which have been previously employed in various studies[15,16] (Fig. 1 and Table 1). This amplification scheme (scheme 1) provided positive results for neither the lung carcinoma specimens (0/16) nor the non-cancerous lung tissues from the same patients (0/16) (Table 3A), but demonstrated positive results in all HPV16-positive cervical cytological samples tested. Amplification was optimized for $MgCl_2$ and primers' (35, 50, 65 and 80 pmol/reaction) concentration and for annealing temperatures for both PCR steps: 50/58,

Table 1
Primer sequences used in PCR of HPV16 E7 amplification schemes 1–4.

Primer	Oligonucleotide sequence	Base position
Lns	CATGCATGGAGATACACCTACA	561
Lns′	GCGAATTCCATGCATGGAGATACACCTACA	561
Mns	ATGTTAGATTTGCAACCAGAGAC	595
Sns	CTGATCTCTACTGTTATGAGC	620
Sa	TAGTGTGCCCATTAACAGGTC	822
La	TGGTTTCTGAGAACAGATG	855
La′	GTCTCGAGTGGTTTCTGAGAACAGATG	855

For forward primers Lns, Lns′, Mns, Sns and for reverse primers Sa, La, La′ the first and the last nucleotides are indicated, respectively.