Biological evidence for a causal role of HPV16 in a small fraction of laryngeal squamous cell carcinoma

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Background: Human papillomavirus (HPV) is a causal factor in virtually all cervical and a subset of oropharyngeal squamous cell carcinoma (OP-SCC), whereas its role in laryngeal squamous cell carcinoma (L-SCC) is unclear.

Methods: Formalin-fixed paraffin-embedded (N = 154) and deep-frozen tissues (N = 55) of 102 L-SCC patients were analysed for the presence of 51 mucosal HPV types. HPV DNA-positive (HPV DNA+) cases were analysed for E6*I mRNA transcripts of all high risk (HR)/probably/possibly (p)HR-HPV identified, and for HPV type 16 (HPV16) viral load. Expression of p16INK4a, pRb, cyclin D1 and p53 was analysed by immunohistochemistry.

Results: Ninety-two patients were valid in DNA analysis, of which 32 (35%) had at least one HPV DNA+ sample. Among the 29 single infections, 22 (76%) were HPV16, 2 (7%) HPV56 and 1 each (4%) HPV45, HPV53, HPV70, HPV11 and HPV42. Three cases harboured HPV16 with HPV33 (twice) or HPV45. Only 32% of HPV DNA+ findings were reproducible. Among HPV16 DNA+ L-SCC, 2 out of 23 (9%) had high viral loads, 5 out of 25 (21%) expressed E6*I mRNA and 3 out of 21 (14%) showed high p16INK4a and low pRb expression (all three HPV16 RNA-positive), immunohistochemical marker combination not identified in any other HPV DNA+ or HPV DNA-negative (HPV DNA−) L-SCC, respectively.

Conclusion: HPV type 16 has a causative role in a small subgroup of L-SCC (<5% in this German hospital series).
ranging from 3 to 60% have been reported (Perez-Ayala et al, 1990; Almadori et al, 1996; Gungor et al, 2007; Morshed et al, 2008); however, consistent biological evidence for viral involvement in L-SCC is still lacking. Varying HPV DNA prevalence reported across different epidemiological studies can be attributed to geographical differences, inadequate separation of laryngeal carcinoma cases from other cancers of the HHN-region, for example, the OP-SCC, differences in analytical sensitivity of HPV-genotyping methods applied and limited spectrum of HPV types analysed. Currently, of 51 mucosal HPV types known today for CaCa (Bernard et al, 2010), 12 are classified as carcinogenic or high-risk (HR)-HPV (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), 8 as probably/possibly carcinogenic or (p)HR-HPV (HPV26, 53, 66, 67, 68, 70, 73 and 82) and 31 as types of low/undetermined carcinogenicity or LR-HPV (IARC, 2011). Most of the studies analysed L-SCC samples for the presence of only the five HR-HPV types 16, 18, 45, 31 and 33 that are most frequent in CaCa (reviews (Kreimer et al, 2005; Torrance et al, 2011) and references therein), with HPV16 predominating. Few case reports identified pHr-HPV73 and 82 DNA in L-SCC by direct sequencing technology (Pannone et al, 2010; Si-Mohamed et al, 2012). The LR-HPV6 and 11 have been reported more frequently especially in association with recurrent respiratory papillomatosis (Syrijan et al, 1987; Salam et al, 1995).

Human papillomavirus DNA alone is not sufficient to identify HPV-driven cancers as has become evident for OP-SCC (Jung et al, 2010; Holzinger et al, 2012). High-risk-HPV DNA has been found in benign and normal tissue of the larynx (Garzia-Milian et al, 1998; Duray et al, 2011), supporting the idea that HPV DNA presence alone cannot demonstrate causality. The concept for causal involvement of mucosal HPV in the pathogenesis of SCC includes: (a) presence of at least one viral genome copy per tumour cell, (b) active transcription of the viral oncogenes E6 and E7 and (c) interaction of the viral oncoproteins with central cellular surrogate markers for HPV transformation. Applying this biological evidence for causal involvement of HPV16 in a small fraction of L-SCC, and suggest a combination of markers to define HPV-driven L-SCC.

**MATERIALS AND METHODS**

**Patients and tissue samples.** This study was approved by the ethics committee of the Medical Faculty of the University of Heidelberg, study code 176/2002. A total of 102 L-SCC patients were enrolled. Patients were diagnosed with invasive L-SCC and treated at the ENT department of the University Hospital Heidelberg, Germany between 1990 and 2006. Patient's age ranged from 39 to 82, with a median of 59 years. An initial study inclusion criterion was the availability of at least one FFPE biopsy of a primary L-SCC tumour (P-TU). For 11 patients P-TU was not available, therefore tumour recurrence (TU-Rec) or L-SCC as a secondary carcinoma (2nd Ca) were included. For 34 patients multiple FFPE biopsies (range 2–11; mean 4, median 3 FFPE biopsies per patient) were analysed. Owing to potential technical challenge imposed by formalin fixation in FFPE biopsies, we also analysed concordant DFT P-TU biopsies available for 55 patients (one DFT biopsy per patient). In total, 209 (154 FFPE and 55 DFT) biopsies were analysed for 102 patients; 91 patients had P-TU, 9 TU-Rec and 2 2nd Ca L-SCC. Per biopsy, tumour histology was independently verified by two study pathologists (CF and BL). Per patient, clinical data were collected from clinical charts and pathological reports.

**Tissue sectioning, DNA and RNA extraction.** To probe the reproducibility of HPV DNA findings, each of 154 FFPE biopsies was sectioned twice and 308 DNA extracts were prepared. Deep-frozen tissue biopsies were sectioned once resulting in 55 DNA extracts. In total, 363 DNA extracts (154 × 2 FFPE and 55 DFT) were analysed for 102 patients.

To prevent cross-contamination during sectioning, each biopsy (FFPE or DFT) was sectioned with a new blade, the sectioning area was cleaned with 70% ethanol and acetone, respectively, and gloves were changed accordingly. Each experimental step included necessary controls (Supplementary Information).

For nucleic-acid extractions, FFPE and DFT tissue ribbons were obtained according to the sandwich method, that is, the first and the last 4-μm-sections were stained by haematoxylin and eosin (H&E) stain to verify the presence of tumour cells. The number of ribbons required for nucleic-acid extraction depended on the biopsy size. For FFPE, 2–8 × 5 μm, and for DFT 2–8 × 16 μm tissue ribbons were cut, and genomic DNA was prepared as described (Halac et al, 2012; Holzinger et al, 2012). All DNA extracts were stored at −20 °C until use. Each FFPE and/or DFT biopsy that yielded HPV DNA + result was sectioned again to obtain tissue ribbons for RNA extraction. Total RNA was extracted as described (Halac et al, 2012; Holzinger et al, 2012). RNA samples were stored at −80 °C until use.

**Human papillomavirus genotyping and measurement of HPV16 viral load.** For genotyping 10 μl, and for HPV16 quantitative real-time PCR (qPCR) 1 μl of DNA were used.

For HPV genotyping, broad-spectrum GP5+/6+/PCR/multiplex HPV genotyping assay (BSGP5+/6+/PCR/MPG) combing the BSGP5+/6+/PCR, which homogeneously amplifies all 51 genital HPV types generating biotinylated amplimers of ~150 bp from the L1 region and 208 bp amplicon of β-globin (Schmitt et al, 2008, 2013), and a Multiplex HPV Genotyping assay with bead-based xMAP Luminex suspension array technology (Qiagen, Hilden, Germany) (Schmitt et al, 2006, 2008) was used. An L-SCC case (including all biopsies from one patient) was considered valid if the β-globin and/or HPV DNA + result was identified at least once. Each HPV DNA invalid (HPV and β-globin DNA − ) result was verified by retesting.

The qPCR assay targeting HPV16-specific sequences of the E6 gene was developed to calculate HPV16 viral genome copy-number per cell. Primer sequences used for qPCR are available upon request (Schmitt et al, manuscript in preparation). Quantitative real-time PCR for β-globin was used to verify the DNA quality and to measure the amount of input DNA. As on average, each tumour specimen contained ~50% of tumour cells, and in HPV-transformed cells at least one genome copy per cell is expected, 0.5 copies of HPV16 genome per cell were set as a cut-off to define samples with high (HPV16high, ≥0.5 copies per cell) or low (HPV16low, <0.5 copies per cell) viral load (Holzinger et al, 2012).
Biological evidence for HPV16-driven laryngeal cancers

Table 1. Cut-offs and criteria for evaluation of p16 INK4a, pRb, cyclin D1 and p53 protein expression

| Antibody reference | Clone Company | Intensity | Localisation | Pattern of protein expression | Percentage cell positive | Protein expression in NOM |
|--------------------|--------------|-----------|--------------|-------------------------------|--------------------------|--------------------------|
| p16 INK4a          | E6H4/MTM     | ≥2        | Diffuse/focal| Low                           | 25%                      | Diffuse or continuous    |
|                    |              |           |              |                               |                          |                          |
| pRb                | 1F8 Novocastra | ≥2        | Diffuse/focal| Low                           | 25%                      | Diffuse or continuous    |
|                    |              |           |              |                               |                          |                          |
| Cyclin D1 (Cyd1)   | DCS-6 DAKO   | ≥2        | Diffuse/focal| Low                           | 25%                      | Diffuse or continuous    |
|                    |              |           |              |                               |                          |                          |
| p53                | DO.7 DAKO    | ≥2        | Diffuse/focal| Low                           | 25%                      | Diffuse or continuous    |
|                    |              |           |              |                               |                          |                          |

Diffuse or continuous—protein expression pattern observed over the whole tumour, and focal is protein expression ... in one part of the tumour. Nuclear pRb and CyD1 were abundant in NOM and found in up to 25% of proliferating cells in the basal and parabasal layers with intensity 2 or 3. Staining of NOM for p16 INK4a and p53 was observed in up to 5% of proliferating cells and with low intensity 1 or 2.

Immunohistochemical analysis on TMA. A tissue microarray (TMA) was constructed carrying the tissue cores obtained from FFPE biopsies of all 102 patients, and cores with normal oral mucosa (NOM) of a cancer-free patient as a staining control. Each L-SCC core was representative of the invasive tumour as assessed by H&E analysis from single sections. Tissue microarray slides were stained manually as described (Halec et al, 2012; Holzinger et al, 2013) and on DAKO autostainer (Glostrup, Denmark). Protein expressions were evaluated separately by four investigators (FXB, DHol, BL and GH). Evaluation involved semi-quantitative scoring of the staining intensity (0 = no expression, 1 or 2 = low intensity and 3 = high intensity) and the percentage of stained tumour cells, as described (Halec et al, 2012; Holzinger et al, 2013).

For the simplicity of evaluation, only two protein expression categories were applied, low and high, with cut-off guided by protein expression in NOM. For p16<sub>INK4a</sub><sup>high</sup>, pRb and CyD1, positivity in >25% of the tumour cells with intensity ≥2 was required to define high protein expression (Table 1). Evaluation of staining for p53 in association with HPV involvement was more complex. In HPV-driven carcinomas, p53 expression is initially upregulated by HPV E7 actions (Massimi and Banks, 1997), but HPV E6 counteracts this upregulation by targeting p53 for ubiquitin-mediated degradation (Scheffner et al, 1990). However, wild-type p53 protein level can increase to immunohistochemical (IHC) detectable levels during, for example, cellular damage caused by HPV infection (Kastan et al, 1991; Gottlieb and Oren, 1996; Skyldberg et al, 2001). Therefore, we defined p53 expression as p53<sub>high</sub> when p53 positivity was found in >50% of tumour cells with intensity = 2, or in >25 of tumour cells with intensity = 3.

Definition of HPV DNA +, HPV16<sub>high</sub>HPV16<sub>low</sub> and HPV-driven L-SCC. An L-SCC case (including all biopsies from one patient) was considered HPV DNA + if at least one DNA extract yielded HPV DNA + result.

To define HPV16 viral load per patient, DNA extract prepared from the first FFPE section, DNA extract prepared from the second, consecutive FFPE section of the same biopsy and DNA extract prepared from the DFT section results were combined. An L-SCC case was considered of high viral load (HPV16<sub>high</sub>) if quantitative results were above cut-off (≥0.5 copies per cell) in ≥50% of findings.

Finally, based on CxCa and HPV-driven OP-SCC data (Halec et al, 2012; Holzinger et al, 2012) and data in this study, we defined combination of HPV DNA +, E6<sub>1</sub> mRNA + and high p16<sub>low</sub>/pRb<sub>low</sub> protein pattern in tumour cells as a critical parameter to define HPV-driven L-SCC. Detailed overview of HPV DNA, E6<sub>1</sub> mRNA and IHC results per biopsy and per patient is provided in Supplementary Table S1.

RESULTS

Human papilloma virus DNA prevalence in L-SCC tissues. In this hospital series, 102 patients were included with their clinical characteristics, as described (Table 2). Altogether, 363 across the E6<sub>1</sub> splice site, as described (Halec et al, 2012). The biotinylated strands of the amplimers were detected by hybridisation with type- and splice site-specific oligonucleotide probes coupled to fluorescence-labelled Luminex beads (Luminex Corp., Austin, TX, USA). The E6<sub>1</sub>* mRNA assays are available for 20 HR-HPV types for which existence of splice sites was demonstrated ((Halec et al, 2012) and references therein), and applicable on both DFT and FFPE biopsies (Halec et al, 2012; Hoffmann et al, 2012). For detection of LR-HPV11 mRNA, the assay with primers designed to amplify 77 bp amplicon of the full-length HPV11 E6 gene, and an oligonucleotide probe for HPV11 E6 full-length detection, was applied.
Table 2. Clinical characteristics of patients stratified by HPV status

| Characteristics          | All (N = 102) | DNA valid (N = 92) | HPV (N = 60) | non-HPV driven (N = 28) | HPV driven (N = 3) |
|--------------------------|---------------|-------------------|--------------|------------------------|-------------------|
|                          | N (%)         | N (%)             | N (%)        | N (%)                  | N (%)             |
| **Gender**               |               |                   |              |                        |                   |
| Male                     | 91 (89)       | 81 (88)           | 55 (93)      | 24 (86)                | 2 (67)            |
| Female                   | 11 (11)       | 11 (12)           | 4 (7)        | 4 (14)                 | 1 (33)            |
| **Age (years)**          |               |                   |              |                        |                   |
| Median                   | 59.0          | 59.0              | 59.0         | 57.0                   | 49.0              |
| **Localisation**         |               |                   |              |                        |                   |
| Supraglottic             | 35 (34)       | 30 (33)           | 19 (32)      | 9 (32)                 | 2 (67)            |
| Glottic                  | 65 (64)       | 60 (65)           | 39 (65)      | 19 (68)                | 1 (33)            |
| Subglottic               | 2 (2)         | 2 (2)             | 2 (3)        | 0 (0)                  | 0 (0)             |
| **Tumour status**        |               |                   |              |                        |                   |
| Primary                  | 91 (89)       | 81 (88)           | 52 (88)      | 23 (82)                | 2 (67)            |
| Recurrence               | 9 (9)         | 9 (10)            | 5 (8)        | 4 (14)                 | 1 (33)            |
| > 2nd Ca                 | 2 (2)         | 2 (2)             | 2 (3)        | 1 (4)                  | 0 (0)             |
| **Tumour size**          |               |                   |              |                        |                   |
| T1–T2                    | 38 (42)       | 35 (44)           | 22 (40)      | 13 (52)                | 1 (50)            |
| T3–T4                    | 52 (58)       | 45 (56)           | 33 (60)      | 12 (48)                | 1 (50)            |
| No data                  | 12            | 12                | 5            | 3                      | 1                 |
| **Lymph nodes**          |               |                   |              |                        |                   |
| N0                       | 64 (73)       | 59 (75)           | 46 (82)      | 20 (80)                | 2 (100)           |
| N1 +                     | 24 (27)       | 20 (25)           | 10 (18)      | 5 (20)                 | 0 (0)             |
| No data                  | 14            | 13                | 3            | 3                      | 1                 |
| **Distant metastases**   |               |                   |              |                        |                   |
| M0                       | 85 (99)       | 77 (99)           | 55 (100)     | 24 (96)                | 2 (100)           |
| M1                       | 1 (1)         | 1 (1)             | 0 (0)        | 1 (3)                  | 0 (0)             |
| No data                  | 12            | 14                | 4            | 3                      | 1                 |
| **Histopathological pattern** |         |                   |              |                        |                   |
| Keratinising             | 98 (96)       | 88 (96)           | 59 (98)      | 26 (93)                | 2 (67)            |
| Non-keratinising         | 4 (4)         | 4 (4)             | 1 (2)        | 2 (7)                  | 1 (33)            |
| **Clinical stage**       |               |                   |              |                        |                   |
| I–III                    | 43 (53)       | 38 (53)           | 15 (29)      | 14 (56)                | 1 (50)            |
| IV                       | 38 (47)       | 34 (47)           | 36 (71)      | 11 (44)                | 1 (50)            |
| No data                  | 21            | 20                | 8            | 3                      | 1                 |
| **Follow-up event**      |               |                   |              |                        |                   |
| Rec                      | 16 (16)       | 15 (16)           | 6 (10)       | 9 (32)                 | 0 (0)             |
| 2nd Ca                   | 10 (10)       | 9 (10)            | 7 (12)       | 1 (4)                  | 1 (33)            |
| Met                      | 17 (16)       | 14 (15)           | 12 (20)      | 2 (7)                  | 0 (0)             |
| No event (censored)      | 59 (58)       | 54 (59)           | 35 (58)      | 16 (57)                | 2 (67)            |
| **Alcohol use**          |               |                   |              |                        |                   |
| Yes                      | 65 (79)       | 57 (78)           | 37 (77)      | 18 (82)                | 1 (50)            |
| No                       | 9 (11)        | 9 (12)            | 6 (12)       | 2 (9)                  | 1 (50)            |
| Former                   | 8 (10)        | 7 (10)            | 5 (10)       | 2 (9)                  | 0 (0)             |
| No data                  | 20            | 19                | 11           | 6                      | 1                 |
| **Tobacco use**          |               |                   |              |                        |                   |
| Yes                      | 76 (85)       | 68 (85)           | 45 (83)      | 24 (92)                | 2 (100)           |
| No                       | 5 (6)         | 5 (6)             | 5 (9)        | 0 (0)                  | 0 (0)             |
| Former                   | 8 (9)         | 7 (8)             | 4 (7)        | 2 (8)                  | 0 (0)             |
| No data                  | 13            | 12                | 5            | 2                      | 1                 |
Table 2. (Continued)

| Characteristics | All (N=102) | DNA valid (N=92)* | HPV (N=60) | non-HPV driven (N=28)** | HPV driven (N=3)^* |
|-----------------|------------|----------------|------------|-------------------------|----------------|
| N (%)           | N (%)      | N (%)          | N (%)      | N (%)                   | N (%)          |
| FFPE            | 154 (74)   | 144 (72)       | 118 (73)   | 44 (27)                 | 22 (71)        |
| DFT             | 55 (26)    | 55 (28)        | 44 (27)    | 9 (29)                  | 3 (60)         |

Abbreviations: DFT = deep-frozen tissue; FFPE = formalin-fixed paraffin-embedded.

*aNumber of patients with valid biopsies, that is, β-globin and/or HPV DNA-positive.

**Number of patients with non-HPV-driven L-SCC (N=28). One patient (HPV16, 45 DNA +) was also HPV16 RNA +; however, expression of proteins could not be assessed owing to poor tissue quality for IHC analysis. Therefore that patient could not be grouped into b or c.

^The three HPV-driven L-SCC patients with HPV DNA +/RNA +/p16high/pRblow pattern.

DNA extracts (154 × 2 FFPE and 55 DFT) prepared from 209 tumour tissue samples (154 FFPE and 55 DFT) were analysed for the presence of all currently defined 51 mucosal HPV types.

At least one valid DNA sample (HPV and/or β-globin DNA +) was obtained from 80 of the 102 patients (78%) with FFPE tissues and from all of the 55 patients with additionally DFT biopsy available, including 12 patients with no valid FFPE-derived sample (Figure 1). Ten patients with FFPE biopsies only, were DNA invalid and excluded from the analysis. Of the 92 L-SCC patients with at least one valid DNA sample, 32 (35%) were HPV DNA +, with a single HPV type found in 29 (91%) and multiple types found in 3 (9%) cases (Table 3). Human papilloma virus DNA positivity in present L-SCC series did not significantly change over the sampling period, with 30% in the first half of cases collected until 1997 and 24% in the second half of cases collected thereafter.

HPV type 16 was the most prevalent type found in 22 out of 29 (76%) single, and in all 3 multiple-type positive cases. High-risk-/pHR-HPV types 45, 53 and 70 were identified as single sequences in one case each and HPV56 in two cases. Two L-SCC cases harboured LR-HPV type 11 and 42, respectively. The three cases with multiple HPV types harboured HPV16 together with HPV33 (two cases) and HPV45 (one case).

Of 23 patients with at least one HPV DNA + FFPE-derived DNA sample, and a valid, second FFPE-derived DNA sample, the same HPV type was found in only 6 (26%) of the second DNA samples. Among 16 valid DNA sample pairs derived from FFPE/DFT tissue pairs with at least one HPV DNA + sample, only 5 (31%) had concordantly positive DNA samples. In total, for only 10 (32%) of 31 patients with at least one HPV DNA + sample, the DNA findings could be reproduced in a second DNA sample (Table 3, Supplementary Table S1).

In viral load analysis, 13 of the 25 HPV16 DNA + samples were HPV16 DNA −, 8 had viral loads <0.5 copies per cell, 2 had viral loads >0.5 copies per cell and for 2 HPV16 viral loads could not be calculated (non-quantifiable) because of β-globin negativity.

Thus, of the 23 HPV16 DNA + tumours analysable by qPCR, 2 (9%) were defined as HPV16high and 21 (91%) as HPV16low (Table 3, Figure 1).

Interestingly, both HPV16high L-SCC cases had reproducible HPV DNA + findings from the consecutive FFPE section and/or DFT biopsies (Table 3). However, only 5 out of 21 (24%)
Table 3. Molecular characteristics of HPV DNA + L-SCC patients

| Patient number | Tissue biopsy | HPV DNA copies/cell | Final HPV DNA and VL | HPV RNA | p16<sub>high</sub> | pRb<sub>low</sub> | CyD1<sub>low</sub> | p53<sub>low</sub> | HPV-driven L-SCC |
|---------------|--------------|---------------------|----------------------|---------|-------------------|----------------|----------------|----------------|----------------|
| 1             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 6769 Invalid 227 | 16 H | 16 | + | + | + | + |
| 2             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 15 18 | 16 H | 16 | + | + | - | + |
| 3             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | Invalid Invalid Invalid <0.001 | 16 L | 16 | + | + | + | + |
| 4             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 0.07 0.004 | 16 L | - | - | + | - | - |
| 5             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | Invalid 16 16 0 16 | 16 L | - | - | - | + | + |
| 6             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid Invalid 0 | 16 L | - | NA | NA | NA | NA |
| 7             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid Invalid 0 | 16 L | - | - | + | - | - |
| 8             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | + | + |
| 9             | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | + | - |
| 10            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | - | - |
| 11            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid Invalid 0 | 16 L | - | - | - | + | - |
| 12            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid <0.001 | 16 L | - | - | + | - | - |
| 13            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L 16 | - | - | - | - | - |
| 14            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | + | + | + |
| 15            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid <0.001 | 16 L | - | - | + | - | + |
| 16            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid Invalid 16 L | - | - | - | + | + |
| 17            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | + | - |
| 18            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | + | - |
| 19            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 <0.001 0 | 16 L | - | - | - | + | + |
| 20            | FFPE<sub>1</sub>, FFPE<sub>2</sub>, DFT | 16 Invalid 0 | 16 L | - | - | - | - | - |
HPV16\textsubscript{low} L-SCC cases had reproducible HPV DNA\textsuperscript{+} results supporting the HPV16\textsubscript{low} findings.

HPV E6\textsuperscript{I} mRNA expression in HPV DNA\textsuperscript{+} L-SCC. E6\textsuperscript{I} mRNA was found in 6 out of 30 (20\%) RNA analysable cases; 5 of them were found harbouring single HPV16 and one HPV70 (Table 3). Among the five HPV16 RNA\textsuperscript{+} cases, viral load was high in two (40\%) cases (18 and 6800 genome copies per cell, respectively) and low in three (60\%) other cases (with 0.20, 0.0001 and non-quantifiable copies per cell, respectively, Table 3). Among all 18 RNA\textsuperscript{+/C0} cases analysable by HPV16 qPCR, viral load was low including 9 (45\%) non-quantifiable HPV16 DNA\textsuperscript{+} samples. This difference was statistically significantly ($\chi^2$-test, $P = 0.005$).

P16\textsubscript{INK4a}, pRb, CyD1 and p53 protein expression in L-SCC. Expression levels of p16\textsubscript{INK4a} and pRb proteins could be analysed for 75, and CyD1 and p53 for 65 and 69 patients, respectively. High expression of p16\textsubscript{INK4a} (p16\textsubscript{high}) was found in 4 out of 75 (5\%) cases, 3 of them were HPV16 DNA\textsuperscript{+} and RNA\textsuperscript{+} and the one remaining case was HPV11 DNA\textsuperscript{+} but RNA\textsuperscript{-}. Thus, three of the five RNA\textsuperscript{+} cases (60\%) analyzable by IHC were p16\textsubscript{high}, and this was statistically significantly different from HPV RNA\textsuperscript{-} and HPV DNA\textsuperscript{-} groups, where only one out of 21 (5\%, $\chi^2$-test, $P = 0.002$) and none of 47 ($\chi^2$-test, $P < 0.0001$) showed p16\textsubscript{high}. The specificity of p16\textsubscript{high} for HPV RNA\textsuperscript{+} group was even higher for HPV16 RNA\textsuperscript{+} cases only, that is, when HPV70 RNA\textsuperscript{+} case was excluded (75\% vs 60\%, Table 4).

Low pRb expression (pRb\textsubscript{low}) was found in 33 out of 75 (44\%) cases. Of the 5 HPV RNA\textsuperscript{+}, IHC analyzable cases, 3 (60\%) were pRb\textsubscript{low}, compared with 8 out of 21 (38\%) HPV RNA\textsuperscript{-} and 22 out of 47 (47\%) HPV DNA\textsuperscript{-} cases with pRb\textsubscript{low}. However, this difference was not statistically significant ($\chi^2$-test, $P = 0.373$).

Low CyD1 expression (CyD1\textsubscript{low}) was found in 33 out of 65 (51\%) cases. Of the 5 RNA\textsuperscript{+} L-SCC, 2 (40\%) were CyD1\textsubscript{low}.
The biological role of HPV in the pathogenesis of L-SCC as compared with OP-SCC has not been sufficiently defined. Many studies in CxCa and its precursors have established individual markers of HPV transformation (Klaes et al., 2001; Tsuda et al., 2003; Sotlar et al., 2004; Schmitt et al., 2010), and recently marker combinations have been comprehensively analysed in CxCa as a basis for stringent definition of HPV-driven mucosal cancers (Halec et al., 2012). Such marker combinations have also been observed among OP-SCC to discriminate HPV-driven from non-HPV-driven tumours, whereas L-SCC (as well as OP-SCC) are HPV-driven in most cases. The marker combination p16high/pRblow, indicative of viral transformation, was found in one of the 47 HPV DNA+ cases. The differences in clinicopathological features among the 3 HPV-driven (one HPV16 DNA+/RNA+ and one HPV16-driven; Table 2) and 28 non-HPV-driven or 60 HPV DNA − cases could not be statistically assessed owing to the small sample numbers in HPV-driven group. Interestingly, the median age of the three HPV-driven patients was 8 years lower than that of those in the non-HPV-driven group, and 10 years lower than in the HPV DNA − group.

**DISCUSSION**

The biological role of HPV in the pathogenesis of L-SCC as compared with OP-SCC has not been sufficiently defined. Many studies in CxCa and its precursors have established individual markers of HPV transformation (Klaes et al., 2001; Tsuda et al., 2003; Sotlar et al., 2004; Schmitt et al., 2010), and recently marker combinations have been comprehensively analysed in CxCa as a basis for stringent definition of HPV-driven mucosal cancers (Halec et al., 2012). Such marker combinations have also been useful in OP-SCC to discriminate HPV-driven from non-HPV-driven tumours with or without HPV DNA (Smeets et al., 2007; Jung et al., 2010; Holzinger et al., 2012; Liang et al., 2012).

We thoroughly analysed both FFPE and fresh, frozen tumour biopsies of 102 L-SCC patients for HPV presence and markers of biological activity. Using a stringent definition, we considered
L-SCC cases as HPV-driven only when a marker combination with HPV DNA+/RNA+/p16high/pRblow was identified. Among the 73 cases with valid DNA, IHC and RNA (HPV DNA+/only) data, we identified only 3 (4%) fulfilling this definition, all 3 harbouring HPV16 as a single infection.

We did not consider the presence of viral transcripts per se as a sufficient marker for HPV transformation. In the cervix, HPV16 E6*I transcripts are also abundantly expressed in infection without transformation (Schmitt et al., 2010); therefore, it is a marker of biological activity but is not transformation specific. Further, HPV16 DNA+ oropharyngeal SCC with HPV16 E6*I transcription have been identified that, however, did not display cellular transformation markers and also did not show the better survival typically associated with HPV-driven OP-SCC (Holzinger et al., 2012). Upregulation of p16INK4a and downregulation of pRb are well established and frequent cellular consequences of HPV transformation, and were added here for a stringent definition of HPV-driven tumours.

In this series, there was no case positive for two functional markers, and 12 cases were positive for one functional marker only (nine DNA+/pRblow, two DNA+/RNA+ and one DNA+/p16high). These data indicate a robust separation of truly HPV-driven L-SCC vs HPV DNA+ cases in which the additional markers did not support the classification as HPV-driven tumours.

Among the 29 cases lacking either valid DNA, RNA or IHC data, 2 were p16high/pRblow (DNA invalid, Supplementary Table 1) and 1 was HPV16 RNA+ (IHC invalid), representing at maximum three more candidates for HPV-driven L-SCC cases in this series.

Reproducibility of HPV DNA+ and HPV16 viral load supported our stringent definition of HPV-driven L-SCC. Among cases with multiple valid tissue sections, only 29% of non-HPV-driven but two of the two HPV-driven cases had reproducible HPV DNA+ findings. Also, all non-HPV-driven HPV16 DNA+ tumours showed viral loads far below 0.5 genome copies per cell, whereas two of the three HPV-driven L-SCC showed high viral loads with 6800 and 18 HPV16 genome copies per cell. The low viral load in the third HPV-driven case remains unexplained.

Taken together, of the 102 L-SCC cases that at least had valid DNA or IHC data, a minimum of 3 (3%) with potentially a maximum of 6 (6%) appear to be HPV driven. This small fraction of HPV-driven tumours in L-SCC is in line with three previous studies that demonstrated HPV16 DNA+/RNA+/p16high in 2 of 27 (7%; Schlecht et al., 2011), 1 of 30 (3%) (Lewis et al., 2012) and in
3 of 60 (5%) L-SCC cases (Chernock et al, 2012). These data demonstrate that HPV16-driven L-SCC do exist but contribute only little to the overall L-SCC burden.

Ascertainment of the HPV-driven tumours as laryngeal and exclusion of any potentially oropharyngeal origin is crucial. The three HPV-driven tumours were clinically classified as primary laryngeal tumours without clinical evidence of OP extension. They were surgically removed in toto allowing pathological analysis of the tumour borders. One of the HPV-driven supraglottic tumours had cartilage, and the second supraglottic tumour had respiratory epithelium evident on H&E slides. The third HPV-driven tumour was a big glottic tumour extending also into supraglottis and subglottis, but importantly, again with no border extending into hypopharynx or base of the tongue.

The overall HPV DNA prevalence in our study was 35%, with HPV16 being the predominant genotype. This is comparable with previous studies from Central Europe where overall HPV DNA prevalence in L-SCC collected mainly between 1990 and 2006 varied between 20 and 35% (Salam et al, 1995; Gorgoulis et al, 1999; Morshed et al, 2008). Although HPV DNA positivity in L-SCC tissue samples is substantial, our data demonstrate that in most (around 90%) of these cases HPV is not the driving force. This separates L-SCC from OP-SCC in Central Europe where HPV prevalences of 15–50% were reported (Smeets et al, 2007; Smith et al, 2008; Jung et al, 2010; Holzinger et al, 2012), and HPV has an aetiologial role in ~45% of HPV DNA+ OP-SCC tumours (Smeets et al, 2007; Jung et al, 2010; Holzinger et al, 2012).

Human papilloma virus DNA positivity in non-HPV-driven tumour biopsies could at least theoretically originate from the cross-contamination in routine pathological tissue processing or from laboratory contaminations. The similar HPV DNA prevalence in DFT-derived samples that had not gone through routine pathological tissue processing, when compared with FFPE tissues, and utmost care we applied to avoid and to detect any laboratory contaminations, appear to argue against such contamination hypothesis. Human papilloma virus DNA positivity could also result from non-transforming infection in tumour or neighbouring tissue, or may represent depositions of material from oral HPV infection at other sites.

The relative fraction of HPV DNA+ OP-SCC or tonsillar cancers appears to have increased in the last decades in the United States and Europe (D’Souza and Dempsey, 2011). In our L-SCC case series, HPV DNA positivity did not significantly change with tumour collection time, and all three HPV-driven and the three non-HPV-driven L-SCC were among the first half of tumours to be collected.

The BSGFP5+/I6+/−PCR/MPG assay identifies all currently defined 51 mucosal HPV types, which allowed us to address the question whether presence of other HPV types in L-SCC may have contributed to the lower fraction of RNA- derived samples could be: (a) longer BSGFP5+/I6+/−PCR/MPG amplicon size (150 bp for HPV and 208 bp for β-globin DNA vs 65–75 bp for HPV and 81 bp for ubiquitin C cDNA) and (b) higher copy-numbers per cell of the HPV and ubiquitin C mRNA.

By using a combination of four viral and surrogate markers, our study provides a robust biological evidence for the existence of truly HPV-driven L-SCC. Despite the relatively large case series analysed here, the low frequency of these HPV-driven tumours did not allow to address the question whether like in OP-SCC also in L-SCC HPV-driven tumours differ significantly in biological and clinical characteristics, especially better response to treatment and better survival. These questions can only be addressed in considerably larger, multicentric, collaborative studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GH, MP, FXB planned the study design and performed data collection and data interpretation. GH, DHol, MS, DH, BL, CF, GD conceived and carried out experiments, collected the data and analysed the data. GH, DHol, FXB, MP performed literature search and generation of figures. All authors were involved in writing the paper and had final approval of the submitted and published versions.

DISCLAIMER

MS, DH, FXB and MP are listed on a DKFZ patent application to the European Patent Office (Europe patent application EP11175242.4).

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