

HPV typing and CGH analysis for the differentiation of primary and metastatic squamous cell carcinomas of the aerodigestive tract

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Abstract. Human papilloma virus (HPV) typing and Comparative Genomic Hybridisation (CGH) analysis can be used in the classification of multiple tumours of the aerodigestive tract for the differentiation between secondary malignancy versus metastasis. We present 3 exemplary cases of patients with multiple squamous cell carcinomas, localised within the head and neck region, cervical lymph node and the lung. In two patients, HPV typing identified HPV type 16 in the tonsillar carcinomas and the corresponding cervical lymph node and lung carcinoma indicating that the latter were metastatic spreads. In case 1, CGH confirmed the clonal relationship. Case two showed a peculiar syncytial growth pattern with lymphocytic infiltration which may constitute a potential morphological marker for HPV infection. In case three, a vallecular carcinoma was HPV negative while a lung cancer was positive for HPV type 6 indicating two independent primary tumours. Our case triplet illustrates the variability of HPV infection in squamous cell cancer of the aerodigestive tract and power as well as limitations of morphology, HPV typing and tumour genetics in the classification of multiple tumours.

Keywords: HPV, CGH analysis, primary tumours, metastases

1. Introduction

Squamous cell carcinoma may arise from the skin, the squamous mucosa of the inner organs or other types of preferentially multilayer epithelium, e.g. the lung respiratory mucosa or the bladder urothelium after squamous metaplasia. Carcinomas of the aerodigestive tract consist of head and neck squamous cell carcinomas (HNSCC) comprising epithelial tumours of the oral cavity, pharynx and larynx as well as lung and oesophageal squamous cell cancer. HNSCC represent 5% of all malignancies worldwide [50] and have a high incidence of local recurrences and metastasis formation, overall survival rates are relatively low [42]. Similar to other aerodigestive tract carcinomas, exposure to tobacco smoke and heavy drinking have been described as the most common risk factors. By field canceriza-

tion the patient has a high risk for the development of second primary tumours [44].

Human papilloma viruses are known to be closely associated with squamous cell carcinomas of the genital tract. In addition, they have been increasingly reported to be involved in the development of HNSCC [17,53] and define a distinct subtype [22]. Classification of human papilloma viruses depends on the homology of nucleic acid sequences [54] and has led to the identification of about 100 subtypes [30]. With regard to their association with benign or malignant tissue, HPV have been subdivided into high or low risk types.

Aneuploidy and chromosomal alterations are early and prevalent genetic events in HNSCC tumorigenesis [47] that may be used to define a clonal relationship between malignant tumours within one patient [1]. Similarly, HPV typing has been used to identify relationships between squamous cell carcinomas [12,38]. For the adequate medical treatment of cancer patients with multiple tumours, the distinction between second

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primary tumours and metastases is of major importance [49].

We report on three clinical cases of patients with multiple squamous cell carcinomas of the aerodigestive tract. They illustrate the diversity of these tumours and how HPV typing and molecular cytogenetic analysis can be used to differentiate between the occurrence of second primaries and metastatic tumours.

2. Materials and methods

2.1. HPV detection and typing

Evidence of HPV infection was accomplished with 2 independent test methods. For detection of HPV in tissue samples, we performed PCR analysis using consensus primers for the amplification of the L1-region as described previously [19].

In order to confirm the results and to classify for HPV subtypes, samples underwent hybridisation analysis, using specially designed microchips (Chipron GmbH, Berlin) with DNA probes of the major high and low risk HPV types (Fig. 2). Hybridisation procedures were performed according to the manufacturer's protocol. Briefly, a complex primer mixture was used to generate biotinylated PCR products from a region of the L1 gene of the HPV genome. PCR products were hybridized to HPV type specific capture probes immobilized as two-dimensional arrays on the surface of a transparent polymer support (chip). Following hybridisation (30 min, 35°C) and high stringency washes (5 min, 24°C), the hybridisation events were visualised by binding of streptavidin-horseradish peroxidase (HRP) conjugate and the precipitation of a chromogenic substrate of HRP (5 min, 24°C). Data analysis was mediated by high resolution transmission light scanning and software supported analysis of the resulting image, using the Analysis Package 1.0 (Chipron GmbH, Berlin, Germany). The LCD-array HPV 2.0 contained primer pairs and capture probes specific for the HPV low risk types 06, 11, 42, 43, 44 and the high risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59 and 68.

2.2. CGH preparation and analysis

Following surgical resection, a tissue sample of the tonsillar tumour underwent formalin fixation and subsequent paraffin embedding. An aliquot of the resected

lung carcinoma was quick-frozen in liquid nitrogen and stored at -80°C.

For further analysis, frozen tissue was prepared using cryotom microdissection. Paraffin-embedded material was obtained by manual microdissection from paraffin-sections.

DNA preparation was carried out via proteinase K digestion and phenol-chloroform-isoamylalcohol-extraction according to standard protocols as described [36].

CGH analysis was accomplished by the hybridisation of biotin-labeled tumour and digoxigenin-labeled reference DNA to normal metaphase chromosome spreads (Vysis, IL, USA), detection by avidin-FITC and anti-digoxigenin-rhodamine followed by the evaluation of chromosomal imbalances represented by the deviation of the fluorescence ratio profiles. Preparation and analysis procedures as DNA labelling, hybridisation, DNA detection, image acquisition and digital image analysis were performed as described previously [25,36]. To assess the degree of concordance, CGH profiles of tonsillar and lung tumour samples were evaluated by applying concordance analysis as previously described [1,51]. A detailed protocol of the CGH procedures can be obtained from our homepage at <http://amba.charite.de/cgh>.

3. Results

3.1. Case 1

A 47-year-old female patient was diagnosed with a moderately differentiated squamous cell carcinoma of the left tonsil. Fourteen months after resection of the tumour, several pulmonary tumours were diagnosed of which one was resected and classified as a moderately differentiated squamous cell carcinoma with lymph vessel invasion. The histomorphology of both tumours is shown in Fig. 1A,B. The tonsillar tumour showed an extensive carcinoma *in situ* component with basaloid but also invasive growth pattern. Cells were relatively isomorphic with predominately small nuclei and multiple mostly typical mitoses. In contrast, the lung cancer was morphologically more pleomorphic with small and medium size nuclei, atypical tripolar mitoses, apoptosis and also extensive necrosis. In the subsequent molecular pathological examination, both malignancies were detected as HPV 16-positive. In addition, the tonsillar and pulmonary tumour indicated a highly similar pattern of chromosomal imbal-

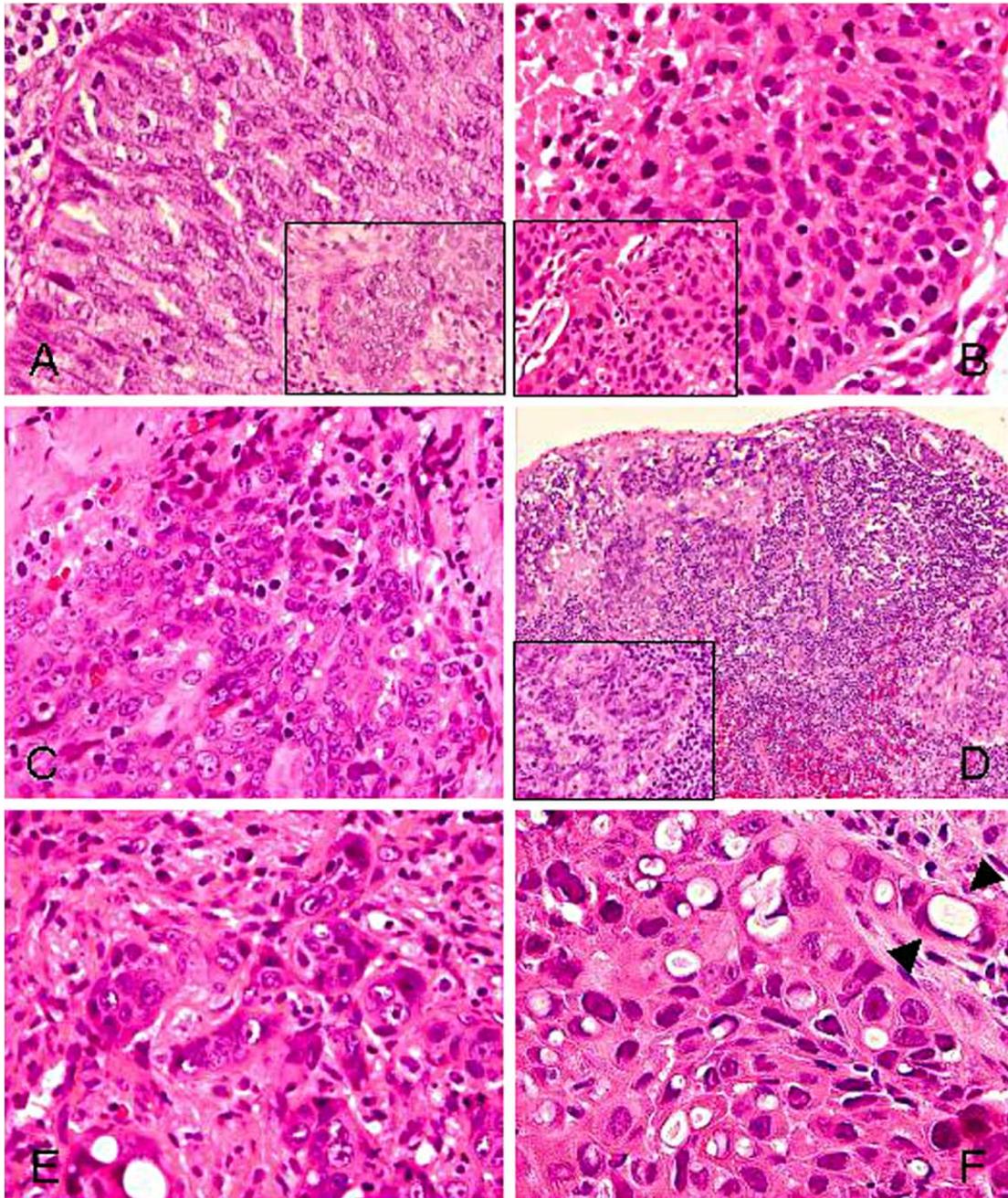


Fig. 1. Histomorphology. In case 1, the histomorphology of the tonsillar tumour (A) showed a squamous cell carcinoma with mostly small nuclei, multiple mostly typical mitoses, basaloid growth pattern and with an extensive carcinoma *in situ* component however with clearly invasive growth at several sites (insert picture). In contrast, the lung cancer was morphologically more heterogeneous with small and medium size nuclei, atypical tripolar mitoses, apoptosis and even extensive necrosis (B). The tonsillar carcinoma of case 2 being almost hidden within the fibrotic and lymphocytic stroma showed a syncytial growth pattern. The diagnosis required a high power magnification indicating nuclear pleomorphism and mitoses (C). Similarly, the squamous cell carcinoma of the lymph node showed a fairly well differentiated epithelium at the inner, almost smooth cystic surface while within the deeper portions cell polymorphism and invasive growth were detectable (D). The vallecular carcinoma of case 3 being the only HPV-negative tumour showed trabecular invasion within a desmoplastic stroma with scattered inflammatory infiltration (E). The squamous cell carcinoma of the lung (F) showed mostly an intraepithelial growth pattern as carcinoma *in situ* (F, lower left part) with only focal invasion (arrow heads). Additionally, the tumour cell indicated a peculiar clear cell appearance reminiscent of koilocytes probably correlating with the detection of HPV low risk type 6 in the PCR chip analysis (H&E stainings, magnification A–C, E, F: 400 \times , D: 100 \times).

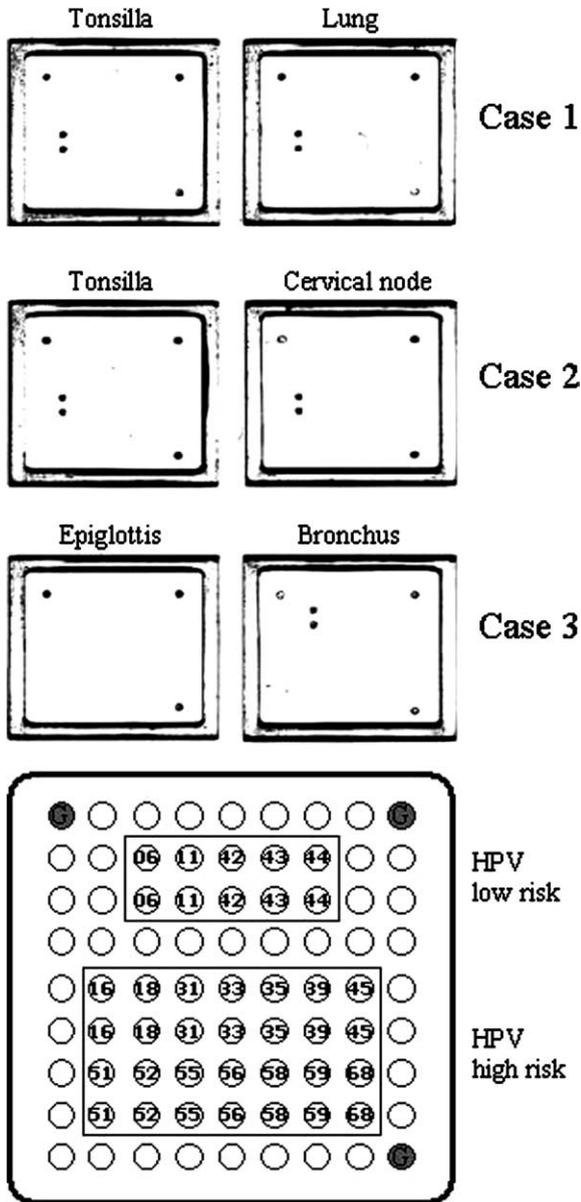


Fig. 2. Detection of HPV types in tissue samples using Chipron microchip analysis. DNA prepared from tissue samples of patients 1–3 were hybridized to microchips harbouring a set of HPV type specific probes. The experiments revealed a congruent HPV 16 status in the tonsillar and lung carcinoma of patient 1, as well as in the tonsillar and lymph node tumour of patient 2. These congruent findings propose a clonal relationship of both tumour pairs. In case 3, HPV typing exhibited HPV 6-positivity for the bronchial carcinoma, while the epiglottic cancer was HPV-negative, thus indicating two independent primaries.

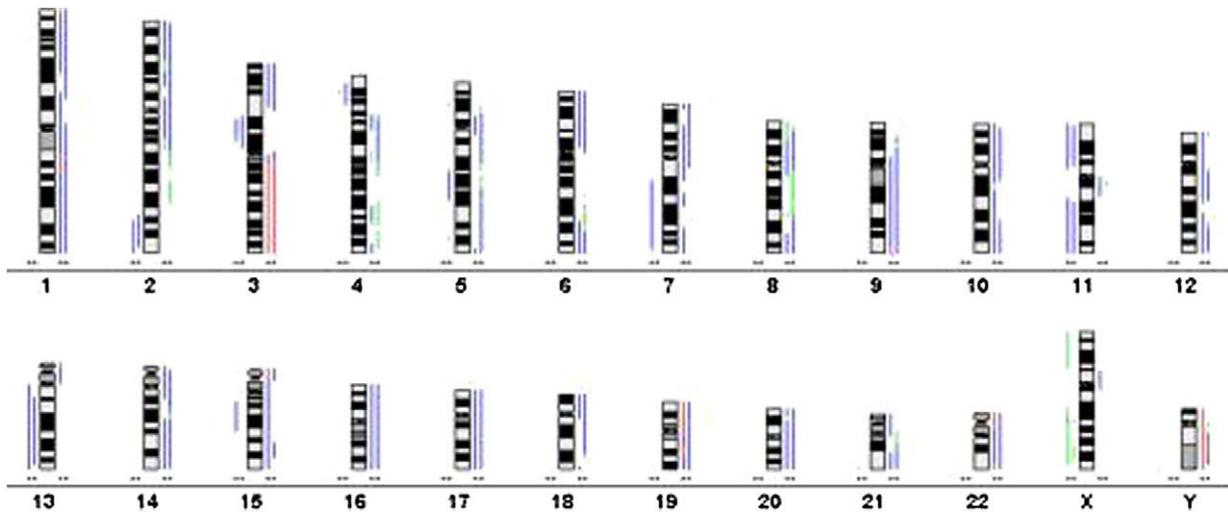
ances in the CGH analysis with a concordance of more than 80% (Fig. 3). The clinical and molecular analysis clearly indicated a tonsillar carcinoma with metastases formation in the lung.

3.2. Case 2

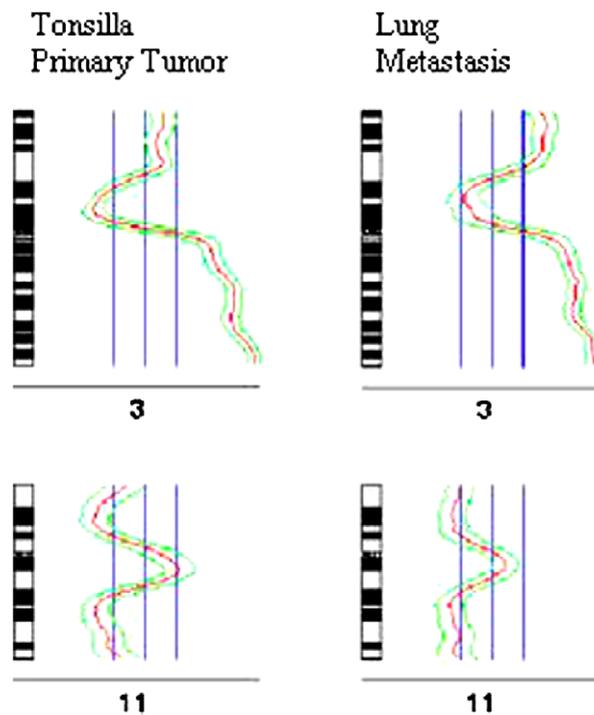
A 54-year-old male patient was clinically diagnosed with lateral cervical cyst that surprisingly revealed a squamous cell carcinoma within cystic lymphoid tissue (Fig. 1D). Towards the inner, smooth surface the tumour showed a fairly well differentiated epithelium while within the deeper portions cell polymorphism and invasive growth were detectable. The lesion was compatible with the malignant transformation of a conventional lateral cervical cyst. In the pathology report, the possibility of a lymph node metastasis of an unknown primary tumour was mentioned which finally led to a bilateral tonsillectomy. Within one tonsil a moderately differentiated squamous cell carcinoma was found (Fig. 1C). This carcinoma was almost hidden within the fibrotic and lymphocytic stroma. The diagnosis required a high power magnification indicating nuclear pleomorphism and mitoses. Additionally, the tumour showed a syncytial growth pattern which was recognisable within the cystic carcinoma of the lymph node. HPV analysis showed positivity for type 16 in both carcinomas. Due to the low amount of tumour tissue in the tonsil and the strong lymphocytic infiltration of the tumours, no CGH analysis was performed. The HPV analysis supported the histopathological diagnosis of a primary tumour of the tonsil with a cystic cervical lymph node metastasis.

3.3. Case 3

A female 52-year-old patient first presented with a squamous cell carcinoma of the vallecula showing solid and trabecular invasion (Fig. 1E). After a period of 11 months, another papillary exophytic growing squamous cell carcinoma was detected in the left pulmonary lobe. This tumour demonstrated an intraepithelial growth as carcinoma *in situ* and a peculiar clear cell morphology compatible with koilocytes (Fig. 1F). The HPV analysis revealed low risk papilloma virus within the lung cancer (Fig. 2F). The pharyngeal carcinoma was negative (Fig. 2E). The histomorphological and molecular analysis indicated two independent primary carcinomas.



(A)



(B)

Fig. 3. Comparison of genetic imbalances in tonsillar and lung carcinoma using CGH-analysis. (A) Line representation of chromosomal aberrations detected by CGH of the tonsillar carcinoma (inner lines) and lung carcinoma (outer lines) of case 1. Lines on the left side of the ideogram represent deletions, lines on the right side overrepresentations. DNA gains and losses were scored by statistical evaluation, i.e. as deviations of the ratio profile together with its confidence interval from the normal value 1.0. Blue lines indicate imbalances using the 99% confidence interval, green those of 95% confidence, red lines (ratio >1.5 or <0.5) illustrate pronounced changes. There is a highly similar distribution of changes for most chromosomes yielding 80% concordance in the statistical analysis being indicative for a clonal relationship of both tumours and thus metastasis formation in the lung. (B) Representative ratio profiles with the 99% confidence intervals are shown for chromosomes 3 and 11. The profiles are again highly similar. Interestingly, there is a shift to the right of the profile of chromosome 3p suggesting that a duplication of the short chromosome arm occurred during metastasis formation.

4. Discussion

The occurrence of multiple tumours within one patient can be related to genetic predisposition, field cancerization and metastasis formation. Correct classification of secondary malignancies plays an essential role for the appropriate therapy of cancer patients. Particularly for tumours with similar histomorphological differentiation, genetic markers provide means to distinguish between the differential diagnoses metastasis and second primary tumour. As carcinogenesis is a mutation-dependent process, it is important to select diagnostic markers which determine molecular genetic characteristics. In conjunction with their application, however, the significance and prevalence of each marker within the tumour subtype need to be critically assessed.

4.1. Assessment of clonal relationship by genetic and chromosomal mutations

Studies focusing on distinguishing features between second primary tumours and metastases have been based mostly on microsatellite analysis [4–6,13,29,40,41] or detection of TP53 tumour suppressor gene mutations using PCR techniques [20,26,39].

TP53 mutations are convenient markers because the gene is affected by a high variety of mutations (<http://p53.free.fr>). So far, 15,000 tumours with TP53 mutations have been published, leading to the description of more than 1500 different TP53 mutants [45]. The frequency of these mutants is highly heterogeneous. However, there are hot spots. While 306 mutants have been reported only once, 11 mutations were found more than 100 times, thus offering the existing, small chance that two independent tumours may carry the same mutation.

CGH analysis has been established as a research tool for comparing primary tumours and their metastases [1–3,24]. These studies generally showed a high similarity between tumour pairs and detected chromosomal imbalances important for tumour progression. There are, however, also studies showing a higher variability of the imbalance pattern suggesting that the accumulation of chromosomal alterations does not necessarily follow a linear progression model [32]. The divergence between related tumour pairs may be more pronounced for certain tumour types like renal cell cancer or breast cancer. It may also depend on the time span between the occurrence of the primary tumour and the development of metastatic disease [28,32].

Furthermore, there are common patterns of chromosomal imbalances which may hamper the establishment of a clonal relationship, e.g. squamous cell carcinoma of the lung and of head and neck both tend to carry gains on 3q, 8q, 17q, 19, 20, 22q and losses on 1p, 4, 5q, 6q, 8p, 9p, 11, 13q, 18q, 21q [2,7,18,35,37,46]. Therefore, we and others used statistical approaches to establish a clonal relationship between two malignancies based on CGH findings. Concordance values above 50% were a strong indicator for clonally related tumours [1]. Thus, the CGH pattern of case 1 with a concordance value of 80% can be regarded as strong support for a clonal relationship of the two carcinomas and thus as evidence for metastasis formation.

4.2. HPV prevalence in SCC of the aerodigestive tract

Papillomaviruses typically show high incidence rates in cancers of the genital tract, such as cervical, penile and vulvar lesions [9,10]. They play an important role in the molecular mechanisms of carcinogenesis by downregulation of tumour suppressor genes and are involved in regulatory steps of the cell cycle, therefore promoting cell proliferation as well as viral replication [21]. Former studies have given evidence that HPV even contributes to the process of metastasis formation. By enhancing the gene expression of metalloproteinases which show enzymatic activity in collagen cleavage, HPV thereby promotes the invasion of malignant cells into the lymphovascular space [14]. In recent reports, HPV has been used as a marker for the assignment of second primary tumour versus subsequent lesions in cancers of the genital tract [12,38].

In general, the detection of HPV via PCR might be considered with caution, as this method detects only the presence of viral DNA that does not necessarily have to be integrated into the tumour genomes – a process which is essential for carcinogenesis [43]. It is conceivable that the dissemination of viral particles to the lymph nodes by immune cells may lead to an unspecific contamination of negative lymph nodes with viral DNA, thereby producing false positive results. However, due to the overwhelming evidence for the association of HPV with cancer, the detection of HPV by PCR in tissue containing a squamous cell carcinoma is in our view a justification to assume that the specific virus type is causally related to tumor development. Additional investigations like viral load, transcript analysis and in particular the confirmation of virus integration into the tumour genome might be used to confirm this assumption.

Besides genital cancer, also HNSCC show an association with papillomaviruses [15,23,31]. The HPV status of infected tumour pairs therefore provides means to distinguish between clonally derived or independent lesions. However, this characterization depends on the incidence of HPV in different tumor sites, populations as well as the prevalence of specific viral subtypes. Thus, as a classification method for clonal relationship it is only recommended for tumour pairs preferably in high and low incidence localizations to provide specificity. In European reports, the infection rates of HNSCC vary upon the sublocalisation and are relatively high in the oropharynx, whereas the occurrence of HPV is moderate in hypopharyngeal and laryngeal lesions, and only sporadic in pulmonary and oesophageal tumours [53]. Particularly tonsillar carcinoma had increased infection rates of more than 50% [23,31] which may be due to their anatomical location at the border of mesodermic and ectodermic epithelium, making the cells more susceptible to metaplastic transformations [52]. Another criterion for their high association rates with HPV may be based on the organization of the tonsillar squamous epithelium, being infiltrated by lymphocytes and therefore initiating the contact to immunologically competent cells which bear pathogenic particles [34,52].

The application of this test method can be useful for the classification of tumour pairs in locations like oropharynx and lung, given their different incidence rates. In geographical regions like East Asia, however, the prevalence of HPV infected pulmonary and esophageal SCC is significantly higher [8]. A classification based on the HPV status can therefore not provide specificity, as HPV positive tumours might be infected independently. Case three of our report illustrated that HPV infection in the lung does also occur in European individuals. However, the history of this patient as well as the detection of HPV low risk suggested that it was a coincidental finding which was not related to the carcinogenic event.

4.3. Morphological characteristics of HPV infected SCC

While HPV typing using molecular genetic techniques reveals definite results for the HPV status, histomorphology may already provide some hints on a potential infection [16,27]. Similar to previous studies, we observed morphological findings like a poor degree of keratinization [52] and a typical basal cell

morphology [11] in tumours infected with HPV. Furthermore, tumours showing a syncytial growth pattern with a lymphocytic and/or fibrotic stroma may harbour the virus as exemplified by case 2. Although the significance of this observation needs to be validated by further studies it is important to note that HPV infection may induce characteristic morphological changes. These may also become detectable by DNA measurement [33,48].

In general, the presented cases show how the combination of molecular cytogenetic, virological and morphological methods can be used to obtain indications on the clonal relationship of multiple tumours. The application on larger collectives will be useful to assess the clinical value of these analyses.

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