Combined human papillomavirus typing and TP53 mutation analysis in distinguishing second primary tumors from lung metastases in patients with head and neck squamous cell carcinoma

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Abstract

Background: In head and neck squamous cell carcinoma (HNSCC), the occurrence of concurrent lung malignancies poses a significant diagnostic challenge because metastatic HNSCC is difficult to discern from second primary lung squamous cell carcinoma (SCC). However, this differentiation is crucial because the recommended treatments for metastatic HNSCC and second primary lung SCC differ profoundly.

Methods: We analyzed the origin of lung tumors in 32 patients with HNSCC using human papillomavirus (HPV) typing and targeted next generation sequencing of all coding exons of tumor protein 53 (TP53).

Results: Lung tumors were clearly identified as HNSCC metastases or second primary tumors in 29 patients, thus revealing that 16 patients had received incorrect diagnoses based on clinical and morphological data alone.

Conclusion: The HPV typing and mutation analysis of all TP53 coding exons is a valuable diagnostic tool in patients with HNSCC and concurrent lung SCC, which can help to ensure that patients receive the most suitable treatment.

KEYWORDS
head and neck cancer metastasis, human papillomavirus (HPV), lung cancer, next generation sequencing, p53
1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world, with an incidence of over 650,000 new cases each year.\(^1,2\) Although the living quality of patients with this devastating disease has been raised in the last decades, the 5-year survival rate still remains low.\(^3,4\) The traditional risk factors for development of HNSCC are smoking and alcohol consumption, but infection with high-risk human papillomavirus (HPV) is also a known risk factor and is becoming more common.\(^5,6\) Currently, HPV DNA is found in approximately 15% of HNSCC cases, whereby HPV-16 is the most commonly detected serotype.\(^8\) In comparison to HPV-negative HNSCC, HPV-positive HNSCC is observed in younger, lighter alcohol-consuming individuals and is associated with a better prognosis, which suggests a distinct disease process in these patients.\(^9\)

It is generally accepted that HPV-negative HNSCC is founded on cumulative genetic alterations, which result in inactivation of tumor suppressor genes and activation of proto-oncogenes.\(^10\) The most frequently affected targets include protein 16 (p16)INK4A and p14ARF, the tumor protein 53 (TP53), cyclin D1, the fragile histidine triad, the ras association domain family 1A, and the epidermal growth factor receptor.\(^11\) The TP53 is mutated in most HPV-negative HNSCC and disruptive TP53 mutations are associated with an invasive tumor growth pattern.\(^12,13\) Therefore, TP53 mutation analysis has been used as a molecular marker in the diagnosis and progression assessment of HNSCC.\(^14\) In contrast to HPV-negative HNSCC, HPV-positive HNSCC is induced by at least 2 viral oncoproteins, E6 and E7, which cause cellular transformation and dysregulation of cell cycle control.\(^15\) The TP53 is usually not mutated in HPV-positive HNSCC, but the viral oncoprotein E6 leads to a reduction of TP53 bioavailability through accelerated degradation.\(^15\)

Squamous cell carcinomas (SCCs) represent 25%-30% of lung cancers\(^16\) and, similarly to HNSCC, TP53 mutations are among the most common genomic alterations in lung SCC, although these alterations alone have been reported not to be core genomic events.\(^17,18\) Moreover, lung SCC is typically not associated with HPV infection.\(^19\) In contrast to HNSCC, however, HPV-infection and TP53 mutation are frequently correlated in lung SCC and it has been suggested that TP53 mutation and HPV infection have a synergistic effect in lung SCC.\(^20\)

Advanced HNSCC tends to form distant metastases in the lungs, a development that is frequently associated with widespread tumor dissemination, a limitation of therapeutic choices to palliative chemotherapy, and a very poor prognosis.\(^21,22\) However, patients with HNSCC also tend to develop second primary SCCs in the lung, which are frequently low-stage lesions and, therefore, amenable to curative resection.\(^23,24\) Due to these strong differences in recommended therapy and prognosis, it is crucial that lung SCC in patients with HNSCC are identified correctly as HNSCC metastases or second primary lung SCC.\(^23-26\) However, this can be challenging in patients who present with singular pulmonary nodules because the morphologic similarities between HNSCC tumor spread and localized lung SCC make them indistinguishable on histopathologic grounds in most cases.\(^27\)

Not surprisingly, considerable effort has been made to develop methods that are capable of discerning between pulmonary HNSCC metastasis and second primary lung SCC, but a robust, cost-effective diagnostic standard has yet to be established.\(^27\) The used concepts include loss of heterozygosity (LOH) pattern assessment, gene expression profiling, HPV infection status analysis, p16 immunohistochemistry as surrogate parameter for HPV infection status, and mutational profile analysis.\(^22,28-32\) Although HPV-typing is readily applicable and robust, the value of this method is limited to cases of HPV-positive HNSCC.\(^33\) By contrast, TP53 has become a favored target for comparative mutational profiling aimed at differentiating metastatic HNSCC from second primary lung SCC, which can be used in the majority of cases.\(^14,23\) The TP53 gene has 11 exons, of which exons 2 to 11 are protein coding. Because most TP53 mutations in HNSCC have been reported to occur on exons 5 to 8, these exons have been the focus of many TP53 mutation-profiling studies.\(^34\) However, this limitation in focus is questionable because it has been reported that cancer driver mutations of TP53 also occur outside of exons 5 to 8.\(^35\)

In search of cost-effective, reliable methods for identifying singular lung nodules as HNSCC metastases or second primary lung SCC, we validated an improved diagnostic tool that uses targeted next-generation sequencing (tNGS) of TP53 exons 2 to 11 in combination with HPV typing in a cohort of 32 patients to (1) assess whether patients received the most suitable therapy in the past and (2) to support further therapeutic decisions.

2 | PATIENTS AND METHODS

2.1 | Patients

We compared HNSCCs and concurrent lung SCCs from 32 patients (see Figure 1). The patients were aged 44 to 72 years (median 60.8 years) and were diagnosed with HNSCC in different primary sites (14 laryngeal, 8 oral cavity, 5 oropharyngeal, 4 hypopharyngeal, and 1 unknown primary [disease diagnosed from a cervical lymph node]) between 2011 and 2016. The median time between the diagnosis of HNSCC and a metachronous lung tumor was 17.5 months. In 3 cases, the patients had synchronous lung and head and neck tumors.

The study was approved by the ethics committee of the Justus-Liebig University of Giessen (AZ 105/16).
2.2 | Histopathology

Specimens (biopsies and resectates) were formalin-fixed and paraffin-embedded. The 2-μm sections of the routinely processed paraffin blocks were stained with hematoxylin-eosin and examined by an expert pathologist.

2.3 | p16 immunohistochemistry

The p16 immunohistochemistry was analyzed as the surrogate parameter for HPV infection status. To do so, 3-μm sections were cut from routinely processed paraffin blocks and immunostained with a primary p16 antibody (clone E6H4 mouse monoclonal; Clonetics; Ventana Medical Systems, Tucson, AZ). Visualization was carried out with a rabbit anti-mouse immunoglobulin G antibody (Bond Polymer Refine Detection Kit; Leica Biosystems, Nussloch, Germany) and staining was carried out using autostaining machines (BOND-III; Leica, Wetzlar, Germany). Immunohistochemical staining was assessed as specific nuclear positivity of tumor cells and always compared with integrated on-slide positive controls.

2.4 | DNA isolation

Using the hematoxylin-eosin stained slides corresponding to the formalin-fixed paraffin-embedded blocks, tumor areas containing >30% of tumor cells were marked, and DNA was obtained from the formalin-fixed paraffin-embedded blocks using manual macrodissection. The DNA isolation process was partially automated using the Maxwell 16 System (Promega, Madison, WI) and the formalin-fixed paraffin-embedded LEV DNA Purification Kit (Promega, Madison, WI).

The material was transferred into a 180 μl incubation buffer and 20 μl proteinase K solution (20 mg/mL) was added. The material was incubated overnight at 70°C, after which 400 μl lysis buffer was added, then transferred to the Maxwell cartridge for automated extraction using the appropriate program (DNA/formalin-fixed paraffin-embedded), in accord with the manufacturer’s instructions.

2.5 | Human papillomavirus typing

The HPV detection and typing were carried out by polymerase chain reaction (PCR) and reverse dot blot in situ hybridization (ISH) using the HPV DNA Array Kit (AID, Straßberg, Germany), in accord with the manufacturer’s instructions. Briefly, the highly conserved E1 DNA fragment of the HPV genome and a glyceraldehyde 3-phosphate dehydrogenase amplification control were amplified from the extracted DNA by PCR using biotin labeled primers (HPV Easy-PN-Mix, AID, Straßberg, Germany). If HPV DNA was amplified, the most frequent anogenital high-risk and low-risk HPV types (HPV-6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and 97) were differentiated by reverse dot blot ISH with half automated detection (ELISPot reader; AID, Straßberg, Germany).

2.6 | Targeted next-generation sequencing of all coding exons of the tumor protein 53 gene

If both tumors from a patient were classified as HPV-negative, mutational profiling by tNGS of all coding exons (2-11) of the TP53 gene was performed using the Ion Torrent platform (Life Technologies, Carlsbad, CA). Briefly, the
primer pool was designed with the Ion AmpliSeq Designer tool (multipool design for 175 bp amplicons; forward and reverse primers are shown in the Supporting Information Tables S1 and S2) and the Ion AmpliSeq Library version 2.0, Ion Sphere Quality Control, Ion PGM Hi-Q Reagent Mix, Ion X-press Barcode Adaptors 1-16 Kits, and the Ion Torrent PGM were used for library generation and sequencing. The \(TP53\) mutations were detected using the Ion Reporter software and the Exome Variant Server (National Heart, Lung, and Blood Institute, Bethesda, MD) was used to exclude single nucleotide polymorphisms. For all samples, a minimal average read depth of 1000 \(\times\) was achieved and only variants with allele frequencies >10\% were further considered.

### 2.7 Assessment

In cases with p16-positive and HPV-positive HNSCC, lung tumors were classified as HNSCC metastases if they were also HPV-positive for the same HPV type and as the second primary if they were HPV-negative or positive for a different HPV type. In cases in which HNSCC was HPV-negative (irrespective of p16-status), the lung tumor origin was assessed by mutational profiling, whereby lung tumors with matching mutations in HNSCC and lung SCC were classified as HNSCC metastases, and lung tumors with different mutational profiles in HNSCC and lung SCC were classified as second primary lung SCCs. In patients with HNSCC that were both HPV-negative and revealed no \(TP53\) mutations, an assessment of the lung SCC origin was not possible. Even if the corresponding lung tumor contained mutations, these could be the result of clonal evolution of the HNSCC or due to the occurrence of a second primary lung SCC. In all cases, HPV typing results and mutational profiles were interpreted in context with the histopathological assessment. In order to assess whether a patient has received an oncologically adequate lung resection, the administered type of lung resection was compared to the standard of care required for the definitive diagnosis provided by the means of molecular genetic analysis. This is, in case of a solitary squamous lung

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**Figure 2** Representative immunohistochemical staining of protein 16 (p16; brown color) in laryngeal resectates (patient 10). Cells were counterstained with hematoxylin (blue color). Scale bar represents 200 \(\mu\)m [Color figure can be viewed at wileyonlinelibrary.com]

**Figure 3** Distribution of tumor protein 53 (\(TP53\)) gene mutations in the coding exons 2 to 11. Mutations of the splice donor and acceptor sites adjacent to exons were counted as mutations of the respective exon. According to previous reports, most mutations are localized on exons 5 to 8, but we found that 25\% of the mutations were located outside of exons 5 to 8 in our patient cohort. This underlines the importance of analyzing all coding exons for mutations.
<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age / sex</th>
<th>HNSCC localization / tumor stadium</th>
<th>p16 IHC / HPV-PCR/ISH HNSCC</th>
<th>p16 IHC / HPV-PCR/ISH lung tumor</th>
<th>Match of TP53 mutation profile in HNSCC and lung tumor</th>
<th>Clinicopathological diagnosis</th>
<th>Molecular genetic diagnosis</th>
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<th>Further therapy</th>
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(Continues)
metastasis, pulmonary wedge resection, whereas lung SCC requires anatomic lung resection (lobectomy, segmentectomy, or pneumonectomy) and systematic lymph node dissection of the peribronchial, interlobar, hilar, and mediastinal nodes.23,36

### TABLE 1 (Continued)

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<th>Patient number</th>
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Abbreviations: CUP, cancer of unknown primary; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; IHC, immunohistochemistry; ISH, in situ hybridization; N/A, not applicable; p16, protein 16; PCR, polymerase chain reaction; TP53, tumor protein 53.

The HPV-typing only facilitated differentiation between metastatic and primary tumor of the lung in 2 cases (patients 12 and 29), whereas mutational profiling facilitated this differentiation in 27 cases, therefore, profiling of exons other than 5 to 8 was necessary in 6 cases (see Supporting Information Tables S1 and S2). Clinicopathological diagnoses represent the combination of clinical diagnosis and pathological diagnosis as made without molecular genetic data. If there was a discrepancy between clinical and pathological diagnosis, the pathological diagnosis was compared with the molecular genetic diagnosis. Clinicopathological diagnoses matched the molecular genetic diagnosis in 13 cases (green highlight), whereas 16 clinicopathological diagnoses were discrepant to the corresponding molecular genetic diagnosis (red highlight). In cases 30 to 32, molecular genetic diagnoses were not available because no TP53 mutations were detected in the HNSCC (grey highlight).

Patient 2 was diagnosed with a cervical lymph node infiltration adherent to his lung tumor. Surprisingly, all 3 tumors (head and neck, lymph node, and lung) harbored different TP53 mutations, wherefore it was concluded that the cervical lymph node was infiltrated by a CUP origin. Curative resection of the lung tumor and curative radiation of the cervical region was carried out.

Patient 15 had different mutations in lymph node metastases of an unknown primary tumor and the lung tumor, but the morphological examination was very suggestive of a metastatic pattern, so that a lung metastasis of an unknown head and neck primary tumor was diagnosed.

### 3 RESULTS

Immunohistochemistry (IHC) for the detection of p16 was successful in 55 tumors. In 10 cases, there was insufficient tumor material to perform both IHC and HPV typing, in
which case HPV typing was prioritized. Eleven tumors (20%) were identified as p16-positive (see Figure 2), whereas HPV-DNA was only amplified from 3 tumors (5%), and HPV-16 was identified in each case. Overall, only 2 patients with HNSCC were positive for both p16 and HPV, which resulted in HPV status assessment only allowing the differentiation of metastatic and primary lung tumors in 2 of 32 cases (6%).

Targeted next-generation sequencing of all coding exons of the \( \text{TP53} \) gene revealed 57 mutations in 54 of 61 tumors (89%) and allowed identification of lung tumors as HNSCC metastases or second primary lung SCC in 27 of 30 cases (90%). Forty-seven of the detected mutations were substitutions (11 of which being nonsense mutations), 3 were splice donor site mutations, 1 was a duplication, 1 was an insertion, and 5 were deletions (1 of which was combined with an insertion). Nine tumors (15%) showed no mutation, whereas 5 (8%) tumors had multiple \( \text{TP53} \) mutations.

Of the 57 detected \( \text{TP53} \) mutations, 43 (75%) occurred in exons 5 to 8 and 14 (25%) were found outside of exons 5 to 8 (see Figure 3). The mutations outside of exons 5 to 8 were found in similar proportions in head and neck tumors (28%), in lung metastases (19%), and in primary lung tumors (25%; data not shown).

Overall, identification of lung tumor origins in HNSCC was achieved in 29 of 32 cases (90%) by combined HPV typing and \( \text{TP53} \) mutational profiling.

A comparison of clinicopathological diagnoses and molecular genetic diagnoses revealed that the origin of lung tumors had only been identified correctly in 13 of 29 cases (45%) based on clinical and morphological data alone (see Table 1). Moreover, analysis of 23 patients for whom surgical treatment was administered before mutational profiling results were available, showed that 11 patients (48%) had not received the most suitable treatment: 3 patients (4, 15, and 17) with pulmonary HNSCC metastases were subjected to pulmonary lobectomy or pneumectomy with excision of regional lymph nodes (Figure 4, patient 17), whereas the second primary lung SCCs from 8 patients (10, 14, 19, 23, 25, 26, and 29) were not excised radically (see Table 1). By contrast, all patients for whom therapeutic decisions were made after molecular profiling results became available were administered the most suitable therapy (Figure 4, patient 16).
4 | DISCUSSION

Patients with HNSCC are at risk of developing metastatic or second primary tumors in the lungs.37 However, the prognosis and therapeutic options for patients with metastatic HNSCC are considerably different from patients with localized primary pulmonary SCC. In HNSCC with concurrent lung SCC, patients with a low-stage second primary tumor of the lung should be considered cured after tumor resection and not be subjected to adjuvant chemotherapy, or worse, palliative care, whereas unnecessary lung resections in metastatic cases should be prevented in order not to further impede respiratory function.23,36 Therefore, establishing a reliable diagnosis is essential in order to ensure patients are administered the most suitable treatment.23–26

In the present study, we used HPV typing and TP53 mutational profiling by tNGS to identify the origin of concurrent lung tumors in patients with HNSCC. Although HPV typing only achieved diagnosis of lung tumor origins in 2 of 32 cases, mutational profiling of all TP53 coding exons (2–11) facilitated an exact diagnosis in 27 of 30 cases. The low rate of HPV detection in our patient cohort was expected because HPV-positive HNSCC does not tend to form distant metastases and primary lung SCC also tends to be HPV-negative.9,10,38 By contrast, the observation that correctly identifying the lung tumor origin depended on mutational profiling of TP53 exons other than 5 to 8 in 6 of 32 cases (19%) puts the common limitation of TP53 mutation analysis to exons 5 to 8 in question and shows that detection of TP53 mutations should comprise all coding exons in order to maximize the sensitivity of the assay.35

Remarkably, a comparison of lung tumor origin diagnoses made with or without molecular genetic data revealed that 16 of 29 patients (55%) for whom a clear diagnosis could be made by HPV typing and mutational profiling had received an incorrect diagnosis of the lung tumor origin when this diagnosis was based on clinical and morphological data alone. As a consequence, 3 patients with metastatic HNSCC were subjected to pulmonary lobectomy or pneumectomy, whereas the second primary lung SCCs from 7 patients were not excised radically. For example, patient 17 was diagnosed with a second primary lung SCC based on clinical and morphological data alone and subjected to extensive pulmonary resection. However, mutational profiling data later revealed that the lung tumor was in fact an HNSCC metastasis and, thus, should not have been removed. In fact, patient 17 developed further metastases in the contralateral lung approximately 7 months later (Figure 4).

Until now, most studies use TP53 mutational profiling to identify lung tumors in HNSCC, whereas HNSCC metastases or second primary lung SCCs have used traditional Sanger sequencing of TP53 exons 5 to 8.32,39 However, this method lacks sufficient sensitivity for detecting clinically relevant low-level mutant alleles in tumor biopsies and has, therefore, never been established as the generally accepted diagnostic standard of care.40 Moreover, limitation of the analysis to exons 5 to 8 is burdened with the risk that a portion of TP53 mutations may escape undetected.35 The growing distribution of next-generation sequencing platforms has changed this situation dramatically, as this method is ideal for detecting mutations in specific cell populations with high sensitivity.41

Our results demonstrate that a combination of HPV typing and TP53 mutational profiling by tNGS facilitates the accurate diagnosis of the lung tumor origin in most HNSCC cases and that analysis of all TP53 coding exons is a significant improvement over analysis of exons 5 to 8 alone. However, it must also be noted that this method cannot be applied in cases in which only a minimal quantity of sample tissue is available for analysis. Moreover, further investigations will be needed to determine whether our results are confirmed in larger patient cohorts. Nevertheless, our study provides strong evidence that the combination of HPV typing and TP53 mutational profiling by tNGS is a powerful diagnostic tool to ensure the most suitable therapy is administered to patients with HNSCC and concurrent lung SCC.42 In particular, our finding that over 40% of patients had not received the most suitable therapy when therapeutic decisions were made without HPV typing and TP53 mutational profiling data shows that molecular genetic typing of multiple tumors by next-generation sequencing is indispensable for ensuring the administration of the best possible treatment to each patient.43

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