Secondary lung lesions after head and neck cancer: Diagnosis, differentiation, screening, survival
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Chapter 3

Differential Diagnosis of Pulmonary Carcinoma Following Head and Neck Cancer by Genetic Analysis

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Abstract

Purpose:
Patients with head and neck cancer often develop a tumor that can be diagnosed as distant metastasis (DM) or second primary tumor (SPT). In this study, we use TP53 mutation analysis for validation of an allelic loss markers panel and a decision algorithm for distinguishing between DM and SPT.

Experimental Design:
Tumor pains of 39 patients were analyzed for TP53 mutations, for patterns of allelic loss and immunohistochemical analysis of p53 expression. Results of these three analyses were compared, using mutation analysis as gold standard.

Results:
Loss of heterozygosity (LOH) analysis indicated DM in 15 and SPT in 23 cases (one inclusive), TP53 mutation analysis was informative in 15 cases. Based on the p53 mutation status alone, nine tumors were diagnosed as SPT and six as DM. In all 15 cases the LOH analysis was in concordance with the TP53 mutation analysis. Immunostaining for p53 showed promise as a first scan to diagnose lung tumors as SPT but cannot be used to diagnose DM.

Conclusion:
The TP53 mutation data validate the suitability of the LOH marker panel and decision algorithm for differential diagnosis of DM and SPT in the lung. LOH analysis can theoretically be exploited in almost all cases and is less laborious than TP53 mutation analysis.
Introduction

Long-term survival in patients with head and neck cancer with locoregional control depends a great deal on the absence of distant metastases (DM) or second primary tumors (SPT). SPT most frequently occur in the head and neck region, with the lungs as the second most common site. The lungs are also the most common site of DM in patients with head and neck squamous cell carcinoma (HNSCC), causing a problem in differential diagnosis. Early detection and treatment of small size primary lung carcinoma improves survival. However, when a lung carcinoma is detected in patients treated for HNSCC, it is not clear whether it is an SPT or DM. Prognosis and treatment options for patients with SPT might be different from patients with DM, but reliable data are missing as there is no convincing differential diagnostic modality. In theory, SPT can be treated curatively, whereas patients with DM may benefit from systemic therapy or limited surgical resection, but this is often considered palliative in these cases. Currently, the differentiation between SPT and DM is made on the basis of clinical criteria, but in an earlier report, we showed that these clinical criteria are not reliable. Therefore, we explored the use of patterns of allelic loss to distinguish DM from SPT, a method that has also been investigated in other studies for this purpose. It has been shown that patterns of allelic loss sometimes differ between tumors and metastasis due to progression or tumor heterogeneity, which might cause a problem for their exploitation in differential diagnosis. In our previous study, we therefore developed a decision algorithm for evaluating the results of loss of heterozygosity (LOH) analysis. However, neither the marker panel to assess allelic loss patterns nor the decision algorithm has been validated against a gold standard.

TP53 mutation analysis has often been promoted as the gold standard for differentiation of SPT from DM, as it can be used as a marker for clonality. The TP53 gene is frequently involved in HNSCC and lung cancer carcinogenesis. It is located on the short arm of chromosome 17, at position 17p13.1 and is 16 to 20 kb in length. The TP53 gene consists of 11 exons, of which, the first is noncoding. Mutations are mostly found in exons 5 to 8. The gene encodes a phosphoprotein that functions as a stress-induced transcription factor, regulating the cell cycle and apoptosis. Loss of p53 function also causes increased genetic instability. However, TP53 mutation analysis is time-consuming and the point mutations are present in only 50% to 60% of HNSCCs. In addition, usually only formalin-fixed paraffin-embedded material is available, which severely hampers efficient and rapid TP53 mutation analyses. LOH analysis can be done more easily on paraffin-
embedded material, is less time-consuming, can in theory be done in all patients, and seems therefore more suitable for clinical daily practice. We therefore decided to validate our LOH marker panel and decision algorithm using TP53 mutation analysis as the gold standard in 39 treated HNSCC patients who developed squamous cell carcinoma in the lung. In addition, we explored the potential diagnostic value of immunohistochemical analysis of p53 expression on the paraffin tissue of the HNSCC and the lung carcinomas.

Patients and methods

Patients
The Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (or the Pathologic Anatomic National Automated Archive of the Netherlands) database was searched for all cases between 1978 and 2002 at the Netherlands Cancer Institute coded as a primary HNSCC followed by a malignant squamous lesion of the lung. Sufficient paraffin-embedded material from both lesions had to be available in our pathology archive to perform LOH analysis and TP53 mutation analysis. The patient series has been reported previously.  

DNA isolation
Paraffin sections were deparaffinized and rehydrated. The region containing >50% tumor cells was microdissected from the glass slide using a scalpel. As a source of reference DNA, tissue that was free of tumor was microdissected. The tissue was transferred to a tube containing digestion buffer [2 mg/ml. proteinase K (Roche) in 10 mmol/l. Tris-HCl (pH 8.0), 50 mmol/l. KCl, 2.5 mmol/l. MgCl₂, 0.5% Tween 80, and 0.1 mg/ml. gelatin] and incubated for 24 h at 55°C. After centrifugation, the supernatant was transferred to a new tube and stored at 4°C until use. Before PCR amplification for TP53 sequencing, the DNA was cleaned by phenol/chloroform extraction and ethanol precipitation, and dissolved in LoTE [3 mmol/l. Tris-HCl (pH 8.3), 0.2 mmol/l. EDTA].

LOH analysis
LOH analysis. LOH analysis was done as previously described. In short, 12 microsatellite markers distributed on 11 chromosome arms were amplified by DNA PCR. One of the amplification primers was labeled with a fluorochrome. The PCR products were run on an automatic sequencer to separate the alleles by capillary electrophoresis and to measure the peak intensities of the fluorescent-labeled PCR products of both alleles. The intensity ratio between the two allele peaks of
heterozygotes was calculated and this ratio was subsequently divided by the ratio of the alleles in the normal tissue for normalization resulting in a LOH index. A LOH index of <0.75 or >1.33 was interpreted as “loss of heterozygosity”. Between these values, it was considered as “retention of heterozygosity” (ROH). The decision algorithm was based on the three following basic rules:

Discordant LOH, e.g., LOH of different alleles, not explainable by progression (LOHx-LOHy or LOH-ROH) is a strong argument for SPT as this pattern is not likely when two tumors are clonally related.

Concordant LOH, e.g., LOH of the same allele (LOHx-LOHx) is an argument for clonal relation, but can also occur by chance especially if the expected frequency of LOH is high.

ROH-LOH is a variant of discordant LOH that could occur as a result of tumor progression and is therefore a less strong argument against clonal relation (metastasis) as the two other forms of discordant LOH (LOHx-LOHy and LOHx-ROH).

These rules were formalized in a decision algorithm, and the 39 tumor pairs were then categorized according to this diagram as described previously.

TP53 mutation analysis

The procedure for TP53 sequencing and mutation analysis was based on the protocol previously described by van Houten et al. Adaptations to this protocol included the use of AmpliTaq Gold as a thermostable DNA polymerase. Exons 5, 6, 7, and 8 of the TP53 gene were amplified by means of PCR using genomic DNA of both the head/neck tumor and the lung tumor of each patient as template, and sequenced using dideoxynucleotides. Only sequence changes that resulted in missense, nonsense, frame shift, or splice site mutations were considered. When a mutation in the lung tumor of a particular patient was the same as the mutation in the corresponding head and neck tumor, it was diagnosed as a DM. When a mutation in one of the tumors was not found in the TP53 sequence of the other tumor, the lung tumor was classified as an SPT.

Immunohistochemical analysis

For 38 patients, immunohistochemical analysis of p53 expression was done using a standard protocol with DO7 (DAKO) in a LabVisionstainer automatic stainer as described previously. In 25 patients, both specimens could be stained and interpreted. Criteria for interpretation of the immunohistochemical p53 expression in terms of underlying p53 mutation are not standardized in the literature. We
analyzed the reliability of p53 immunostaining as a potential marker using an independent panel of 62 HNSCCs with known mutation status, and 8 HNSCCs with corresponding lymph node metastases. All immunostainings were evaluated by two independent investigators and a definitive score defined in a consensus review. Based on these results, we decided to define very strict criteria to exploit p53 immunostaining for differential diagnosis: when <50% of the tumor cells show staining, the tumor is considered wild-type or a deletion mutant (undetectable by the antibody). In case of 50% to 75% staining, no reliable diagnosis can be made. More than 75% staining is considered as a mutation. When immunostaining indicated a different mutation status in the two tumors (e.g., del mutant/wild-type 0-50% versus mutant >75%), the lung tumor was considered an SPT. When immunostaining was identical (both <50% or >75%), it was considered as not discriminating. Immunohistochemistry cannot be used to diagnose a metastasis.

Results

Patients
Thirty-nine patients with a SCC of the head and neck followed by SCC of the lung, with sufficient paraffin-embedded material from both lesions to perform LOH and TP53 mutation analyses, were identified. Of these 39 patients, 33 were male and 6 were female. The average age at the time of the primary HNSCC was 61 years (range, 25-85 years). Twenty-one patients had laryngeal carcinomas, 8 had oral carcinomas, 7 had oropharyngeal carcinomas, and 3 had hypopharyngeal carcinomas. The following stages (International Union Against Cancer tumor-node-metastasis classification of malignant tumors, 2002) were included: stage I (n = 5), stage II (n = 9), stage III (n = 11), and stage IV (n = 14). Based on the LOH analysis, we differentially diagnosed 20 lung tumors as SPT, 3 as likely SPT, 8 as DM, 7 as likely DM, and 1 remained undiagnosed (Table 1).

TP53 mutation analysis
Analysis of six tumor pairs failed due to poor DNA amplification (n = 3; patients 34, 35, and 37) or unreadable exons (n = 3; patients 36, 38, and 39). In 18 cases (patients 16-33), neither HNSCC nor lung tumor mutations were found (Table 1), and these cases were not discriminating. Mutations were found in either one or both tumors in 15 patients, which allowed differentiation between SPT and DM. Nine patients were classified as SPT (patients 1-9) and six as DM (patients 10-15).
Table 1. Overview of all 39 patients in which the lung carcinomas are differentially diagnosed by three different methods

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Diagnosis p53 mutation</th>
<th>Diagnosis LOH</th>
<th>IHC HNSCC (%)</th>
<th>IHC lung (%)</th>
<th>IHC</th>
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<td>SPT</td>
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<td>90</td>
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<td>x</td>
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</tr>
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<td>ND</td>
<td>DM</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
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</table>

Abbreviations: HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemical; NM, no mutation found; ND, not determined (one of the tumors could not be analyzed); NDi, no discrimination by p53 immunostaining; NP, not performed; SPT, second primary tumor; DM, distant metastasis
Genetic analysis of pulmonary carcinoma following HNSCC

In patients 1, 2, 5, 6, 7, and 9, we found a TP53 mutation in the HNSCC and wild-type TP53 in the lung carcinoma. In patients 3 and 4, we found wild-type TP53 in the HNSCC and a TP53 mutation in the lung carcinoma. In patient 8, we found a deletion in the HNSCC and a missense mutation in the lung carcinoma. These cases were therefore classified as SPT.

The remaining patients were diagnosed as DM because the same mutation was found in both tumors (for details, see Table 2). In all 15 cases in which TP53 mutation analysis was diagnostic, the diagnosis using LOH analysis was in concordance with the TP53 mutation data (see Table 1).

Immunohistochemical analysis of p53 expression

We also explored whether we could use p53 immunostaining as an initial quick scan to differentially diagnose lung cancers as SPT’s (Table 1). As a first step, we immunostained 62 independent tumors, of which, the TP53 mutation status was known by sequence analysis. In general, we confirmed that missense mutations lead to overexpression and nonsense mutations to absence of detectable p53. Tumors with wild-type TP53 revealed both the absence and overexpression of p53 (data not shown). As a second step, we immunostained eight independent primary HNSCC and corresponding lymph node metastases for p53. Both tumors with missense mutations, nonsense mutations and wild-type p53 were included. In all cases, both the pattern and intensity of p53 immunostaining in the lymph node metastases closely resembled that of the primary tumors (data not shown). Based on these data, we concluded that immunostaining does not always resemble the mutational status, but a given immunostaining pattern seems to be conserved in the primary tumors, and its metastases might be exploited as an exclusion marker. We reasoned that when TP53 is wild-type or a deletion mutant, immunostaining will be generally weak and will occur in less than 50% of the cells. When there is a missense mutation, it will be intense and higher than 75%. Hence, when one tumor shows staining of <50% of the cells, and the other >75% of the cells, we assume that the TP53 mutation status will differ, and consequently, that the lung tumor is an SPT. In all other combinations, the staining will not be discriminating and genetic analysis is required. The immunohistochemical analysis of p53 expression was informative in 25 patients. Using our strictly defined criteria, 5 out of 25 cases could be classified as SPT (patients 1, 2, 6, 17, and 23; see Table 1) as the p53 immunostaining pattern of the primary HNSCC and the lung carcinoma differed tremendously, excluding a clonal relationship. All other cases were considered as “not discriminating.”
Table 2. Analyses in the 15 patients with a TP53 mutation

<table>
<thead>
<tr>
<th>Patient no</th>
<th>E/C/P HNSCC</th>
<th>E/C/P lung cancer</th>
<th>Mutation HNSCC</th>
<th>Mutation lung cancer</th>
<th>Mutation analysis diagnosis</th>
<th>LOH analysis diagnosis</th>
<th>p53 IHC HNSCC (%)</th>
<th>p53 IHC Lung cancer (%)</th>
<th>IHC analysis diagnosis</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>7/134/b</td>
<td>7/134/b</td>
<td>A → G (Tyr → Cys)</td>
<td>Wild-type</td>
<td>SPT</td>
<td>SPT</td>
<td>99</td>
<td>0</td>
<td>SPT</td>
</tr>
<tr>
<td>2</td>
<td>8/278/b</td>
<td>8/278/b</td>
<td>C → G (Pro → Arg)</td>
<td>Wild-type</td>
<td>SPT</td>
<td>SPT</td>
<td>95</td>
<td>50</td>
<td>SPT</td>
</tr>
<tr>
<td>3</td>
<td>5/151/b</td>
<td>5/151/b</td>
<td>Wild-type</td>
<td>C → deletion (Pro → deletion)</td>
<td>SPT</td>
<td>SPT</td>
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<tr>
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<td>5/144/a</td>
<td>Wild-type</td>
<td>C → T (Gln → Stop)</td>
<td>SPT</td>
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<td>99</td>
<td>ND</td>
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<td>5/153/b</td>
<td>5/153/b</td>
<td>C → T (Pro → Leu)</td>
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<td>Wild-type</td>
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<td>0</td>
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<tr>
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<td>Wild-type*</td>
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<td>99</td>
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<td>95</td>
<td>80</td>
<td>NDi</td>
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</table>

Abbreviations: E/C/P, exon/codon/position; IHC, immunohistochemistry; ND, not determined; NDi, no discrimination by p53 immunostaining; G, guanine; A, adenine; T, thymine; C, cytosine; Ala, alanine; Arg, arginine; Asn, asparagine; Cys, cysteine; Glu, glutamate; Met, methionine; Phe, phenylalanine; Stop, stop codon; Thr, threonine; Tyr, tyrosine; Val, valine.

* Mutation was absent; not all exons could be sequenced.
Discussion

In view of the prognosis and treatment options, it seems essential to reliably distinguish SPT from DM in the lung. As suggested in the literature, TP53 mutation analysis can be considered the gold standard for differential diagnosis. However, the method is very laborious, time-consuming and can only be used in a limited number of patients because the mutations are only found in 50% to 60% of patients with HNSCC. LOH analysis, on the other hand, can be easily done on paraffin-embedded material, is, in theory, usable in all patients with HNSCC, and is therefore more suited to perform in clinical practice. In a previous study, we described a LOH marker panel and a decision algorithm for the differentiation of SPT and DM. The present study shows that the LOH marker panel and algorithm was completely concordant with the TP53 mutation analysis in 15 patients in which both methods could be applied.

In 13 of 33 (39%) analyzable HNSCC samples, a mutation was found, which is very close to 24 of 55 (44%) mutations in these same exons from a previous HNSCC study. Some mutations might have been missed due to fact that ~10% of the p53 mutations occur in exons other than exons 5 to 8, which were not analyzed. However, we cannot exclude that we missed some mutations due to the poor quality of the archival paraffin DNA, although this did not seem to hamper LOH analysis.

Although our LOH panel and algorithm seems to perform well, there is one important consideration. Tabor et al. have investigated the reliability of both LOH analysis and TP53 mutations as clonal markers by comparing primary HNSCC and their accompanying lymph node metastases and DM at sites other than in the lung. For TP53 mutations, the concordance of the primary HNSCC with the lymph node metastases was 100%. When the tumor contained a mutation, the metastases showed the same mutation, and when a tumor was TP53 wild-type, the metastases were also TP53 wild-type, again demonstrating the potential of TP53 mutations as a marker of clonality. In the same study, the patterns of allelic loss were also evaluated as clonal markers, and in tumors with TP53 mutations, the patterns were very concordant (as expected). However, more differences were seen in the tumors that were TP53 wild-type. Hence, the group of TP53 wild-type HNSCC tumors might show more discordant LOH markers in metastases, which might lead to false SPT diagnosis of lung tumors when the patterns of allelic loss are used as differential diagnostic criterion. This potential problem in TP53 wild-type tumors cannot be addressed well as long as there is no better or comparable marker of clonality than TP53 mutations. Hence, extrapolation of our validation
data to all tumor pairs, including those that are TP53 wild-type, should be done with caution, and this is also a reason that our algorithm for interpretation of LOH results allows some discordant LOH. It has been suggested that in multiple chromosomes, breakpoints should be relied upon instead of LOH patterns. However, in our previous study, we showed that even breakpoint analyses might lead to misinterpretations, particularly when TP53 is wild-type. In 7 of 34 metastases (metastases in lymph nodes or at distant sites, lung metastases excluded), the breakpoints differed from those detected in the primary tumors, indicating that breakpoints also have their limitations as indicators of clonality. With this in mind, we can safely conclude that differential diagnosis on the basis of genetic criteria using allelic loss of 12 microsatellite markers on 11 chromosomes is highly reliable and superior over clinical and histologic criteria. It should be noted, however, that these data concern a single institute retrospective analysis on a relatively small number of patients. Additional, preferably prospective, studies with independent validation of the proposed algorithm are needed to confirm clinical utility and its relevance for outcome.

We further evaluated whether immunohistochemical analysis of p53 expression in the SCC of both the head and neck and lung could be of help, at least as an initial quick screen. Positive immunostaining for p53 is presumptive evidence for gene mutation, as more than 90% of missense mutations will result in abnormal p53 protein accumulation. Immunohistochemistry is a simple and fast technique, and easily implemented at any pathology laboratory. Although we also showed that p53 immunostaining is associated with mutational status, it is not a straightforward one-to-one correlation. Overexpression of p53 when the gene is wild-type can be due to ongoing DNA damage, and this is easily confused with abnormal accumulation caused by mutations. Also, a high proliferation rate in the tumor might cause increased immunostaining, easily misinterpreted as “mutation.” All these events hamper the correlation of TP53 mutation analysis and p53 immunohistochemistry. Therefore, we also analyzed the staining pattern of eight primary HNSCCs and corresponding metastases and showed that p53 immunostaining is very concordant between tumors and metastases, both with respect to the pattern and the intensity. This allows differential diagnosis of lung carcinomas in patients with HNSCC as SPT by exclusion of clonal relationship using p53 immunostaining. When using our stringent interpretation criteria, the diagnosis of SPT could be established reliably in 5 of 25 cases analyzed. In these five cases, the primary HNSCC and the lung carcinoma showed an extreme difference in the staining pattern and intensity, excluding a clonal relationship. For this reason, immunohistochemistry with these stringent interpretation criteria seems usable in everyday
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clinical practice to identify a small number of SPTs, and might be exploited as the first echelon in the decision algorithm. The criteria we chose to use are quite strict [<50% weak staining (likely wild-type or nonsense mutation) versus intense nuclear staining of >75% of the tumor cells (likely missense mutation)], which is possibly the reason that we could exploit it successfully in this small series. Larger series are required to confirm its utility, using genetic analysis as the gold standard. On the basis of our studies, we decided to place p53 immunostaining in the algorithm, but still with a question mark (Figure 1).

![Classification diagram for interpretation of LOH results](image)

Figure 1. Classification diagram for interpretation of LOH results. The first step evaluates the outcome of immunohistochemistry. When immunostaining indicated a different mutation status in the two tumors (del mutant/wild-type 0-50% vs. mutant >75%), the lung tumor was considered an SPT. We left a question mark in the decision because of the limited validation. The second step evaluates the number of alleles on different chromosome arms with discordant LOH not explainable by progression (LOHx-LOHy or LOH-ROH). The third step examines the number of chromosome arms with concordant LOH (LOHx-LOHx). The fourth step examines all forms of discordant LOH thus including ROH-LOH (which might be explained by tumor progression).

In our series, a relatively high number of lung carcinomas were diagnosed as SPT (20 of 28 that could be differentially diagnosed reliably). Other studies showed lower percentages of SPT compared with DM (3 of 9 that could be differentially diagnosed reliably). The high ratio of SPT in our study is likely due to the selection criteria of the patient group and the retrospective nature of the study. We included patients with SCC of the lung following HNSCC with enough available...
material to allow LOH analysis and TP53 mutation analysis. This material was obtained either by resection or biopsies of the lung carcinoma. These procedures are done particularly in patients when scheduled for resection with curative intent or when biopsies are taken to find out whether there is a possibility to treat. Patients who are highly suspected to have DM by clinical criteria (e.g., multiorgan metastasis) are not routinely biopsied or treated. A second explanation might be the relatively large number of laryngeal cancers in the patient group (n =21; 54%). These carcinomas are less likely to metastasize and are more often accompanied by second primary lung carcinomas.18

In conclusion, with TP53 mutation analysis as the gold standard, LOH analysis with the marker panel and algorithm proposed based on our data seems reliable and may be applicable in clinical practice (figure 1). However, these data concern, at present, a single institute retrospective analysis on a relatively small number of patients. Planning of a large prospective multicenter trial will be required to both proof the clinical utility of this approach and determine the association with clinical outcome. Based on such data, the management of treated HNSCC patients might be adapted.

References