# Molecular approaches to the staging of head and neck carcinomas (Review)

DANIELA GASPAROTTO and ROBERTA MAESTRO

Experimental Oncology 1, CRO-IRCCS, Aviano National Cancer Institute, I-33081 Aviano (PN), Italy

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**Abstract.** Despite the efforts during the last decades to improve clinical management and therapy, carcinomas of the head and neck still represent a disease with an unfavourable course. Over 50% of the patients die from their disease within 5 years after diagnosis, with tumour recurrence, metastasis, and development of second primary neoplasms as major causes of treatment failure. In addition, surgical treatment of locally advanced disease often results in invalidating and disfiguring conditions that heavily affect patients' quality of life. Current criteria of tumour staging, essentially based on clinical and pathological assessment, fail to prove effective in providing reliable information on tumour prognosis or supporting an optimized planning of the therapies, prompting the identification of new and more accurate staging criteria. This review focuses on the impact of molecular biology in HNSCC staging, namely differentiation of second primary lesions from recurrence/metastasis and detection of lymph node micrometastasis, and highlights how the integration of the histopathological diagnosis with molecular analyses may result in a better management of HNSCC patients.

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## 1. Introduction

Head and neck tumours, consisting mainly of squamous cell carcinomas of the oral cavity, pharynx, and larynx (HNSCC), account for approximately 3% of new cancer cases, with an incidence rate of approximately 500,000 new cases per year worldwide. These tumours represent the fourth most common tumour type in developing countries and the sixth in Western countries, where they are essentially associated to alcohol abuse and cigarette smoking (1,2).

HNSCCs include an array of pathological entities with variable presentation patterns and biological behaviour. Despite efforts being made to improve clinical management of this neoplasm in the last decades, HNSCC remains an invalidating and disfiguring disease associated with a high mortality rate (3-5). Size and site of the neoplasm, degree of differentiation, lymph node involvement, and presence of distant metastases or second primary tumours are the major factors affecting HNSCC prognosis (3). Unfortunately, the majority of HNSCC are already loco-regionally advanced (stages III and IV) at the diagnosis, and surgery followed by chemo/radiation therapy represents the conventional approach for these tumours. Aggressive surgical strategies, which involve removal of essential components of the phonatory and masticatory apparatuses, besides heavily affecting the quality of life of patients, only marginally impact on long-term control of the disease. Thus, over 50% of patients die within 5 years because of local relapse, tumour spreading, or development of second primary tumours in the aerodigestive tract (3,6-8). The set up of integrated chemo-radiotherapeutic approaches, whenever possible associated with reconstructive surgery (organ preservation trials), although allowing a large fraction of patients to avoid laryngectomy, failed to significantly improve the overall survival (3-5). These discouraging results are only in part due to the intrinsic aggressiveness of this type of tumour. *De facto*, current HNSCC staging criteria fail to differentiate patients who are likely to recur or metastasize (and hence might benefit from more aggressive approaches) from patients that are affected by more benign forms of cancer. Taken together, these facts urge the identification of new and more reliable diagnostic markers that may allow clinicians to plan optimised risk-adjusted strategies.

One of the major problems that clinicians face in treating an HNSCC patient who relapses after an apparent complete remission is to discriminate whether the outgrowth is actually a local recurrence or a new, completely independent entity. This dilemma is a consequence of the fact that the epithelium of the aerodigestive tract in HNSCC patients shows signs of 'field cancerization', a condition in which the mucosa is primed to the development of multiple and independent transformation events, as a consequence of alcohol and tobacco smoke-associated carcinogens (9). It is estimated that at least 3-7% of

*Correspondence to:* Dr Roberta Maestro, Experimental Oncology 1, CRO-IRCCS, Aviano National Cancer Institute, via F. Gallini 2, I-33081 Aviano (PN), Italy
E-mail: maestro@cro.it

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HNSCC patients develop second primary tumours, synchronous or metachronous (10-14). Therefore, the appearance of a new lesion proximal to the site of the first tumour poses a problem of differential diagnosis.

The same diagnostic uncertainty holds true when HNSCC patients exhibit lung lesions. In fact, the lung is the principal site of metastatic spreading for HNSCC. On the other hand, a large fraction of pulmonary neoplasms, similar to HNSCC, are of squamous histology, and the major risk factors for HNSCC (i.e., tobacco smoke and alcohol) are also risk factors for lung cancer. Therefore, these types of tumours may easily co-develop in the same patient. On these grounds, a differential diagnosis of metastasis of HNSCC versus primary lung carcinomas is hardly feasible on the basis of the sole clinical and pathological examinations. This is not trivial, as the therapeutic approach to metastatic disease differs from that to limited disease. In fact, a second primary lesion may justify either a surgical targeted approach or the repetition of the very same type of therapeutic regimen which has proven curative in the first instance; rather, a more aggressive approach is advised in the case of metastatic disease.

At present, no clear-cut diagnostic criteria that allow a reliable classification of multiple tumours exist. The criteria originally suggested by Warren and Gates in 1932 (tumours must be both malignant and distant enough to be considered distinct; moreover, the possibility of being a metastasis one of the other must be excluded) and subsequent refinements (tumours arising in the same anatomical region should be distant to each other by at least 2 cm and separated by normal mucosa; a minimum of a 3-year interval should elapse between primary and secondary lesion) are definitively ill-defined and arbitrary, and hence a source of confusion and mistakes (15,16).

Because of the subjective decision making, it is clear that the current clinical definition of tumour multiplicity carries the risk of misclassification and mistreatment. Thus, the identification of more sensitive markers for the differentiation of second primary tumour, recurrence or metastasis is mandatory for an appropriate management of HNSCC patients.

## 2. Molecular staging of HNSCC

Cell transformation is considered the result of the progressive accumulation of molecular defects affecting genes involved in the control of cell proliferation, death and differentiation, namely oncogenes and tumour suppressor genes. In the last two decades a number of studies have identified several gene alterations involved in HNSCC development and have provided insight on the frequency and timing of these events during HNSCC progression (17-19). These studies have laid the ground for the development of more specific and sensitive diagnostic procedures and more effective therapeutic approaches. In particular, the value of molecular techniques in the diagnosis of HNSCCs has been validated by several recent studies.

In this review, we will summarize two of the most significant applications of molecular analyses in the diagnosis and staging of HNSCCs: i) the differentiation of tumour recurrence/metastasis from second primary tumours, ii) the detection of lymph node micrometastasis.

*Molecular assessment of tumour clonality: discrimination of tumour recurrence/metastasis versus second primary tumour.* Several different molecular strategies have been proposed to tackle the problem of the differential diagnosis between recurrence/metastasis and independent primary tumours in the context of HNSCC.

Sidransky *et al* used the pattern of X chromosome inactivation to assess the clonal relationship between primary and secondary neoplasms in a series of female patients affected by bladder cancer (20). Unfortunately, since most HNSCC patients are male, this strategy has limited usefulness in the context of tumours of the upper respiratory tract. Other cytogenetic techniques (tumour karyotyping and FISH) proved to be complex and scarcely informative (21,22).

A step forward was represented by p53 mutation analysis. Mutations of p53 affect over 60% of HNSCCs, occur very early during tumour development and are maintained throughout HNSCC progression (23-30). Moreover p53 mutational spectrum is extremely polymorphic, involving over 200 different amino acids (mostly between exons 5-8). Thus, two HNSCC with a different p53 gene status are likely to be two independent lesions. On the whole, these features render p53 mutational spectrum apt to be used as a clonality marker. On this ground, Noguchi *et al* (31) compared the mutational spectrum of a series of matched HNSCC/lung and lung/lung synchronous or metachronous lesions and were able to make a differential diagnosis of metastatic disease versus second primary cancer in 6 out of 9 tumour pairs analyzed. Similarly, by this approach our group carried out a retrospective study in a series of metachronous HNSCC tumours (32). In approximately 40% of the cases analyzed suitable for molecular diagnosis we were able to appraise the independent origin of the two lesions, based on the discordance of p53 mutational spectrum. In approximately 60% of the cases the same mutation was detected in the primary and secondary neoplasm, allowing a molecular diagnosis of recurrence. Intriguingly, since in one of these cases the recurrence occurred 9 years after the initial diagnosis, it had been classified by clinicians as an independent tumour according to Warren and Gates' criteria. The same analysis was performed on 2 lung cancers arisen 2 and 6 years, respectively, after the initial diagnosis of HNSCC. In both cases the discrepancy of p53 mutation pattern allowed us to rule out the metastatic nature (32).

Also the analysis of the pattern of chromosome deletion has proven very useful in the appraisal of tumour multiplicity. During tumour growth, cells often lose chromosome segments, with some regions undergoing loss of material very precociously. For instance, the loss of the short arm of chromosomes 3, 9, and 17 is a common feature of the hyperplastic and dysplastic mucosa of the aerodigestive tract (3,18, 33-35), while other chromosome regions, such as chromosome 13 or 18, tend to be lost late in HNSCC progression (36-43). Chromosomes 3, 9, and 17 harbour tumour suppressor loci whose loss of function is involved in the early phases of tumour development. The extent of each chromosome loss and the involvement of either maternal or paternal chromosome (which can be distinguished on the bases of DNA population polymorphisms) produce a distinct genetic signature, featuring as a tumour marker. To this end, analysis of microsatellite polymorphisms has proven very effective.

Microsatellites are non-coding DNA regions constituted by repetitive oligonucleotidic motifs whose length may be different from individual to individual, although genetically inherited. The variable length of these regions produces an elevated number of possible alternative alleles that accounts for the common condition of heterozygosity in the population. On these grounds, microsatellite analysis proves highly informative for tracking tumour-specific chromosome losses (or loss of heterozygosity, LOH). Moreover, since the pattern of LOH represents a tumour signature, a concordant LOH pattern is highly suggestive of clonal relationship, while a discordant pattern supports an independent origin for two tumours.

Leong *et al* (16) used microsatellite-based analysis of LOH at 10 polymorphic loci on chromosomes 3p and 9p to assess the clonal relationship between HNSCCs and squamous cell carcinomas of the lung. In 10 out of the 16 cases analyzed, HNSCC and matched lung lesion showed an identical pattern of LOH for all the informative loci, suggesting metastatic spread; in contrast, in three patients the paired tumours had discordant patterns of loss, therefore supporting an independent origin. Molecular data were substantiated by the clinical and pathological findings.

Although LOH analysis has been widely used to assess tumour multiplicity (44-47), several authors warned on the accurate selection of type and number of microsatellite markers to be used for clonality studies. Tabor *et al* (48) demonstrated that while assessment of HNSCC clonality provided reliable results if performed using a certain number of loci mapping on chromosomes 3p, 9p and 17p, it failed to be sound when chromosomes 8p, 13q and 18q were analyzed. These chromosome regions harbour genes involved in tumour progression (49). Therefore, the ascertainment of tumour multiplicity should be performed by using genetic events, including LOH, that mark the early phases of cell transformation, excluding those that tend to be acquired during tumour spreading.

In line with this concept, Geurts *et al* (50) developed an accurate interpretation scheme for the use of LOH as a clonality marker in the context of HNSCC. By this scheme, these authors analyzed a series of patients with a previous diagnosis of HNSCC, who subsequently developed a squamous cell carcinoma of the lung. Thirty-eight out of 44 cases had been classified as metastatic according to clinical-pathological criteria, while 6 were diagnosed with second primary tumours. Strikingly, the diagnosis of metastatic disease was confirmed only in 19 out of the 38 informative cases by LOH analysis; in the remaining 19 cases the molecular analysis supported, instead, an independent origin. Thus, this study provides evidence that a considerable number of HNSCC patients with lung lesions (50%) are erroneously treated by clinicians as metastatic, while a surgical approach with curative intent would be justified, according to molecular data.

*Molecular assessment of lymph node involvement*. Proximal lymph nodes are the major route of dissemination for HNSCC, and the nodal status represents the most important predictor of disease outcome. Accurate staging of cervical lymph nodes is therefore fundamental for prognosis and planning of therapeutic treatments.

Clinical staging (which relies on physical examination and imaging techniques) and pathological examinations (tumour and node stage, histological grade, and perineural invasion) have limited sensitivity and can miss early nodal disease, i.e., isolated cancer cells and micrometastases. On this ground, lymph node resection is performed not only in the presence of evident signs of cancer dissemination but also on the basis of a theoretical risk of metastatic disease, deduced from clinical and histopathological parameters of the primary tumour (51). Thus, currently also a fraction of patients devoid of metastases undergo lymph node resection. The sentinel lymph node (SLN) strategy has been developed to avoid the morbidity of unnecessary neck dissection. By this approach a large number of serial lymph node sections are scored for cytological and immunohistochemical (e.g., expression of cytokeratins) evidence of epithelial tumour cells. Unfortunately, this procedure is very time consuming and, therefore, can hardly be employed in routine applications. Moreover, the reliability of SLN examination significantly decreases as tumour cell numbers decrease. In fact, a worrying discrepancy among different laboratories has been observed as for the detection of small numbers of breast cancer metastatic cells in SLN evaluation (52).

In the attempt to increase accuracy and sensitivity in the detection of micrometastases and to overcome the issue of the subjectivity in assessment procedures, molecularly-based approaches have been implemented. The approaches hinge on the application of the polymerase chain reaction (PCR), a fast and highly sensitive technique, for the detection of tumour-specific markers in the nucleic acids extracted from lymph nodes.

One of the first approaches consisted of the screening of lymph nodes for tumour cells by searching tumour-specific gene alterations. Among these, one of the first to be evaluated was p53. Brennan *et al* (53) assessed the diagnostic power of p53 gene mutation analysis in detecting occult tumour cells in surgical margins and lymph nodes. Noteworthy, approximately 50% of surgical margins and 20% of lymph nodes which were negative at the pathological inspection revealed instead the presence of cells sharing the very same p53 mutation of the index tumour at the molecular analysis. Thus, molecular analysis was more sensitive and accurate than pathological analysis in assessing the presence of infiltrating tumour cells. Accordingly, patients scoring positive at molecular analysis had a significant high risk for recurrence (53). The diagnostic power of p53 gene analysis in assessing the nodal status of HNSCC has been further corroborated by recent studies (54,55) in which an improved mutation detection assay (phage plaque assay) was employed. Molecular diagnosis by mutational analysis of p53 is definitively a highly specific and sensitive approach, but it is affected by a number of caveats including the fact that it is technically cumbersome and time consuming besides being applicable only to a fraction of HNSCCs (only 60% of HNSCCs carry p53 mutation). Therefore, such a 'qualitative' approach hardly meets the requirements for routine diagnosis, particularly in the case of intraoperative analyses.

To overcome these limitations, 'quantitative assays' have been implemented. The rationale of these approaches is the detection in the lymph nodes of gene transcripts typically

expressed by the carcinoma cells, while normally absent or expressed at very low levels in normal lymphoid organs. Thus, the detection of a tumour-specific RNA in a lymph node biopsy is highly suggestive of nodal invasion. These assays hinge on the application of the real-time PCR (RT-PCR), a technique that allows a fast and automated quantisation of specific transcripts. Recent technical improvements (GeneXpert) have rendered this procedure easier and faster, and therefore compatible with an intraoperative molecular tumour staging (56,57).

Hamakawa *et al* (58-60) and Onishi *et al* (61) applied this strategy to assess the presence of HNSCC micrometastases. In particular, these authors evaluated the nodal positivity at RT-PCR for a number of epithelial markers, such as cytokeratins (K13, K19, and K20), SCC antigen (SCCA), CD44v6 and EGFR. Although the occasional presence of ectopic salivary glands might give rise to false positives (thus affecting specificity), the adoption of RT-PCR, particularly for SCC antigen, increased by approximately 20% the sensitivity in the detection of micrometastases with respect to cytological/immunohistochemical examination.

Garrel *et al* (62) found a strong correlation between expression of cytokeratin 17, as assessed by RT-PCR, and the extent of node involvement, further confirming the sensitivity of the molecular approach. Once again, the occasional presence of K17-positive dendritic cells in the healthy lymphatic tissues was occasionally a source of false positives, thus limiting the specificity of K17 as a marker. Similar conclusions were reached by Shores and co-workers (63). They compared the sensitivity and specificity of immunohistochemistry and RT-PCR in the detection of cytokeratin 14-positive cells and concluded that K14 RT-PCR was very sensitive and detected micrometastases in lymph nodes that had been classified as negative by routine pathological examination. Nevertheless, the relatively high rate of false positives prompted the identification of markers more specific to tumour cells.

To this end, Ferris and co-workers (57) screened 40 genes, previously reported to be expressed at high levels in HNSCCs and low levels in normal lymph nodes, and assessed their diagnostic power by RT-PCR. These authors identified 4 transcripts that discriminated between positive and benign nodes with accuracy over 97%: SCCA1/2, PVA, TACSTD1 and PTHrP. Strikingly, the expression of the sole PVA showed a discrimination power of 100%, supporting the use of this molecular marker for routine staging of cervical lymph nodes in HNSCCs.

Nieuwenhuis and co-workers (64) showed that nodal positivity for the gene transcript encoding for the squamous cell specific marker E48 (Ly-6D) was significantly associated with poor survival. The same authors (65) demonstrated that RT-PCR for E48 (Ly-6D) increased the sensitivity and the frequency of reached diagnosis, compared with cytology, in the detection of micrometastases in lymph node aspirates. They concluded that PCR for E48 (Ly-6D) should routinely complement cytological examination. Subsequently, similarly to that reported for other tumour types (66), Colnot *et al* (67) demonstrated that the E48 (Ly-D6) marker could be employed for the evaluation of bone marrow involvement and the identification of a group of HNSCC patients with poorer prognosis.

## 3. Conclusions

Management of HNSCC patients has been long complicated by the lack of effective criteria of tumour staging. Accurate staging is critical since it will determine the therapeutic strategies employed to cure the disease. Recent progresses in the field of molecular biology have disclosed the possibility of improving current criteria of diagnosis and staging of HNSCC.

This review highlights recent studies on the application of molecular approaches to the assessment of tumour multiplicity, otherwise based on arbitrary evaluations, and the detection of occult cancer cells, increasing the sensitivity of 2-3 logs with respect to standard techniques.

The choice of the tumour marker to be used is crucial. An ideal tumour marker is a genetic event that is commonly detected in HNSCC and exclusive of the tumour cell population (absent in normal tissues); if acquired during tumour development, it occurs very early and follows the tumour growth in all phases of progression. Recent technical innovations, that have made molecular analyses relatively quick and cost-effective, will allow a rapid translation of these procedures into the diagnostic routine, laying the foundations for a better management of HNSCC patients.

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