REWIEW

Molecular metastases markers in head and neck squamous cell carcinoma: review of the literature

Markers molecolari di metastasi nei carcinomi squamosi della testa e del collo: revisione della letteratura

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Parole chiave

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Summary

It is now widely accepted that the presence of lymph node metastases is a negative prognostic factor in head and neck squamous cell carcinoma. It follows that the ability to determine the presence of micro-metastases or the metastatic potential of a tumour at an early stage would condition the therapeutic strategy and evolution of this type of tumour. Prediction of the metastatic potential of head and neck squamous cell carcinoma is still, today, entrusted to clinical and histological evaluation of the tumour. However, the high percentage of relapse in this tumour shows the inadequacy of these parameters in predicting metastatic potential. Furthermore, progress made over the last ten years in understanding the molecular mechanisms involved in the process of neoplastic tumour progression has led to the identification of molecules that can be used as potential prognostic markers of head and neck squamous cell carcinoma. There are many molecules involved in the process of forming metastases. This process represents the final stage of a multistep model, in which alterations occur to genes that are important for growth, proliferation and migration, to which are added variations in the expression of molecules involved in the process of homeostasis of the extra-cellular matrix, of angiogenesis and lymphangiogenesis, favouring tumour invasion and the formation of metastases. This review of the literature shows that the tumour invasion process is associated with numerous molecular alterations that might be used as potential prognostic molecular markers. However, none of these alterations is univocally associated with the metastasization used in clinical practice. Further studies on larger series and on a larger scale, such as genome studies, and preclinical studies on markers used as targets in specific therapies, will provide a valuable contribution to their use in clinical practice in the short term.

Riassunto

È ormai ampiamente accettato che la presenza di metastasi linfonodali rappresenta un fattore prognostico negativo nei carcinomi squamosi della testa e del collo (HNSCC). Ne consegue che la precoce individuazione del potenziale metastatico di un tumore mediante l'identificazione di micrometastasi condiziona in maniera favorevole la strategia terapeutica e l'evoluzione di questo tipo di tumori. Ancora oggi, la predizione del potenziale metastatico degli HNSCC è affidata alla valutazione clinica ed istologica del tumore. Tuttavia l'elevata percentuale di recidive di questi tumori dimostra l'inadeguatezza di tali parametri nel predire il potenziale metastatico. Inoltre, i progressi fatti negli ultimi dieci anni nella comprensione dei meccanismi molecolari coinvolti nel processo di progressione neoplastica dei tumori hanno portato all'identificazione di alterazioni geniche e molecolari, utilizzabili come potenziali markers prognostici degli HNSCC. Le alterazioni geniche e molecolari coinvolte nel processo di formazione di metastasi sono numerose. La metastatizzazione rappresenta la tappa finale di un modello di carcinogenesi "multistep", in cui avvengono e si accumulano nel tempo alterazioni in geni importanti per la crescita, la proliferazione e la migrazione ed a cui si aggiungono variazioni dell'espressione di molecole coinvolte nel processo di omeostasi della matrice extracellulare, di angiogenesi e linfoangiogenesi, favorenti l'invasione del tumore e la formazione di metastasi. Lo scopo che questo studio si pone è quello di cercare di identificare, tramite una revisione della letteratura e di contributi personali, quelle alterazioni molecolari più frequentemente associate al processo di invasione tumorale e metastatizzazione, che possono essere utilizzate come potenziali markers prognostici. Tuttavia, ciò che emerge da questa breve analisi ci porta a concludere che, non essendo stata riscontrata alcuna associazione univoca tra un'alterazione genica o molecolare ed il processo di metastatizzazione, al momento, nessuna di esse può essere utilizzata nella pratica clinica. Ulteriori studi su casistiche più ampie e su larga scala, quali quelli di gnomica e proteomica e studi preclinici sull'utilizzo di tali marcatori molecolari come target di terapie specifiche, sicuramente forniranno un vantaggioso contributo al loro utilizzo nella pratica clinica entro breve termine.

Review

The poor prognosis for patients affected by advanced head and neck squamous cell carcinoma (HNSCC) is often related to frequent local and regional recurrence, and it is widely accepted that the presence of lymph node metastases is the most prognostic factor for these tumours. It therefore follows that assessment of cervical lymph nodes is an important step both in pre-operative and post-operative management of HNSCC patients. At present, from the clinical standpoint, some evidence linked to the histological evaluation of the primary tumour and of the loco-regional lymph nodes, such as lymph node capsular rupture (R) and tumoural intra-vascular embolization (E) may be considered absolutely predictive of the metastasization process [1 2]. Also of prognostic value, with regard both to loco-regional recurrence and distant metastasis, are tumour thickness, degree of differentiation, the pattern of tumour invasion within the stroma, the host inflammatory response, bone and/or cartilage invasion by the tumour and positive margins after surgical excision [3 4]. However, these clinical prognostic parameters often underestimate the presence of involved lymph-nodes and the frequency of early metastatic relapse indicates that a number of disseminated tumour cells are undetectable by current methods. It is, therefore, mandatory to find both a method and a marker to detect and to diagnose microscopic and occult metastases. In the last decade, the improvement in molecular biology techniques has allowed studies to proliferate in which the predictive significance of tumour-related molecular factors, in regard to the metastasization process, has been sought. It is now well known that the process of neoplastic progression, which culminates with metastasization, is favoured, from the genetic standpoint, by the activation of oncogenes and/or by the inactivation or inhibition of onco-suppressor genes. These events, which accumulate progressively over time following the "multistep" model of carcinogenesis [5], are the causes of a "genetic-molecular imbalance" applicable also to the biology of HNSCC tumours, which is also favoured by some host-linked conditions, such as smoking and drinking or HPV infection [6 7]. Although activation of oncogenes and/or inhibition of onco-suppressor genes is closely associated with the neoplastic progression process, nevertheless neither of these alterations appears to be univocally linked to the metastasization process. If anything, metastasization is the result of the acquisition of a more invasive phenotype by some cell clones, which gives them a stronger migratory capability. For this reason, the formation of metastases, once what we might call the oncogenetic phase is completed, to which a greater proliferative capability is associated, appears to be closely conditioned by the capability of some cells to penetrate into blood and lymphatic vessels at the level of the primary tumour and to leave these vessels at the level of the loco-regional or distant metastases. These latter events are related, from the molecular standpoint, to the alteration of the expression of adhesion molecules, to the secretion of proteolytic enzymes, metalloproteinases and cytokines having angiogenetic and immunosuppressive action. In brief, the steps of the metastasization process may be outlined as follows:

1. genetic alterations able to induce the invasive-metastatic phenotype during tumour progression (oncogene activation and/or tumour suppressor gene inactivation);
2. breakdown in cell-cell and cell-matrix adhesion (tumour cell detachment from the primary tumour and basement membrane invasion);
3. tumour cell migration;
4. modulation of extra-cellular matrix (ECM) and proteolysis;
5. angiogenesis.

Genetic alterations capable of inducing the invasive-metastatic phenotype during tumour progression

The most common change, occurring as an early event during HNSCC development, is the loss of chromosomal region 9p21, which contains the p16 gene, an inhibitor of cyclin-dependent kinase (CDK) [8 9]. Others genomic alterations correlated with HNSCC are the LOH of 17p13 and the amplification of 11q13, coding respectively for p53 and cyclin D1 proteins. Although some studies report genomic alterations of 3p, 4q, 6p,11q, and 14q, as predictor of progression to malignancy, none of these alterations has been definitely linked to metastases [10-15].

Breakdown in cell-cell and cell-matrix adhesion

After tumour initiation, one of the first steps in the metastatic process is the loss of epithelial integrity through a reduced cell-cell and cell-matrix adhesion involving the adhesion molecules [16]. The adhesion molecules involved in the metastatic process belong to four major protein families: the cadherins, integrins, selectins and immunoglobulins. These proteins interact with basement membrane molecules and regulate the cell-cell and cell-extracellular matrix spatial and metabolic interactions.

E-cadherins

The E-cadherin molecules are expressed by all normal epithelia and mediate the cell-cell adhesion in a

calcium-dependent manner through the formation of complex homodimers/homodimer complexes. In patients with HNSCC, the loss of expression of components of the E-cadherin complex is a common abnormality, and reduced or aberrant expression of E-cadherin protein has been associated with the presence of cervical metastases [17-19]. A recent study by Gasparoni et al. [20] showed higher levels of E-cadherin in normal cell line cultures than in HNSCC cell cultures**.** In a study on 200 cases of primary HNSCC, reduced E-cadherin expression was a prognostic factor, as determined by multivariate analysis, independent of tumour stage, site or histological grading. Notably, the E-cadherin level was not significantly associated with tumour stage and nodal status at the initial diagnosis, but during follow-up was strongly associated with subsequent metastatic spread to regional lymph nodes and distant sites. Moreover, E-cadherin was also very significantly associated with local recurrences ($p < 0.0001$) [18].

Integrins

Integrins are a family of heterodimeric transmembrane glycoproteins, composed of non-covalently associated transmembrane alpha and beta subunits, expressed by diverse cell types, mainly of epithelial origin. Integrins function as major receptors, either for extracellular matrix components mediating interaction between cell and substrate (SAM – substrate adhesion molecules) or as cell-to-cell adhesion molecules (CAM – cell adhesion molecules). To date, approximately 16 alpha and 8 beta subunits have been identified [21]. More recently, integrins have been shown to play significant roles in malignant tumour migration, extension, and metastasis [22-24]. Additionally, aberrant integrin expression has been implicated in HNSCC tumour invasion and metastasis. The most commonly expressed integrin in the basal layer of the normal squamous cell epithelia is $\alpha v\beta 6$, and its altered expression has been reported in the development of HNSCC [25 26]. In HNSCC, Cortesina et al. [27] described the different patterns of expression of integrins ($\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$) and extracellular matrix proteins (laminins 1 and 5, collagen type IV and two fibronectin isoforms: ED-A and ED-B) in normal and transformed mucosa of laryngeal and oropharyngeal carcinomas from 40 patients. They found an altered topographical pattern of integrins in both groups, although the extent of changes was significantly more marked in oropharyngeal tumours, which are known to have a poor prognosis. Their findings suggest that the molecular patterns of expression of integrins may be used as an additional prognostic factor of biological tumour aggressiveness in oropharyngeal tumours. A Japanese study on 65 biopsy specimens of oral HNSCC reported similar results [28]. The study demonstrated a statistically significant correlation between the expression of integrins $\alpha 2$, $\alpha 3$, and $\alpha 6$ and histologic invasiveness ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively), degree of differentiation ($p < 0.05$, $p < 0.05$, $p < 0.001$) and nodal involvement ($p < 0.001$, $p < 0.05$, $p < 0.001$).

Tumour cell migration

The acquisition of cell motility plays a central role in the complex multi-step process of metastasis. Increased cell motility (migration) is a critical component of cancer-cell invasion at the primary site (allowing cells to gain access to the vasculature), and it also allows cells to penetrate the host tissue at distant sites. The process of tumour progression to the metastatic state is facilitated by mutations in signalling molecules, which transduce signals necessary to stimulate cell movement, thus increasing cell motility.

MET oncogene

An example of a molecule influencing metastatic potential by affecting cell motility is the MET proto-oncogene encoding the tyrosine kinase receptor for Scatter Factor (also known as Hepatocyte Growth Factor, SF/HGF). HGF is a growth factor that, besides promoting cell proliferation and survival, can dissociate epithelial sheets and stimulate cell motility. A direct role of MET receptor signalling in the metastatic behaviour of human cancers has been proposed, based on experimental evidence [29-31]. The deregulated control of the invasive-growth phenotype by oncogenically activated MET might give cancer cells invasive and metastatic proprerties. The role of the MET gene in the progression of HNSCC was thoroughly investigated by Cortesina et al., in a series of studies. In a preliminary study, they analyzed a large number of HNSCC and their metastases, and found that the MET oncogene was overexpressed in most specimens [32]. A total of 151 lymph nodes from 20 patients were studied with both in-depth histology and real-time quantitative reverse-transcription-polymerase chain reaction (RT-PCR) [33]. MET-encoded sequences were found in 61/151 nodes (40%), 24 (16%) of which were found to be metastatic at histopathology. Parallel routine histopathologic analysis of 654 lymph nodes from the same cases identified 36 metastases (5%). These data indicate that the MET gene product might be a valuable marker with which to detect occult tumour cells in lymph nodes, thanks to its high expression in metastatic cells. Interestingly, Di Renzo et al. [34], found mutations in the MET oncogene in lymph node metastases of HNSCC; they demonstrated that transcripts of the mutant alleles were highly represented in metastases but barely detectable in primary tumours, suggesting that cells carrying the MET mutations were selected

during metastatic spread. Taken together, these data indicate that cells expressing mutant MET undergo clonal expansion during HNSCC progression, and suggest that MET might be one of the long-sought oncogenes controlling progression of primary cancers to metastasis.

Epidermal growth factor receptor

The EGFR family has four members (EGFR/c-erbB-1, c-erbB-2/Her-2, *neu*, c-erbB-3/Her-3 and c-erbB4/Her-4), all transmembrane proteins, which have tyrosine kinase activity and are involved in tumour growth and malignant transformation [35 36]. The role of the EGFR family in HNSCC has been thoroughly investigated [37-41]. Several studies report an independent correlation of EGFR members with the presence of nodal metastases and poor clinical outcome [42-44]. The most widely accepted hypothesis is that the cooperative signalling of all four EGFR receptor members may play a significant role in the metastatic potential of HNSCC. Indeed, although HER2/*neu* is an orphan receptor, its ability to form heterodimers with other EGF receptor family members, also over-expressed in HNSCC, enhances proliferation and invasion in HNSCC cells, thus activating the MAPK and PI3K downstream signalling pathways involved in the transcriptional regulation of proteases and cytokines [45].

Modulation of extra-cellular matrix (ECM) and proteolysis

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that play an important role in tumour invasiveness: they remove physical barriers by degrading ECM macromolecules including the basement membrane (BM). There is strong evidence that MMP-1, MMP-2, MMP-9 and MT-1 MMP are related to the metastatic potential in HNSCC, but the prognostic value of these regulators remains unclear [46]. Several studies have associated MMP-2 and MMP-9 with lymph node metastasis and poor outcome in laryngeal cancer [47 48]. Furthermore, micro-array gene expression studies on whole HNSCC tumour samples have identified over-expression of MMP-1, MMP-2, and MMP-3 in HNSCC [49-51]. In an attempt to clarify the involvement of MMPs in the HNSCC metastatic process, Wiegand et al. [52], performed a meta-analysis comprising 14 studies and totaling 710 patients. Their aim was to determine whether there is a correlation between MMP expression and lymphatic metastatic spread that would help in predicting the presence of lymph node metastases. The Authors concluded that, despite the heterogeneity of the studies published, the results suggested an increased risk for lymph node metastases in patients whose HNSCC tumours are positive for MMP-2, MMP-3 and MMP14. Moreover, considerable evidence supports the hypothesis that the up-regulation of MMPs in HNSCC is mediated by epidermal growth factor receptor (EGFR) signalling, uPA and integrins [42 53]. Other molecules, such as tissue inhibitors of metalloproteinases (TIMPs) or plasminogen activator inhibitors (PAIs), may function as metastasis suppressor proteins in HNSCC by inhibiting tumour-cell invasion of the ECM. A significant correlation between expression of TIMP-2 and metastatic lymph node status has been reported [48 54].

Cathepsin D

Cathepsins are lysosomal endopeptidases, and their altered secretion in malignant cells is presumed to function in the digestion of ECM components. Cathepsin D (Cath-D) is over-expressed in various types of human carcinomas, its concentration in the primary tumour being closely correlated with an increased risk of metastasis. In HNSCC, the variable expression of Cath-B and -D correlates with highly invasive and metastatic phenotypes of oral cancer. High levels of Cath-D expression were observed in oral carcinomas (OSCC) with regional lymph node metastasis (pN1/pN2) compared with node-negative tumours (pN0), in a series of 63 patients examined by immunohistochemical analysis [55]. Gandour-Edwards et al. [56] found a close association between the expression of Cath-D and cervical lymph nodal metastasis ($p = 0.008$) in a series of 34 HNSCC patients. In a study on 63 patients with primary laryngeal squamous-cell carcinoma, followed up for a median of 33 months after surgery, Maurizi et al. [57], showed that Cath-D levels were correlated with neck lymph node metastasis; they also demonstrated, by multivariate analysis, that Cath-D status is an independent factor for predicting short metastasis-free survival, and suggest that Cath-D assay may prove to be particularly useful in identifying laryngeal cancer patients who, with or without neck lymph node involvement at presentation, are at high risk of metastatic disease and poor outcome.

Angiogenesis

The process of angiogenesis is the result of an imbalance between pro- and anti-angiogenic factors produced both by tumour and normal cells, which may promote or limit endothelial proliferation and the formation of new blood vessels [58]. These new blood vessels provide the principal route by which tumour cells leave the primary tumour site and enter the circulation. It therefore follows that angiogenesis is essential for the progression of malignant tumours

and the development of metastases [59]. Although angiogenesis is difficult to measure directly in human tumours, there is increasing evidence that microvessel density (MVD) may be considered an indirect marker of neo-angiogenesis [60]. The most common antibodies used for microvessel staining thus far are those against Von Willebrand Factor (Factor VIII), CD31 and CD34. However, the accuracy of MVD assessment with these markers may not be the highest possible, as their sensitivity and specificity are not optimal: these pan-endothelial markers cannot distinguish proliferating endothelium in tissue undergoing angiogenesis from normal pre-existing blood vessels. In addition, either the anti-endothelial marker and antigen retrieval method used or the system used to count the microvessels may lead to conflicting results. Consequently, some studies of HNSCC have found a significant association between MVD, unfavourable clinico-pathological parameters and a poor prognosis, whereas others have failed to demonstrate any relationship [61-68]. Interestingly, a number of studies have recently shown the prognostic impact of CD105 in tumour angiogenesis for HNSCCs. CD105 (endoglin) is a homodimeric membrane glycoprotein that binds transforming growth factor-β1 (TGF-β1) and TGF-β3 [69], and is preferentially expressed in proliferating vessels during angiogenesis in tumour areas, while it is absent or weakly expressed in blood vessels of normal tissues [70 71]. Schimming and Marmè [72] were the first to analyze CD105 expression in 51 SCCs of the oral cavity and in adjacent normal mucosa; they showed that endoglin expression in neoplastic tissue was significantly higher than in normal mucosa, and that it was correlated with tumour size (endoglin expression by T1 tumours was significantly lower than that expressed by T2, T3 and T4 tumours). The study also showed that oral cavity SCCs with histologically proven regional metastases presented higher endoglin expression than SCCs without metastases. Martone et al. [73], analysed both CD105 and CD34 expression in 127 consecutive cases of head and neck primary SCCs. CD105-assessed MVD was significantly higher in N+ tumours. Patients with high MVD had significantly shorter disease-free and overall survival. Multivariate analysis showed that high CD105-MVD was the only independent marker of tumour recurrence or death. On the contrary, Marioni et al. [74], investigated both the relation between CD105-assessed MVD and pathological features (pT, pN, G) and the relation between MVD and prognostic features (locoregional SCC recurrence, post-treatment outcome) in a total of 26 patients with oral and oropharyngeal SCCs. Multivariate logistic regression that considered MVD, pT and pN showed that CD105-assessed MVD was the only feature significantly related to the patient's post-treatment outcome ($p = 0.049$). They did not find any significant correlation between CD105-assessed MVD and N lymph node status. An alternative approach to the study of angiogenesis is to assess the expression of angiogenic molecules in human tumours. The most important angiogenic factor appears to be the vascular endothelial growth factor (VEGF), which is known to stimulate the proliferation of endothelial cells. At least six members of the VEGF family have been identified (VEGF-A, -B, -C, -D, and -E). In HNSCC, increased expression of VEGF-A and VEGF-C has been correlated with tumour progression and lymph node metastasis [75 76]. Furthermore, co-expression and up-regulation of both VEGF and interleukin-8 (IL-8) have been associated with aggressive tumour growth and decreased survival in HNSCC [77]. Other evidence suggests that the formation of tumour-associated lymphatic vessels may play an active role in the metastatic spread of several human malignancies, including HNSCC [78 79]. One report [80] investigated tumour lymphangiogenesis in a series of 52 HNSCC patients, and assessed whether the extent of tumour lymphangiogenesis, determined within the tumour and in the peritumoural area, was correlated with the presence of lymph node metastasis and with the patient's clinical outcome. The results have supported the hypothesis that tumour-associated lymphangiogenesis is an active process that plays a clinically relevant role as the route for lymphatic dissemination in HNSCC, because peritumoural LVD (lymphatic vessel density) and relative vascular area were found by multivariate analysis to be independently related to lymph node metastasis.

Chemokine receptors

Chemokine molecules constitute a super-family of inducible, secreted, pro-inflammatory proteins involved in a variety of immune responses, acting primarily as chemo-attractants and activators of specific types of leukocytes [81]. Recent studies have shown the involvement of chemokine receptors in cancer metastasis [82-84]. In HNSCC, it may be hypothesized that, during the process of lymph node metastasis, tumour cells use a chemokine-mediated mechanism because, in general, metastases are cervical, and at a single site, whereas distant metastases are infrequent. In particular, a recent study [85] has demonstrated, by quantitive RT-PCR analysis, high levels of expression of CCR7 and CCR6 down-regulation in both HNSCC metastatic cell lines and primary tumour tissues. The Authors argue that metastatic HNSCC cells down-regulate CCR6 and up-regulate CCR7, exploiting an immune mechanism common to dendritic cells and memory T lymphocytes, to migrate to the regional lymph nodes. They also link receptor expression to the metastatic destination of tumour cells.

Conclusion

Although clinical staging (TNM) and pathological grading of differentiation remain the most important factors in predicting the prognosis of HNSCC, over the last decade several clinical studies have demonstrated a prognostic role of several molecules involved in the tumour progression process and metastasization. Many of these molecules have been shown to be potentially useful markers of poor prognosis, on account of their ability to assess or predict the presence of metastases. However, none of them is currently employed in clinical practice, probably because the analysis of their expression, generally through immunohistochemical or quantitative techniques, has shown an involvement of these molecules in tumour progression, without, however, being able to attribute to them, with any certainty, a specific marker role for metastases of HNSCC. This is plausible, if we think that the development of metastases depends on the migration of tumour cells, the destruction of barriers, the development of new blood vessels, the availability of lymphatic vessels and the presence of phenotypic characteristics that allow for growth, survival, resistance to apoptosis and evasion of immune defenses. All these processes involve the alteration of numerous pathways, and to evaluate them requires the contemporary evaluation and analysis of many molecular markers. In addition to this, it must be borne in mind that the data reported are very mixed, leading to contrasting results. This lack of data uniformity may be ascribed to the wide variation in the methods used, such as the use of different antibodies that recognize different epitopes of the proteins analyzed, the inevitable lack of uniformity when assays are performed in different laboratories, different interpretation of results, e.g. selection of different cut-off points, leading to qualitative and quantitative differences, and lastly to the small number of cases analyzed.

However, we believe that in the short term, development of increasingly accurate molecular analysis on a large scale, such as gene expression profiling of human cancers, by DNA micro-array technologies that enable the simultaneous expression of thousands of genes to be compared in patients with and without metastases, may provide a "metastasis signature" that may predict the metastatic potential of the primary tumour, also based on anatomic tumour subsites. We also believe that the inability to find a specific marker of metastases in HNSCC must also be attributed to the lack of animal models of HNSCC metastases and of pre-clinical studies in which these markers are used as specific targets. The discovery of a significant molecular marker able to predict cervical lymph node metastasis from a primary tumour biopsy, in addition to the traditional clinico-pathological parameters, might provide clinicians the opportunity to establish new diagnostic strategies, therapeutic integration and more careful follow-up.

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