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Gene Expression Signatures of Lymph Node Metastasis in Oral Cancer: Molecular Characteristics and Clinical Significances

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Abstract

Even though lymph node metastasis accounts for the vast majority of cancer death in patients with oral cancer (OC), the molecular mechanisms of lymph node metastasis remain elusive. Genome-wide microarray analyses and functional studies *in vitro* and *in vivo*, along with detailed clinical observations, have identified a number of molecules that may contribute to lymph node metastasis. These include lymphangiogenic cytokines, cell adhesion molecules, basement membrane-interacting molecules, matrix enzymes and relevant downstream signaling pathways. However, defined gene signatures from different studies are highly variable, which hinders their translation to clinically relevant applications. To date, none of the identified signatures or molecular biomarkers has been successfully implemented as a diagnostic or prognostic tool applicable to routine clinical practice. In this review, we will first introduce the significance of lymph node metastasis in OC, and clinical/experimental evidences that support the underlying molecular mechanisms. We will then provide a comprehensive review and integrative analysis of the existing gene expression studies that aim to identify the metastasis-related signatures in OC. Finally, the remaining challenges will be discussed and our insights on future directions will be provided.

Keywords

Oral cancer; gene expression profiling; lymph node metastasis; microarray; diagnosis and prognosis; biomarker

1. INTRODUCTION

1.1. Oral Cancer and Lymph Node Metastasis

Oral cancer (OC) is the sixth most common malignancy in humans, occurring with an increasing frequency worldwide. This increase in frequency may be due to greater general life-expectancy and an increase in alcohol and tobacco consumption [1], and involvement of new etiologic factors (e.g., HPV infection). Histologically, over 90% of OCs are classified as squamous cell carcinoma (OSCC) and are derived from the epithelium lining of the oral cavity. Despite current advancements in surgery, radiation and chemotherapy, OSCC treatment still has a high failure rate as many patients present with loco-regional metastasis at the time of presentation, or subsequently develop metastasis after initial diagnosis. Postoperative histological examination shows that approximately one third of patients clinically diagnosed with negative neck (cN0) have metastasis-positive lymph nodes in the neck [2–4]. Based on our survey of 11 published studies, the incidence of false negative diagnoses of nodal metastasis can be as high as 47% (with an average of 32.4%) for T1-T2 oral tongue SCC [5–15]. The 5-year survival for patients with lymph node metastasis at presentation dramatically decreases to 25–40%, compared to approximately 90% of patients without metastasis [16]. Clearly, early diagnosis of lymph node metastasis is essential for improved clinical outcomes of OC patients.

OSCC patients generally undergo surgery to remove primary tumors. Patients clinically diagnosed with lymph node metastasis (cN+) require additional surgical treatment of the neck, followed by radiation and chemotherapy. However, significant controversy exists regarding the management of OSCC patients without lymph node metastasis based on clinical diagnosis (cN0) [17–20]. Currently, there are several different strategies for treating patients diagnosed with cN0 [20]. The predominant opinion is that node-negative patients should have a neck dissection if the risk of occult metastasis exceeds 15–20% [21, 22]. This opinion implies that this procedure may well be unnecessary for the remaining 80–85% of patients. Thus, a better diagnostic technique is needed to identify subclinical cervical metastases and to provide a guide for the treatment of these patients.

The clinical diagnosis of lymph node metastasis is currently based on imaging techniques and sentinel lymph node biopsy (SLNB). However, current imaging tests have been proven to be unreliable, especially in the detection of early nodal diseases. The concept of sentinel lymph node, which is defined as the lymph node to which a tumor first metastasizes, was first proposed by Gould *et al.* in the 1960s [23]. Numerous pilot studies have evaluated SLNB in cN0 patients with OSCC [2, 4, 24–31], but the applicability of SLNB for oral cancer is still controversial. Another main handicap of SLNB for oral cancer is “skip metastasis,” in which the disease bypasses the nodal basin of known cervical lymphatic drainage (level I–III) and goes directly to a different nodal group found usually at lower levels (level IV–V) [32–34]. Furthermore, the difficulty for localization of SLN in patients with certain cancers such as floor-of-mouth carcinoma and the difficulties with detection of micrometastasis in frozen sections [25] further limit SLNB as an adequate guide to clinical decision-making. There is therefore a strong need for a better understanding of the lymphatic metastasis development as well as new diagnostic strategies to predict the clinical behavior of the disease.

1.2. Selected Molecular Features of Lymph Node Metastasis in Oral Cancer

Cancer cell metastasis involves a series of sequential and interdependent events that include initial growth, local invasion, angiogenesis, intravasation of invading cells into vasculature or lymphatic systems, extravasation and subsequent deposition and proliferation at a second site. While for most cancer types, metastasis involves spreading of the disease through both

blood stream and lymphatic drainage, OSCC is unique in that the tumor cells mainly spread to regional lymph nodes through the draining lymphatics, while involvement of distant sites is relatively uncommon. This is believed to be determined by both anatomic location and the molecular characteristics of OSCC. In recent years, considerable advances have occurred in our understanding of OSCC metastasis. Herein, we place particular emphasis on the new and evolving views of this specific aspect. Selected novel and evolving molecules involved in lymphatic metastasis are shown in Table 1.

1.1.1. Lymphangiogenesis—Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing lymphatic vessels. It plays important physiological roles in homeostasis, metabolism and immunity. It also contributes to a number of pathological conditions including tumor cell metastasis. Lymphangiogenesis has traditionally been overshadowed by the greater emphasis placed on the blood vascular system (angiogenesis). This is in part due to the lack of identified lymphangiogenic markers.

Recently, the lymphatic system has received renewed interest due to the discovery of a number of specific lymphatic markers, including podoplanin, LYVE-1, PROX-1, desmoplakin, VEGF-C, VEGF-D and their receptor VEGFR-3. VEGF-C and VEGF-D are classic lymphangiogenic cytokines which primarily activate VEGFR-3, and have been firmly linked to lymphatic metastasis of OSCC [51, 70, 98–104]. It is not surprising that hypoxia inducible factor-1 α (HIF-1 α), which induces VEGF-C, also plays a role in OSCC metastasis. While hypoxia is believed to stimulate angiogenesis by regulating VEGF-A, HIF-1 α expression was significantly associated with VEGF-C expression and lymph node metastasis in esophageal cancer [105], and with VEGF-C expression and lymphangiogenesis in breast cancer [106]. Similarly, HIF-1 α has also been suggested to play a role in lymphangiogenesis and lymph nodal metastasis in OSCC by regulating lymphatic expression of VEGF-C [51]. While not all studies show positive correlations between lymphangiogenic cytokines expression and lymph nodal metastasis, there is certainly enough evidence, to suggest VEGF-C as an important contributor to lymph node disease in OSCC. Of note, VEGF-C has also been proposed as a biomarker to predict lymphatic metastasis in early OSCC. Kishimoto *et al.* found that higher expression of VEGF-C in early stage OSCC (stages I-II) is associated with lymph node metastasis, suggesting that VEGF-C expression can act as a predictor for regional lymph node metastasis [107]. In contrast, a recent study indicated that VEGF-C level is not predictive for occult lymph node metastasis in the early stages of OSCC [108]. While these apparent contradictions may reflect the differences in patient groups examined in these studies, a better understanding of the molecular mechanisms of VEGF-C/VEGFR will be helpful for addressing this issue.

Lymphangiogenic cytokines may be derived from tumor or host cells, the latter including stromal fibroblasts, inflammatory cells and macrophages. Two important signaling molecules, including hepatocyte growth factor (HGF) and stromal cell-derived factor-1 (SDF-1), have been involved in lymphatic metastasis of OSCC. HGF, also known as scatter factor, is a pleotropic growth factor that regulates cell proliferation, survival, migration, invasion, angiogenesis, and cancer cell metastasis. The diverse biological effects of HGF are mediated through interaction with its receptor, c-Met protein. Both HGF and c-Met expression were shown to be correlated with OSCC progression [56, 109]. The elevated expression of HGF and c-Met in the tumor invasion front, were significantly associated with nodal status [109]. More importantly, recent experiments showed that enhanced production of HGF and SDF-1 by oral fibroblasts, specifically the “activated” fibroblasts, myofibroblasts, can stimulate OSCC cell invasion *in vitro* [57].

In addition to co-opting vessels and subsequent passive entry into the lymphatics, tumors can actively interact with and influence lymphatic vessel formation through the production

of various growth factors. However, evidence for direct casual relationships is not easy to establish due to the lack of reliable means to identify functionality or activation status of lymphatic vessels. By using a new marker, PA2.26 for staining of lymphatic endothelium, Muñoz-Guerra *et al.* observed that 54.1% (33/61) patients with early-stage OSCC had intratumoral lymphangiogenesis (IL), and a strong association was found between IL and locoregional recurrence [110]. This finding suggested that intratumoral lymphatic vessels may also form, at least in part, by vascular mimicry or transdifferentiation.

1.1.2. Chemotaxis—Chemotaxis is one of the most basic cell physiological responses, and plays a major role in cancer cell metastasis. The major role of chemokines is to act as a chemoattractant to guide this response. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of that chemokine. Chemokines are a family of more than 40 small cytokines that bind to G protein-coupled receptors expressed on target cells, allowing these cells to migrate following concentration gradients into selected tissues [111, 112]. Some chemokines control cells of the immune system during processes of immune surveillance, such as directing lymphocytes to the lymph nodes to participate in pathogen screening. Some chemokines have roles in development; they promote angiogenesis, or guide cells to tissues that provide specific signals critical for cellular maturation. Other chemokines are inflammatory and are released from a wide variety of cells in response to infection, physical damage. Inflammatory chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage. It became apparent that similar mechanisms that allow chemotaxis in normal physiological conditions can play a major role in cancer metastasis. Indeed, it has been suggested that chemokine-receptor interactions determine the destination of the invasive tumor cells in several types of cancer, including OSCC. The involvement of chemokines in lymph node metastasis is also supported by clinicopathological data. For example, it has been reported that the expression of CCR7 was detected in the draining lymph nodes of metastatic oral and oropharyngeal SCCs, and correlated with nodal metastasis [89]. In the past years, the involvements of SDF-1/CXCR4 system in lymph node metastasis have been widely recognized [36–40, 42–44], and they will be introduced in the following sections.

1.1.3. Cell Adhesion and Extracellular Matrix Remodeling—In the metastatic process, reduction in cell to cell adhesion, particularly the E-cadherin-catenin cell adhesion complex, is an essential step. Classical cadherins are transmembrane glycoproteins and mediate cell-to-cell adhesion *via* homotypic interactions in the extracellular space [113]. In addition, they mediate connections to the cytoskeleton by means of their association with catenins. A decrease in cadherin-mediated cell adhesion has been identified as an important step of tumorigenesis and tumor progression by multiple mechanisms. In primary OSCCs, decreased expression of E-, P-cadherin and the aberrant N-cadherin expression closely correlate with invasiveness and nodal metastasis [64, 66–72]. The adhesive function of cadherins is dependent on their interaction with a group of cytoplasmic regulatory proteins, including actin cytoskeleton proteins such as catenins [67, 88], cell membrane glycoprotein dysadherin [114], DNA repair and synthesis protein p53R2 [35], plasmin protease [65], S100A4 [85, 86] and SIP1 [115]. Moreover, recent studies showed that certain epigenetic alterations, such as CpG methylation of E-cadherin, may also play a role in lymphatic metastasis [67, 116, 117].

CD44 is one of the cell adhesion molecules that play an important role in cancer metastasis. In the context of oral carcinoma, a general down regulation of CD44 expression is associated with tumor progression [73, 118–122]. *In vitro* experimental data has shown that down-regulation of a specific CD44 variant (CD44v9) by treatment with an anti-CD44v9 antibody increased the invasive potential of tumor cells. On the other hand, transfecting

CD44v9 gene into tumor cells with low CD44v9 expression resulted in an enhanced cell-cell adhesion and therefore, inhibited the invasive potential [73]. Also, CD44 is one of the markers used to define cancer stem cells from a variety of tumor types. Boldrup *et al.* [118] showed that up-regulation and differential splicing of CD44 following p63 over-expression play key roles in the regulation of adhesion, metastasis and maintaining cancer stem cell phenotype.

In addition to the molecular process discussed above, it should be pointed out that many well-recognized candidate genes reported previously have important roles in tumor invasion and metastasis of OSCC. These include EGFR, nm23, matrix metalloproteases and tissue inhibitors of metalloproteases, integrins, laminins, collagens and immunoglobulin superfamily members [see review: [123, 124]]. Despite our current understanding of the molecular and cellular mechanisms responsible for the nodal metastasis of oral cancers, the vast majority of the predisposing genetic factors is still unknown. Hence, at present, no reliable markers have been included in clinical work-up strategies for predicting the presence of or potential for lymph node metastasis.

2. EXPRESSION PROFILING STUDIES AIM TO IDENTIFY METASTASIS-RELATED GENES IN ORAL CANCER

It has been well recognized that metastasis is a multi-step event. Given that multiple genetic and/or epigenetic alterations might be required to accomplish individual steps of metastasis, it is apparent that a systematic genome-wide approach is required to capture such complex and highly interactive processes at the molecular level. Recently, microarray analysis has been used extensively to examine the global gene expression changes typical in non-metastatic versus metastatic human tumor samples. Comparing the gene expression profiles of primary tumors from these two groups of patients resulted in a list of genes whose expressions were significantly different in these patient groups. Several gene expression studies have successfully generated “metastasis signatures” in primary tumors for predicting lymph node metastasis of OSCC [125–135]. Moreover, studies have also shown that gene expression patterns in the primary tumors are co-determined by the genetic background that pre-exist in normal tissues, and are maintained in their resultant lymph node metastatic disease tissues.

As illustrated in Table 2, there are currently three main approaches for exploring gene expression differences that contribute to primary OSCCs committing to either metastatic or non-metastatic progression routes: i) by comparing gene expression profiles from primary tumors with normal oral mucosa; ii) by comparing the transcriptional profiles of primary tumors from metastasis-free patients with those of metastasis patients to pinpoint differences in primary tumors that may be indicative of the metastatic potential; iii) by comparing expression signatures from primary tumors with those obtained from matched cervical lymph node metastases in order to elucidate mechanisms of metastasis formation, i.e. the putative secondary alterations acquired in the transition from primary tumors to regional metastasis site.

In the following sections, the value of these metastasis signatures will be explored. First, we will summarize the clinical studies of metastasis-related gene expression profiling in oral cancers. Then, we will highlight the gene expression studies that involve cancer cells versus normal oral mucosa, metastatic primary tumors versus non-metastatic tumors, and primary tumors versus the paired lymph node metastases, and studies of functional genomics in metastasis.

2.1. Differential Expression Profiling Between Tumor and Normal Mucosa

It is believed that expression signatures with predictive values for metastasis may in part be due to genetic background [143], and can be defined by differential expression profiling based on comparison of tumor and normal tissue. A limited number of studies (including 4 studies using cDNA and oligonucleotide microarrays [132, 140–142] and 1 study using a spotted array with 557 known cancer genes [128]) attempted to identify metastasis-related genes based on this design. Schmalbach and colleagues identified a group of 57 deregulated genes between metastatic tumors and normal mucosa [132]. These genes appear to belong to broad functional categories include extracellular matrix, adhesion, motility, inflammation, intracellular signaling and angiogenesis. The second microarray-based study [140] identified 234 genes whose expression is significantly different during progression from normal tissue to primary tumor and subsequently to metastatic disease. Several adhesion molecules such as moesin (a member of the ezrin/radixin/moesin [ERM] family of cytoskeletal proteins), fibronectin 1 (FN1), laminin 5 gamma 2, ECM glycoprotein tenascin and osteopontin, appear to be up-regulated during progression from normal tissue to locally invasive cancer to nodal metastasis. Recently, by employing MAPPFinder, a component of Gen-MAPP, Sticht *et al.* generated a statistically ranked list of molecular signaling pathways by comparing OSCCs with healthy mucosa [142]. Among others, as expected, cancer-related pathways are found to be deregulated in the OSCCs. These include mitogen-activated protein kinase (MAPK) signaling, transforming growth factor-beta signaling, and apoptosis-related signaling pathways. Notably, little overlap was found based upon the listed genes among these studies described here. The discrepancy was also observed in a study by Nagata *et al.* [128] in which expression analysis was performed by ‘low-complexity’ (557 genes) cancer-related cDNA arrays. In the up-regulated cluster, proteolytic enzymes MMP1, MMP3 and uPA, cell-ECM adhesion molecules FN1 and tenascin C, transcription factor STAT1, and superoxide dismutase 2 (SOD2), were all identified.

However, it’s noteworthy to point out that not all investigations support the hypothesis that lymph node metastasis status can be accurately predicted from the gene expression patterns of primary OCs. In a supervised analysis of the gene expression on 60 head and neck SCCs, a “metastasis predictor” for LN metastasis was developed [141]. When simultaneously considered with the actual pathological nodal status, the accuracy of the ‘metastasis predictor’ was only about 60%. Further work on individual samples identified that about half of the mistakes were made on the oral cavity-derived tumors, indicating that the oral cavity cancers were more heterogeneous than other subsites in regards to expression patterns. Thus, genes involved in tumorigenesis may also play a role in metastasis and therefore, lymph node metastasis could be predicted from the gene expression patterns of primary tumors. Through these studies, oral cancers display a distinct pattern of gene expression during progression from histologically normal mucosa to primary carcinoma to nodal metastasis. Some genes, for example, matrix enzymes, signaling components and cell adhesion molecules contribute to metastasis.

2.2. Differential Expression Profiling Between Primary Tumors With or Without Metastasis

The potential to predict lymphatic metastasis based on the molecular characteristics of primary cancer represents substantial clinical/translational significance. The majority of the published studies reporting ‘metastasis’ signatures and their validations were performed based on differential profiling between primary tumors with or without metastasis [125–135].

While there is limited overlap among gene lists identified from these studies, these metastasis-associated genes tend to over-represent a number of gene families, such as cell adhesion, keratin, signaling, matrix metalloprotease and their regulators. The apparent

discrepancy among gene lists can be contributed to by various external factors, including variations in study design, patient selection, gene selection criteria, data mining and classification. Again, differences in microarray platforms and sample isolation protocols are likely to underlie these discrepancies. Taking into account these limitations, we are highlighting the results from an excellent study carried out by Roepman and colleagues [131]. In this study, 82 oral/oropharyngeal cancer patients with and without nodal involvement were used as a training set, and a 102-gene signature using a comparable, supervised method, was identified to predict the nodal metastasis status. The predictor genes, which were clustered based on their similarities across the 82 tumors, correctly determined all of the 61 individuals with metastatic disease; and the performance increased gradually as more recent samples were analyzed. More strikingly, in a subsequent validation group with 22 patients the predictor genes performed better than current clinical diagnostic methods. The predictor genes had an overall accuracy of 86% compared with 68% for clinical diagnosis. No false negative predictions were made, which is most important for the goal of achieving clinical relevance. While many of the predictors have previously been implicated in metastasis (including COL5A1, PLA1, SERPINE1 and ECM1), more than half of them have not been directly associated with tumorigenesis or metastasis before. Among those genes, many of them are involved in stromal and immune-regulatory components which are in agreement with previous notions [144, 145]. This result also highlighted the importance of considering cell-cell interactions in the study design and data analysis of gene expression profiling studies.

To avoid the possible effects of sampling, array platform, and laboratory-related influences on expression signature, we recently performed a secondary analysis on four existing independent microarray datasets [129, 130, 132, 135]. All of the four studies were carried out using Affymetrix microarray platform. In the first, 57 differentially expressed genes were identified in metastatic tumors [132]. This metastatic profile includes genes related to extracellular matrix, cell growth and differentiation, cellular adhesion, immune response and protease inhibition. Most of the listed genes, such as COL11A1, require activation from surrounding nontumor cells. Similarly, O'Donnell *et al.* identified a 116-gene signature capable of predicting lymph node metastasis [130]. In consideration of complexity of the tumor system, non-microdissected tissue samples were used in this study. Consequently, the interactions with immune and stromal cells have been shown to play a role in tumor aggressiveness. Also, by selection of cancer tissues containing > 80% tumor cells, our laboratory group generated a gene set for pN- and pN+ cases. Logistic models with specific combinations of genes (CTTN, MMP9 and EGFR for pN and CTTN, EEF1A1 and MMP9 for extracapsular spread (ECS, an important negative prognostic factor for HNSCC)) achieved 85% prediction accuracy [135].

It's known that the actual genes present in a metastasis-related expression signature strongly depend upon the study design, primary tumor locations and the dataset used to develop the signature. For this reason we do not claim that the 102-gene signature developed by Roepman *et al.* is necessarily more important than others. Yet the fact that the expression values of genes encoding extracellular proteins identified by the comparison of primary tumors with or without metastasis in these unrelated studies makes their functional involvement in the process of lymphatic metastasis very likely. Moreover, these results, at least partially, support the hypothesis that genes regulating tumor and microenvironment interactions can be used for predicting the lymphatic metastasis status of OSCCs. In contrast to these previous papers, a study on 30 OSCC cases (training set) identified a predictive gene signature [129]. Eight of the 85 differentially expressed genes, including IL-15, DCTD, PTHLH and c9orf46, were selected for prediction of the nodal status, and the results in 12 of 13 cases (92.3%, test set) were predicted correctly. Interestingly, only three of the genes (including IL-15, DCTD and PTHLH) in this signature have been previously associated with

an aggressive phenotype in other types of cancers. It is worth knowing that laser captured microdissection was used in this study to yield cancer cells.

2.3. Differential Expression Profiling Between Primary Tumor and Metastatic Disease

It was generally believed that a subpopulation of cells in the primary tumor acquire the metastatic phenotype by acquiring specific genetic mutation and/or epigenetic changes. Only rare cells within a primary tumor were thought to accumulate the multiple alterations necessary for metastasis [146]. In contrast to the traditional model, Bernards and Weinberg [147] proposed that the ability of cancer cells to metastasize was acquired early in tumorigenesis and was to a large extent determined by the nature of the mutations acquired during carcinogenesis. In the past few years, evidence has been accumulating supporting the model of Bernards and Weinberg, and additionally suggesting that the ability to metastasize was present in the bulk of primary tumors rather than in rare cells as previously implied. Recently, Méndez *et al.* [139] showed that a gene signature obtained from the differential expression between lymph node metastases and unmatched, node-negative primary OSCCs can accurately distinguish primary tumors with or without lymph node metastasis. These results support the current hypothesis. They suggest that the metastatic tumor cells in the lymph nodes have changes in gene expression similar to those in the tumor cell in the primary site of node-negative patients from which the metastases derived. Once again, many of the expression changes found in the metastatic cells from the lymph nodes were also found in the matched node-positive primary tumors.

Two additional studies also attempted to identify metastatic signatures by directly comparing differentially expressed genes between primary tumors and the matched lymph node metastases. Roepman *et al.* [137] demonstrated that gene expression profiles of metastatic primary tumor (OC/OP) are largely maintained in their corresponding lymph node metastases. Notably, only a single gene, metastasis-associated gene 1 (MTA1), was found to show consistently changed expression between matched primary tumor/lymph node metastasis pairs. Similarly, a study of 40 primary HNSCC cases (38 of these were OSCCs) and paired lymph node metastases generated a 301-gene signature [138]. Functional analysis *in vivo* and *in vitro* suggest that some of the identified genes and pathways, including CCL19, EGR2, FUCA1, SELL/IGFBP6 and KLK8 play a role in lymphatic metastasis. These findings indicate that the disseminated primary tumor cells do not need to undergo additional developmental changes to survive and proliferate in metastatic sites. These findings, therefore, also support the model of Bernards and Weinberg. More recently, Belbin *et al.* [140] identified a 234-gene set that was consistently deregulated in expression during progression from normal tissue to invasive tumor and to subsequent metastasis of the lymph node. This result supports the hypothesis that the capacity of cancer cells to metastasize was acquired early in tumorigenesis. Given the above data, it is possible to conclude that, in some situations, the genes activated early in tumorigenesis may eventually contribute to lymph node metastasis, while in other situations, acquiring additional genetic alterations may be necessary for metastatic phenotype. Indeed, given the heterogeneity of oral cancers, it would not be surprising if multiple gene pathways or mechanisms were involved in the development of lymph node metastasis in OSCC.

3. HIGHLIGHTS OF THE ADVANCES IN THE UNDERSTANDING OF LYMPH NODE METASTASIS IN ORAL CANCER

Microarray expression profiling has proven to be a powerful approach for characterizing the changes associated with biological processes such as in OSCC metastasis. However, as described above, although the broad functional categories identified by the differentially expressed genes seem to be common among studies, little overlap, if any, is found when

individual genes are taken into consideration. This may partly be due to the multifactorial nature of the oncogenesis process and the heterogeneity in oncogenic pathways. These are two major biological difficulties that confront the task of molecular classification/prediction of tumor phenotypes (e.g. metastatic versus non-metastatic). The multifactorial nature of oncogenesis is best described with the multi-hit model, in which most tumors, especially those of adults, result from an inter-dependent series of genetic alterations, rather than a single decisive event. This multi-hit model complicates the task of prediction because a single abnormality may lead to a specific tumor phenotype in some cases (e.g., those in which a complementary genetic alteration already exists) but not in others (e.g. in cases where a required collaborating genetic alteration has not occurred). A second biological issue complicating the development of oncology prediction models is heterogeneity in oncogenic pathways. Distinct genetic lesions may give rise to a common malignant phenotype (i.e. there may be several different “ways” to get lymph node metastasis in oral cancers). If there are many distinct etiologic possibilities, and only one is necessary to produce lymph node metastasis, then many other etiologic variables may remain “normal” despite the fact that nodal metastasis develops. Therefore, instead of focusing on specific genes, it may be more fruitful if we focus our attention on a higher level of biological information (e.g., alterations of group of genes, certain pathways, or biological processes in lymph node metastasis of OSCC). The following sections highlight the recent advances in the understanding of OSCC metastasis based on the knowledge gained through this genome-wide system biological approach.

3.1. Matrix Metalloproteases and Tissue Inhibitors of Metalloproteases

Hyper-activation of matrix metalloproteases (MMPs) is a hallmark of invasive cancers for which it constitutes a mechanistic prerequisite for the degradation of the basement membrane and extracellular matrix (ECM) thus allowing tumor cells to leave the primary tumor site and enter blood or lymphatic vessels for dissemination [[148]. The role of MMPs in metastasis of OSCCs is well established [90, 123, 124, 149–154] and highlighted by their presence in metastasis-related gene signatures [120, 125, 127, 128, 131, 135, 141, 155].

Four endogenous protease inhibitors for MMPs are known to exist, i.e TIMP1, TIMP2, TIMP3 and TIMP4 [156]. Since specific MMPs can promote cancer progression, it is reasonable to hypothesize that high levels of endogenous TIMPs would prevent cancer progression, and consequently, tumors with high TIMPs levels would have a better prognosis than those with low TIMPs levels. In OSCCs, several studies have shown that elevated expression of TIMPs, especially TIMP1 and TIMP2, in metastatic carcinoma have a good prognosis [157–159]. However, contradictory findings have also been reported [81, 90, 91]. These different findings may be because TIMPs are multifunctional proteins and that their effects on tumor progression are context-dependent and concentration-dependent. Nevertheless, further studies are warranted to fully explore the roles of TIMPs in OSCC metastasis.

Both expression of MMPs and their endogenous inhibitors are regulated by a variety of cytokines, growth factors, and transcription factors that participate in events associated with tissue remodeling. TIMP2 is an essential factor for efficient activation of pro-MMP2. TIMP2 accomplishes this activation by acting as a bridge between MMP2 and membrane type 1-MMP (MT1-MMP) on the cell membrane. This trimolecular complex allows a second MT1-MMP molecule to cleave the pro-domain of MMP2 [160]. In two independent studies, MT1-MMP was found to be up-regulated by laminin 5 [79] and E1AF (an ets-oncogene family transcription factor) [80] and therefore, activated the expression of MMP2 in tumor cells. Using a xenograft model, Miyazaki *et al.* [161] demonstrated that both MMP2 and MMP9 levels were increased under hypoxic condition. MMP2 was predominantly expressed in the hypoxic region of tumor tissue, while MMP9 was mainly

detected in neighboring stromal tissues containing blood vessels. Interestingly, the actions of MMPs and their inhibitors also depend on their concentrations. Baker *et al.* [157] reported that tissue concentrations of a subset of these factors correlated with tumor progression, suggesting that it is the balance between MMPs and their corresponding TIMPs that control tissue degradation at each stage of tumor invasion and metastasis. These findings support the hypothesis that specific TIMPs, under specific conditions and at concentrations founded *in vivo*, may play a role in promoting rather than inhibiting cancer progression. More studies are currently underway to investigate the role of the MMP/TIMP system in tumor invasion and metastasis.

3.2. Urokinase-Type Plasminogen Activator and its Receptor

Recently, the critical roles of the serine protease urokinase-type plasminogen activator (uPA) in ECM remodeling, tumor invasion and metastasis have become evident. The protease uPA binds to a surface-anchored receptor (uPAR), focalizing proteolytic activity to the pericellular milieu. Furthermore, uPA and uPAR interact with transmembrane proteins to regulate multiple signal transduction pathways and influence a wide variety of cellular behaviors, including cell adhesion, migration, chemotaxis and tissue remodeling [48, 162, 163]. uPAR expression levels in tumor tissues and their prognostic values have been studied in several cancer types, including breast [164], lung [165], prostate [166], ovarian [167] and colorectal cancers [168]. Results from these studies have shown that the elevated uPAR expression correlate with poor prognosis, thereby making it a potential biomarker for molecular classification of cancers. Enhanced expression of uPA and uPAR has also been found in OSCC, and correlated with tumor differentiation grade, lymph node metastasis and prognosis [45, 46, 48, 49, 169]. Functional study has shown that silencing the endogenous uPAR expression in highly malignant OSCC cells resulted in a dramatic reduction of tumor cell proliferation, adhesion, migration and invasion *in vitro* [47]. Furthermore, two recent genome-wide profiling studies have identified the uPA as a strong biomarker for predicting poor disease outcome of OSCC using a 'gene signature' approach [124, 128]. Of note, Ghosh *et al.* demonstrated that uPA expression and uPAR relocalization are regulated by $\alpha 3 \beta 1$ integrin-activated Src/MEK/ERK signaling pathway in oral keratinocytes [170]. Conversely, blocking uPAR- $\alpha 3 \beta 1$ integrin interaction results in significant inhibition of uPA expression, suggesting a functional relevance of uPAR- $\alpha 3 \beta 1$ integrin association in proteinase regulatory pathways [171]. This evidence implicates an important role for the uPA-uPAR system in invasion and metastasis of OSCC.

3.3. SDF-1/CXCR4 Signaling Axis and Epithelial-Mesenchymal Transition (EMT)

It has been demonstrated that the G-protein-coupled seven-span transmembrane receptor CXCR4 is expressed in numerous types of embryonic cells and the α -chemokine stromal-derived factor 1 (SDF-1) has chemoattractant effects on these cells [172–174]. Animal models in which SDF-1/CXCR4 signaling have been interrupted exhibit a number of phenotypes that can be explained by inhibition on SDF-1-mediated chemoattraction of stem/progenitor cells [175–178]. Also, the expression patterns for both SDF-1 and CXCR4 are highly consistent with the possibility that they have shifted developmental patterns in the formation of many different tissues. These observations suggest a crucial role for the SDF-1/CXCR4 signaling axis in regulating the migration of different types of stem/progenitor cells. It is believed that cancer stem cells, much like normal stem/progenitor cells, can give rise to tumor cells in primary tumors and can also metastasize to seed tumors in a second site. In this case, one may postulate that the SDF-1/CXCR4 signaling axis may influence the biology of tumors and direct the metastasis of CXCR4-expressing tumor cells by chemoattracting them to organs that express high levels of SDF-1 (e.g., lung, liver, bones and lymph nodes). Supporting this notion, it has been recently reported that several CXCR4-expressing cancers, including breast, prostate, ovarian cancer and neuroblastoma [179–182],

metastasize to specific organs in a SDF-1-dependent manner. The role for the SDF-1 / CXCR4 signaling axis involved in lymphatic metastasis of OSCC was also investigated in the past several years [36–40, 42–44]. SDF-1 alpha expression was detected mainly in the stromal cells, but also occasionally in the tumor cells metastasized to the regional lymph nodes [42]. CXCR4 expression in metastatic cancer tissues was significantly higher than that in non-metastatic cancer tissues, and its expression was strongly associated with invasion, recurrence and lymph node metastasis. Additional studies have shown that SDF-1alpha rapidly activated extracellular signal-regulated kinase (ERK) 1/2, Akt/protein kinase B (PKB) and Src family kinases (SFKs) in CXCR4-expressing cancer cells [40, 42]. More importantly, recombinant SDF-1 alpha stimulates *in vitro* invasiveness and scattering in CXCR4-expressing OSCC cells, and induces metastasis of these cells to the cervical lymph node in an orthotopic nude mice model [43]. Taken together, these results indicate that SDF-1/CXCR4 signaling mediates the establishment of lymph node metastasis in OSCC *via* ERK1/2 and/or Akt/PKB pathway.

Epithelial-mesenchymal transition (EMT), in which epithelial cells lose their polarity and become motile mesenchymal cells, occurs during the development process and is also a key step in the tumor progressing towards metastasis, including metastasis of OSCC [40, 76, 183, 184]. Accumulating evidence supports that EMT can contribute to metastasis by changing the adhesive properties of tumor cells and promoting their motility, thereby increasing their invasiveness. More strikingly, a variety of EMT markers, including downregulation of E-cadherin and cytokeratin [40], increased expression of N-cadherin [70], MMPs [125, 131, 135, 185] and transcription factors such as Snail 1 (Snail) [186], SIP1/ZEB2 [115], NF- κ B [187], and occludins [188], have also been found in the metastatic OSCC cells in lymph nodes. Moreover, cadherin switching, which has been known to play a central role in the EMT, has also been implicated in OSCC metastasis [70]. The presence of the EMT markers in tumor tissues indicates an important role for EMT in driving invasion and metastasis of OSCCs.

4. SUMMARY AND CONCLUDING REMARKS

Lymph node metastasis in OSCC is an important negative prognosis factor. The risk of metastatic spread can sometimes be predicted by clinical parameters (e.g., tumor size) and molecular features (e.g., gene expression pattern) [131, 135]. DNA microarray technology has significantly improved efficiency of genome-wide analysis on gene expression and opened up new avenues for clinical investigation. A significant amount of experimental results point to the existence of metastatic signatures in tumor cells. This signature codifies not only for the metastatic ability but also for organ-specificity. To date, many studies have provided gene expression profiles that are relevant to lymph node metastasis of OSCC. A number of cellular and biological events and relevant molecular regulators have been identified. They represent novel therapeutic targets for OSCC, and also provide a model system for lymphatic metastasis formation in other cancers. However, several critical issues still need to be addressed before these findings can be fully utilized in patient care.

First, system(s) to measure reproducibility, consistency, and validity of the results need to be implemented. It's clear from our discussion that gene expression profiling has great potential for predicting OSCC metastasis and for improving our understanding on this disease. However, most of these gene expression studies are retrospective studies with small sample size. Variations in study designs, genes selection criteria and data analysis approaches also contribute to the discrepancies observed in studies described here. In fact, as mentioned above, the genes differentiating metastatic from non-metastatic tumor had limited overlap even though there are performed on the same microarray platforms. It will be ideal to replicate these studies in a single, high-powered, prospective, randomized controlled trial or

more practically a comprehensive meta-analysis on several well-designed studies. Most importantly, it would be of great value to test these molecular targets in the context of OSCC and study the effects that they would have on lymphatic metastasis. Collectively, a combination of better-defined and larger sample size patient cohorts; improved microarray platforms; and a standardized data analysis approach, will ensure the successful application of gene expression profiling in oral cancer research, and lead to validatable and clinically useful biomarkers.

Second, considering the multifactorial nature of oncogenesis process and heterogeneity in oncogenic pathways, it is unlikely, from the evidence provided, that individual genes or single pathways would be sufficient to account for lymphatic metastasis in all OSCC cases. Furthermore different etiological factors and extracellular stress that play a role in the formation and progression of OSCC may also contribute to the observed gene expression difference that is not directly related with the metastasis phenotype. For example, epidemiological and molecular data indicate that, in addition to tobacco and alcohol consumption, high-risk human papillomaviruses (HPV) plays an important etiological role in a subset of head and neck cancer, especially in oral and oropharyngeal carcinogenesis [15, 189–191]. Accumulating evidence suggests that HPV-positive and -negative HNSCCs may represent different cancer lineages formed through separate etiological pathways of multi-stage tumourigenesis [192]. Also, based on a recent expression profiling study which demonstrated that a subset of genes involved in viral defense and immune response, including interleukins and interferon-induced proteins, was found to be down-regulated in HPV-positive HNSCCs [193]. This result supports a characteristic and unique transcriptional profile in HPV-induced HNSCC. It's conceivable that multiple gene expression classifiers, involving multiple molecular pathways, could be combined with traditional clinicopathological parameters as well as different etiological factors.

In summary, a microarray-based gene expression profiling approach greatly improved our understanding of the molecular mechanisms underlying lymph node metastasis of OSCC. Genes and pathways involved in adhesion, invasion, angiogenesis and cell growth have already been linked to this multi-step process. A number of gene signatures with predictive values for lymph node metastasis have been identified. However, it should be emphasized that gene expression profiling is likely to improve and refine, rather than replace, current diagnostic and prognostic methods. Integrative models that combine clinical and pathological parameters together with multidimensional molecular classifiers, such as gene expression patterns and functional proteomic signatures, are being developed to evaluate the likelihood of OSCC metastasis, and will lead to dramatic improvement in the management of OSCC patients in the near future.

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Table 1

Selected Novel and Evolving Molecules in Lymphatic Metastasis of OSCCs

ene Symbol	Gene Name	Putative Function in Metastasis	Ref.
2	Ribonucleotide reductase small subunit	DNA repair and synthesis	[35]
CXCL12	Chemokine, cxc motif, ligand 12	Chemotaxis	[36]
CXCR4/SDF1	Chemokine, cxc motif, ligand 4/ Stromal cell-derived factor 1	Chemotaxis, basal extravasation	[36–44]
uPA/uPAR	Urokinase-type plasminogen activator receptor	Extracellular proteolysis, migration and remodeling	[45–49]
MTA1	Metastasis-associated protein 1	Invasion	[50]
HIF-1 α	Hypoxia inducible factor – 1 α	Lymphangiogenesis	[51, 52]
P75 NTR	Nerve growth factor receptor	Signaling pathway	[53]
CCL2	Chemokine, cc motif, ligand 2	Inflammation, migration	[54]
Ep-CAM	Epithelial cell adhesion molecule	Migration, proliferation, cell signaling	[55]
HGF	Hepatocyte growth factor	Motility, invasion	[56–58]
CCL3	Chemokine, cxc motif, ligand 3	Chemotaxis, migration	[59]
iNOS	Inducible nitric oxide synthase	Invasion	[60, 61]
COX-2	Cyclooxygenase-2	Migration, angiogenesis	[62, 63]
Cadherins		Cell adhesion	[64–72]
CD44	CD44 antigen	Cell adhesion	[73, 74]
POSTN	Periostin	Invasion, angiogenesis	[75] [76]
PDPN	Podoplanin	Lymphangiogenesis	[77]
Ln-5	Laminin-5	Migration	[78, 79]
MT1-MMP	Membrane type 1-matrix metalloproteinase	Migration	[79–81]
Survivin		Apoptosis	[82–84]
S100 CBP	S100 calcium-binding protein	Cell motility	[85–87]
CTNN	Catenin	Cell adhesion	[88]
CCR7	Chemokine, cc motif, receptor 7	Chemotaxis, migration	[89]
TIMPs	Tissue inhibitors of matrix metalloproteinase	ECM remodeling	[81, 90–92]
CTSB	Cathepsin B	Cell motility	[93, 94]
HMGA2	High mobility group AT-hook 2	Cell motility	[95]
MSN	Moesin	Cell adhesion, motility	[96]
HMOX1	Heme oxygenase-1	Signaling pathway	[97]

Table 2

List of Published Gene Expression Microarray Studies Relating to Lymphatic Metastasis

Study Design	Tumor Cells	Site	Array Platform	Ref.
primary tumors with metastasis vs. without metastasis		OC	IntelliGene (cDNA)	Nagata, 2003 [128]
primary tumors with metastasis vs. without metastasis	$\geq 70\%$	OC, OP	Affymetrix	Schmalbach, 2004 [132]
primary tumors with metastasis vs. without metastasis	$\geq 80\%$	OC	cDNA	Warner, 2004 [133]
primary tumors with metastasis vs. without metastasis		OC	Affymetrix	O'Donnell, 2005 [130]
primary tumors with metastasis vs. without metastasis	$\geq 50\%$	OC, OP	oligo	Roepman, 2005 [131]
primary tumors with metastasis vs. without metastasis		OC	AceGene (oligo)	Kato, 2006 [126]
primary tumors with metastasis vs. without metastasis	$\geq 50\%$	OC, OP	oligo	Roepman, 2006 [136]
primary tumors with metastasis vs. without metastasis	$\geq 80\%$	OC	Affymetrix	Zhou, 2006 [135]
primary tumors with metastasis vs. without metastasis	100% (LCM)	OC	Affymetrix	Nguyen, 2007 [129]
primary tumors with metastasis vs. without metastasis	$\geq 60\%$	OC	IntelliGene (cDNA)	Kondoh, 2008 [127]
primary tumors with metastasis vs. without metastasis	$\geq 50\%$	OC	cDNA	Kashiwazaki, 2008 [125]
primary tumors with metastasis vs. without metastasis	$\geq 50\%$	OC	oligo	Watanabe, 2008 [134]
primary tumor vs. metastatic lymph node disease	$\geq 50\%$ in primary, $\geq 25\%$ in lymph node	OC, OP	oligo	Roepman, 2006 [137]
primary tumor vs. metastatic lymph node disease	100% (LCM)	OC	Agilent (CDNA)	Liu, 2006 [138]
primary tumor vs. metastatic lymph node disease	100% (LCM)	OC	Affymetrix	Mendez, 2007 [139]
normal vs. primary tumor vs metastatic lymph node disease	$\geq 70\%$	OC	cDNA	Belbin, 2005 [140]
normal vs. tumor		OC, OP, HP, L	Agilent (CDNA)	Chung, 2004 [141]
normal vs. tumor	$\geq 80\%$	OC	oligo	Sticht, 2008 [142]

Table 3

Deregulated Key Genes and Pathways Compared Between Metastatic and Non-Metastatic Primary Tumors

Study Design	Results	Key Genes/Pathways	Ref.
20 OC/OPs, 7 pN- and 13 pN+	23 up-regulated genes and 34 down-regulated genes in metastatic tumors	ECM 1, TIMP-1, collagen COL11A1	[132]
20 OCs, 7 pN- and 13 pN+	23 gene set distinguished nodal status	Claudin 1	[133]
15 OCs, 7 pN- and 8 pN+	14 up-regulated genes and 19 down-regulated genes in metastatic tumors	Proteolytic enzymes, keratins, ECM remodeling and cell adhesion molecules	[128]
43 OCs, 23 pN- and 20 pN+	85 genes deregulated in the metastatic group, 8 gene set gave 92.3% accuracy	IL-15, PTHLH	[129]
12 OCs, 6 pN- and 6 pN+	57 marker genes associated with invasion	ECM remodeling, adhesion and cytoskeleton molecules, signal transduction	[127]
18 OCs, 7 pN- and 11 pN+	116 genes differentiated metastatic tumors	Keratin 13, AP-2, GIRK1	[130]
23 OCs, 15 pN- and 8 pN+	20 gene set gave 80% accuracy in pN- patients, and 100% accuracy in pN+ patients	Ribosomal proteins, cadherin 1, STAC	[126]
41 OCs, 32 pN- and 9 pN+	30 up-regulated genes and 9 down-regulated genes in metastatic tumors	Adhesion, angiogenesis and intracellular signaling molecules, MMPs, cell cycle regulators	[125]
104 OC/OPs, 49 pN- and 55 pN+	102 predictor genes gave 100% accuracy in pN- patients, and 77% accuracy in pN+ patients	Epithelial markers, adhesion, ECM remodeling and immune regulatory components, MMP	[131]
40 OCs, 22 pN- and 18 pN+	19 genes differentially expressed and gave a predictive accuracy of 76%	MSR1, RET, Mucin 15, Wnt 11	[134]
25 OCs, 14 pN- and 11 pN+	Specific combinations of genes achieved 85% accuracy for predicting both pN and ECS	CTTN, MMP9, EGFR, BMP2, EEF1A1	[135]