Review Article

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Role of angiogenetic markers to predict neck node metastasis in head and neck cancers

ABSTRACT

Angiogenesis plays a key role in the initiation of growth and metastatic process in cancers. The angiogenic switch may be one of the earliest events in conferring a metastatic potential to the tumor. Further evolution in this multi-step cascade is controlled by the positive and negative regulators of angiogenesis. Recent advances in molecular biology have given a better insight into the mechanisms governing head neck cancer with promising data elaborating the role of angiogenesis. Metastasis to neck nodes is a very important determinant of prognosis, and is more frequently encountered than distant metastasis in head and neck cancers. Systematic PUBMED search of English-language literature of studies involving humans between 1990 and 2008 using the Mesh terms 'pathologic neovascularization', 'head and neck neoplasms', 'lymphatic metastasis' was performed. Quality assessment of selected studies included clinical pertinence, publication in peer reviewed journals, adequate number of enrolled patients. The present article reviews the utility value of various angiogenic parameters and markers that have been utilized to predict regional metastasis including micro vessel density, positive and negative regulators of angiogenesis, and genetic markers for angiogenesis. Although there seems promising preclinical and clinical evidence paving way for novel diagnostic and therapeutic interventions, the implicit role of angiogenesis in metastatic head and neck cancers needs further substantiation.

KEY WORDS: Angiogenesis, head and neck neoplasms, neck

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INTRODUCTION - ANGIOGENESIS IN H AND N - EMERGING ROLE

Autopsy studies conducted over a century of humans who died of trauma revealed an interesting insight: Women 40-50 years, 39% had *in situ* carcinomas but breast cancer was diagnosed in only 1%. Similarly in men between age groups of 50-70 years, 46% had *in situ* prostrate carcinoma but only 1% manifested during life. A comparable presentation was also seen in people of age groups of 50-70 years, of whom > 98% showed small carcinomas of thyroid of which 0.1% discernible disease.[1,2] This subset of people never manifested with disease through their live times. What keeps these tumors under check? These revelations witnessed the dawn of conceptualizing the role of angiogenesis in cancers. These showed that the tumors that failed to manifest have been attributed to either host derived factors (immunity) that prevent the angiogenic switch or endogenous inhibitors of angiogenesis.

Nutrients and gas exchange are mandatory for survival of every living cell. Practically, every living cell in the body lives adjacent to a capillary blood vessel, or at least no further than the mean oxygen diffusion distance of 100-200 μm.[1] Hence, angiogenesis forms an integral part of growth and development in any cell. Under normal circumstances, vast majority of these pertain to physiological functionality, and is regulated in an orderly fashion. Physiologic angiogenesis is a well acknowledged entity, lasting days in ovulation to weeks in wound healing or months in development of fetus or placenta. These processes have a time bound down regulation.

Intrinsic behavior of any malignant tumor in the body is to grow, invade and metastasize. Hence, malignant tumors also utilize the phenomenon of angiogenesis potentially in a similar order, but in uncontrolled and persistent manner. Most tumors at the commencement are not conferred with an angiogenic potential. Hence, these tumors exist *in situ* as microscopic foci ranging 0.2-2 mm^3 for varying periods. Without adequate vascularization, tumor larger than 1mm^3 may undergo necrosis.[2]

Genetic alterations that manifest during the transformation of a normal cell into a cancer cell, such as acquiring a malignant phenotype, although necessary are not sufficient for the tumor to grow and metastasize. Consequently, it is quite obvious

that a majority of the tumors that present to us clinically have overcome this phase to undergo an angiogenic switch.

The angiogenic switch may be one of the earliest events in a metastatic process, in that stromal fibroblasts in a prospective site may be induced to become proangiogenic by tumor even prior to the arrival of tumor cells. Recent studies have shown that vascular endothelial growth factor (VEGF) receptor cells from the bone marrow arrive at a specific site of future metastasis even prior to arrival of metastatic cells.[1]

Tumor cells enter the circulation by penetration through proliferating capillaries that have fragmented basement membranes. Further progress in this multi-step cascade is controlled by the positive and negative regulators of angiogenesis. Hence, the process of metastasis to a large extent is angiogenesis dependent.

Literature Search: Systematic PUBMED search of English-language literature of studies involving humans between 1990 and 2008 using the Mesh terms 'pathologic neovascularization', 'head and neck neoplasms', 'lymphatic metastasis' was performed. Quality assessment of selected studies included clinical pertinence, publication in peer reviewed journals, adequate number of enrolled patients.

HEAD AND NECK CANCERS WITH N_0 NECK- CURRENT MANAGEMENT SCENARIO

Lymph node involvement is one of the most important prognostic factors which influence the therapy. Therefore, pretreatment staging should be as exact as possible. While it is obvious that the positive neck must be treated, controversy has always delimited the clinically node negative neck with respect to the ideal treatment policy.

There are presently three policies advocated in the management of an N 0 neck, which include elective neck irradiation, prophylactic neck dissection or close observation. The choice of therapy often takes into consideration T stage, site of primary, grade, compliance for follow up, or the probability for occult metastasis [>20%]. Treatment of the neck, even when included with the primary treatment, often confers additional costs, morbidity and prolonged treatment time to the patient. Most often, a single modality treatment is used to treat the primary site and neck . The choice of which is dictated by the treatment of the primary site.

The issue is further confounded when the primary site is treated surgically without violating the neck such as in early cancers of the oral cavity [T1/T2]. These cancers are usually treated with surgery where excision is through the per-oral route. In such cases, elective neck dissection in such a situation is an additional surgical procedure with its associated costs, prolonged hospitalization and may be unnecessary in as high as 80% of patients who finally turn out to be pathologically node negative. There is also concern about additional morbidity of a staging neck procedure with some degree of shoulder dysfunction as a result of dissection in the region of the accessory nerve.

In addition to the above, there is no conclusive evidence to show if this elective neck treatment approaches contribute to improved overall survival for the patients with HNSCC and clinically negative neck.

DILEMMAS IN ASSESSMENT OF N_0 NECK

The assessment of cervical lymph nodes is known to be extremely difficult clinically. Despite recent advances in the fields of radio diagnosis, its utility to detect occult neck metastasis still lacks considerable power. Owing to the high number of undersized lymph node metastases, the non-invasive neck staging methods are limited to a maximum accuracy of 76%.[3]

In view of the lacunae present in conventional imaging, functional imaging was sorted to eliminate this uncertainty. Several studies have evaluated fluorodeoxyglucose (FDG) Positron emission tomography (PET) in this setting, attempting to identify the patients who need neck dissection. In 3 studies totaling 48 patients, in which a sentinel node biopsy with immunohistochemistry was used as the gold standard, the detection rate of PET was between 0% and 30%, making PET an unreliable modality in this clinical setting.[4-6] This is not unexpected, given that 40% of cervical nodal metastases are less than 1 cm in size and PET detection rate for nodes less than 1 cm is reported at 71%.[7]

Numerous promising pilot studies have evaluated sentinel node biopsy (SNB) in this group, although statistically significant validation such as is available for melanoma remains to be acquired. Furthermore, up to 16% patients required additional immunohistochemistry (IHC) on the sentinel nodes to detect metastasis.[8] In addition, SNB follows the preformed angiogenetic pathway to study the patterns of metastasis.

Owing to these inadequacies in detection of occult nodal metastasis, surgical dissection and serial histologic examination are the currently accepted "yardsticks".

ROLE OF ANGIOGENESIS - N_0 NECK

Recent advances in molecular biology have given a better insight into the mechanisms governing head neck cancer. This has consequently let to developments of novel techniques in the detection of occult neck metastasis. Role of angiogenesis to predict metastasis has been widely utilized in several sites.

Micro vascular density

Micro vascular density (MVD) by far is the most commonly

used and reliable predictor for metastasis. Angiogenesis is quantified through the staining of blood vessels with various endothelial cell markers. These measurements have correlated well with metastasis for patients with cancers of the breast, ovaries, prostrate and digestive tract. Several studies have also looked at micro vascular density in relation to clinical outcome in head and neck but with conflicting results.[9-11] Some of the *Markers used - CD 31, CD34, CD105, FACTOR VIII*

Reasons for the lack of correlation in this study may be because of the following:

a. The small number of patients in these studies may have contributed to the lack of statistical significance for tumor angiogenesis as a predictor of nodal metastasis.
b. The early cancers may be less reliant on micro vessels for growth
c. No study to compare the various staining techniques, and demonstrate the ideal immunostaining technique.
d. Micro vessel density may be an inadequate measure of tumor angiogenesis for head and neck cancers and alternate techniques may need to be developed. Distinguishing preexisting micro vessels from neovascularization is difficult, if not impossible;
e. The head and neck is a highly vascular region. Tumors in this area may therefore be less reliant on neovascularization for growth;
f. A highly angiogenic aggressive clone of a heterogeneous primary tumor may not be the dominant clone at the primary site, but may then be the cause of the tumor metastasis or recurrence. Evaluation for microvessel density at the primary site would therefore reveal low microvessel densities, but the tumor would have high angiogenic potential.[9]

Several recent studies have utilized anti CD105 to look into MVD. These studies suggest that the anti-CD105 mAb would specifically react with proliferating endothelial cells in tissue undergoing active angiogenesis, including tumor tissues, whereas it would stain no or weakly with blood vessels within normal tissues, thus suggesting the hypothesis that the anti-CD105 mAb could be a more specific marker in evaluation of tumor angiogenesis.[10]

Certain other studies have looked at not only assessing total number of vessels, but also included diameter categories of vessels. They found that especially very small caliber staining was found in nonmetastasized tumours, whereas larger vessels were dominant in metastasized tumors. It is theorized that the larger vessels are functional and may contribute to metastasis, while the small ones presumably represent single endothelial cells without perfusion capability.[11]

There has been a prodigious volume of data on angiogenesis that have shown promising results. However, unfortunately at the head neck front we are yet to gather substantial evidence to use these novel approaches into mainstream clinical practice. The failure of micro vessel density to correlate with tumor aggressiveness in these cancers does not disprove the possibility that neovascularization contributes to tumor growth and spread. Majority of these have been retrospective, studies that have been underpowered to reach any meaningful conclusions. Hence further quantification of this may prove valuable as a prognostic indicator.

REGULATORS OF ANGIOGENESIS

Multiple gene products are involved in angiogenesis, all of which have been demonstrated to be critical for regulating angiogenic phenotype. This has raised the need for comprehensive analysis of the angiogenic phenotype using microarray analysis and global proteomic approaches.[2] Complex interplay between positive and negative regulators determines the degree of neovascularization in and around the tumor.

Various markers assessing the role of regulators have been studied.

Matrix metalloproteinases (MMP) has the ability to degrade connective tissues such as the basement membrane which is a crucial step in the initiation of metastatic process, thus serving as a negative regulator of metastasis. Similarly, E-cadherin is an important molecule that promotes cell to cell adhesion which serves as a positive regulator of metastasis. A recent study indicated that angiogenesis and M/E ratio (matrix metalloproteinases and E-cadherin) were specific predictors for metastases of renal cell carcinoma, especially to the lung or lymph node. Therefore, matrix metalloproteinases (MMP) and E-cadherin were considered to be relevant targets for novel therapeutic strategies to control or prevent the metastasis of renal cell carcinoma.[12] These results have supported exploring the role of angiogenetic regulators in head and neck cancer.

Expression levels of molecules involved in tissue remodeling and extracellular matrix (ECM) adhesion, especially *MMP-1* and *integrin-3,* can provide an accurate biomarker system for predicting the risk of cervical lymph node metastasis in oral squamous cell carcinoma.[13] Low expression of E-cadherin should be considered as a high-risk group for late cervical metastasis when a wait-and-see policy for the neck is adopted.[14]

Vascular endothelial growth factor [VEGF] promotes angiogenesis in many different tumor types. VEGF is a highly potent angiogenic agent that acts to increase vessel permeability and enhance endothelial cell growth, proliferation, migration and differentiation.[15] VEGF levels may affect tumor growth, metastatic potential, and response to radiotherapy. VEGF positivity was the most significant predictor of poor prognosis. VEGF status may prove to be an important prognostic factor in head and neck cancer.[16] Role of VEGF in oral cancer has been the subject of numerous

studies, because it is a powerful promoter of angiogenesis in many tumor types. A recent review of the literature found that the contribution of VEGF to the development of oral dysplasia and invasive carcinomas is not well understood.[15] In addition, the potent role of VEGF in angiogenesis has spurred interest in using this molecule as a therapeutic target in anti angiogenetic therapy.

Endostatin, which exhibits specific inhibitory action on the proliferating endothelial cells of newly formed blood vessels, represents one of the better defined and most potent negative regulators of angiogenesis.[17] Earlier studies have shown that plasma levels of endostatin in patients with head and neck squamous cell carcinoma (SCC) have been associated with histologic grade, recurrence, and survival rate.[18] However, the immunohistochemical expression of endostatin and collagen XVIII in SCC tissues and their significance for the growth and metastatic potential of these tumors have not been widely studied. In a recent study, the levels of endostatin were lower in the primary tumors of cases with multiple metastatic lymph nodes compared with non metastatic tumors [node negative group]. The differences in endostatin expression between these tumors corresponded well with the levels of collagen XVIII, suggesting that the reduction in endostatin expression in the node positive group is because of decreases in the production of the precursor molecule collagen XVIII. On the other hand, these results contradict those of Homer *et al*,[18] who observed a positive trend between higher levels of endostatin and nodal metastasis and an association between increased endostatin expression and higher tumor grade, recurrence, and death in patients with head and neck SCC. The authors attributed this discrepancy to differences in methods, as these investigators measured the circulating levels of endostatin, whereas this study assessed the levels in tissue samples.[19]

The MMPs are a large group of secreted proteinases that require zinc for catalytic activity. MMP-2 and MMP-9 are the largest members of this gene family. They are able to degrade connective tissue, among other substrates, the basement membrane collagen, which appears to be very crucial in tumor cell invasion and in the process of metastasis.

The association of the expression of MMP-9 and MMP-2 with mode of tumor invasion and nodal involvement has previously been found in squamous cell carcinoma, and recently its utility has been proven in oral cancers.[20,21] However, some studies have shown that the activation of MMP-2 was more prominent as compared with MMP-9 in malignant oral SCCs. Elevated activation ratio of MMP-2 has also correlated significantly with lymph node metastasis in oral SCCs. Accordingly, MMP-2 was considered by some investigators as more selective molecular marker for prediction of metastatic potentials of oral SCCs.[21] Certain other studies have shown results favoring the use of MMP-9 as a prognostic indicator.[22]

Association between MMP-9 and vascular endothelial growth factor expression or micro vessel density has been found in head and neck carcinoma.[23]

GENETIC MARKERS OF ANGIOGENESIS

At the molecular level, the angiogenic switch operates as a shift in the balance of production by tumor cells of molecules that positively and negatively regulate angiogenesis. Over expression of positive factors and down regulation of inhibitors during the early tumor development are triggered by genetic mutations that control angiogenesis, such as:

1. over expression of RAS oncogene increases production of angiogenetic protein VEGF
2. deletion of Tp53 down regulates production of angiogenetic inhibitor protein - Thrombospondin-1

Although the expression levels of p53 and proliferating cell nuclear antigen (PCNA) were also investigated at different cut points, there was no significant correlation between their levels and incidence of occult neck metastasis.[24] A recent study indicates that the expression of cyclin D1 correlates with the presence of occult cervical metastases in head and neck carcinoma patients, thus suggesting that its immunohistochemical evaluation in biopsy samples may be used as an additional tool for identifying patients to be treated with elective neck dissection. However, this study had included advanced lesions in the node positive cases and early lesions in node negative cases. Hence in addition to the retrospective nature of the study, this could have possibly led to a misleading result.[25]

LYMPHANGIOGENESIS

The terms "lymphangiogenesis" and "hemangiogenesis" were introduced to distinguish lymph vessel from blood vessel formation. The presence and potential function of lymphatic vessels in tumors have remained controversial, mostly due to the absence of specific molecular markers that could distinguish between lymphatic and blood vessels. Emerging evidence suggest that the lymphangiogenic factors may also have to play an important role in lymph node metastasis in many cancers.

Intratumoral lymphangiogenesis is associated with locoregional disease recurrence in early-stage oral carcinoma and has been recently emerging.[26] The presence of IL is a useful discriminator in predicting the outcome of patients with absence of lymph node metastasis.

Various studies has stressed on the impact of tumor thickness as a significant factor that had predictive value for local disease recurrence survival and neck metastasis. The rationale was that the depth of invasion would determine proximity to blood and lymphatic vessels and facilitate the ability of the tumor to expand. In most cases, metastasis in squamous cell carcinoma occurs via the lymphatic vessels and dilation of lymphatic

vessels is frequently found in oral tumors with lymph node involvement. However, the influence of intratumoral or peritumoral lymphangiogenesis on in squamous cell carcinoma of the oral cavity is still controversial.

An association between lymphangiogenic growth factors, intralymphatic growth and tumor metastasis has been suggested. However, the role of intratumoral lymphangiogenesis [IL] in the progression of squamous cell carcinomas has not been studied.

Several markers have been utilized in the study of lymphangiogenesis. The main disadvantage of this method is that it relies on quantitative rather than qualitative differences between lymphatic and blood vessels and therefore requires a certain amount of subjective interpretation. In addition, most used antibodies react both with blood vessels and lymph vessels.[11] Some of these studies have correlated the presence of VEGF-C in the tumor cells, with an increased likelihood of lymph node metastasis in oral SCC with promising results.[27-31]

CONCLUSION

The validity of these hypotheses could prove valuable for the assessment of prognosis and design of new therapeutic approaches, hence needs further more systematic studies. While there are numerous studies assessing a plethora of histologic and molecular parameters in primary head and neck tumors, few studies have attempted to evaluate these parameters in the corresponding nodal metastases. Comparison of the levels of angiogenesis between primary and metastatic tumors in different types of cancer, including head and neck SCC, has generated conflicting results, which can be due, at least in part, to the use of different methods.

Taking these factors into consideration and, in view of the present evidence on the role on angiogenesis to predict occult angiogenesis being sparse, it seems apt to tap the potential utility of angiogenesis as a predictive marker for cancers of the head and neck with N0 Neck.

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