Intratumoral Lymphatics Are Essential for the Metastatic Spread and Prognosis in Squamous Cell Carcinomas of the Head and Neck Region

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ABSTRACT

Head and neck squamous cell carcinomas (HNSCCs) frequently disseminate to regional lymph nodes. To investigate the possible mechanisms involved, we studied the expression of cancer cell adhesion molecules together with lymphatic vascular and blood vascular markers in a panel of 97 primary HNSCC tumors and correlated expression levels with conventional clinicopathological parameters and with long-term prognosis. In particular, we measured the density of intratumoral and peritumoral lymph vessel lymph nodes as assessed with the marker lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and the density of tumor CD44, a receptor up-regulated in many metastatic cancers. Intratumoral LYVE-1+ lymphatic vessels were clearly associated with a higher risk for local relapse as well as with poor disease-specific prognosis (P = 0.02 and 0.0009, respectively). In contrast, a high density of peritumoral LYVE-1+ vessels was a sign of favorable survival (P = 0.05). Strong primary tumor CD44 expression was associated with a poor prognosis, an increased risk of local recurrence (P = 0.03 and 0.02, respectively), and an increase in resistance to radiation therapy (P = 0.03). CD44 was the only factor with an independent prognostic value for the disease-specific overall survival (P = 0.04). Our results suggest that intratumoral lymphatics play a greater role than peritumoral lymphatics in nodal metastasis of HNSCC and that tumor CD44 levels can predict sensitivity to radiation therapy. These parameters may be useful predictive and prognostic tools in the clinical management of HNSCC.

INTRODUCTION

At present, HNSCC1 holds the seventh position in the worldwide cancer statistics, largely as a consequence of abundant tobacco smoking and alcohol consumption in industrialized countries (1, 2). This heterogeneous group of squamous cell cancers arising from different anatomical locations in the oral cavity, pharynx, and larynx forms an oncologically challenging entity. Any form of recurrence is strongly associated with high mortality, and the total 5-year survival rate today is only 50%. The accuracy of established prognostic methods is frequently questionable and is insufficient for the design of personal treatment protocols. Indeed, clinical stage at presentation remains the most powerful tool for estimating the course of the disease. HNSCCs are known to metastasize predominantly via lymphatics (3), and treatment options range from different combinations of surgery and irradiation to chemotherapy and, more recently, chemoradiation (4, 5).

On the basis of recent studies, cDNA microarray analysis promises to be a powerful tool for future cancer prognostics (6–9). Nevertheless, there remains a need to identify functionally relevant markers not only for the purpose of conventional prognostic analyses but also for the design of more specific arrays. To date, the list of such markers includes proteolytic enzymes essential for invasion and metastasis (10–13), proteins involved in the growth and branching of tumor blood vessels (14–16), and adhesion proteins necessary for intercellular communication and tumor dissemination (17, 18). One of the important adhesion molecules involved in malignant processes is CD44, a widely expressed cell surface glycoprotein family also essential for numerous physiological events (17). Since the first evidence of a role of CD44 in malignant behavior was reported, expression patterns of CD44 variant forms on primary tumor cells have been correlated with known clinicopathological parameters and prognosis in many malignant diseases. However, in most cases, tumor expression of CD44 or its variants appears to be insufficient alone to determine the prognosis of a particular cancer (19).

Tumor-associated blood vessels not only nourish the primary tumor but also serve as an important route for metastasis; hence the levels of tumor angiogenic factors have frequently been measured and shown to correlate with cancer prognosis (10). However, only half of all malignancies are thought to use the blood vasculature as the primary route for tumor spread. The remaining half, which includes HNSCC, metastasize mainly via the lymphatics, a route that is presently not well understood. The reason for this situation has for long been the lack of immunohistochemical markers for the detection of lymphatic vessels. In the last few years, however, several lymphatic endothelial markers have been discovered, and the true role of the lymphatics in tumor metastasis has begun to emerge. Today, of several lymph endothelial molecules, LYVE-1 is considered to be one of the most valuable (20, 21). CD44 and LYVE-1 share both structural and functional similarities but show mutually exclusive expression in the blood vessels (14–16), and adhesion proteins necessary for intercellular communication and tumor dissemination (17, 18).

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expression to assess their utility as prognostic indicators and to test their statistical association with LYVE-1+ lymph vessel density in light of their proposed role in tumor metastasis.

**MATERIALS AND METHODS**

**Patients.** A total of 97 patients with histopathologically defined SCC of the head and neck region treated at the Department of Oncology and Radiotherapy and the Department of Otorhinolaryngology—Head and Neck Surgery at Ankara University Hospital during March 1989–March 1995 were included in the study. The median age of the study patients was 66 years (range, 25–97 years) at the time of diagnosis, representing that of the general population with HNSCC. Most of the patients were treated with preoperative radiation therapy followed by radical surgery. All tissue specimens used for staining were primary diagnostic samples from the tumors taken before any therapy. Details of the pretreatment characteristics are displayed in Table 1. Treatment and follow-up are described more closely in Table 2.

**Antibodies.** Hermes-3 and 1F1 are both mouse mAbs directed against different epitopes in the constant region of human CD44. 20E6 is a rat mAb that specifically recognizes the human CD44 variant exon v6 encoding domain. TK10/79, a rat mAb against mouse VAP-1, cross-reacts with human VAP-1 on paraffin-embedded sections. TK10/79 recognizes vascular endothelium and smooth muscle in vascular structures. The specificity and production of each of the above antibodies have been described elsewhere (28–31). The mAbs were used as ammonium sulfate-precipitated suspensions at a final concentration of 10 µg/ml. For the staining of lymphatic vessels, a polyclonal rabbit antihuman LYVE-1 antibody (20) was used. A commercial mAb against M5 8 6 0/paraffin-embedded serial sections of the primary tumor margin and inside the tumor cell islets (two categories). The number of TILs was jointly reviewed, and a consensus was sought. The number of TILs was semiquantitatively with light microscopy by two independent observers (S-M. M. and R. R.). The surface expression of CD44 and CD44v6 was graded into one of four categories: – (completely negative), +, ++, or +++ (strongly positive). In addition, borderline cases (−/+ and ++/+++ and −/−) were jointly reviewed, and a consensus was sought. The number of TILs was visually evaluated and scored as either virtually absent or clearly abundant at the tumor margin and inside the tumor cell islets (two categories). The vascularity (CD31+ vessels, VAP-1+ vessels, and LYVE-1+ vessels) was assessed by counting cross-sections of the stained vessels with a standardized method using a 0.25-mm2 microscopic ocular grid. Nine representative fields were analyzed, both within and at the immediate vicinity of the tumor cell islets. The vessel density was then calculated as vessel diameters observed/mm2. Vessels were also scored by location as IT or PT.

**Immunohistochemical Techniques.** A standard immunoperoxidase staining procedure was carried out to detect tissue expression of the studied molecules. Briefly, 5-µm paraffin-embedded serial sections of the primary tumor samples were stained with the Vectastain Elite ABC kits; the method is based on amplification of the biotin-streptavidin-horseradish peroxidase reaction (mouse or rabbit; Vector Laboratories, Inc., Burlingame, CA). Nonspecific binding was blocked with normal goat serum (for rabbit antihuman LYVE-1) or with normal horse serum (for all of the mAbs produced in mice or rat). 3,3-Diaminobenzidine in Tris-buffered saline containing 0.03% H2O2 was used as the substrate for the peroxidase-mediated reaction, and the sections were counterstained with Mayer’s hematoxylin. After staining, the sections were dehydrated, cleared in xylene, and permanently mounted in DePex (BDH Limited, Poole, United Kingdom).

**RESULTS**

**Table 1 Pretreatment characteristics of the 97 patients with HNSCC**

<table>
<thead>
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- **Follow-up time of living patients (months)**
  - Median: 24
  - Range: 1.5–87
- **Radiation therapy, n (%)**
  - Preoperative (60–70 Gy): 62 (64)
  - Definitive curative: 21 (22)
  - Postoperative: 2 (2)
- **Palliative**
  - No radiation therapy: 11 (11)
- **Surgery, n (%)**
  - Curative: 67 (70)
  - Palliative: 7 (7)
  - No operation: 22 (23)
- **Chemotherapy**
  - None: 13 (13)
  - Local recurrence, n (%): 34 (35)
  - Persisting disease, n (%): 20 (21)

The surface staining of tumor cells (CD44 and CD44v6) was verified semiquantitatively with light microscopy by two independent observers (S-M. M. and R. R.). The surface expression of CD44 and CD44v6 was graded into one of four categories: – (completely negative), +, ++, or +++ (strongly positive). In addition, borderline cases (−/+ and ++/+++ and −/−) were jointly reviewed, and a consensus was sought. The number of TILs was visually evaluated and scored as either virtually absent or clearly abundant at the tumor margin and inside the tumor cell islets (two categories). The vascularity (CD31+ vessels, VAP-1+ vessels, and LYVE-1+ vessels) was assessed by counting cross-sections of the stained vessels with a standardized method using a 0.25-mm2 microscopic ocular grid. Nine representative fields were analyzed, both within and at the immediate vicinity of the tumor cell islets. The vessel density was then calculated as vessel diameters observed/mm2. Vessels were also scored by location as IT or PT.

**Cheluminescence Enzyme Immunoassay.** The enzyme immunoassay (EIA) is a sensitive method for detection of small amounts of a specific protein. The EIA is based on the principle that an antibody reacts with an antigen in a specific manner. The antigen-antibody complex is then detected by a second antibody that is labeled with a radioactive isotope or a fluorescent dye.

**Statistical Analyses.** Statistical analyses were performed with the SAS 8.0 computer program for personal computers (SAS Institute Inc., Cary, NC). To test associations between different biological variables and between the biological markers and clinicopathological parameters, we used the χ2 test for categorical variables and Pearson’s correlation coefficient test for continuous data. Cumulative survival between the groups for each variable studied was compared with the log-rank test. The relative importance of different variables was analyzed by the Cox’s proportional hazards model.

**RESULTS**

**Low LYVE-1+ Lymphatic Vessel Density Correlates with All Conventional Markers of Poor Prognosis.** Representative sections with at least a 0.5-mm tumor margin were available from 71 speci-
mens. LYVE-1 expression was essentially restricted to thin-walled vessel structures but was occasionally seen in tissue macrophages and in some connective tissue. LYVE-1 vessels were predominantly located as clusters in the inflammatory front between the tumor tissue and the surrounding normal tissue. The median density of LYVE-1 vessels was 13/mm² (range, 0–52/mm²) in areas where they were found. Nevertheless, some tumors were totally devoid of LYVE-1 (n = 22). Occasionally, LYVE-1 vessels were observed to contain tumor cells, suggesting invasion. In statistical tests for correlations between LYVE-1 vessel density and the conventional clinicopathological parameters (the total LYVE-1 median vessel density was used as the cutoff value in the following comparisons), we found first that the density of lymphatic vessels was lower in high-stage tumors (P = 0.0004). In addition, both large tumor size and the degree of regional lymph node involvement correlated individually with low lymphatic vessel density (P = 0.01 and P < 0.0001, respectively). No significant association was seen between lymphatic vessel density and sex, age, number of relapses, histological grade, or disease-specific death (P > 0.1 for all of the above comparisons).

**Localization of LYVE-1**<sup>+</sup> **Vessels in Tumors Is Critical.** In view of reports that IT lymphatics may in some cases be nonfunctional, we examined the precise location of LYVE-1<sup>+</sup> lymphatic vessels in HNSCC in more detail. Specifically, we encoded the tumors by their LYVE-1<sup>+</sup> vessel localizations into two groups, IT and PT. IT LYVE-1<sup>+</sup> vessels are defined as those within the tumor cell islets and PT LYVE-1<sup>+</sup> lymphatic structures as those strictly located outside of the carcinoma tissue at the tumor margin. IT LYVE-1<sup>+</sup> vessels were observed in 13% (n = 9) of the samples studied. The presence of IT LYVE-1<sup>+</sup> lymphatics was strongly associated with disease-specific death: 41% of the patients who died of their HNSCC had LYVE-1<sup>+</sup> IT lymphatics compared with 6% of those who died of other reasons or were alive (P = 0.005) at the end of the follow-up period. PT LYVE-1<sup>+</sup> vessels were associated with all traditional clinical parameters that predict more favorable prognosis. PT vessels positive for LYVE-1 were found predominantly in tumors with low clinical stage (stages I and II; P = 0.008). Similarly, both components of the clinical staging, i.e., the tumor size and the cervical lymph node status, were independently associated with the localization of LYVE-1<sup>+</sup> vessels (P = 0.04 and 0.0006, respectively), so that IT vessels were found in large tumors and in tumors that had already spread to the regional lymph nodes. Examples of vascular stainings are shown in Fig. 1.
High VAP-1⁺ Vessel Density Is Associated with Tumor Inflammation. A VAP-1⁺ vessel density above the median level was observed more commonly in low-grade tumors than in high-grade tumors (P = 0.02). Moreover, there was a direct trend toward a correlation between VAP-1⁺ vessel density and the number of TILs seen in the tumor sections (P = 0.07), supporting the idea that VAP-1 may participate in the immune response against the tumor. As expected, VAP-1⁺ vessel density was in concordance with that of CD31⁺ vessel density (P < 0.0001). The density of total tumor vasculature was lower in small tumors when CD31 was used as the pan-endothelial marker (P = 0.03). Additionally, tumors with more VAP-1⁺ vessels also had more LYVE-1⁺ vascular structures (trend toward a correlation; P = 0.07). We found no significant associations between VAP-1⁺ vessel density and age at diagnosis, sex, number of relapses, disease-specific death, the conventional clinical parameters, or CD44 (P > 0.1 for all of the above comparisons). For the above comparisons, the tumors were divided into two groups by the median VAP-1⁺ or CD31⁺ vessel density/mm².

Strong Tumor Cell CD44 Expression Is Associated with Decreased Response to Radiation Therapy in HNSCC. All SCCs expressed CD44 family members either moderately or strongly [n = 37 (38%) and n = 56 (58%), respectively], and only 4 tumors (4%) were weakly positive for CD44. In the case of CD44 variants containing the v6 exon, 22% (n = 19) of the tumors were negative or only very faintly positive. Moderate expression was the most common level found [n = 43 (44%)], and 34% (n = 33) of the tumors had strong expression. Serum levels of soluble CD44 measured at diagnosis were available for 38 patients. The median serum concentration of soluble CD44 was 30.6 ng/l (range, 1.6–603.8 ng/l). The most interesting finding concerning the role of CD44 in HNSCC tumor biology was that malignancies with strong CD44 expression at the time of diagnosis still contained active live cancer cells at surgery that was performed after an intense course of radiation therapy [n = 22 (67%); P = 0.03]. We found a weak trend toward a correlation between the primary tumor CD44 expression and soluble CD44 concentrations (P = 0.07, Pearson’s correlation test) but found no other statistical associations between serum CD44 and clinicopathological parameters or any of the histological markers. Primary tumor CD44v6 expression was associated with CD44 expression (P = 0.0025) but with no other parameters (P > 0.1 for all of the comparisons). See Fig. 2 for examples of the CD44 staining intensities.

Factors Associated with the Risk for Local Recurrence. SCC with detectable IT LYVE-1⁺ vessels had a significantly higher risk for relapse of the cancer (P = 0.02). Furthermore, strong primary tumor CD44 expression was seen as a marker of a higher relapse probability (P = 0.03). Logically, patients whose tumors presented with active malignant residues at the time of surgery had a higher risk for developing local recurrences (P = 0.03). As expected, high clinical stage, large tumor size, and regional lymph node involvement all predicted a higher risk for relapses (P = 0.0009, P = 0.0005, and P < 0.0001, respectively). In multivariate analysis, however, none of the above factors established an independent prognostic value for the incidence of local recurrence (Fig. 3).

Factors Prognostic for Disease-specific Survival. HNSCC patients with detectable LYVE-1⁺ lymphatics (either IT or PT) had a more favorable survival compared with those with tumors totally devoid of LYVE-1 (P = 0.05). However, IT localization of the LYVE-1⁺ vessels in particular was strongly associated with poor survival (P = 0.0009). In addition, strong primary tumor CD44 expression was a sign of poor survival (P = 0.02). All of the conventional clinicopathological parameters, i.e., occurrence of recurrences, large tumor size, lymph node metastases at the time of diagnosis, and high clinical stage, were all very strong disease-specific prognostic markers (P = 0.002 for clinical stage; P < 0.0001 for all others; Fig. 4).

Independent Prognostic Factors in HNSCC. We performed a multivariate analysis to determine which of the studied variables might be suitable as independent prognostic factors for disease-specific overall survival. In addition to the biological parameters that showed correlation in univariate analyses, clinical stage, tumor size (T), node involvement (N), and the presence or absence of recidivisms were included in the Cox regression model. The only parameter with independent prognostic value was CD44 expression of the primary tumor (P = 0.04; hazard ratio, 4.7; 95% confidence interval, 1.0–20.8). Absence of LYVE-1⁺ lymphatics gave a trend toward association with poor survival (P = 0.06; hazard ratio, 0.27; 95% confidence interval, 0.07–1.06).

DISCUSSION

HNSCC is one of the most common cancers. Although potentially curable by local radiotherapy and surgical resection, the overall 5-year survival rate is only 50%, largely because of the propensity of some HNSCC tumors to disseminate via the lymphatics. Indeed, a finding of lymph node involvement is one of the strongest predictors of poor prognosis. The tailoring of individual treatment programs to aggressively treat those cancers at greatest risk of dissemination would likely improve long-term survival. Hence, there is an urgent need to identify characteristics of the primary tumor that might predict nodal metastasis. One of the main aims of the present study was to assess tumor lymph vessel density as a predictive indicator by examining its relationship with prognosis. Surprisingly, we found that the presence of LYVE-1⁺ lymphatics was associated with longer overall survival. Inspection of the tumor-associated lymph vessel revealed that in many cases these were present within the tumor body (IT lymphatics) in addition to the tumor margin (PT lymphatics). Furthermore, when the numbers of each type of vessel were analyzed, it became clear that the presence of LYVE-1⁺ IT lymphatics was associated with poor 5-year survival and an increased tendency for nodal metastasis. In contrast, a high density of PT lymphatics was associated with higher 5-year survival rates. These results confirm earlier findings that IT lymphatics are present in HNSCC and further strengthen the suggestion that IT vessels act as a conduit for nodal metastasis (32).

There has been considerable debate about the functional significance of IT lymphatics. Many investigators have suggested that tumors do not possess a lymphatic supply. In addition, in cases where IT lymphatics have been detected, these have been reported to be non-functional, based on the results of dye uptake measurements (33). Although we cannot comment on the capacity of lymph vessels in HNSCC to take up fluorescent dyes, the strong implication is that they are involved in nodal metastasis. Beasley et al. (32) have published results similar to ours in HNSCC. Nevertheless, in the study by Padera et al. (33), metastases were discovered despite of no detectable IT LYVE-1⁺ vessels. They proposed that functional lymphatics available at the tumor margin are sufficient for promoting metastasis by offering a larger area for tumor cell escape. In contrast, in our more clinically oriented study on HNSCC, we found that PT lymphatics offer a markedly better survival capacity for the patient. Moreover, we discovered that the presence of LYVE-1⁺ lymphatic vessels in the PT region was more favorable for the patient than a total absence of LYVE-1⁺ lymphatic vessels. A question therefore arises concerning the role of PT lymphatics in the pathology of HNSCC. One possibility is that they facilitate recruitment of antigen-presenting cells, such as dendritic cells, which then cross-prime cytotoxic T cells in draining lymph nodes. Such a scenario would explain the correlation between PT lymph vessel density and positive prognosis. Schoppmann et al. [35]
recently showed that tissue macrophages support the growth of a lymphatic network in the PT region by expressing lymphatic growth factors. They propose that the rationale behind this would be enhanced antigen presentation by blood monocyte-derived dendritic cells.

The second aim of the study was to investigate the role of tumor cell CD44 in the behavior of HNSCC. Changes in CD44 expression have been successfully correlated with the prognosis in several tumor types. It has become quite clear that CD44 has very different roles in the behavior of different tumors (19). Here we report for the first time in clinical material that high CD44 expression is evidently associated

Fig. 2. Examples of CD44 expression on tumor cells. 3G6 against avian T cells was used as the negative control (neg. co.). Magnification, ×200.
with the mechanisms malignant cells use to survive the radiation effect. This may further contribute to the higher risk for relapse and thus to poor prognosis. Recently, Yasuda et al. (35) published an experimental report on their finding that apoptosis is reduced in lung cancer cells after CD44 stimulation by down-regulation of Fas. They proposed that hyaluronan in the extracellular matrix would be responsible for the stimulation of CD44. On the basis of our results, the same mechanism may be involved in HNSCC as well. Furthermore, CD44 has been reported to be a docking molecule in HNSCC (36), and this may explain the higher recurrence rate seen in tumors with strong CD44 expression in our study. CD44 has an acknowledged role not only in anchoring tumor cells but also in tumor dissemination, and these processes involve differential regulatory mechanisms. Locally, CD44 may dock tumor cells to the surrounding tissue structures and thus support cancer growth at the primary location. In contrast, CD44-mediated invasion and consequent metastasis formation is, at least, partially attributable to the biological association of CD44 with the proteolytic form of matrix metalloproteinase 9 on the tumor cell surface (37). How the balance between local growth support and tumor cell detachment is controlled, however, remains unclear.

In conclusion, IT localization of LYVE-1^+ lymphatic vessels is a novel indicator of poor prognosis in HNSCC, whereas PT LYVE-1^+ lymphatics indicate a positive prognosis. Moreover, tumors that strongly express CD44 are more resistant to radiation therapy and have higher incidence of local recurrences. These results suggest that both LYVE-1 and CD44 may be useful prognostic markers in HNSCC.

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