Expression Pattern of Chemokine Receptor 6 (CCR6) and CCR7 in Squamous Cell Carcinoma of the Head and Neck Identifies a Novel Metastatic Phenotype

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ABSTRACT

Squamous cell carcinoma of the head and neck (SCCHN) metastasizes predictably to cervical lymph nodes, with low rates of distant metastases. Tumor cells can express various receptors that facilitate such metastatic spread to lymph nodes and other nonlymphoid organs. Chemokine receptors (CCR), normally expressed on lymphocytes, control immune and inflammatory cell migration, providing a link between innate and adaptive immunity. Chemokine receptor expression was evaluated in SCCHN, using paired primary and metastatic tumors cell lines, and paired primary and metastatic biopsies from the same patients. Quantitative reverse transcription-PCR showed a consistent pattern of CCR6 down-regulation and up-regulation of CCR7 in metastatic cells and tissues. Chemotaxis assays, ligand-induced receptor down-regulation, and specific antibody blocking experiments supported the quantitative reverse transcription-PCR results, indicating that these surface receptors were functional on metastatic tumor cells. Cells derived from a highly metastatic mouse model of SCCHN were used to confirm CCR7 up-regulation in tumor cells with higher metastatic potential. CCR6 down-regulation is consistent with its decreased expression in cells emigrating from peripheral mucosal sites, whereas CCR7, important for homing of immune cells to secondary lymphoid organs, was significantly up-regulated. Thus, CCR6, CCR7, and their ligands, normally important in controlling immune cell trafficking in response to inflammatory stimuli, may have an important role in determining the metastasis of SCCHN cells in vivo.

INTRODUCTION

Head and neck cancer is the sixth most common tumor in the United States, with 40,000 new cases annually (1). The 5-year survival is only 30%, mainly due to the frequent presence of metastasis at diagnosis, and the pattern of regional cervical metastasis (as opposed to distant organs) in this disease is remarkably consistent (2). Because metastasis is the best predictor of prognosis, an urgent goal in head and neck oncology is to develop improved antimetastatic agents for clinical use. A better understanding of this unique metastatic process is necessary to enable the development of therapies designed to prevent tumor dissemination.

The predilection of metastases for specific organs may depend on a variety of factors (3–4). According to the one theory, all of the migrating tumor cells can enter any tissue but would form a metastasis only if all requirements for their growth were met (5). The second suggests that tissue-specific adhesion molecules on endothelial cells select migrating cells able to attach and form a premetastatic nucleus of cells (6). The most recent theory proposes that chemotactic agents, produced by stromal or immune cells, lead invasive cancer cells to the tissue of their potential secondary growth (7).

Chemokine molecules constitute a superfamily of inducible, secreted, proinflammatory proteins (8–11) involved in a variety of immune responses, acting primarily as chemoattractants and activators of specific types of leukocytes (12–14). Recent studies have shown the involvement of chemokine receptors in cancer metastasis (15–17). Chemokine ligand (CCL19)/MIP3-β and CCL21/SLC, two chemokines constitutively expressed by lymph nodes (LN) and other immune cells, share a common chemokine receptor, CCR7 (16). This receptor is also expressed on mature dendritic cells (DC), naïve, and some memory T cells. A recent study reported high levels of expression of CCR7 and CXCR4 in breast cancer cells and linked the receptor expression to the metastatic destination of tumor cells (17).

Because of the unique site of cervical metastasis seen in squamous cell carcinoma of the head and neck (SCCHN) without frequent distant metastases, we hypothesized that SCCHN cells might also use a chemokine-mediated mechanism during the process of LN metastasis of cancer cells, similar to those regulating lymphocyte trafficking. However, we found that CCR7 up-regulation alone did not appear sufficient, rather loss by tumor cells of surface CCR6 enabled their LN metastasis. Interestingly, these results show that SCCHN cell metastasis displays a similar receptor pattern as manifested by immune cells, during the normal maturation and trafficking to LN away from peripheral sites of inflammation. Metastatic tumor cells appear to down-regulate CCR6 expression and up-regulate CCR7, enabling migration to secondary lymphoid tissues. SCCHN is thought to metastasize through afferent lymphatic channels, as opposed to the hematogenous route. Our data suggest that CCR7-mediated metastasis, using established gradient expression of the cognate ligand on vascular channels infiltrating tumors, may enable SCCHN cells to access the cervical LN.

MATERIALS AND METHODS

Human Tumor Samples and Cell Lines. Fourteen sets of paired human head and neck primary tumors and tumor-containing metastatic LNs were harvested at surgery and immediately snap frozen at −80°C until RNA extraction. Institutional Review Board-approved, written informed consent was obtained from all of the patients donating specimens for this study, through the Department of Otolaryngology, University of Pittsburgh. Clinical and demographic data for paired tumor/metastatic cell lines and fresh tumor/metastasis specimens are shown in Table 1. Cell lines PCI-4T/M, PCI-6T/M, PCI-15T/M, PCI-57/T/M (T: primary tumor, M: metastatic LN) were derived from the patient lesions (see Table 1) at the University of Pittsburgh (18–20). The panel of cell lines TU686, 686LN, derived from a SCCHN patient’s primary (TU) and metastatic (LN) tumor, and 686LN-M3a2, 686LN-M3b2, and 686LN-M3b3 (3 different clones derived from a highly metastatic derivative of 686LN cells passed three times in nude mice; Refs. 20–22), were used to compare CCR7 expression by quantitative reverse transcription-PCR (qRT-PCR).

Cells were cultured in DMEM (Invitrogen Corp., Carlsbad, CA), which contained 8% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen Corp.).

Immunohistochemistry. Immunostaining was performed by the avidin-biotin-peroxidase method; color reaction was developed in diaminobenzidine.
solution, and counterstaining was performed with Mayer’s hematoxylin solution. Tissues were treated using 2H4 primary antibody (Ab), mouse IgM, anti-human CCR7 (BD Biosciences Inc., San Diego, CA), or 53103111 mouse IgG2B, anti-human CCR6 (R&D Systems Inc., Minneapolis, MN). Ab titrations and isotype control Ab determined optimal staining conditions.

**DC Cultures.** DCs were generated from human peripheral blood mononuclear cells (24). Briefly, peripheral blood mononuclear cells were isolated from leukocyte-enriched buffy coats by density-gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences AB, Uppsala, Sweden). Peripheral blood mononuclear cells (5 × 10^6) were resuspended in 10 ml of warm AIM-V medium. After 1 h at 37°C, nonadherent cells were removed, and adherent cells were cultured in 1 ml of DC medium supplemented with interleukin 4 (1 × 10^5 units/ml) and granulocyte macrophage colony-stimulating factor (1 × 10^5 units/ml) for 35°C, 30 s, total for 33 cycles. DC maturation was induced using AIM-V plus 10 ng/ml tumor necrosis factor α, 10 ng/ml interleukin 1β, and 10 ng/ml interleukin 6 for 18 h. Both immature DCs and mature DCs were stained by flow cytometry, using Abs to CD80, CD86, HLA-DR, CD40, and CD83 for staining as described previously.

**Isolation of RNA and Reverse Transcription (RT-PCR) Analysis.** Total RNA was isolated from tumor lines and biopsies using RNeasy Mini kit (Qiagen Inc.) according to the manufacturer’s protocol. All of the RT-PCR was done by using Thermoscript One-step System kit (Invitrogen Corp.), using primers as described (25). Reverse transcription was performed at 55°C for 35 min and 95°C for 5 min. PCR was performed at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, total for 33 cycles.

**Quantitative Real-Time RT-PCR.** RNA from normal cervical LN was used as positive control and a calibrator of relative CCR7 expression. This calibrator RNA sample was amplified in parallel on all of the RT-PCR plates to allow comparison of samples run at different times. Reverse transcription was performed with random hexamer primers and Superscript II (Invitrogen Corp.) or rat antihuman CCR7 antibody, IgG2b (BD Biosciences Inc.) for 1 h at 4°C. After washing the cells twice in 4-ml aliquots of the same PBS buffer, cells were resuspended and fixed in 200 μl 1% (v/v) paraformaldehyde for 30 min. The filter was carefully removed, and cells in each lower well were counted under a light microscope in at least five different fields (original magnification, ×200).

For CCR-blocking experiments, CCR specific monoclonal Abs (mouse antihuman CCR6 or antihuman CCR7 antibody) were used. The cells were incubated with monoclonal Ab (4 μg/10^6 cells) for 1 h at room temperature before loading onto the filter, using an isotype control, and the chemokine assay was performed as above.

**Statistical Analysis.** Data are presented as mean ± SD of repeated assays. The signed rank test was used to test differences among samples that were matched by patient, cell line, or cell type.

**RESULTS**

**Chemotaxis and CCR Blocking Assays.** Migration studies used disposable 96-well chemotaxis chambers (ChemoTx Neuroprobe, Gaithersburg, MD; Ref. 28) with a 5-μm pore size and 6-mm width/well, in triplicate. Cells and chemokina blockers were resuspended in DMEM (Invitrogen Corp.) supplemented with 0.5% (w/v) BSA (Invitrogen Corp.). Human immature DCs and mature DCs derived were used as controls. CCR6 ligand (MIP-3α) and CCR7 ligands (MIP-3β and 6Ckine/SLC; R&D Systems, Inc.) were used. Aliquots (29 μl) of the CCL were added to the wells at a concentration of 50 or 500 ng/ml, and 50 μl of the cell suspension (1 × 10^5 cells) were placed on top of the filter. After 4 h or 18 h of incubation at 37°C in the presence of 5% CO_2, the cells were removed with a cell harvester, and the filter was washed with medium. A 50-μl aliquot of trypsin-EDTA (Invitrogen Corp.) was added to the filter for 3 min at 37°C; 5% CO_2 before centrifuging at 1500 rpm for 10 min. The filter was carefully removed, and cells in each lower well were counted under a light microscope in at least five different fields (original magnification, ×200).

**Differential Chemokine Receptor Expression in Primary and Metastatic Tumor Cells.** To determine whether chemokine/chemokine receptor interactions are involved in the metastatic process of SCCHN, we tested the expression of various chemokine receptors (CCR1 to CCR10 and CXCR1 to CXCR5) by RT-PCR in four pairs of SCCHN cell lines (data not shown), derived from the primary (T) or metastatic (M) tumor of each patient (Table 1). Comparing the semiquantitative RT-PCR results of CCR expression in each pair of cells, a consistent pattern of CCR6 down-regulation compared with metastases and higher CCR7 expression was found in metastatic tumor cell lines (Fig. 1A). Importantly, the same pattern of CCR6 down-regulation and CCR7 up-regulation in metastatic as compared with primary tumor tissues was confirmed by standard RT-PCR analysis in 14 sets of paired primary and metastatic biopsy specimens, obtained from the same patients (Fig. 1B). Fig. 1 shows data for the first 12 patients, whereas the 7/M biopsy samples of 2 other patients demonstrated similar results (data not shown). Whereas other CCR genes were expressed to various extents in tumor cells and tissues, no other consistent expression pattern could be observed for the other CCRs or between individual patients.

These findings were not absolute. We observed no loss of CCR6

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**Table 1.** Clinical and demographic data of cell lines and fresh tumors obtained from squamous cell carcinoma of the head and neck (SCCHN) patients in this study.

<table>
<thead>
<tr>
<th>Cell lines/patients</th>
<th>Age</th>
<th>Sex</th>
<th>Primary site</th>
<th>Stage</th>
<th>Grade</th>
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<tr>
<td>PCI-4</td>
<td>51</td>
<td>Male</td>
<td>Larynx</td>
<td>T1N1M0</td>
<td>Mod well</td>
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<tr>
<td>PCI-6</td>
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<td>Oropharynx</td>
<td>T3N3M0</td>
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<tr>
<td>PCI-15</td>
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<tr>
<td>P-1</td>
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</tr>
<tr>
<td>P-2</td>
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<td>Mod well</td>
</tr>
<tr>
<td>P-3</td>
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<tr>
<td>P-4</td>
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<td>Oropharynx</td>
<td>T2N2M0</td>
<td>Poor</td>
</tr>
<tr>
<td>P-5</td>
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</tr>
<tr>
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<td>Oral cavity</td>
<td>T4N1M0</td>
<td>Mod well</td>
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</table>

* Grade refers to degree of differentiation; mod well, moderately well; mod, moderate.
expression on 686LN cells, the human parental metastatic cell line used in the metastatic SCCHN mouse model (21), developed by passing these cells through several serial metastases in nude mice. Standard RT-PCR analysis of these cells (TU686 and 686LN, which were both derived from a human primary and metastatic tumors) showed expression of both CCR6 and CCR7 in each cell line. CCR6 down-regulation was not measured quantitatively, due to its apparent lack of expression in M cells in our data obtained in the 14 human primary and metastatic tumor biopsies.

Significant Up-Regulation of CCR7 Expression in Metastatic Tumor Cells by qRT-PCR. As described above, CCR7 mRNA was detected in both primary and metastatic tumor lines and fresh specimens by RT-PCR analysis (see Fig. 1), raising the question as to whether levels of expression were similar between primary and metastatic tumor cells. Therefore, using real time qRT-PCR, we determined the relative CCR7 expression between primary tumors and metastases, in paired cell lines and fresh tumor tissues. We carefully dissected tumor from surrounding nodal architecture to remove contaminating immune cells. Because CCR6 expression was not detected in metastases, it was not analyzed using qRT-PCR. These experiments showed up to 50-fold higher expression level (P < 0.001) of CCR7 in metastases than in primary tumors (Fig. 2).

To determine whether up-regulation of CCR7 was associated with metastasis in vivo, we studied three highly metastatic cell lines, derived from the poorly metastatic human SCCHN cell line, 686LN, by qRT-PCR. These three randomly selected, subcloned cells were isolated from the third passage of increasingly metastatic tumor derivatives in a metastatic mouse model (21, 22). CCR7 expression in parental SCCHN cells, TU686 and 686LN, derived from a primary (TU) and metastatic (LN) human SCCHN tumor (29) was compared with that determined in these three highly metastatic subclones (686LN-M3a2, 686LN-M3b2, and 686LN-M3b3), produced by serial passages of metastases in a well-described metastatic nude mouse model (21–23). As shown in Fig. 3, CCR7 up-regulation was determined quantitatively to be significantly associated with increased metastatic potential when comparing TU686 with 686LN (P < 0.05), as well as comparing TU686 with any of the three subcloned, highly metastatic cells (P < 0.005). The three subcloned, highly metastatic cells were randomly selected to ensure that this up-regulation was a generally observed phenomenon. Because of the homology between murine and human CCR7 ligands and cross-reactivity with human CCR7 (30, 31), functionally relevant signaling can explain this significant CCR7 up-regulation.

Confirmation of Chemokine Receptor Expression Pattern by Flow Cytometry. To analyze the T/M cell lines by flow cytometry, surface CCR6 but not CCR7 was stained on primary tumors (T), whereas metastases (M), had low if any expression of this receptor (Fig. 4). On the other hand, metastatic tumors expressed surface CCR7, but not CCR6 (see Fig. 4A). Increased expression of CCR7 in 686LN and 686LN-M3 cells compared with TU686 cells was also observed using this technique (data not shown). Fig. 4B also shows CCR7 responses after treatment with its ligand, MIP-3β, at a concentration of 500 ng/ml for 4 h by flow cytometry. Metastatic (M) cells manifest the expected CCR7 down-regulation only after treatment with the appropriate ligand (MIP-3β). However, down-regulation of CCR6, after treatment of primary (T) tumor cells, was observed after MIP3α treatment (data not shown). Treatment of each M cell line with the CCR6 ligand, MIP-3α, as a control did not result in CCR7 down-regulation, as shown in Fig. 4B.

Fig. 1. Loss of CCR6 expression and up-regulation of CCR7 expression in squamous cell carcinoma of the head and neck (SCCHN) cell lines and human tumors. CCR6 and CCR7 mRNA expressions were determined by reverse transcription-PCR. A, in 4 sets of paired SCCHN cell lines (see Ref. 19; T, cell line from primary tumor; M, cell line from metastasis); and B, in 12 sets of paired tumor specimens.

Fig. 2. Quantitative reverse transcription-PCR analysis of CCR7 expression was performed and analyzed. A and B, in cell lines. C and D, in paired tumor specimens. Expression of CCR7 was calculated relative to the endogenous control housekeeping gene, β-glucuronidase (GUS), using the ΔΔCT method (Relative expression = 2^−ΔΔCT), where ΔΔCT = ΔCT (CCR7) − ΔCT (GUS). A and B, mean of two experiments in duplicate, n = 4; bars, ± SD. E, mean; bars, ± SD; n = 4. F, mean; bars, ± SD, n = 12. * P < 0.001 indicating significant differences.

Fig. 3. Quantitative reverse transcription-PCR (qRT-PCR) of CCR7 expression in 686 derived metastatic tumor cells. Squamous cell carcinoma of the head and neck (SCCHN) cells 686 TU and LN, derived from a primary (TU) and metastatic (LN) human SCCHN tumor, were compared with three subclones of a highly metastatic derivative tumor (686LN-M3a2, 686LN-M3a3, and 686LN-M3b3), produced by serial passages of metastases in a metastatic nude mouse model. CCR7 up-regulation was quantitatively determined in these cells, indicating the association of CCR7 up-regulation with increased metastatic potential. Mean, bars, ± SD, n = 8. * P < 0.05; ** P < 0.005.
Immunohistochemical Staining of CCR6 and CCR7 in Fresh Tumor Biopsies Shows That a Novel Metastatic Expression Pattern Is Present in Vivo. To study chemokine receptor expression in vivo, paraffin tumor blocks were stained for CCR6 and CCR7 (Fig. 5). Using immunohistochemistry, differential CCR6 and CCR7 expression in primary versus metastatic head and neck tumors (n = 4 patients) was confirmed (P1-P4 in Table 1) from patient tumors studied using qRT-PCR. Immunostained tissue sections indicated that primary tumors expressed high levels of CCR6, but metastatic tumors expressed high levels of CCR7 (Fig. 5, A–F). These results rule out the possibility that high CCR7 expression in metastatic tumors was due to lymphoid cells in tumor-metastatic LN specimens, and is consistent with the retention of different migratory responses of primary (T) and metastatic (M) tumor cell lines, even after culture in vitro for months.

Fig. 5. Immunohistochemical staining of CCR6 and CCR7 expression in tumor specimens indicates coordinate expression pattern between primary and metastatic tumors in vivo. A–C, primary tumor; D–F, lymph node metastasis. Original magnification was ×200. A and D, anti-CCR6; B and E, anti-CCR7; C and F, isotype control monoclonal antibody (Ab). The results from a representative staining experiment are shown from the same patient (P3).

Tumor Cell Chemokine Receptors Mediate Chemotaxis in Response to Selective Chemokines. We next analyzed the capability of paired T/M cells to migrate in vitro in response to the respective ligands. Transwell migration assays followed by blocking of the receptors with receptor-specific monoclonal Abs showed that both CCR6 (on T cells) and CCR7 (on M cells) surface receptors are functionally active. Media-pulsed and isotype-matched monoclonal Ab-pulsed wells (data not shown) were used to control for CCR-specificity of the blocking effect. Increase in migration of primary tumor cells was also observed in all three of the cell lines examined, in response to MIP-3α (Fig. 6A). Enhanced responsiveness to both CCR7 ligands, MIP3β and SLC (Fig. 6B), was observed in three of three cell lines tested, consistent with CCR7 up-regulation seen by qRT-PCR.

MATRIGEL invasion assays using 686LN (poorly metastatic) cells and 686LN-M3a2 (highly metastatic derivative) as described previously (22), indicated a correlation between increased CCR7 expression detected by qRT-PCR in 686LN-M3a2 cells, with invasive potential.

DISCUSSION

Metastatic sites have been correlated with chemokine receptor expression in some metastatic tumor cells (15, 17, 31, 32, 34, 35). Our report indicates a novel role for loss of expression of the inflammatory chemokine receptor, CCR6, in addition to CCR7 up-regulation, during the metastatic process. Whether metastasis takes place via hematogenous (similar to T cells) or lymphatic channels, is not presently clear from our findings, although certainly both mechanisms may be important, depending on angiolymphatic structures invaded in proximity of CCR7-expressing tumor cells.

The consistent loss of expression of CCR6 in our system is reminiscent of the response of immune cells to inflammatory stimuli. For example, after antigen uptake or other stimuli that activate their progression to mature DCs, CCR6 expression is lost, whereas CCR7 is significantly up-regulated. Emigration from peripheral sites and trafficking to regional LNs usually leads to interaction with other immune cells. Whereas CCR7 is also used by circulating naïve and memory T cells, we would expect that metastatic SCCHN cells are likely to access cervical LNs using afferent lymphatics, similar to DCs. However, our data do not currently distinguish between vascular pathways used for this phenomenon, as our studies only used DCs as controls in qRT-PCR and migration studies. Lymphangiogenesis is
CHEMOKINE RECEPTORS IN HEAD AND NECK CANCER METASTASIS

Fig. 6. Migration assay of squamous cell carcinoma of the head and neck (SCCHN) cells in response to chemokines in vitro. Chemotaxis of three sets of paired SCCHN cell lines was measured in 96-well chemotaxis chambers at the concentrations determined previously to give optimal migration (500 ng/ml). Immature dendritic cells and mature dendritic cells were used as controls. CCR6 ligand (MIP-3α) in A and CCR7 ligand (MIP-3β and 6Cln/SLC) in B were used as chemotractants (500 ng/ml), in 4 h assays. Mean, bars, ± SD; n = 6; * P < 0.005.

not well understood currently, including the ligand-mediated signals related to embolism and implantation into regional LN.

The abundant expression of the homeostatic chemokine SLC (a ligand of CCR7) in LNs makes it a likely candidate to attract CCR7+ tumor cells (32–35). On the other hand, CCR6 down-regulation in metastatic tumor cells indicates that the coordinate expression of CCR6 and CCR7 may be important in orchestrating SCCHN metastasis (31). Remarkably, our observations in fresh tumor specimens were maintained in cell lines cultured in vitro for months at a time. This also helps rule out that, in fresh metastatic tumor biopsies, CCR7 up-regulation was simply the result of immune cell contamination of our tumor RNA. In addition, the pattern of this coordinate CCR expression pattern in metastases indicates that the up-regulation of CCR7 in these tumor cells is not due to the LN microenvironment in fresh tissues, because even in metastatic tumor cell lines, this effect is maintained over long periods in culture. The finding that increasingly metastatic tumor derivatives in a metastatic mouse model showed quantitatively increased CCR7 expression demonstrates the in vivo importance of this phenomenon, in a completely separate experimental system.

Thus, a specific and differential expression pattern of CCR6, CCR7 is manifested by metastatic tumor cells, as their migration appears to be regulated by the loss of CCR6 expression, acting as the brake, and gain of expression of CCR7, acting as the accelerator, leading to migration of tumor cells to proximal lymphoid tissues. Although the organ predilection of metastatic cells might ultimately depend on multiple chemokine receptor-ligand interactions as we encountered here, rather than a single interaction, our data suggest that inhibition of CCR7 signaling could be a clinically useful target for therapy in the prevention of SCCHN metastasis. Additional work is under way to determine metastatic pathways responsible for tumor cell chemokine receptor-associated metastasis, proinflammatory, nuclear factor κB-mediated signaling pathways (36–38). Preventing such a poor prognostic feature in this disease would likely enable more successful locoregional tumor control and improve survival, even with current surgical and/or chemoradiotherapeutic modalities.

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