Tumor-Associated Lymphangiogenesis in the Development of Conjunctival Melanoma

Ludwig M. Heindl,1 Carmen Hofmann-Rummelt,1 Werner Adler,2 Jacobus J. Bosch,3 Leonard M. Holbach,1 Gottfried O. H. Naumann,1 Friedrich E. Kruse,1 and Claus Cursiefen1

PURPOSE. To analyze whether tumor-associated lymphangiogenesis is concurrent with the progression of premalignant conjunctival melanocytic intraepithelial neoplasia (C-MIN) into invasive conjunctival melanoma (CM) and to study its association with prognosis.

METHODS. Twenty patients with CM were closely matched with 20 patients with C-MIN with atypia and 20 with C-MIN without atypia regarding tumor size, tumor location, tumor extension, and patient’s age. All conjunctival specimens were analyzed for the immunohistochemical presence of proliferating lymphatic vessels, with LYVE-1 and podoplanin used as specific lymphatic endothelial markers and Ki-67 as a proliferation marker.

RESULTS. Intratumoral and peritumoral proliferating lymphatic vessels were detected in none of the C-MINS without atypia, in 10 of the 20 C-MINS with atypia, and in all 20 CMs. Invasive CM vessels were detected in none of the C-MINs without atypia, in 10 of the 20 C-MINs with atypia, and in all 20 CMs. Invasive CM showed a significantly higher intratumoral density of lymphatic vessels than did C-MIN with atypia (P ≤ 0.001). Patients with high intratumoral lymphatic density revealed significantly lower recurrence-free survival rates (P = 0.041) in C-MIN with atypia and significantly lower recurrence-free (P = 0.006), lymphatic-spread–free (P = 0.041), distant-metastasis–free (P = 0.029), and melanoma-specific survival rates (P = 0.029) in CM.

CONCLUSIONS. Development of CM from premalignant precursors is concurrent with the outgrowth of lymphatic vessels. This active lymphangiogenesis seems to be associated with an increased risk of local recurrence in patients with C-MIN with atypia and with an increased risk of local recurrence, lymphatic spread, distant metastasis, and tumor-related death in patients with invasive CM. (Invest Ophthalmol Vis Sci. 2011;52: 7074–7085) DOI:10.1167/iovs.11-7902

Malignant melanoma of the conjunctiva (CM) is the second most common and one of the most fatal malignancies of the ocular surface, showing an increased trend in its age-adjusted incidence up to 0.8 per million in Caucasian populations during the past three decades.1–3 Despite diverse advances in the treatment of the primary tumor, including surgical excision, cryotherapy, brachytherapy, and adjuvant topical chemotherapy (mitomycin C, 5-fluorouracil, and interferon-alpha), the long-term rate of local and systemic failure remains high.4–21 Local recurrence is reported to be 26% to 60% at 5 years and 38% to 52% at 10 years.2–5,9,11–15 Clinically evident regional lymph node metastases appear in 12% to 25% of patients and systemic metastases in 9% to 25% of patients.2–5,9,11–15 Conjunctival melanoma-related mortality is 7% to 24% at 5 years and 13% to 38% at 10 years.2–5,9,11–15

CMs may arise most commonly from primary acquired melanosis (PAM) with atypia,4–25 which can be termed histologically more accurately as conjunctival melanocytic intraepithelial neoplasia (C-MIN) with atypia.4 More rarely, they appear de novo or evolve from a preexisting nevus.4–25 In a large clinical series of 382 cases, CM was preceded or accompanied by clinically detectable PAM in 74% and by nevus in 7%.15 By histopathologic examination, 50% to 75% of CMs feature associated C-MINS with atypia, and 17% to 26% feature associated nevi.1,6–20 From a reverse perspective, conjunctival nevi are reported to have a 0.4% risk of evolution into melanoma.22,23 C-MIN without atypia a 0% risk,24,25 and C-MIN with severe atypia a 13% to 46% risk of malignant transformation.24,25 Nonetheless, the biological mechanisms leading C-MIN lesions to progress to an invasive phenotype with a propensity for lymphatic spread into the regional lymph nodes are obscure.

Tumor-associated lymphangiogenesis (i.e., outgrowth of new from preexisting lymphatic vessels) is considered as the initial step in lymphogenic metastasis of several tumors (e.g., malignant melanoma of the skin,26,27 head and neck squamous cell carcinoma,28,29 squamous cell carcinoma of the uterine cervix,30,31 ciliary body melanoma with extracocular extension,32–34 and conjunctival squamous cell carcinoma35–37). It can be induced by a variety of tumor cells as well as by peritumoral macrophages with the help of the lymphangiogenic growth factors vascular endothelial growth factor (VEGF) C and D and their specific VEGF receptor 3.36–38

Although the role of peri- versus intratumoral lymphangiogenesis remains controversial, its role as a decisive risk factor for tumor metastasis is now established.27–39,40 However, as described in squamous cell carcinoma of the uterine cervix and of the conjunctiva, the lymphangiogenic stimulus is initiated early in premalignant precursors, even before the invasive phenotype.34,35
Since we have recently shown that tumor-associated lymphatic vessels can be detected within and around the tumor in CM, and lymphatic spread is known to occur in CM, but not in C-MIN, we were interested in analyzing whether tumor-associated lymphangiogenesis occurs with the transition from premalignant C-MIN into invasive CM and in studying its association with prognosis and other clinicopathologic tumor characteristics.

**METHODS**

**Patient Population and Clinical Data**

A case-controlled, matched-pair cohort study was arranged from the archives of the Ophthalmic Pathology Laboratory at the Department of Ophthalmology, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany. Of a total of 763 conjunctival specimens excised for TABLE 1. Clinical Characteristics of the Three Study Groups

<table>
<thead>
<tr>
<th></th>
<th>C-MIN without Atypia (n = 20)</th>
<th>C-MIN with Atypia (n = 20)</th>
<th>Invasive CM (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at diagnosis, y</strong></td>
<td>65 ± 11 (31–87)</td>
<td>64 ± 11 (31–88)</td>
<td>66 ± 11 (32–89)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
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<tr>
<td>Male, n (%)</td>
<td>9 (45)</td>
<td>10 (50)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>11 (55)</td>
<td>10 (50)</td>
<td>11 (55)</td>
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<tr>
<td><strong>Laterality</strong></td>
<td></td>
<td></td>
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<tr>
<td>Right eye, n (%)</td>
<td>13 (65)</td>
<td>9 (45)</td>
<td>12 (60)</td>
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<tr>
<td>Left eye, n (%)</td>
<td>7 (35)</td>
<td>11 (55)</td>
<td>8 (40)</td>
</tr>
<tr>
<td><strong>Largest basal tumor diameter, mm</strong></td>
<td>5.0 ± 1.4 (2.0–8.1)</td>
<td>5.0 ± 1.4 (2.1–8.5)</td>
<td>5.0 ± 1.3 (2.2–8.1)</td>
</tr>
<tr>
<td>2.0–5.0, n (%)</td>
<td>9 (45)</td>
<td>8 (40)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>5.1–8.0, n (%)</td>
<td>10 (50)</td>
<td>11 (55)</td>
<td>10 (50)</td>
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<tr>
<td>&gt;8.0, n (%)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>1 (5)</td>
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<tr>
<td><strong>Location of tumor center</strong></td>
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<tr>
<td>Superior, n (%)</td>
<td>2 (10)</td>
<td>2 (10)</td>
<td>2 (10)</td>
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<tr>
<td>Temporal, n (%)</td>
<td>3 (15)</td>
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<tr>
<td>Inferior, n (%)</td>
<td>7 (35)</td>
<td>7 (35)</td>
<td>7 (35)</td>
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<tr>
<td>Nasal, n (%)</td>
<td>8 (40)</td>
<td>8 (40)</td>
<td>8 (40)</td>
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<tr>
<td><strong>Invasion of adjacent structures</strong></td>
<td></td>
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<tr>
<td>Limbus, n (%)</td>
<td>11 (55)</td>
<td>11 (55)</td>
<td>11 (55)</td>
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<tr>
<td>Cornea, n (%)</td>
<td>3 (15)</td>
<td>3 (15)</td>
<td>3 (15)</td>
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<tr>
<td>Fornix, n (%)</td>
<td>7 (35)</td>
<td>7 (35)</td>
<td>7 (35)</td>
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<tr>
<td>Palpebral conjunctiva, n (%)</td>
<td>5 (25)</td>
<td>5 (25)</td>
<td>5 (25)</td>
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<tr>
<td><strong>Tumor-to-limbus distance, mm</strong></td>
<td>0.3 ± 1.7 (–3.5 to 3.3)</td>
<td>0.3 ± 1.7 (–3.4 to 3.2)</td>
<td>0.3 ± 1.8 (–3.5 to 3.1)</td>
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<tr>
<td>≤ −3.0, n (%)</td>
<td>2 (10)</td>
<td>2 (10)</td>
<td>2 (10)</td>
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<tr>
<td>–2.9 to 0, n (%)</td>
<td>9 (45)</td>
<td>9 (45)</td>
<td>9 (45)</td>
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<tr>
<td>0.1 to 3.0, n (%)</td>
<td>8 (40)</td>
<td>8 (40)</td>
<td>8 (40)</td>
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<tr>
<td>&gt;3.0, n (%)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>1 (5)</td>
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<tr>
<td><strong>Tumor-to-fornix distance, mm</strong></td>
<td>0.3 ± 2.4 (–4.2 to 3.4)</td>
<td>0.2 ± 2.4 (–4.0 to 3.3)</td>
<td>0.3 ± 2.4 (–4.0 to 3.4)</td>
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<tr>
<td>≤ −3.0, n (%)</td>
<td>4 (20)</td>
<td>4 (20)</td>
<td>4 (20)</td>
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<tr>
<td>–2.9 to 0, n (%)</td>
<td>3 (15)</td>
<td>3 (15)</td>
<td>3 (15)</td>
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<tr>
<td>0.1–3.0, n (%)</td>
<td>11 (55)</td>
<td>12 (60)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>&gt;3.0, n (%)</td>
<td>2 (10)</td>
<td>1 (5)</td>
<td>2 (10)</td>
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<tr>
<td><strong>Tumor pigmentation, n (%)</strong></td>
<td></td>
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<tr>
<td>Amelanotic,</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Weak</td>
<td>3 (15)</td>
<td>2 (10)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6 (30)</td>
<td>8 (40)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Strong</td>
<td>11 (55)</td>
<td>10 (50)</td>
<td>12 (60)</td>
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<tr>
<td><strong>Clinical TNM classification, n (%)</strong></td>
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<tr>
<td>N/A</td>
<td>20 (100)</td>
<td>13 (65)</td>
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<tr>
<td>T(is) N0 M0</td>
<td>—</td>
<td>7 (35)</td>
<td>—</td>
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<tr>
<td>T1a N0 M0</td>
<td>—</td>
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<td>8 (40)</td>
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<tr>
<td>T1b N0 M0</td>
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<td>5 (25)</td>
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<tr>
<td>T2a N0 M0</td>
<td>—</td>
<td>—</td>
<td>4 (20)</td>
</tr>
<tr>
<td>T2b N0 M0</td>
<td>—</td>
<td>—</td>
<td>3 (15)</td>
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<tr>
<td><strong>Therapeutic management, n (%)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Excision</td>
<td>9 (45)</td>
<td>5 (25)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Excision+cryotherapy</td>
<td>2 (10)</td>
<td>3 (15)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Excision+topical mitomycin C</td>
<td>9 (45)</td>
<td>12 (60)</td>
<td>13 (65)</td>
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<tr>
<td><strong>Follow-up</strong></td>
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<tr>
<td>Follow-up-time, mean mo ± SD (range)</td>
<td>169 ± 73 (60–288)</td>
<td>170 ± 67 (60–272)</td>
<td>168 ± 69 (60–280)</td>
</tr>
<tr>
<td>Local recurrence, n (%)</td>
<td>2 (10)</td>
<td>6 (30)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Transformation into melanoma, n (%)</td>
<td>—</td>
<td>1 (5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Lymphatic spread, n (%)</td>
<td>—</td>
<td>—</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Distant metastasis, n (%)</td>
<td>—</td>
<td>—</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Tumor-related death, n (%)</td>
<td>—</td>
<td>—</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>

* Distance between central tumor margin and limbus, with negative values if the tumor extended into cornea.
† Distance between peripheral tumor margin and fornix, with negative values if the tumor extended into palpebral conjunctiva.
an epibulbar melanocytic tumor between 1986 and 2005, 20 primary invasive CMs (with pathologic tumor origin from C-MIN) were closely matched with 20 C-MINs with atypia and 20 C-MINs without atypia, regarding tumor size (basal diameter, ±0.5 mm), tumor location, tumor extension (tumor-to-limbus distance, ±1.0 mm; tumor-to-fornix distance, ±1.0 mm), and patient’s age. Specimens of complete resections rather than biopsies were selected so that both normal and tumor tissue were present on each slide. Exclusion criteria were (1) bilateral or multifocal lesions; (2) other treatments (e.g., radiotherapy, chemotherapy, or immunotherapy) before excision; (3) invasion of intraocular compartments, lacrimal punctum, canaliculi, plica, caruncle, eyelid margin, orbit, paranasal sinuses, and central nervous system; and (4) missing follow-up data for at least 5 years after surgical excision. This retrospective, nonrandomized, clinicopathologic single-center study was performed in conformance with the tenets of the Declaration of Helsinki. Institutional Review Board/Ethics Committee approval was not required in this instance.

Patients with C-MIN without atypia, C-MIN with atypia, and invasive CM showed comparable distributions of clinical tumor characteristics (Table 1). All lesions were classified according to the 2009 TNM classification system.10 In none of the patients did general physical examination and oncologic workup at the time of diagnosis reveal any evidence of another primary tumor, hematogenous metastasis, or lymph node metastasis. Sentinel lymph node biopsy was not performed as a standard procedure. All patients had surgery as their first line of management, with some receiving additional cryotherapy and adjuvant topical mitomycin C chemotherapy (two 14-day cycles of 0.02% eye drops, five times a day with a 14-day break).

The follow-up regimen for all patients included regular ophthalmic examinations at least every 6 months and, for CM patients, ultrasound

<table>
<thead>
<tr>
<th>C-MIN without Atypia (n = 20)</th>
<th>C-MIN with Atypia (n = 20)</th>
<th>Invasive CM (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-MIN score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (range)</td>
<td>1 ± 0 (1–1)</td>
<td>5.2 ± 2.9 (1–10)</td>
</tr>
<tr>
<td>5, n (%)</td>
<td>20 (100)</td>
<td>10 (50)</td>
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<tr>
<td>5, n (%)</td>
<td>—</td>
<td>10 (50)</td>
</tr>
<tr>
<td><strong>Horizontal spread of melanocytes, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal proliferation</td>
<td>20 (100)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Pagetoid spread</td>
<td>—</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Nonconfluent nests</td>
<td>—</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Confluent nests</td>
<td>—</td>
<td>5 (15)</td>
</tr>
<tr>
<td><strong>Vertical spread of melanocytes with atypia, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal layer of epithelium</td>
<td>20 (100)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>&lt;50 epithelial thickness</td>
<td>—</td>
<td>9 (45)</td>
</tr>
<tr>
<td>&gt;50% epithelial thickness</td>
<td>—</td>
<td>4 (20)</td>
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<tr>
<td>&gt;90% epithelial thickness</td>
<td>—</td>
<td>3 (15)</td>
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<tr>
<td><strong>Grade of cytological atypia, n (%)</strong></td>
<td></td>
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<tr>
<td>Vesicular nuclei (≥ basal squamous cells)</td>
<td>—</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Abundant cytoplasm (≥ basal squamous cells)</td>
<td>—</td>
<td>9 (45)</td>
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<tr>
<td>Prominent nucleoli</td>
<td>—</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Presence of mitotic figures</td>
<td>—</td>
<td>6 (30)</td>
</tr>
<tr>
<td><strong>Tumor thickness, mm</strong></td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Mean ± SD (range)</td>
<td></td>
<td></td>
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<tr>
<td>5, n (%)</td>
<td>20 (100)</td>
<td>14 (70)</td>
</tr>
<tr>
<td><strong>Predominant tumor cell type, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid cell type</td>
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<td>3 (15)</td>
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<tr>
<td>Mixed cell type</td>
<td>—</td>
<td>2 (10)</td>
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<tr>
<td>Spindle cell type</td>
<td>—</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Indeterminate cell type</td>
<td>20 (100)</td>
<td>14 (70)</td>
</tr>
<tr>
<td><strong>Mitotic count (per 10 high-power fields)</strong></td>
<td></td>
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<tr>
<td>Mean ± SD (range)</td>
<td>0 ± 0 (0–0)</td>
<td>0.5 ± 0.9 (0–3)</td>
</tr>
<tr>
<td>0, n (%)</td>
<td>20 (100)</td>
<td>14 (70)</td>
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<tr>
<td>1, n (%)</td>
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<td>3 (15)</td>
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<tr>
<td>≥2, n (%)</td>
<td>—</td>
<td>3 (15)</td>
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<tr>
<td><strong>Ki67 proliferation index (%)</strong></td>
<td></td>
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<tr>
<td>Mean ± SD (range)</td>
<td>0 ± 0 (0–1)</td>
<td>8 ± 4 (1–17)</td>
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<tr>
<td>&lt;10, n (%)</td>
<td>20 (100)</td>
<td>14 (70)</td>
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<tr>
<td>10–20, n (%)</td>
<td>—</td>
<td>6 (30)</td>
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<tr>
<td>&gt;20, n (%)</td>
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<tr>
<td><strong>Tumor involvement of surgical margins, n (%)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Tumor-free margins</td>
<td>18 (90)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>Tumor-involved margins</td>
<td>2 (10)</td>
<td>3 (15)</td>
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<tr>
<td><strong>Pathologic T categories of TNM classification, n (%)</strong></td>
<td></td>
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<tr>
<td>N/A</td>
<td>20 (100)</td>
<td>13 (65)</td>
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<td>pT(is)</td>
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<td>7 (35)</td>
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<tr>
<td>pT1a</td>
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<td>pT1b</td>
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<td>pT1c</td>
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<td>pT2a</td>
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<tr>
<td>pT2c</td>
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</table>
imaging of the regional lymph nodes by an otorhinolaryngologist at least every 6 months and a general physical examination and oncologic workup at least every 6 months. In a standardized telephone interview in 2010, all the specialized physicians were asked for data on survival, new onset of lymph node or distant metastasis, local recurrence, and, in the patients with C-MIN, transformation into melanoma.

**Histologic Analyses**

All conjunctival specimens were fixed in a buffered 10% formaldehyde solution, dehydrated, and embedded in paraffin. Multiple serial sections cut at 4 μm were stained with hematoxylin-eosin, periodic acid-Schiff, S-100 protein (rabbit, 1:400; Dako, Hamburg, Germany), anti-melanosome HMB-45 (mouse, 1:400; Dako), and Melan-A (mouse, 1:50; Dako) and analyzed by three independent investigators by microscope (Axioskop; Carl Zeiss, Oberkochen, Germany), to confirm the diagnosis histopathologically (Table 2). Conjunctival melanocytic intraepithelial neoplasia (C-MIN) without atypia, widely referred to as primary acquired melanosis (PAM) without atypia, was defined as intraepithelial melanocytic proliferation with increased numbers of normal or hypertrophic melanocytes confined to the basal layer of the conjunctival epithelium showing no cytological features of atypia.5,8 C-MIN with atypia, synonymously called PAM with atypia, was defined as intraepithelial melanocytic proliferation with significant cellular pleomorphism, but without penetration of the basal membrane.5,8 C-MIN score developed by Damato and Coupland 5 was used for grading C-MIN lesions according to the pattern of horizontal spread, degree of vertical spread, and grade of cytologic atypia.5-8 Invasive CM shows atypical melanocytes that have penetrated the epithelial basement membrane and spread into the conjunctival stroma.4-8 Greatest tumor thickness was measured on hematoxylin-and-eosin–stained sections using digital analysis (AxioVision 4.6; Carl Zeiss). Predominant tumor cell type was classified as epithelioid, mixed, or spindle, and if no typical epithelioid or spindle cells were present, as indeterminate.18 Mitotic count was assessed by counting the number of mitotic figures in 10 randomly selected high-power fields.18 The Ki-67 proliferation index was detected as percentage of tumor cells immunopositive with the monoclonal Ki-67 antibody (mouse, 1:100; AbD Serotec, Kidlington, Oxford, UK). In all 20 of the CMs, remnants of C-MIN with atypia were recognizable, suggesting pathologic tumor origin from C-MIN. Surgical margins of excision were closely evaluated for tumor involvement.

**Immunohistochemical Detection of Lymphatic Vessels**

To identify lymphatic vessels, immunohistochemistry was performed in all 60 cases as described previously.42 For the differentiation between preexisting and proliferating lymphatic vessels, all paraffin-embedded sections were double-stained with (1) a polyclonal antibody against the human lymphatic vascular endothelium–specific hyaluronic acid receptor LYVE-1 (rabbit, 1:100; ACRIS, Herford, Germany) as a specific marker for lymphatic vascular endothelium43 and a monoclonal antibody against Ki-67 (mouse, 1:100; AbD Serotec) as a specific marker for proliferating cells28,44 and (2) a monoclonal antibody

![Figure 1](https://example.com/figure1.png)
against the human lymphatic vascular endothelial-specific glycoprotein podoplanin D2-40 (mouse, 1:40; AbD Serotec) as another specific marker for lymphatic vascular endothelium55 and a monoclonal antibody against Ki-67 (rabbit, 1:200; Medac, Hamburg, Germany). Positive controls were performed on corneoscleral ring specimens for LYVE-1 and podoplanin and on tonsil tissue for Ki-67; negative controls were performed with control immunoglobulin G.

Alternating serial sections were evaluated for evidence of proliferating lymphatic vessels by three independent investigators in a masked fashion using a microscope (Axiohot; Carl Zeiss) combined with a computer-aided image analysis system (AxioVision 4.6; Carl Zeiss). Proliferating lymphatic vessels were identified as LYVE-1+ and podoplanin+ structures with a clearly identifiable erythrocyte-free lumen and with at least three lymphatic endothelial cells showing nuclear Ki-67 positivity. Tumor invasion into proliferating lymphatic vessels was considered to have occurred if at least one tumor cell cluster was clearly visible inside an LYVE-1+/Ki-67+ and podoplanin+/Ki-67+ vessel.

Main outcome measures included lymphatic vascular density (LVD), which is the number of vessels per square millimeter, and the relative lymphatic vascular area (RLVA), which is the percentage of positively stained area, determined for proliferating LYVE-1+/Ki-67+ and podoplanin+/Ki-67+ lymphatic vessels. The LVD and RLVA were measured within the mass (intratumoral LVD/RLVA), within an area ≤500 μm from the tumor border (peritumoral LVD/RLVA) and within a more distant area of conjunctiva (tumor-distant LVD/RLVA). If the tumor-adjacent conjunctival area was not completely represented on the specimen, we evaluated as much of the area as was represented.

When the association with other clinicopathologic tumor features and prognosis was analyzed, the median value of the intratumoral LVD for proliferating LYVE-1+/Ki-67+ and podoplanin+/Ki-67+ lymphatic vessels was used as a cutoff to separate tumors with high versus low intratumoral lymphatic density.

Statistical Analyses
The statistical programming language R version 2.10.1 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses. Comparisons between the intratumoral lymphatic density and the other clinicopathologic variables were performed using the nonparametric Mann-Whitney U test, Fisher exact test, and Pearson’s χ2 test.

RESULTS
Identification of Lymphatic Vessels
In none of the 20 C-MIN lesions without atypia were proliferating LYVE-1+/Ki-67+ and podoplanin+/Ki-67+ lymphatic vessels identified within the tumor mass or in the peritumoral area (Figs. 1A–E). Ten of the 20 C-MIN lesions with atypia (50%; Figs. 1F–J) and all 20 CMs (100%; Figs. 1K–O) revealed both intratumoral and peritumoral proliferating lymphatic vessels. Intratumoral proliferating lymphatics increased in density from discrete hotspots concentrated within sheets of tumor cells in C-MIN with atypia (Figs. 1G, 1H) toward a branching network in invasive CM (Figs. 1I, 1M). Peritumoral proliferating lymphatics, mostly located in the inflammatory area surrounding the tumor, also increased in density from C-MIN with atypia (Figs. 1I, 1J) toward invasive CM (Figs. 1N, 1O).

Using LYVE-1/Ki-67 and podoplanin/Ki-67 double staining, multiple lymphatic endothelial cells showing nuclear Ki-67 positivity identified tumor-associated lymphatic vessels as active and proliferating (Figs. 2A, 2B). Significantly more of the intratumoral lymphatics were proliferative than of the peritumoral (P = 0.047) or tumor-distant ones (P < 0.001; Fig. 2C). In none of the C-MINs, without or with atypia, were tumor cell clusters detectable in intra- or peritumoral lymphatic vessels. In CM tumors, invasion of intratumoral lymphatic vessels by tumor cells was observed in five (25%) melanomas, invasion of peritumoral lymphatics in four (20%), and synchronous invasion of intratumoral lymphatic vessels in three (15%). Notably, 75% of the lymphatic vessels showing tumor emboli were proliferative (Fig. 2B).

Quantitative Evaluation of Lymphatic Vessels
By computer-assisted morphometric analysis of proliferating lymphatic vessels, invasive CM revealed significantly higher survival rates with SE were determined for the time to first local recurrence, lymphatic spread, distant metastasis, and tumor-related death, according to the Kaplan-Meier method, and compared using the log rank test. P < 0.05 was considered statistically significant.

Figures

FIGURE 2. Tumor-associated active lymphangiogenesis in C-MIN without atypia, C-MIN with atypia, and invasive CM. (A) LYVE-1 (brown)/Ki-67 (red) and (B) podoplanin (brown), Ki-67 (red) double staining of multiple lymphatic endothelial cells showing nuclear Ki-67 positivity (arrowbeads) identified lymphatic vessels as active and proliferating. Note the tumor cells (asterisks) invading an intratumoral proliferating lymphatic vessel (B). (C) Significantly more lymphatic vessels were found proliferating within the tumor mass (intratumoral) compared with an area ≤500 μm from the tumor border (peritumoral) and a more distant area of conjunctiva (tumor-distant) (Mann-Whitney U test, P = 0.047 and P < 0.001, respectively). Original magnification: (A, B) ×800.
values of intratumoral LVD and RLVA than did C-MIN with atypia ($P < 0.001$, respectively) or C-MIN without atypia ($P < 0.001$, respectively; Figs. 3A, 3B). Peritumoral LVD and RLVA of proliferating lymphatics were significantly higher in invasive CM than in C-MIN with atypia ($P \leq 0.001$, respectively) or C-MIN without atypia ($P < 0.001$, respectively; Figs. 3C, 3D). Morphometric analysis of the tumor-distant lymphatic vessels revealed no significant differences.

Overall, 10 (50%) of all 20 invasive CM tumors were included in the group with high intratumoral lymphatic density (intratumoral LVD $> \text{the median value of 21.0 per mm}^2$), and 10 (50%) melanomas were included in the group with low intratumoral lymphatic density (intratumoral LVD $\leq \text{the median value of 21.0 per mm}^2$).

Association with Other Clinicopathologic Tumor Features

In C-MINs with atypia, presence of intratumoral proliferating lymphatic vessels was significantly associated with larger tumor diameter ($P = 0.010$), higher C-MIN score ($P = 0.010$), and pT(is) category of TNM classification ($P = 0.013$). No significant association was observed with age, sex, laterality, location of tumor center, invasion of the limbus, invasion of the cornea, invasion of the fornix, invasion of the palpebral conjunctiva, tumor-to-limbus distance, tumor-to-fornix distance, tumor pigmentation, predominant tumor cell type, higher mitotic count, Ki-67 proliferation index, or tumor involvement of the surgical margins.

In invasive CMs, high intratumoral lymphatic density was significantly associated with larger tumor diameter ($P = 0.010$), invasion of the fornix ($P = 0.013$), invasion of the palpebral conjunctiva ($P = 0.045$), lower tumor-to-fornix distance ($P = 0.010$), higher T categories of clinical TNM classification ($P = 0.025$), and higher pT categories of pathologic TNM classification ($P = 0.025$). No significant association was observed with age, sex, laterality, location of tumor center, invasion of the limbus, invasion of the cornea, tumor-to-limbus distance, tumor pigmentation, tumor thickness, predominant cell type, and Ki-67 proliferation index.

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**Figure 3.** Computer-assisted morphometric analysis of tumor-associated lymphangiogenesis in C-MIN without atypia, C-MIN with atypia, and invasive CM. Invasive CM demonstrated significantly higher intratumoral (A) and peritumoral (C) LVD and significantly higher intratumoral (B) and peritumoral (D) RLVA of proliferating lymphatic vessels compared with C-MIN, with and without atypia (Mann-Whitney U test, $P \leq 0.001$, respectively).
tumor cell type, mitotic count, Ki-67 proliferation index, or tumor involvement of surgical margins.

All P values were adjusted for multiple testing using the method of Benjamini and Hochberg. 46

Association with Tumor Prognosis

Within a mean follow-up time of 169 ± 68 months (range, 60–288 months) after surgical excision, the tumor recurrence at least once in two (10%) patients with C-MIN without atypia, in six (30%) patients with C-MIN with atypia, and in eight (40%) patients with CM. The mean time interval to first local recurrence was 32 ± 17 months (range, 15–75 months). One C-MIN with atypia progressed to invasive melanoma within 3 years after surgical excision. Regional lymph node metastases occurred in six (30%) patients with CM, with a mean delay of 40 ± 16 months (range, 18–60 months). Submandibular lymph nodes were involved in four patients, preauricular nodes in one patient, and cervical nodes in one patient. The clinical diagnosis of lymphatic spread was histopathologically confirmed in four patients. Four (20%) patients with CM developed distant metastases with a mean time interval of 44 ± 10 months (range, 33–56 months) after primary treatment. Systemic metastases were detected after lymphatic ones in all four cases. The sites of distant metastases included lung in two cases and liver in two cases. Systemic spread was histopathologically confirmed in two patients. Of the 20 patients with CM, 4 (20%) died of melanoma within a mean time interval of 49 ± 11 months (range, 36–60 months) after primary treatment.

In C-MINs with atypia, patients developing at least one local recurrence revealed significantly higher intratumoral lymphatic densities (P = 0.041). The 5-year cumulative recurrence-free survival rate was 50% (SE 16%) for melanomas with a high intratumoral lymphatic density and 90% (SE 10%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower recurrence-free survival rates (P = 0.006; Fig. 5A).

Patients with CM who developed lymphatic spread into the regional lymph nodes had significantly higher intratumoral lymphatic densities (P = 0.002). The 5-year cumulative lymphatic-spread-free survival rate was 50% (SE 16%) for melanomas with a high intratumoral lymphatic density and 90% (SE 10%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower lymphatic-spread-free survival rates (P = 0.041; Fig. 5B). No significant association was observed between the risk of regional lymph node metastasis and the detection of tumor invasion into intra- and/or peritumoral lymphatic vessels.

Patients with CM developing distant metastases had significantly higher intratumoral lymphatic densities (P = 0.003). The 5-year cumulative distant-metastasis-free survival rate was 60% (SE 16%) for melanomas with a high intratumoral lymphatic density and 100% (SE 0%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower distant-metastasis-free survival rates (P = 0.029; Fig. 5C).

Patients who died of melanoma had significantly higher intratumoral lymphatic densities (P = 0.003). The 5-year cumulative melanoma-specific survival rate was 60% (SE 16%) for melanomas with a high intratumoral lymphatic density and 100% (SE 0%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower melanoma-specific survival rates (P = 0.029; Fig. 5D).

Discussion

The present study revealed two novel important findings: (1) The development from the premalignant precursor C-MIN with atypia into invasive CM is concurrent with the outgrowth of lymphatic vessels. This active tumor-associated lymphangiogenesis begins early in premalignant intraepithelial lesions and continues to increase toward invasive malignancy. (2) The intratumoral density of proliferating lymphatic vessels seems to be associated with an increased risk of local recurrence in patients with C-MIN with atypia and with an increased risk of local recurrence, lymphatic spread, distant metastasis, and tumor-related death in patients with invasive CM. This result may establish the immunohistochemical detection of tumor-associated lymphangiogenesis as a novel prognostic indicator in patients with intraepithelial and invasive melanocytic lesions of the conjunctiva.

The biological mechanisms leading C-MIN lesions to progress to invasive CM with a propensity for lymphatic spread into the regional lymph nodes are obscure.2–25 Our results suggest that the molecular armamentarium of lymphatic neovascularization begins early in the intraepithelial lesions’ development and continues to increase toward invasive CM. This supports the concept, noted in squamous cell carcinoma of the uterine cervix31 and of the conjunctiva,32 that the lymphangiogenic stimulus is initiated before the invasive phenotype. However, it remains unclear whether this lymphangiogenesis is the cause of or more likely a result of the malignant transformation process.

The hypothesis of an active lymphangiogenesis is supported by our observation that most of the intratumoral lymphatics

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Tumor-associated lymphangiogenesis and prognosis in C-MIN with atypia. There was a significantly lower recurrence-free survival rate in patients with lesions showing intratumoral proliferating lymphatic vessels than in those without intratumoral lymphangiogenesis (log rank test; P = 0.041).

In invasive CMs, patients developing at least one local recurrence revealed significantly higher intratumoral lymphatic densities (P = 0.016). The 5-year, cumulative, recurrence-free survival rate was 40% (SE 16%) for melanomas with a high intratumoral lymphatic density and 90% (SE 10%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower recurrence-free survival rates (P = 0.006; Fig. 5A).

Patients with CM who developed lymphatic spread into the regional lymph nodes had significantly higher intratumoral lymphatic densities (P = 0.002). The 5-year cumulative lymphatic-spread-free survival rate was 50% (SE 16%) for melanomas with a high intratumoral lymphatic density and 90% (SE 10%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower lymphatic-spread-free survival rates (P = 0.041; Fig. 5B). No significant association was observed between the risk of regional lymph node metastasis and the detection of tumor invasion into intra- and/or peritumoral lymphatic vessels.

Patients with CM developing distant metastases had significantly higher intratumoral lymphatic densities (P = 0.003). The 5-year cumulative distant-metastasis-free survival rate was 60% (SE 16%) for melanomas with a high intratumoral lymphatic density and 100% (SE 0%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower distant-metastasis-free survival rates (P = 0.029; Fig. 5C).

Patients who died of melanoma had significantly higher intratumoral lymphatic densities (P = 0.003). The 5-year cumulative melanoma-specific survival rate was 60% (SE 16%) for melanomas with a high intratumoral lymphatic density and 100% (SE 0%) for those with a low intratumoral lymphatic density. A high intratumoral lymphatic density was significantly associated with lower melanoma-specific survival rates (P = 0.029; Fig. 5D).

Discussion

The present study revealed two novel important findings: (1) The development from the premalignant precursor C-MIN with atypia into invasive CM is concurrent with the outgrowth of lymphatic vessels. This active tumor-associated lymphangiogenesis begins early in premalignant intraepithelial lesions and continues to increase toward invasive malignancy. (2) The intratumoral density of proliferating lymphatic vessels seems to be associated with an increased risk of local recurrence in patients with C-MIN with atypia and with an increased risk of local recurrence, lymphatic spread, distant metastasis, and tumor-related death in patients with invasive CM. This result may establish the immunohistochemical detection of tumor-associated lymphangiogenesis as a novel prognostic indicator in patients with intraepithelial and invasive melanocytic lesions of the conjunctiva.

The biological mechanisms leading C-MIN lesions to progress to invasive CM with a propensity for lymphatic spread into the regional lymph nodes are obscure.2–25 Our results suggest that the molecular armamentarium of lymphatic neovascularization begins early in the intraepithelial lesions’ development and continues to increase toward invasive CM. This supports the concept, noted in squamous cell carcinoma of the uterine cervix31 and of the conjunctiva,32 that the lymphangiogenic stimulus is initiated before the invasive phenotype. However, it remains unclear whether this lymphangiogenesis is the cause of or more likely a result of the malignant transformation process.

The hypothesis of an active lymphangiogenesis is supported by our observation that most of the intratumoral lymphatics
were actively proliferating vessels rather than trapped preexisting or hyperplastic lymph vessels. The existence of proliferating lymphatics was detected with LYVE-1/Ki-67 double staining, as described by Beasley et al. and for reasons of specificity confirmed using podoplanin/Ki-67 double staining. Since multiple immunohistochemistry was not feasible on paraffin-embedded sections to detect both LYVE-1 and podoplanin, comparison of serial sections revealed the presence of both markers on the lymphatic endothelium, but clearly showed their absence on vascular endothelium. Unlike lymphatic vessels of the normal conjunctiva, tumor-associated lymphatics mostly contained Ki-67, dividing nuclei in the lymphatic endothelial cells constituting considerable evidence for the presence of active lymphangiogenesis in intraepithelial and invasive melanoma of the conjunctiva.

The main significance of proliferating intratumoral lymphatic vessels is that they could provide a route for the lymphatic spread of CM into the regional lymph nodes. Indeed, we observed the presence of tumor cell clusters within proliferating lymphatics in invasive CM, although it should be noted that there are limitations to detecting what may be a rare event, by examining small sections of archival tissue at a single point in time. In addition, we showed a significant association between the intratumoral lymphatic density and an increased risk of lymphatic spread, distant metastasis and melanoma-related death in CM patients. However, these data suggest, but do not prove that intratumoral proliferating lymphatics actually are functional or are, indeed, involved in transport of tumor cells to the regional lymph nodes, since CMs already have access to the preexisting lymphatic vessels of the conjunctiva.

**FIGURE 5.** Tumor-associated lymphangiogenesis and prognosis in invasive CM: There were significantly lower recurrence-free (A, logrank test, \( P = 0.006 \)), lymphatic-spread-free (B, logrank test, \( P = 0.041 \)), distant-metastasis-free (C, logrank test, \( P = 0.029 \)), and melanoma-specific (D, logrank test, \( P = 0.029 \)) survival rates in patients with melanomas showing a high intratumoral lymphatic density (>21.0 per mm²) than in those with a lower intratumoral lymphatic density.
In squamous cell carcinoma of the head and neck as well as of the conjunctiva, tumor-associated lymphangiogenesis is documented to have prognostic value, not only for metastasis but also for local recurrence. This is in line with our finding that high intratumoral lymphatic density was a significant prognostic predictor of local recurrence in C-MIN with atypia and in invasive CM. Tumor-associated lymphangiogenesis may contribute directly to higher recurrence rates via facilitation of immune cell access to the tumor environment resulting in altered immune responses to the tumor, or indirectly via larger size and increased aggressiveness of the tumor.

However, the limitation of the present study, especially regarding conclusions for prognosis, is the small number of included patients, unsuitable for multivariate statistical testing. Therefore, these results should be verified in larger prospective studies. Particularly, the cutoff value separating tumors with high versus low intratumoral lymphatic density and the necessity of a complex double-staining technique instead of a single lymphatic endothelial staining will have to be defined in detail.

Nevertheless, our data suggest that patients showing an increase in tumor-associated lymphangiogenesis should be followed up closely for local recurrence, and, in the case of CM, also for lymphatic and hematogenic spread. The present study confirms findings of other investigations that C-MIN with atypia should be considered malignant and treated as melanoma. Implications for sentinel lymph node biopsy cannot be drawn, since it was not performed as a standard procedure in our series.

In the future, novel combined antihemangiogenic and anti-lymphangiogenic therapies, such as bevacizumab, or specific antilymphangiogenic therapies, such as anti-VEGF receptor 3 antibodies or integrin α5 blocking peptides, may help to minimize the risk of malignant transformation, local recurrence, and lymphatic spread in these patients. In conclusion, the development of CM from premalignant precursors is concurrent with the outgrowth of lymphatic vessels. This active-tumor-associated lymphangiogenesis seems to be related to an increased risk of local recurrence in patients with C-MIN with atypia and with an increased risk of local recurrence, lymphatic spread, distant metastasis, and tumor-related death in patients with invasive CM.

Acknowledgments

The authors thank Sarah E. Coupland, MB, BS, PhD, for expert advice in C-MIN scoring that was used to grade the C-MIN lesions.

References


