Prognostic Value of Lymphocyte Homing Receptor and S Phase Fraction in Non-Hodgkin’s Lymphoma

By Sirpa Jalkanen, Heikki Joensuu, and Pekka Klemi

Lymphocyte homing receptors (HRs) mediate lymphocyte binding to high endothelial venules, and control their circulation between the blood and the lymphoid organs. The role of HRs and nuclear DNA content in the spread and prognosis of non-Hodgkin’s lymphoma was studied from paraffin-embedded tumor sections of 104 patients followed-up for the minimum of 5 years after the diagnosis. HR expression was analyzed by staining with a monoclonal antibody, Hermes-3, and DNA content by flow cytometry. Ten (10%) lymphomas were HR negative (HR−), 14 (13%) weakly (HR+/-), and 80 (77%) strongly positive (HR+). The 5-year survival rate corrected for intercurrent deaths was 61% in lymphomas with SPF less than 12% or with HR−, but only 15% if SPF was greater than 12% and HR+ (P < .0001). In multivariate analysis stage (P < .001), SPF (P = .002) and HR (P = .003) were the only independent prognostic factors.

Unlike Hodgkin’s disease, the non-Hodgkin’s lymphomas form a heterogeneous group of diseases with variable biologic behavior, ranging from well-differentiated lymphocytic lymphomas, which may be followed-up without treatment, to very aggressive forms of the disease, such as lymphoblastic lymphoma or Burkitt’s lymphoma, which may be fatal within a few months. The type of treatment is chosen based on factors such as patient age and condition, presence of B symptoms, stage, and histologic subtype of lymphoma. However, these factors may fail to predict the biologic behavior, aggressive chemotherapy with its hazards may be given to patients not needing it, and follow-up without treatment, single agent chemotherapy, or radiotherapy may be selected for a patient in need of combination chemotherapy. None of the several histologic classifications available is universally accepted, and all staging examinations may miss small deposits of lymphoma.

Mature lymphocytes circulate continuously between the blood and lymphoid organs in the body. In lymphatic tissues and at sites of chronic inflammation, lymphocytes leave the blood by selectively binding to specialized venules, so called high endothelial venules (HEV). This binding is mediated through homing receptor (HR) molecules that have been identified in the mouse, rat, and human. Although functionally mediating lymphocyte-endothelial cell interaction and therefore named as homing receptors, recent cloning data indicate that MEL-14-defined homing receptor in mouse and Hermes-defined human receptor are structurally distinct. In humans, 85 to 95 Kd glycoprotein class (CD44), defined by a Hermes-series of monoclonal antibodies (MoAbs), mediate lymphocyte binding to peripheral lymph node, mucosa-associated lymphatic tissues, and inflamed joint tissue (synovium). Furthermore, expression of this glycoprotein class correlates well with the HEV-binding capacity of in vitro growing human lymphoma and lymphoblastoid cell lines.

An animal study has indicated that lymphomas, which bind well to HEV and thus possess functional HRs, disseminate hematogenously to all lymph node groups when injected intramuscularly into syngenic recipients, whereas nonbinding lymphomas do not spread via the blood, and involve only the nodes draining local tumor at the site of injection. A small preliminary study with human lymphoma also suggested that HR expression correlates with the extent of lymph node involvement. Picker et al found that Hermes-defined HR expression was higher in disseminated diffuse large cell lymphoma than in nondisseminated disease, but the difference did not achieve statistical significance, and no follow-up was available. Pals et al, on the other hand, found a highly significant correlation between the HR expression and dissemination of large cell lymphomas.

The nuclear DNA content of thousands of cells can be determined in a few minutes by flow cytometry. By this technique, an abnormal DNA content (DNA aneuploidy) has been found in about 30% of non-Hodgkin’s lymphomas. Unlike in many epithelial cancers, DNA aneuploidy is probably not a major determinant of survival in lymphomas; but a large percentage of S phase cells, calculated by a simple procedure from the DNA histogram, has correlated well with poor prognosis.

In this study, tumors of 104 patients with non-Hodgkin’s lymphoma were analyzed for the expression of lymphocyte HRs, using Hermes-3 MoAb, and for the nuclear DNA content, and the data was correlated with histologic classification, clinical behavior of lymphoma, and the final outcome of the patients. The results indicate that HR negative (HR−) lymphomas seldom disseminate and have good prognosis even if they have a large fraction of proliferative cells, and that both HR and S phase fraction (SPF) are
important and independent prognostic factors in non-
Hodgkin’s lymphoma, together with stage of the disease.

MATERIALS AND METHODS

Patients. One hundred four consecutive adult cases (age over 16
years) with histologically diagnosed non-Hodgkin’s lymphoma,
treated in Turku University Central Hospital (Finland) from 1970 to
1980, with adequate staging examinations done and sufficient
paraffin-embedded tumor material available were analyzed. Fifty-
six (54%) of the patients were men and 48 women, the mean age at
the diagnosis was 59 years (range, from 20 to 86 years). Twenty-two
(21%) patients had B symptoms. All patients were followed-up for
the minimum of 5 years (range, from 5 to 17 years) or until death.

Histology, staging, and treatment. Formalin-fixed and paraffin-
embedded tissue blocks were sectioned and stained with the Giemsa,
hematoxylin and eosin, periodic acid-Schiff, methyl green and
pyronin, and van Gieson methods. The original diagnoses were
confirmed with an MoAb against human common leukocyte antigen
(OKT3) with 1,1-diaminobenzidine as the chromogen.

Avidin-biotin complex technique (Vector Laboratories, Burlingame,
CA) with 1,1-diaminobenzidine was the chromogen.

The production and specificity of this antibody have been
described earlier.11 Hermes-3 staining was found to be the only antibody in
the Hermes series that worked on paraffin-embedded tissue sections,
but the staining patterns of Hermes-3 were comparable when tested
on normal tonsils and over 20 different lymphoma specimens both on
frozen and paraffin-embedded tissues (Fig 2). Hermes-3 staining
was scored –, +/−, and + (+, negative; +/−, weakly positive, a
definitive population of tumor cells positive; +, clearly positive, the
majority or all tumor cells positive). In all cases, variable number of
normal tumor infiltrating lymphocytes were seen, easily recognizable with
MT1 antibody in B-cell lymphomas (positive with MB2 antibody; MT1 and MB2 antibodies were from Clonab, Viereich,
Germany). They all stained intensely with Hermes-3 and served as a
tumor marker.

Stage I and II patients were usually treated with radiotherapy
(RT) or with the combination of radio- and chemotherapy, and stage
III and IV patients were usually treated with chemotherapy only.
Involved fields, the mantle, and the “inverted Y” fields were used,
and the tumor dose was usually in the range from 4,000 to 4,500
cGy. The most commonly used chemotherapy combinations were
COP (cyclophosphamide, vincristine, and prednisone) and CHOP
(cyclophosphamide, doxorubicin, vincristine, and prednisone).

Flow cytometry. A representative paraffin embedded tissue
block was chosen for flow cytometry. A 5-μm control section was cut
immediately adjacent to the 50-μm section used for DNA content
analysis for light microscopy to ascertain that tumor tissue was being
analyzed. Paraffin was removed with xylene, and after rehydration in
a series of decreasing concentrations of ethanol, a single cell
suspension was prepared with 0.5% pepsin as described earlier.20
DNA was stained with propidium iodide.21 The resulting suspension
was filtered through a silk mesh before flow cytometry.

Flow cytometry was done with a FACStar flow cytometer (Becton-
Dickinson Immucytometry Systems, Mountain View, CA). A
488-nm argon ion laser line run at 600 mW was used for fluorescence
excitation. For each histogram, 20 × 10^5 particles were measured.
Fig 2. Hermes-3 antibody gives comparable staining patterns on frozen and formalin fixed/paraffin embedded sections. Frozen (A, C, and E) and formalin fixed/paraffin embedded (B, D, and F) sections of three different types of B-cell lymphomas stained with Hermes-3 antibody using immunoperoxidase method and hematoxylin counterstain. (A and B) Hermes-3+ Burkitt's lymphoma. Practically all cells in these fields are tumor cells. (C and D) Large cell immunoblastic lymphoma, in which both tumor cells and tumor infiltrating normal T lymphocytes are Hermes-3+. (E and F) Hermes-3+ diffuse small lymphocytic lymphoma; both normal T cells and tumor cells are Hermes-3+.

useful internal staining standard. Immunocytochemical staining of two different kinds of follicular lymphomas are presented in Fig 3.

Coding. The patients were provided with a numerical code to prevent clinical data or survival information to interfere with histologic, DNA histogram, or HR expression classifications. The classifications were also done without knowledge of the results of the other analyses.

Statistical analyses. Survival analysis was done using a BMDP computer program (BMDP Statistical Software, Department of Biomathematics, University of California Press, Los Angeles). Survival was estimated with the product-limit method, and comparison of survival between groups was done using the generalized Wilcoxon test (BMDP 1L). Both crude survival rate and survival rate corrected for known intercurrent deaths were calculated. The relative importance of prognostic factors was analyzed using Cox's proportional hazard model (BMDP 2L). Frequency tables were analyzed using the chi-square test and Fisher's exact test. The SPF distributions of different groups were compared using Kruskal-Wallis's analysis of variance and Mann-Whitney's U-test. All $P$ values are two-tailed.
RESULTS

Homing receptor expression and DNA content. Ten (10%) lymphomas were HR negative (−), and 14 (13%) were weakly (+/−), and 80 (77%) clearly positive (+) for HR. Three of the 10 HR− and 66 of the 94 HR+/− or HR+ lymphomas disseminated hematogenously during the follow-up (P = .03). Nine (64%) of the 14 patients with HR+/− and 57 (71%) of the 80 patients with HR+ disease had evidence of hematogenous spread during the follow-up. Six HR− lymphomas were Ann Arbor stage I at the time of the diagnosis (P = .06). The patients with HR− lymphoma are described in detail in Table 1.

Table 1. Characteristics of Patients With HR− Lymphoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/Age</th>
<th>Histology</th>
<th>SPF (%)</th>
<th>Stage</th>
<th>Hematogenous Spread</th>
<th>Treatment</th>
<th>Follow-up, Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/55</td>
<td>Lymphoblastic B cell</td>
<td>38.4*</td>
<td>3B</td>
<td>Yes</td>
<td>COP, RT</td>
<td>Dead, 23</td>
</tr>
<tr>
<td>2</td>
<td>F/75</td>
<td>Small cleaved cell, diffuse, B cell</td>
<td>22.5</td>
<td>1AE</td>
<td>No</td>
<td>RT</td>
<td>Dead, 105 (cardiac death)</td>
</tr>
<tr>
<td>3</td>
<td>M/53</td>
<td>Lymphoblastic B cell</td>
<td>21.9</td>
<td>2AE</td>
<td>No</td>
<td>RT</td>
<td>Alive, 65</td>
</tr>
<tr>
<td>4</td>
<td>M/61</td>
<td>Large cell, diffuse, B cell</td>
<td>21.1</td>
<td>2BE</td>
<td>No</td>
<td>Surgery</td>
<td>Alive, 133</td>
</tr>
<tr>
<td>5</td>
<td>F/33</td>
<td>Large cell, diffuse, B cell</td>
<td>17.7</td>
<td>1A</td>
<td>No</td>
<td>RT</td>
<td>Alive, 125</td>
</tr>
<tr>
<td>6</td>
<td>M/74</td>
<td>Small cleaved cell, diffuse, unidenti- fied</td>
<td>8.1</td>
<td>1AE</td>
<td>Yes†</td>
<td>RT</td>
<td>Dead, 40</td>
</tr>
<tr>
<td>7</td>
<td>F/70</td>
<td>Small cleaved cell, follicular, B cell</td>
<td>2.8</td>
<td>1B</td>
<td>No</td>
<td>RT</td>
<td>Alive, 87</td>
</tr>
<tr>
<td>8</td>
<td>F/30‡</td>
<td>Small lymphocytic B cell</td>
<td>2.6</td>
<td>4A</td>
<td>Yes</td>
<td>Cyclophosphamide</td>
<td>Alive, 213</td>
</tr>
<tr>
<td>9</td>
<td>F/63</td>
<td>Mixed, small cleaved and large cell, follicular, B cell</td>
<td>2.5</td>
<td>1A</td>
<td>No</td>
<td>RT</td>
<td>Alive, 106</td>
</tr>
<tr>
<td>10</td>
<td>F/60</td>
<td>Small cleaved cell, follicular, B cell</td>
<td>2.1</td>
<td>1A</td>
<td>No</td>
<td>Surgery</td>
<td>Alive, 83</td>
</tr>
</tbody>
</table>

*Highest SPF measured in the series.
†Dissemination uncertain. Stage I parotid lymphoma, the suspected recurrent tumor in the abdomen was never histologically examined.
‡Biopsies from the recurrent tumors were positive for HR.
LYMPHOCYTE HOMING RECEPTORS IN LYMPHOMA

Sixty-four (61.5%) of the lymphomas were diploid and 12 (11.5%) near-diploid. Twenty-eight (27%) lymphomas were clearly aneuploid (includes five tetraploid tumors). The DNA indices ranged from 1.00 to 2.07, and the most aneuploid peaks were in the hyperdiploid region (Fig 4). SPF ranged from 1.4% to 38.4%, mean 8.8%, SD 7.0%. HR- and HR+/- lymphomas had higher SPFs than HR+ lymphomas \((P = .02)\). Four of the 10 HR- lymphomas had SPF greater than 20% as compared with only 4 of the 86 HR +/− or HR+ lymphomas \((P = .004)\).

SPF correlated well with Working Formulation. In low-, intermediate-, and high-grade malignant lymphomas the mean SPFs were 4.3% (SD, 2.8%), 8.4% (SD, 5.4%) and 14.6% (SD, 8.5%), respectively (Table 2). Only 1 (3%) of the 35 low-grade malignant and 7 (21%) of the 34 intermediate malignant lymphomas had a large SPF (>12%, see below), whereas 15 (56%) of the 27 high-grade malignant lymphomas had SPF greater than 12% \((P < .0001)\). HR expression or DNA ploidy did not correlate significantly with the histologic classification used.

**HR expression and DNA content analysis in predicting survival.** DNA aneuploidy was not associated with survival if the near-diploid cases were grouped together with the aneuploid lymphomas \((P = .84)\), or if they were classified together with the diploid lymphomas. None of the DNA index values were useful in prediction of the final outcome. However, SPF was strongly associated with both crude and corrected survival rate. After a series of calculations, the SPF value 12% was found to be associated with the most predictive power. Only 26% of the patients with lymphoma with SPF greater than 12% were alive 5 years after the diagnosis, whereas 51% of the patients with a lymphoma with less than 12% S phase cells were alive \((P = .006, \text{Fig 5A})\).

HR expression was also a significant factor determining the overall survival. Eighty percent of patients with HR− lymphoma survived for 5 years as compared with 44% of HR+− and HR+ lymphomas \((P = .03, \text{Fig 5B})\).

Prognosis of HR− lymphomas was good even if the lymphoma had a large SPF, suggesting a rapid proliferation rate (Fig 6). Eleven of the 12 patients with an HR+ or a HR+− lymphoma with greater than 15% S phase cells

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**Table 2. DNA Ploidy, Lymphocyte HR Expression, and S Phase Fraction of 104 Lymphomas Correlated With Working Formulation**

<table>
<thead>
<tr>
<th>Working Formulation</th>
<th>Ploidy</th>
<th>Homing Receptor</th>
<th>SPF: Mean %, (SD%), Range %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic, consistent with</td>
<td>N</td>
<td>DI</td>
<td>ND</td>
</tr>
<tr>
<td>CLL</td>
<td>22</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Follicular, predominantly small cleaved cell</td>
<td>13</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Follicular, mixed small and large cell</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate-grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular, predominantly large cell</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diffuse, small cleaved cell</td>
<td>17</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse, mixed small and large cell</td>
<td>8</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Diffuse, large cell</td>
<td>12</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>High-grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cell, immunoblastic</td>
<td>10</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>13</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Small noncleaved cell</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Total 104 | 64 | 12 | 28 | 10 | 14 | 80

Abbreviations: DI, diploid; ND, near-diploid; AN, aneuploid (includes tetraploid tumors).
HR and SPF were found to be valuable prognostic factors predicting survival in non-Hodgkin's lymphoma. Paraffin-embedded tissue was used for analysis, because then long follow-up was available. However, this puts some limitations for detailed phenotyping of lymphomas, because many antibodies working on frozen tissues do not stain paraffin-embedded samples. This is the case also with Hermes series of MoAbs. Only Hermes-3 antibody, which identifies different epitopes of HR molecule(s) than Hermes-1 and -2, stains HRs in paraffin-embedded tissue. Another difficulty in retrospective series is that the known final outcome may influence sample classification. This was solved by sample coding.

Only 10% of lymphomas stained negatively for HR. This may reflect the fact that most normal counterparts of malignant lymphocytes have HRs, and only nonmigratory lymphocytes, eg, most germinal center cells and cortical thymocytes, lack HRs. However, the HR- lymphomas form an important subgroup because they usually remain local, even if they have an unfavorable histologic type and a large SPF (Table 1, Fig 6). Therefore, many of these lymphomas may be curable with local therapy, such as radiotherapy (Table 1), and aggressive chemotherapy often recommended for high-grade malignant lymphomas can be avoided.

At least 2, possibly 3, of the 10 HR- lymphomas gave rise to hematogenous metastases (Table 1). Tumor heterogeneity may partially explain this, since in one such case staining for HR was repeatedly negative from the primary sample, but repeatedly positive from a sample taken from a recurrent
LYMPHOCYTE HOMING RECEPTORS IN LYMPHOMA

Fig 7. Crude (A) and corrected (B) survival for intercurrent causes of 78 patients with a lymphoma with HR- or SPF ≤12%, and 18 with HR + or HR +/- and SPF greater than 12%.

REFERENCES

8. Stamenkovic I, Amiot M, Pesando JM, Seed B: A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56:1057, 1989

Human lymphocytes and lymphoma cells express a member of the cartilage link protein family, the Hermes antigen (HR), which plays a role in lymphocyte homing to lymphoid tissues. HR is expressed on the cell surface of lymphocytes and is involved in lymphocyte recognition and recruitment to lymph node high endothelium. HR+ lymphomas have a higher survival rate compared to HR- lymphomas, suggesting that HR expression is associated with better prognosis. The regulation of lymphocyte traffic is complex and involves various molecules, including the human equivalent of mouse lymphocyte homing receptor (MEL-14) and the CD11a/CD18 complex (LFA-1). These molecules, along with others, are essential for lymphocyte binding to high endothelial venules in non-organ-specific manner. Further, immunohistologic detection of a molecule does not assure its normal function; the HRs in these lymphomas may be nonfunctional.

However, a major reason for some of the HR+ lymphomas not giving rise to hematogenous metastases is probably the relatively short follow-up. All patients with HR+ lymphoma with a high SPF died within a few years (Fig 6). Despite its protracted clinical course, low-grade lymphoma is often an eventually incurable disease, and many of the HR+ lymphomas with low SPF are likely to recur later. It will be of interest to see if HR- low SPF lymphomas form a genuinely curable subgroup of low-grade lymphoma.

In conclusion, the present study indicates that meaningful lymphocyte HR and SPF analyses can be done from paraffin-embedded archival material, even the fresh tissue would be ideal. HR - non-Hodgkin's lymphomas only seldom disseminate hematogenously, although they are often highly malignant and have a large fraction of proliferative cells. They also may be curable by local treatment. Both lymphocyte CD44 molecule involved in lymphocyte binding to mucosal HEV, and CD11a/CD18 complex is needed for lymphoma dissemination in these cases.


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