Abstract. Background: Matrix-metalloproteinases (MMP) are involved in a broad spectrum of physiological processes. Moreover, they also play a key role in tumour invasion and metastatic spread. The induction of MMPs is mediated via the extracellular matrix-metalloproteinase inducer (EMMPRIN). EMMPRIN is expressed in a variety of epithelial tumours, but expression in non-Hodgkin lymphomas has not been studied yet. Materials and Methods: Therefore we studied 201 non-Hodgkin lymphomas (NHL) for EMMPRIN expression by immunohistochemistry using a newly developed tissue microarray (TMA). This new approach to TMA-technology entails the great advantage that areas of interest (for instance with high tumour cell content) are selected by means of serial sections, thus maintaining appropriate samples of each case on the array. The samples were evaluated with regard to the number of positive tumour cells and staining intensity. Results: All specimens on the arrays contained a sufficient amount of tumour cells. Immunohistochemistry yielded satisfactory results in 196 out of 201 cases. EMMPRIN was expressed in a significantly higher number of tumour cells in high-grade NHL compared with low-grade NHL. Furthermore, the staining intensity was significantly stronger in high-grade NHL. Conclusion: We report on a new type of TMA that allows effective parallel analysis of a large number of tissue samples. Our data indicate that the expression of EMMPRIN is strongly associated with a more aggressive lymphoma type. However, additional studies are required to elucidate the role of EMMPRIN in the tumour biology of NHL. Possibly, EMMPRIN could be a new target in the therapy of NHL.

Matrix-metalloproteinases (MMP) comprise a family of 23 proteolytically active enzymes, which are able to degrade extracellular matrix (ECM) and which are important in many physiological processes such as fetal development and tissue repair (1). Furthermore, MMPs play a central role in tumour progression due to their capacity to degrade ECM, especially components of the basement membrane, permitting metastatic spread of tumour cells (2,3).

The extracellular matrix-metalloproteinase inducer (EMMPRIN), also designated CD147, induces the expression of MMPs in surrounding fibroblasts and inflammatory cells. The latter may explain why expression of MMPs in malignant epithelial tumours is mostly restricted to the stromal cells whereas the tumour cells by themselves express only small amounts of MMP (4). While the expression of MMPs in non-Hodgkin lymphoma (NHL) is well documented (5-8), nothing is known about the expression of EMMPRIN in NHL. We recently published data on cDNA expression profiles of NHL and Hodgkin lymphoma cell lines, which indicate a significant expression of EMMPRIN in both Hodgkin and non-Hodgkin lymphoma (9,10). Moreover, at the protein level we have recently reported on the expression of EMMPRIN in Hodgkin lymphomas (11,12). The aim of the present study was to determine the expression of EMMPRIN in high-grade and low-grade NHL.

In cooperation with Euroimmun Medizinische Labordiagnostica AG, Luebeck, Germany (W.S. and E. M-K.), we developed a new type of tissue microarray (TMA) in order to save time and costs. One of the predominant advantages of this new tissue array is the possibility of checking tissue samples and selecting separately for sufficient tumour cells before transferral onto the array.
Materials and Methods

Samples. We studied 201 non-Hodgkin lymphoma samples consisting of 46 T-cell lymphomas (TCL) and 155 B-cell lymphomas. The former comprised 32 angioimmunoblastic lymphomas (AILT), 2 anaplastic large cell lymphomas (ALCL) and 12 T-cell lymphomas of NOS-type. The latter consisted of 100 high-grade lymphomas (69 centroblastic, 16 immunoblastic, 13 diffuse large B-cell lymphoma not otherwise specified and 2 Burkitt lymphomas) and 55 low-grade lymphomas (7 lymphocytic, 16 follicular, 30 mantle cell and 2 nodal marginal zone lymphomas). The mantle cell lymphomas included 8 cases with a high proliferation index (>40% MIB-1-positive cells), while the follicular lymphomas included 6 grade 3 lymphomas according to the WHO classification and can therefore also be classified as high-grade lymphomas.

Morphology and immunohistochemistry. The morphological features were assessed on haematoxylin and eosin-, periodic acid-Schiff-, Giemsa- and Gomori-stained sections of formalin-fixed, paraffin-embedded tissue.

Immunohistochemical stainings were performed according to a standard three-step immunoperoxidase technique with diaminobenzidine as chromogen. The following antibodies were used for routine diagnostic procedure: CD20, OPD4, Bcl6, CD3, kappa and lambda light chain, Cyclin D1 and Mib1, all of which were purchased from Dako (Denmark). CD5, CD10, CD30, CD15, CD2, CD56, CD5, CD8 and CD4 were obtained from Biocarta (Hamburg, Germany). CD23 was purchased from Novocastra (Newcastle, UK), OCT2 from Santa Cruz (Santa Cruz, CA, USA), and TIA1 from Immunotech (Marseille, France). The polyclonal EMMPRIN antibody was purchased from R&D-systems (Minneapolis, USA). All secondary antibodies and all further reagents were obtained from Biocarta (Hamburg, Germany) or Dako (Denmark) depending on the primary antibody.

All stainings were performed at least twice. For the assessment of staining intensity we used a semiquantitative score as follows: 0=no staining; 1=weak staining; 2=moderate staining; 3=strong staining. Tissue-arrays. The principles of this technique were described by one of the authors (W.S.) as early as 1985 (13,14). We modified the technique and developed the present TMA in cooperation

Figure 1. View of the scanned coverslide with the complete donor sample and grid-lines. Each quadrate can be examined, selected and transferred separately to the tissue array.

Figure 2. H&E-stained tissue-array with 95 samples.
Figure 3. a) Lymphocytic lymphoma with weak (=1) positivity. b) Immunoblastic lymphoma with strong (=3) positivity.
with Euroimmun Medizinische Labordiagnostica AG. At first, 3-5 μm sections of routinely formalin-fixed paraffin-embedded material were prepared on chemically-activated cover-slides. Subsequently, the cover-slides were scanned to create a computer image, which was then overlayed with grid lines. Thus, every sample was split in defined square areas measuring between 3.24 to 4 mm² in size. By means of the computer screen, representative areas of the lymphoma were chosen and marked by a pathologist (C.T.). Afterwards, the cover-slides were cut into square sections along the grid lines (Figure 1). Only the marked square sections were then used to equip the tissue arrays. These square sections were transferred to conventional glass-slides and fixed in a defined position using a custom-made spotting machine. Every glass-slide (tissue array) contains up to 100 of the 4-mm² sections, thereby the number of sections originating from a single donor cover-slide is eligible. Thus, up to 100 distinct tumour samples can be placed on one tissue array. For the present study, the lymphoma samples were arranged in combination with samples from tonsils and placenta as negative and positive controls (Figure 2).

Depending on the size and tumour content of the donor paraffin section, a single cover-slide can serve to equip a huge number of tissue arrays, where the respective tumour sample gets its determined position on every new array.

**Statistics.** For statistical comparison of the groups the non-parametric unpaired two-tailed Mann-Whitney U-test was applied. P-values < 0.05 were considered as significant.

**Results**

All samples on the arrays were representative of the tumour as was assessed on conventional Giemsa-stained sections and contained an adequate amount of tumour cells. The immunohistochemical stainings gave reproducible results in 196 out of 201 cases. Four samples of DLBCL and one case of follicular lymphoma yielded unreliable staining results, probably due to a prolonged fixation period. The arrays were evaluated independently by two of the authors (C.T. and H.-
W. B.) and consensus was achieved on a multi-observer microscope. The samples were evaluated with regard to the number of positive tumour cells and to the intensity of the staining. The results are summarized in Table I. Example of different staining intensities are given in Figure 3.

The number of positive tumour cells in high-grade B-NHL ranged from 0 to 100% with a mean value of 81.9%, whereas in low-grade B-NHL significantly fewer tumour cells stained positive (mean 35%; Figure 4). This difference was statistically significant (p<0.001). The same was true with regard to the intensity of staining. The average intensity in high-grade B-NHL was 2.4 as compared to 1.1 in low-grade B-NHL (p<0.001). Interestingly, the samples of grade 3 follicular lymphomas and mantle cell lymphomas with a high proliferation rate had a higher expression of EMMPRIN than low-grade B-NHL and lower values than high-grade B-NHL (Figure 4). This difference was significant for both the number of positive cells (p=0.017 and p=0.007) and the intensity of staining (p=0.029 and p=0.002). However, although there was a trend for a higher expression of EMMPRIN in mantle cell lymphomas with a high proliferation rate in direct comparison to classical MCL, the difference lacked significance (p=0.169 and p=0.1). The same was true comparing grade 3 follicular lymphomas with grade 1 and grade 2 follicular lymphomas (p=0.181 and p=0.328), but this is probably due to the relatively small number of grade 3 follicular lymphomas in this study. It is noteworthy that expression of EMMPRIN was stronger and more frequently observed in immunoblastic lymphomas than in centroblastic lymphomas. This phenomenon may, in part, explain the generally more aggressive clinical course of immunoblastic lymphomas. However, the difference did not reach statistical significance (p=0.102).

T-cell lymphomas had a mean value of 55.1% EMMPRIN-positive cells and a mean intensity of 1.9. Thereby, the expression of EMMPRIN in TCL is in between low-grade and high-grade B-NHL. The differences were statistically significant with regard to both the number of positive cells and the staining intensity (TCL vs. low-grade B-NHL p=0.006; p<0.001 and TCL vs. high-grade B-NHL p<0.001; p=0.003). No significant difference was observed between T-cell lymphomas NOS and angioimmunoblastic lymphomas.

**Discussion**

We developed a new type of tissue microarray and used this technique to verify data obtained with cDNA arrays on cell lines of NHL (9,10). The aim of this study was to determine the expression of EMMPRIN in malignant NHL at the protein level.

The tissue arrays developed in cooperation with Euroimmun Medizinische Labordiagnostica AG have the great advantage that samples can be checked for a sufficient amount of tumour cells before transferral onto the arrays. This is especially important in cases with a highly inhomogeneous tumour growth. Therefore, it is guaranteed that each array contains representative areas of all tumour samples. This is not the case for the common TMA, that are produced on the basis of tissue cores obtained from several donor paraffin blocks and transferred to a recipient paraffin block. Depending on the different thickness of each donor block, the sections from the recipient block will differ from each other. Moreover, the exact tissue composition of samples in deeper levels of the TMA block is hard to determine. Recent studies have shown that immunohistochemical studies on TMA and whole paraffin sections yield comparable results (15,16). The major disadvantage of our newly developed TMA in comparison to conventional TMA is the fact that its production is more time consuming, since all squares are checked separately for a sufficient number of tumour cells. The use of the newly developed tissue arrays proved to be very effective for the immunohistochemical study of a huge number of paraffin-embedded tissue samples.

Our data clearly show that EMMPRIN is expressed in malignant non-Hodgkin lymphoma of the B- and T-cell type. Whereas the expression of EMMPRIN is well
documented in epithelial tumours (17,18), there are currently no data on the expression of EMMPRIN in malignant non-Hodgkin lymphomas. EMMPRIN is able to induce the expression of MMPs in surrounding fibroblasts. Recent data indicate that the tyrosine MAP kinase p38 and 5-lipoxygenase are involved in the regulatory pathways of MMP-induction via EMMPRIN (19,20). The main target of EMMPRIN is the induction of MMP-1, MMP-2 and MMP-3 (21-24). Conversely, MMP-9 and membrane-type-1-MMP (MT1-MMP) are not induced by EMMPRIN according to data from Caudroy et al. (25). However, recent data from Yang et al. (26) provide indirect evidence for the induction of MMP-9-expression by EMMPRIN. These authors were able to demonstrate that the level of MMP-9 decreased after the medium was supplemented with an inhibitory antibody against EMMPRIN.

The role of EMMPRIN in the development and progression, especially the invasion and metastatic spread, of epithelial tumours is easily plausible due to its effects on the extracellular matrix via the induction of various MMPs. MMPs are able to degrade essentially all components of the ECM, especially the basement membrane, and are therefore important in the process of tumour spread and invasive growth. In many epithelial tumours MMPs are strongly expressed and are part associated with worse prognosis or tumour stage (27-30). Consequently, several synthetic MMP-inhibitors have been developed. However, the first results of phase I clinical trials are overall discouraging (31-34), although recent phase II and III clinical trials have shown some benefit in patients with advanced pancreatic and gastric cancer (35,36) [for review see (37)]. Recently, it was shown by Davidson et al. that the expression of EMMPRIN in ovarian carcinoma is associated with a poor overall survival (38). Yang et al. found that EMMPRIN was overexpressed in multidrug-resistant cancer cells, which led to an overexpression of MMP-1, MMP-2 and MMP-9. Interestingly, the more aggressive invasive growth properties of multidrug-resistant cancer cells in comparison to sensitive cancer cells could be inhibited by antibodies against EMMPRIN in vitro (26).

In contrast to epithelial tumours, the biology of malignant NHL differs in that NHL are systemic diseases rather than a primarily localized process. This is at least true for the nodal manifestations of NHL, but may be different for extranodal marginal zone lymphomas of the MALT. Therefore it seems to be unlikely that the role of EMMPRIN in NHL is the same as in epithelial tumours. Regardless of these differences we did find a strong positive correlation between the level of EMMPRIN expression and the aggressiveness of the lymphoma type. Irrespective of the type of NHL, the EMMPRIN staining was mostly found on blast cells, but only occasionally on small lymphatic cells. This finding is not only true for malignant lymphomas but also for reactive lymphatic tissue in tonsils and lymph nodes. Here we found elevated EMMPRIN expression on numerous germinal center blasts as well as on interfollicular blasts, whereas there was no positive staining on centrocytes, mantle/marginal zone B-cells and interfollicular T-lymphocytes. It therefore may be argued that the correlation between the aggressiveness of the lymphoma and the number of EMMPRIN-positive cells is not a causal relationship but rather an epiphenomenon due to the fact that the more aggressive NHL generally do contain a higher number of blasts. However, some data indicate that there may be a causal role for EMMPRIN in the development or progression of malignant lymphoma. We have recently described the interactions between tumour cells and surrounding fibroblasts involving EMMPRIN and different MMPs in Hodgkin lymphoma (12). In contrast to NHL, the hallmark of Hodgkin lymphoma is the paucity of tumour cells, which are embedded in a background of non-neoplastic fibro-histiocytic and inflammatory bystander cells, often accompanied by prominent sclerosis. Therefore, the interactions between tumour cells and surrounding tissue in Hodgkin lymphomas may be, in part, different from those in malignant non-Hodgkin lymphomas. On the other hand, there is also good evidence for a correlation between aggressiveness of lymphoma type and the expression of MMPs: Kossakowska et al. describe the expression of mainly MMP-9 in malignant non-Hodgkin lymphomas (5). So far, however, there are no data as to whether the increase in MMP-levels in high-grade lymphomas is indeed caused by EMMPRIN. As mentioned above, the data on MMP-9 induction via EMMPRIN are contradictory. Therefore further in vitro and in vivo studies are necessary to elucidate whether the expression of EMMPRIN is capable of increasing the aggressiveness of malignant NHL.

In conclusion, the present study provides the first data on the expression of EMMPRIN in malignant NHL and shows that EMMPRIN is strongly expressed in high-grade lymphoma and only weakly or not at all in low-grade lymphoma. This possibly makes EMMPRIN an attractive target for new therapeutic approaches for high-grade lymphomas for instance in terms of siRNA or antibody-based therapies.

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