EXPRESSION OF THE ONCOFETAL ED-B CONTAINING FIBRONECTIN ISOFORM IN HEMATOLOGIC TUMORS ENABLES ED-B TARGETED 131I-L19SIP RADIOIMMUNOTHERAPY IN HODGKIN LYMPHOMA PATIENTS

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Abstract

Current treatment of hematologic malignancies involves rather unspecific chemotherapy, frequently resulting in severe adverse events. Thus, modern clinical research focuses on compounds able to discriminate malignant from normal tissues. Being expressed in newly formed blood vessels of solid cancers but not in normal mature tissues, the extra-domain B of fibronectin (ED-B FN) is a promising target for selective cancer therapies. Using immunohistology with a new epitope retrieval technique for paraffin-embedded tissues, ED-B FN expression was found in biopsies from more than 200 Hodgkin and Non-Hodgkin lymphoma patients of nearly all entities, and in patients with myeloproliferative diseases. ED-B FN expression was nearly absent in normal lymph nodes (n=10) and bone marrow biopsies (n=9). The extent of vascular ED-B FN expression in lymphoma tissues was positively correlated with grade of malignancy. ED-B FN expression was enhanced in lymph nodes with severe lymphadenopathy and in some hyperplastic tonsils. The in vivo accessibility of ED-B FN was confirmed in three lymphoma patients, in whom the lymphoma lesions were visualized on scintigraphy with $^{131}$I-labeled L19 small immunoprotein ($^{131}$I-L19SIP). In two relapsed Hodgkin lymphoma patients, $^{131}$I-L19SIP radioimmunotherapy induced a sustained partial response, qualifying ED-B FN as a promising target for antibody-based lymphoma therapies.
Introduction

Most anti-cancer therapies cannot discriminate between malignant and normal tissues, since their action depends on the increased proliferation rate of tumor versus benign cells. Furthermore, high systemic doses of cytotoxic/cytostatic drugs are generally required to achieve therapeutically active concentrations within the tumor, thus frequently resulting in severe adverse effects. The approach of antibody-based therapies to selectively target therapeutically active molecules to tumor tissues is highly promising. Few of such immunotherapy agents are currently in clinical development or have been approved for treating solid cancers or hematologic malignancies (Mylotarg, EMD273063). The basic prerequisite to apply this strategy is tumor-selective expression of the specific target structure. The extra-domain B of fibronectin (ED-B FN) has been suggested as a potential target for novel anti-cancer therapies in patients with solid tumors.

Fibronectin (FN) is a ubiquitously expressed high-molecular weight extracellular matrix glycoprotein which exists in various isoforms. Sequence analysis has revealed that FN consists of three different sequence homologies designated as type I, II, and III repeats, respectively. Three sites of alternative splicing within the type III repeats of the FN molecule have so far been identified: ED-A (type III homology extra domain A), ED-B (type III homology extra domain B), and IIICS (type III homology connecting segment).

Since the ED-B domain is preferentially expressed by transformed cells in vitro, ED-B FN was designated as oncofetal or embryonic FN, reflecting its expression mainly in fetal tissues and solid tumors. ED-B FN is synthesized, secreted and deposited to the extracellular matrix structures by numerous cell types such as endothelial cells of newly formed blood vessels, myofibroblasts and, most notably,
tumor cells. It can be detected at the abluminal site of endothelial linings of newly formed blood vessels and between stromal structures. Using polyclonal, monoclonal and recombinant antibodies for antigen detection by immunohistochemistry (IH), ED-B FN expression can be demonstrated in a variety of human tissues. ED-B FN expression is tightly controlled and appears to be restricted to embryonic tissues, a few normal adult organs and wound healing. However, the functional significance of ED-B-FN is still to be established. In vitro studies have yielded contradictory results, and studies with in vivo models on single deletion of EIIIA or EIIIB have failed to provide significant insight into the possible functions of these splice variants. In fact, EIIIA-null or EIIIB-null mice are viable, fertile, and maintain normal physiological angiogenesis after birth. Nevertheless, EIIIA/EIIIB double-null embryos display multiple cardiovascular defects, thus indicating a crucial involvement in angiogenesis and in cardiovascular development of the EIIIA and EIIIB-containing splice variants of FN.

While physiologic expression of ED-B FN is rare in healthy adults, it can occur in chronic pathological conditions associated with new blood vessel formation such as ocular-retinal diseases, severe atherosclerosis, and inflammatory rheumatoid disease. ED-B FN is abundant in tissues of almost all human solid cancers, irrespective of histotype.

Since ED-B FN expression has been more extensively evaluated in solid cancers, we thought it worthwhile to explore this biologic event also in a broad range of hematologic malignancies and normal lymphoid and hematopoietic tissues. Moreover, in vivo targeting of ED-B FN was evaluated in three lymphoma patients using a radio labeled ED-B FN binding antibody (131I-L19SIP), as was the clinical response in these patients to a subsequent therapeutic dose of 131I-L19SIP.
Materials and Methods

Specimens

Paraffin-embedded and shock-frozen specimen of hematopoietic malignancies were drawn from the files of the Institute of Pathology, Campus Benjamin Franklin, Charité, Berlin, Germany (Table 1). Additionally, 3 lymph node biopsies with the diagnosis of EBV-associated lymphoproliferation, 13 tonsils with follicular hyperplasia, 10 tumor-free lymph nodes harvested from the drainage of carcinomas, 12 enlarged lymph nodes with severe inflammation, and 9 bone marrow biopsies with non-neoplastic alterations were included in the study (Table 2). Finally, frozen specimens of 2 CLL, 6 mantle cell, and 3 follicular lymphomas were analyzed. The hematopoietic tumors were classified according to WHO classification. Paraffin-embedded and frozen specimens of 6 cases of clear cell renal cell carcinoma were included as positive controls.

The mouse embryonal teratocarcinoma cell line F9 was purchased from ATCC (Manassas, Virginia, USA). Athymic nude mice (8-week-old nude/nude CD1 mice, females) were obtained from Harlan, Italy (Correzzana, Milan, Italy). Nude mice were subcutaneously implanted with F9 cells as described. F9 tumors, known for their strong ED-B FN expression, were harvested when approximately 10 mm in diameter and split in half. One half was paraffin-embedded, while the other half was shock-frozen in liquid nitrogen. All animal experiments were conducted in accordance with the UKCCCR regulations for the welfare of animals and the German animal protection law. Additionally, approval was granted by local authorities from the government of Berlin LaGeSo.
Immunohistochemistry

To retrieve the ED-B FN epitope, paraffin-embedded specimens were pretreated by steam pressure boiling in 2 mM EDTA, pH 8.0, 5 minutes. Immunohistochemistry of cryostat sections and pretreated paraffin sections was performed using the APAAP method. ED-B FN was detected employing the dimeric L19IL2 fusion protein which consists of the human anti-ED-B scFv L19 fused to IL-2 and MX1, an ED-B-specific murine IgG2a monoclonal antibody binding to a different epitope than L19 (Dr. Andreas Menrad unpublished results). In each case, 10 microscopic fields were examined for ED-B FN expression in blood vessels and stroma at 400x magnification. ED-B FN expression was semi-quantitatively scored by assigning all cases to four categories based on the fraction of ED-B FN-positive blood vessels, i.e., more than 90%, between 50% – 90%, between 10% – 49%, or less than 10%, respectively. These scores were applied by 2 different investigators (S.S., H.D.), reaching best inter-observer agreement.

For ED-B FN/CD34 double fluorescence staining, ED-B FN was detected by MX1 and goat anti-mouse Cy3-conjugated antisera (Dianova, Hamburg, Germany), employing a confocal microscope (Leitz, Wetzlar, Germany). After blocking the murine immunoglobulins with an excess of goat-anti-mouse serum, CD34 (QBEND, Immunotech, Marseille, France) was stained and subsequently detected by goat anti-mouse Cy2-conjugated antisera (Dianova). Nuclei were depicted by TOTO-3 (Molecular Probes, Paisley, UK).

Competition experiments were performed by titrating recombinant ED-B FN against MX1 mAb and L19IL2 constructs. Subsequently, the ED-B FN immunohistological staining properties of these mixtures were tested.
\(^{131}\)I-L19SIP SPECT-CT images and \(^{18}\)F-FDG PET scans

Within the framework of a Health Care Authority-approved clinical trial sponsored by Philogen S.p.A (Siena, Italy), both the pharmacokinetic profile, the dosimetry estimates and safety of \(^{131}\)I-L19SIP are being investigated in lymphoma patients at the Regional Center of Nuclear Medicine, University of Pisa Medical School, (Pisa, Italy) and at the European Institute of Oncology in Milan (IEO, Italy). Institutional board approval was obtained from all participating institutions for these studies and patient informed consent was obtained in accordance with the Declaration of Helsinki. According to this study, \(^{131}\)I-L19SIP was intravenously injected in a 73-year-old male patient with advanced relapsed small lymphocytic non-Hodgkin lymphoma (SLL NHL; NHL1), a 28-year-old male (HL1) and a 27-year-old female patient (HL2) with classical Hodgkin lymphoma of nodular sclerosis (HL). These patients had failed multiple chemo/chemoradio- and immunotherapies and presented with relapsed disease as documented by CT and \(^{18}\)F-FDG PET-CT scans. One-hundred-eighty-five MBq (5 mCi) of \(^{131}\)I-L19SIP were first administered for biodistribution and dosimetry evaluations. Whole body scans and single photon emission computed tomography (SPECT) images (GE Infinia™ Hawkeye®) were acquired at different time-points after \(^{131}\)I-L19SIP injection, as described. In particular, SPECT-CT images were acquired 48, 96 hours and 8 days post-injection. Blood effective half-lives (\(T_{1/2}^\alpha\) and \(T_{1/2}^\beta\)) were calculated and dosimetry to normal organs and to lesions was estimated as absorbed dose (Gy). Based on selective uptake into tumor lesions and on adequate bone marrow dosimetry (lesion/red-marrow absorbed dose ratio \(\geq 10\)), patients NHL1 and HL1 subsequently received a dose of \(^{131}\)I-L19SIP of 5.55 GBq (150 mCi), whereas patient HL2 received a \(^{131}\)I-L19SIP dose of 3.7 GBq (100 mCi). Whole-body and SPECT-CT images were obtained 8-12 days post-
administration of this dose to confirm specific tumor targeting.

**Statistical analyses**

Statistical analyses were performed with the U/H-ranking tests using 13.0 Base System SPSS for Windows (SPSS GmbH Software, Munich, Germany).
RESULTS
Reliability and localization of ED-B FN detection in paraffin-embedded specimens

This study presents the first systematic data about the detection of the ED-B epitope of FN in paraffin-embedded tissues. To verify the expression pattern of ED-B FN in paraffin-embedded tissues, we used the mAb MX1 and the fusion protein L19IL2. Both anti-ED-B FN constructs detected the same ED-B FN expression pattern in paraffin-embedded and frozen specimens of renal carcinoma (n=6), lymphocytic lymphoma (CLL; n=2), mantle cell lymphoma (n=6), and follicular lymphoma (n=3). Identical ED-B FN expression patterns were also found in the paraffin-embedded half and the frozen half of the split F9 tumor specimens (Fig. 1).

To confirm the binding specificity of the MX1 mAb and the L19IL2 fusion protein, these reagents were pre-incubated with an excess of recombinant ED-B FN. The mixtures were incubated on human clear cell renal cell carcinomas and murine F9 teratocarcinoma with subsequent immunohistological detection of their binding pattern. Recombinant ED-B FN was capable of inhibiting the immunohistological binding of these reagents (Fig. 2).

Double stainings of diffuse large B cell lymphoma, marginal-zone lymphoma, lymphocytic lymphoma, plasmacytoma, follicular lymphoma (grade 1 and 3), and classical Hodgkin lymphoma (mixed cellularity and nodular sclerosis) revealed that ED-B FN is mostly co-localized with blood vessels, which were identified by their CD34-positive endothelia (Fig. 3). By investigating 1 normal lymph node, 1 lymph node with lymphadenopathy, 1 hyperplastic tonsil, and 2 normal bone marrow samples we also confirmed that the mostly small ED-B FN deposits are localized perivascular in non-malignant lymphoid and hematopoietic tissues.
ED-B FN expression in normal and hyperplastic lymphoid tissue and normal bone marrow

Blood vessels of normal lymphoid tissue rarely and weakly express ED-B FN (Fig 4). ED-B FN expression in hyperplastic tonsils was heterogeneous. More than 90% of all blood vessels were ED-B FN-positive in 7/13 tonsils, whereas 5/13 tonsils showed very low perivascular ED-B FN expression (Table 2). Most of the ED-B FN-positive blood vessels of hyperplastic tonsils were found at the rim of germinal centers inside the inner mantle and marginal zone surrounding the highly proliferating follicular center cells. A few ED-B FN-positive vessels were situated in the fibrous fascicles of tonsils formed during chronic inflammation. ED-B FN expression was found in blood vessels of every diameter in lymphoid tissues.

Normal bone marrow samples revealed ED-B FN expression in less than 10% of all medium sized and large blood vessels. The few ED-B FN-positive medullar vessels bore a fibrous wall. ED-B FN expression was not found in normal sinusoids lacking fibrous support.

ED-B FN expression in nodal and extranodal lymphomas

Nodal and extranodal lymphomas showed significantly more ED-B FN-positive blood vessels than normal lymphoid tissues (p<0.001; Table 1). This finding was true for both Non-Hodgkin and Hodgkin lymphomas. The degree of ED-B FN expression did not depend on the infiltrated organ, as lymphoma infiltration increased ED-B expression to almost the same extent in both nodal and parenchymal sites. High-grade malignant lymphomas (DLBCL, follicular lymphoma Grade 3, and ALL) exhibited significantly higher expression of ED-B FN in blood vessels than small B
cell lymphomas (follicular lymphoma grade 1 and 2, CLL, mantle cell lymphoma, marginal cell lymphoma, plasmacytoma, and lymphoplasmocytic lymphoma; p=0.008). ED-B FN expression was found in blood vessels of all diameters and locations. Vessels inside the tumor, in the tumor stroma, and the tumor capsule bear ED-B FN, while ED-B FN expression was not detectable in vessels at the rim of tumor necrosis. ED-B FN expression did not significantly differ between classical Hodgkin lymphoma and lymphocytic-predominant Hodgkin lymphoma.

Three cases of EBV-associated lymphoproliferation (resembling diffuse large B cell lymphoma) showed high perivascular ED-B FN expression roughly to the same amount as in high-grade malignant lymphoma.

However, lymphoid tissue with severe inflammation, such as lymph nodes with lymphadenopathy or hyperplastic tonsils causing angina, revealed perivascular ED-B FN expression to almost the same extent as in malignant lymphoma.

**ED-B FN expression in neoplastic infiltrates of the bone marrow**

In comparison to normal bone marrow, ED-B FN expression was enhanced in the bone marrow inflicted by myeloproliferative disease (p<0.001). The amount of ED-B FN-positive blood vessels did not significantly differ between acute myeloblastic leukemia and chronic myeloproliferative diseases, such as CML, polycythaemia vera, essential thrombocythaemia, or chronic idiopathic fibrosis.

Infiltration of bone marrow by plasmacytoma or lymphoma led to significantly increased ED-B FN expression in comparison to normal bone marrow (p<0.001).

ED-B FN expression in bone marrow biopsies infiltrated by hematologic malignancies was restricted to small and medium sized blood vessels consisting of
an endothelial layer and a tiny wall of connective tissue. Sinusoids of the bone marrow were also ED-B FN-negative in the presence of neoplastic infiltrates.

**131I-L19SIP SPECT-CT images and 18F-FDG PET scans**

As the radiopharmaceutical 131I-L19SIP contains the very same binding moiety, L19, used for immunohistochemical determination of ED-B FN in lymphoma samples, this radio labeled agent maintains both high-affinity for the target antigen and binding properties typical to other L19 fusion proteins.\(^{35}\) In the patient with advanced SLL NHL, transaxial, coronal and sagittal 131I-L19SIP SPECT-CT images demonstrated selective ED-B FN targeting in a palpable, enlarged lymph node conglomerate in the left cervical region, which corresponded to high 18F-FDG uptake on the baseline PET-CT scan (Fig. 5). The absorbed dose in the target lesion was estimated at approximately 18 Gy, whereas it was 0.99 Gy and 0.42 Gy in the bone marrow and kidney, respectively. The patient was subsequently treated with a dose of 5.55 GBq of 131I-L19SIP. He achieved disease stabilization at 1 month after therapy with the sum of involved lymph node diameters unchanged from baseline (403 vs 417 mm; baseline to 1 month after treatment). However, this patient experienced no clinical benefit from 131I-L19SIP radioimmunotherapy and went off study to receive palliative treatment.

Both HL patients also showed favorable lesion/bone-marrow dosimetry estimates after receiving diagnostic 131I-L19SIP, with an absorbed dose of radioactivity to the target lesions – a pulmonary lymphoma lesion (patient HL1) and a left basal pulmonary lesion and a right axillar lymphoma conglomerate (patient HL2) – estimated to be approximately 14 and 22.7 Gy, respectively. The absorbed dose to the red bone marrow was calculated to be 1.3 and 0.85 Gy for HL patients HL1 and
HL2, respectively. In HL patient HL1, the SPECT-CT images acquired 12 days after a dose of 5.55 GBq of $^{131}$I-L19SIP demonstrated selective ED-B FN targeting in multiple parenchymal lung lesions and in enlarged supraclavicular and lumbo-aortic lymph nodes with all such sites corresponding to high $^{18}$F-FDG uptake on baseline PET-CT scans (Fig. 6). In the second HL patient (HL2), the SPECT-CT images acquired 12 days after a dose of 3.7 GBq of $^{131}$I-L19SIP demonstrated selective ED-B FN targeting in multiple enlarged axillar and supraclavicular (both sides), paratracheal, subcarinal, pleural, as well as peritoneal and iliacal (both sides) lymph nodes. ED-B FN expressing lymphoma lesions were also found in the right and left basal lobes of the lung. All ED-B FN expressing lymphoma lesions corresponded to high $^{18}$F-FDG uptake on baseline PET-CT scans (Fig. 7). Both HL patients experienced a partial clinical response according to RESCIST criteria at 1 month after therapy with shrinkage of the sum of diameters of the measurable lymphoma lesions of 44% (134 to 75 mm; baseline to 1 month after treatment) and 39% (417 to 256 mm) for patients HL1 and HL2, respectively. This partial response was confirmed at 2 and 3 months after $^{131}$I-L19SIP therapy for both HL patients. The three lymphoma patients did not experience any acute toxicity during or after $^{131}$I-L19SIP therapy. Mild and transient thrombocytopenia was observed in both HL patients, but not in the SLL NHL patient, with a nadir of 22’000 and 52’000 platelets /μl for patients HL1 and HL2, respectively, at 6 weeks after $^{131}$I-L19SIP injection.
Discussion

The identification of suitable targets for more selective and less toxic therapies for advanced malignant neoplasias is crucial for the success of innovative treatment approaches. Consequently, great emphasis has been put on the identification of unique molecular pathways or specific cellular antigens of malignant cells. Selective treatments interacting with some of these tumor cell-associated targets were found to be highly beneficial, including the monoclonal antibodies rituximab in combination with chemotherapy for B cell lymphoma,\textsuperscript{36} and trastuzumab for Her2/neu overexpressing breast cancer.\textsuperscript{37} However, for the majority of advanced solid cancers and for many hematologic malignancies, successful tumor-targeting therapies are still lacking.

Recently, it became clear that stromal structures, e.g. blood vessels and extracellular matrix proteins supporting cancer growth and proliferation, are strikingly different from their normal counterparts. As demonstrated by Folkman et al.,\textsuperscript{38} neovascularization is crucial for tumor growth beyond 2 mm\textsuperscript{3}, and was found structurally and functionally altered in comparison to blood vessels of normal mature organs. Vascular shunts, uneven vessel diameters, unusual fenestration with wide interendothelial junctions, discontinuous basement membranes,\textsuperscript{39,40} and dysfunctional or lack of pericytes,\textsuperscript{41,42} are key features of tumor blood vessels rendering them leaky. These characteristics may not be specific for tumor blood vessels, since there is evidence of similar changes in blood vessels associated with periodic tissue remodeling,\textsuperscript{40,43} chronic inflammatory disease,\textsuperscript{28} and severe atherosclerosis.\textsuperscript{27} However, targeting anticancer compounds selectively to epitopes that are typically expressed or over-expressed in tumor blood vessels or stroma is
attractive for two reasons: (i) tumor blood vessel or stromal epitopes are stably expressed, and (ii) such epitopes are typically found in all kinds of tumors.

The extra domain B of fibronectin (ED-B FN) represents such a stromal tumor tissue target.\(^9\) Further validating this concept, the ED-B FN targeting antibody L19 acting as a vehicle for tissue factor or TNF\(\alpha\) (tumor necrosis factor \(\alpha\)) to the tumor vasculature, was found to induce tumor necrosis.\(^{44,45}\)

ED-B FN expression was found to a variable extent and with different expression patterns by immunohistochemistry on cryostat tissue sections in almost all solid cancers studied thus far.\(^4\) To extend these studies to a reliable sample of hematologic malignancies and to include decalcified bone marrow biopsies, we developed an antigen retrieval technique to stain ED-B FN on paraffin-embedded tissues. This technique lead to equivalent immunohistological staining results on paraffin sections as compared with the immunohistological results on cryostat sections, while preserving tissue histology. The loss of ED-B FN staining in tissue sections after addition of soluble recombinant ED-B FN to the anti-ED-B FN reagents demonstrated the specificity of the immunohistochemical procedure. Thus, we are able to present the first systematic report of ED-B FN expression on normal lymphoid and hematopoietic tissue and in a large series of lymphoid and hematologic malignancies, including decalcified bone marrow biopsies.

We found expression of ED-B FN in almost all examined lymphoma samples, irrespective of their histopathologic classification. ED-B FN-positive vessels were significantly more tightly packed in lymph nodes with lymphoma infiltration than in the tumor-free lymph nodes draining carcinomas. Low grade Non-Hodgkin lymphomas contained less ED-B FN-positive vessels than high grade lymphoma, indicating that neoangogenesis is correlated with the proliferation rate of tumor cells. The same holds
true for bone marrow samples infiltrated by lymphoma or leukemia, revealing high perivascular ED-B FN expression while normal bone marrow biopsies are almost ED-B FN-negative. However, the strong ED-B FN expression in lymph nodes with severe inflammation and in hyperplastic tonsils causing angina, as a consequence of the fast expansion of lymphoid tissue in response to viral or bacterial infection confirms that ED-B FN expression is a general feature of rapidly proliferating and remodeling tissues, independent from the cause inducing these processes.

We could not detect cytoplasmatic ED-B FN expression in megakaryocytes. This finding is only partially consistent with the results of Vogel et al. who found ED-B FN in megakaryocytes by immunocytological analyses of bone marrow smears but could not confirm these findings by flow-cytometric analysis or reverse transcription PCR.

Wagner et al. and Blum et al. found weak ED-B FN expression on the surface of CD3- and IL-2-stimulated T cells by flow cytometry. Since immunohistochemistry is less sensitive than flow cytometry, our results may be interpreted as a consequence of a very low ED-B FN expression level in stimulated T cells.

To confirm perivascular ED-B FN expression, we performed immunofluorescence double stainings of ED-B FN and CD34-positive endothelia. These investigations indicate that ED-B FN in hematologic malignancies is almost always associated with the wall of blood vessels and the perivascular connective tissue. In all specimens of lymphoid and hematopoietic tissues, there was only a small amount of ED-B FN displaying an unequivocally extravascular localization. From the perspective of designing antibody-based tumor targeting therapies, ED-B FN in newly formed blood vessels is of particular relevance as compared to stromal ED-B FN, since it is easily
accessible from the blood stream due to the frequent fenestrations and leakiness of tumor blood vessels.\textsuperscript{40}

These considerations were confirmed by our exploratory proof-of-mechanism investigations, demonstrating the accessibility of lymphoma-associated ED-B FN expression via the blood stream as well as respective ED-B antibody binding properties \textit{in vivo}.\textsuperscript{131I-L19SIP} was intravenously injected to patients suffering from relapsed advanced SLL-NHL and HL (n=2). Subsequently performed scintigraphic \textit{in vivo} images demonstrated selective expression and accessibility of lymphoma-associated ED-B FN in these three patients, confirming and extending previously published immunoscintigraphic studies with radio-isotope-labeled ED-B antibodies in patients with solid cancer. In a small study of five patients with malignant brain tumors, planar and SPECT-CT imaging using the \textsuperscript{99mTc}labeled ED-B monoclonal antibody BC-1 disclosed the brain tumors in all cases.\textsuperscript{49} In another study with 20 patients suffering from advanced lung, colorectal or brain cancers, selective localization of ED-B-binding \textsuperscript{123I}-L19(scFv)\textsubscript{2} to tumor lesions was demonstrated.\textsuperscript{50} In our lymphoma patients, the uptake of \textsuperscript{131I-L19SIP} to lymphoma lesions was not only selective and long-lasting (up to 12 days after injection), but also surprisingly high with an absorbed dose to the target lesion of approximately 14-22.7 Gy, sparing radiosensitive organs such as red bone marrow and the kidneys. Furthermore, the two HL patients experienced a sustained partial remission according to RECIST, which was associated with clinical benefit, lasting for at least 2 and 3 months, respectively. In contrast, the SLL-NHL patient had only short disease stabilization and did not benefit from treatment.

In conclusion, developing an antigen-retrieval method for immunohistochemistry on paraffin sections we were able to demonstrate ED-B FN depositions in the
extracellular matrix on newly formed blood vessels in hematologic malignancies. The application of the highly ED-B FN specific radiolabeled antibody $^{131}$I-L19SIP in three lymphoma patients confirms the \textit{in vivo} accessibility of this target in man. The induction of a partial remission in two relapsed HL patients shows that therapeutic doses of radioactivity can be selectively delivered by $^{131}$I-L19SIP to Hodgkin lymphoma lesions. Together, these data suggest that the molecular composition of newly formed vessel walls in hematologic malignancies (i) is equivalent to those of solid cancers, and (ii) is accessible for intravenous antibody treatments. Insofar, these data qualify ED-B FN as a very promising target for L19-based cancer therapies (e.g. $^{131}$I-L19SIP) in lymphoma patients. For solid cancer patients (renal cell and pancreatic cancer), the clinical evaluation of similar approaches is ongoing (e.g. L19IL2).$^{51}$

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\textbf{Individual Contributions to Authorship:}

Contributions: S.S. analyzed the tissue sections for ED-B expression; P.A.E. performed the clinical research on the lymphoma patients; A.M. contributed vital new reagents and analytical tools and analysis; P.A.E., M.P., G.M., C.G., and G.P. cared for the patients, designed the clinical research part and collected the $^{131}$I-L19SIP SPECT-CT images; L.G., B.H., L.Z., and D.N. analyzed the data and reviewed the manuscript; H.D. and H.D.M. designed the research, supervised the workflow,
analyzed and interpreted the results, and wrote the paper. H.D. and H.D.M. contributed equally to the study, as did S.S. and P.A.E.

**Conflict-of-interest disclosure:** H.D.M owns few regular stocks since 2/08. H.D. received a research grant from BayerSchering Pharma, Berlin, Germany.
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Legends to Tables:

Table 1. **ED-B FN expression in hematopoietic malignancies.** Semiquantitative analysis of ED-B FN expression in lymphoid and hematopoietic tumors.

Table 2. **ED-B FN expression on blood vessels in normal lymphatic tissues and lymphatic tissues altered by non-neoplastic disease.** Semiquantitative analysis of ED-B FN expression in normal and inflammatory altered lymphoid tissues. The extent of ED-B expression was classified according to the indicated per cents of ED-B FN-positive vessels.
### Table 1: ED-B FN expression in hematopoietic malignancies

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<td>3/7</td>
</tr>
<tr>
<td>Chronic myeloproliferative disease</td>
<td></td>
</tr>
<tr>
<td>chronic myelogenous leukemia</td>
<td>0/5</td>
</tr>
<tr>
<td>chronic idiopathic myelofibrosis</td>
<td>2/4</td>
</tr>
<tr>
<td>essential thrombocythaemia, polycythaemia vera</td>
<td>0/4</td>
</tr>
<tr>
<td>Acute myeloblastic leukemia</td>
<td>0/7</td>
</tr>
</tbody>
</table>
Table 2: ED-B FN expression on blood vessels in normal lymphatic tissues and lymphatic tissues altered by non-neoplastic disease

<table>
<thead>
<tr>
<th>Entities</th>
<th>Perivascular ED-B FN expression (per cent EB-D+ vessels)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Normal lymphoid and hematopoietic tissues</td>
<td></td>
</tr>
<tr>
<td>normal lymph nodes</td>
<td>0/10</td>
</tr>
<tr>
<td>normal bone marrow biopsies</td>
<td>0/9</td>
</tr>
<tr>
<td>hyperplastic lymphoid tissues</td>
<td></td>
</tr>
<tr>
<td>hyperplastic lymph nodes</td>
<td>5/12</td>
</tr>
<tr>
<td>hyperplastic tonsils</td>
<td>7/13</td>
</tr>
</tbody>
</table>
Legends to Figures:

Figure 1. ED-B FN expression is similarly detectable in cryostat and paraffin-embedded tissues by immunohistology. Cryostat and paraffin-embedded specimens stemmed from the same lymph node biopsy of a patient with follicular lymphoma grade 2. One half of the biopsy was shock-frozen while the other half was paraffin-embedded. Both specimens were stained with the anti-ED-B FN MX1 antibody (magnification of the left pane: 30x; magnification of the right panel: 50x).

Figure 2. Competition of ED-B FN staining by soluble recombinant ED-B FN.

ED-B FN immunostaining was performed on paraffin-embedded serial sections of a clear cell renal cell carcinoma, using L19IL2, with (right) or without (left, positive control) preincubation of L19IL2 with an excess of soluble recombinant ED-B (magnification: 150x).

Figure 3. Double fluorescence staining of CD34-positive endothelia (green label) and ED-B FN (red label) indicates co-localization of both antigens. The co-localization of CD34-positive endothelia and ED-B FN was investigated by double fluorescence confocal microscopy of paraffin-embedded specimens of diffuse large B cell lymphoma, follicular lymphoma (Grad 3a), and classical Hodgkin lymphoma. Co-localization of ED-B FN and CD34-positive endothelia was found as indicated by the yellow label. Nuclei were stained blue (TOTO3). The asterisk in the right panels (classical Hodgkin lymphoma) indicates a large vessel lumen.
Figure 4. **ED-B FN expression is abundant in biopsies of hematologic malignancies.** ED-B FN expression of paraffin-embedded specimens of normal lymph node (A; magnification: 100x, left insert: 20x), and lymph node biopsies of diffuse large B-cell lymphoma (B; 250x, insert 20x); follicular lymphoma grade 2 (C; 20x, insert 100x), and classical Hodgkin lymphoma of mixed cellularity subtype (D; 150x). ED-B FN expression of bone marrow biopsies of normal bone marrow (E; 50x, insert 200x), multiple myeloma (F; 100x), unspecified peripheral T-cell lymphoma (G; 200x), and chronic myelogenous leukemia (H; 200x). All ED-B FN stainings were performed with the MX1 antibody.

Figure 5. **131I-L19SIP uptake in lymphoma lesions in a patient (NHL1) with SLL NHL.**

(A) 18F-FDG PET scans demonstrate intense glucose metabolism in multiple enlarged lymph nodes, particularly in the left latero-cervical region. Coronal images are shown on the left and transaxial images of the cervical regions are displayed at the right side panel.

(B) The same patient received an intravenous infusion of 185 MBq and, subsequently, 5.55 GBq of 131I-L19SIP. Transaxial, coronal, and sagittal SPECT-CT images of the cervical regions (1st, 2nd and 3rd row, respectively) were acquired 8 days after the dose of 5.55 GBq. Left column shows scintigraphic images, central column CT images and right column CT-scintigraphy fused images.

Figure 6. **18F-FDG PET scans and 131I-L19SIP SPECT-CT images from an advanced Hodgkin lymphoma patient (HL1)**. 18F-FDG PET scans show intense...
glucose metabolism in multiple enlarged mediastinal lymph-nodes, intrapulmonary lesions (left-most column, first four images; intrapulmonary lesion marked), as well as in lumbo-aortic lymph-nodes (left-most image in lowest row). The same patient received intravenous injections of 185 MBq and, subsequently, 5.55 GBq of $^{131}$I-L19-SIP. SPECT-CT coronal (upper right panel) and transaxial images of the thorax (rows 2-4) as well as the upper-abdomen (lowest row), are shown, demonstrating selective uptake of $^{131}$I-L19SIP into the $^{18}$F-FDG-labeled lymphomatous lesions.

**Figure 7. Objective partial remission in another advanced Hodgkin lymphoma patient (HL2) induced by $^{131}$I-L19SIP radioimmunotherapy**

(A) $^{18}$F-FDG PET scans show intense glucose metabolism in multiple enlarged lymph nodes, particularly in right and left axillary and supraclavicular nodes, in paratracheal, subcarinal, peritoneal and iliacal nodes as well as in lymphoma lesions located in the basal lobes of both lungs (left-most image). One (center) and two months (right-most image) after application of therapeutic $^{131}$I-L19SIP (3.7 GBq), the number and size of active lymphoma lesions decreased substantially.

(B) $^{18}$F-FDG PET scans and $^{131}$I-L19SIP SPECT-CT images from the same patient. Transaxial SPECT-CT images (upper row) show selective $^{131}$I-L19SIP uptake to a pulmonary lymphoma lesion still detectable 12 days after injecting a therapeutic dose (3.7 GBq). Transaxial $^{18}$F-FDG PET scans captured prior to (2nd row), 1 month (3rd row) and 2 months (lower-most row) after application of therapeutic $^{131}$I-L19SIP, show a significant shrinkage in size and a complete disappearance of metabolic activity in the initially large pulmonary lymphoma lesion, indicative of lymphoma response to $^{131}$I-L19SIP radioimmunotherapy.
Fig. 1

cryostat section

paraffin-embedded section
<table>
<thead>
<tr>
<th>Diffuse large B cell lymphoma</th>
<th>Follicular lymphoma grade 3A</th>
<th>Hodgkin lymphoma nodular sclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD34</strong></td>
<td></td>
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<tr>
<td>![CD34 image]</td>
<td>![CD34 image]</td>
<td>![CD34 image]</td>
</tr>
<tr>
<td><strong>ED-B FN</strong></td>
<td></td>
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<td>![ED-B FN image]</td>
<td>![ED-B FN image]</td>
<td>![ED-B FN image]</td>
</tr>
<tr>
<td><strong>Overlay</strong></td>
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<td>![Overlay image]</td>
<td>![Overlay image]</td>
<td>![Overlay image]</td>
</tr>
</tbody>
</table>

Fig. 3
Fig. 4

Lymph node

A: normal
B: diffuse large B-cell lymphoma
C: follicular lymphoma
D: Hodgkin lymphoma, mixed cellularity

Bone marrow

E: plasmacytoma
F: peripheral T-cell lymphoma, unspecified
G: chronic myelogenous leukaemia
Figure 5 A:
Figure 5 B:
Fig 7A:
Fig 7 B:
Expression of the oncofetal ED-B containing fibronectin isoform in hematologic tumors enables ED-B targeted $^{131}$I-L19SIP radioimmunotherapy in Hodgkin lymphoma patients

Stefanie Sauer, Paola A. Erba, Mario Petrini, Andreas Menrad, Leonardo Giovannoni, Chiara Grana, Burkhard Hirsch, Luciano Zardi, Giovanni Paganelli, Giuliano Mariani, Dario Neri, Horst Durkop and Hans D. Menssen

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