INTEGRIN ALPHA 10, CD44, PTEN, CADHERIN-11 AND LACTOFERRIN EXPRESSIONS ARE POTENTIAL BIOMARKERS FOR SELECTING PATIENTS IN NEED OF CENTRAL NERVOUS SYSTEM PROPHYLAXIS IN DIFFUSE LARGE B-CELL LYMPHOMA

Siri A. Lemma1,2,*, Milla Kuusisto1,2, Kirsi-Maria Haapasaa1,2,3, Raija Sormunen3,4, Tuula Lehtinen5,6, Tuula Klaavuniemi5,6, Mine Eray7, Esa Jantunen8, Ylermi Soini9,10, Kaija Vasala6, Jan Böhm11, Niina Salokorpi12, Peeter Karihtala1,2, Jussi Vuoristo3,4, Taina Turpeenniemi-Hujanen1,2 and Outi Kuittinen1,2

1Department of Oncology and Radiotherapy, Medical Research Center Oulu, Oulu University Hospital, University of Oulu, Kajaanintie 50, 90220 Oulu, Finland, 2Department of Oncology, Institute of Diagnostics, Medical Research Center Oulu, Oulu University Hospital, Kajaanintie 50, 90220 Oulu, Finland, 3Department of Pathology, Institute of Diagnostics, Medical Research Center Oulu, Oulu University Hospital, Kajaanintie 50, 90220 Oulu, Finland, 4Biocenter Oulu, University of Oulu, Kajaanintie 50, 90220 Oulu, Finland, 5Department of Oncology and Radiotherapy, Tampere University Hospital, Teiskontie 35, 33521 Tampere, Finland, 6Department of Oncology and Radiotherapy, Central Finland Central Hospital, Keskussairaalanlahti 19, 40620 Jyväskylä, Finland, 7Department of Pathology, FIMLAB, Tampere University Hospital, Teiskontie 35, 33521 Tampere, Finland, 8Department of Medicine, Kuopio University Hospital, Puijonlaaksontie 2, 70210 Kuopio, Finland, 9Department of Clinical Pathology and Forensic Medicine, Cancer Center of Eastern Finland, University of Eastern Finland, Puijonlaaksontie 2, 70210 Kuopio, Finland, 10Department of Oncology, Kuopio University Hospital, Puijonlaaksontie 2, 70210 Kuopio, Finland, 11Department of Pathology, Central Finland Central Hospital, Keskussairaalanlahti 19, 40620 Jyväskylä, Finland, 12Department of Neurosurgery, Medical Research Center Oulu, Oulu University Hospital, University of Oulu, Kajaanintie 50, 90220 Oulu, Finland and 13Department of Otorhinolaryngology, Biocenter Oulu, University of Oulu, Kajaanintie 50, 90220 Oulu, Finland

*To whom correspondence should be addressed. Tel: +358(0)50 4130022; Fax: +358(0)8 3155423; Email: siri.lemma@student.oulu.fi

Abstract

Central nervous system (CNS) relapse is a devastating complication that occurs in about 5% of diffuse large B-cell lymphoma (DLBCL) patients. Currently, there are no predictive biological markers. We wanted to study potential biomarkers of CNS tropism that play a role in adhesion, migration and/or in the regulation of inflammatory responses. The expression levels of ITGA10, CD44, PTEN, cadherin-11, CDH12, N-cadherin, P-cadherin, lactoferrin and E-cadherin were studied with IHC and IEM. GEP was performed to see whether found expressional changes are regulated at DNA/RNA level. IEM included two PCNSL, one sCNSL, one sDLBCL and one reactive lymph node samples. GEP was performed on two DLBCL samples, one with and one without CNS relapse. CNS disease was associated with enhanced expression of cytoplasmic and membranous ITGA10 and nuclear PTEN (P < 0.0005, P = 0.002, P = 0.024, respectively). sCNSL presented decreased membranous CD44 and nuclear and cytoplasmic cadherin-11 expressions (P = 0.001, P = 0.006, P = 0.048, respectively). In PCNSL lactoferrin expression was upregulated (P < 0.0005). IEM results were mainly supportive of the IHC results. In GEP CD44, cadherin-11, lactoferrin and E-cadherin were under-expressed in CNS disease. Our results are in line with previous studies, where gene expressions in extracellular matrix and adhesion-related pathways are altered in CNS lymphoma. This study gives new information on the DLBCL CNS tropism. If further verified, these markers might become useful in predicting CNS relapses.

Received: January 18, 2017; Revised: March 15, 2017; Accepted: June 21, 2017

© The Author 2017. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma subtype, representing about 30% of all lymphomas. In addition to systemic disease, it can also manifest in the central nervous system (CNS) in the form of secondary CNS involvement of systemic DLBCL or as primary CNS lymphoma (PCNSL). The latter is a rare disease, representing 1–2% of all non-Hodgkin lymphomas (1).

Of systemic DLBCL patients, around 5% will have disease involvement in the CNS either during diagnosis, or more commonly, a few months afterwards (2,3). The inclusion of rituximab in the treatment has increased the effective control of the systemic DLBCL, but the data considering its effect on the risk of a CNS relapse is contradictory. Before the rituximab era, CNS involvement was usually seen simultaneously with refractory, widespread systemic disease (4,5). This paradigm has changed and currently the emergence of solitary CNS relapses has become an increasing problem. Patients with a CNS relapse have a dismal prognosis. High-dose methotrexate and cytara-bine included in the primary treatment of systemic DLBCL seem to reduce the risk of a CNS relapse. However it is associated with considerable toxicity and the identification of patients at high risk of CNS relapse still remains problematic today (6,7).

Currently the patient selection for CNS prophylaxis is carried out by clinical risk assessment based on the following clinical factors: high International Prognostic Index (IPI) score, advanced stage, elevated lactate dehydrogenase, age > 60 years and involvement of extranodal sites (such as paraspinal or paranasal location, testes or breast). At best, this clinical evaluation is able to identify a patient population with a 20% risk of CNS disease, thus the number needed to treat to prevent one relapse is high. There is also a small group of patients who will later develop CNS disease and fail to be detected by this clinical risk score. There is an unmet clinical need for biological markers that could be used for predicting CNS recurrence (8).

Based on our previous study and literature, we hypothesized that CXCR4/CXCL12 signalling mediates the CNS homing of lymphoma cells and provokes an epithelial–mesenchymal transition (EMT)-like process (9). CXCR4/CXCL12 signalling has been linked to the expression and/or activity of integrins, CD44, PTEN, cadherin-11, N-cadherin and E-cadherin (10–14). Integrin alpha 10 (ITGA10), CD44, PTEN, cadherin-11, cadherin-12 (CDH12), N-cadherin and E-cadherin have also been linked to EMT and associate with the expression of EMT transcription factors (15–21). Lactoferrin levels have been reported to be elevated in the cerebrospinal fluid of patients with CNS involvement of leukemia and lymphoma (22). In addition lactoferrin was of interest as it has been shown to downregulate the expression of intercellular adhesion molecule-1 (ICAM-1), this way possibly reducing the recruitment of leukocytes to the inflammatory site (23). In this work, we performed a screening of the above-mentioned molecules with an aim of finding possible biomarkers in order to identify patients with a high risk of CNS recurrence. The expression levels of these molecules were studied by means of immunohistochemistry (IHC) and immunoelectron microscopy (IEM). Gene-expression profiling (GEP) was performed to identify whether the expressional changes seen are regulated at gene-expression level.

Materials and methods

Patients and samples

Patients included in the study were diagnosed and treated at the University Hospitals of Oulu, Jyväskylä, Kuopio and Tampere between 1997 and 2013. The median age of patients at diagnosis was 65 (range 27–82). The control group was selected from patients who did not have any signs of CNS disease during diagnosis, therapy or follow-up from the time of diagnosis until the end of the year 2015. Also the control group did not receive CNS prophylaxis. Patient information was collected from the hospital records, blinded from the laboratory results and used as encrypted data. Clinical data was collected from patients with primary systemic disease, including GC/non-GC phenotype, age, lactate dehydrogenase, IPI and the number of extranodal lesions. Permission to use patient samples included in the study was granted by the Finnish National Supervisory Authority for Welfare and Health (6622/05.01.00.06/2010). This study was approved by the Ethics Committee of Oulu University Hospital (4/2010, 23.6.2010).

The study material for IHC consisted of diagnostic biopsy samples from 96 patients with newly diagnosed DLBCL, including 15 patients with PCNSLs, 31 with secondary CNS lymphomas (sCNSLs) and a control group of 50 patients with systemic DLBCLs (sDLBCL) that did not receive CNS prophylaxis. The sCNSL samples were diagnostic lymph node samples from primary diagnosis. IEM included two PCNSL samples, one sCNSL (brain biopsy), one sDLBCL and one reactive lymph node sample. GEP included two diagnostic lymph node fresh frozen tissue samples, one sDLBCL and one sCNSL sample from primary diagnosis.

Immunohistochemistry

Slide preparation was performed as described earlier except that before deparaffinization the slides were incubated overnight at +37°C (24). The antigen retrieval and the primary antibodies and their concentrations are presented in Supplementary Table 1, available at Carcinogenesis Online. Sample preparation continued as described in our previous study (9). To determine the germinal centre (GC) phenotype of each lymphoma sample, Hans’ algorithm was used with the same antibodies as in our previous study (9,25).

Immunoelectron microscopy

Tissue pieces were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with 2.5% sucrose for 6 h at RT then immersed and stored in 2.3 M sucrose in PBS. Tissue pieces were placed on specimen stubs and frozen in liquid nitrogen. Ultrathin sections (70 nm) were cut with a Leica EM UC7 cryo-ultramicrotome. For immunolabelling, ultrathin sections were incubated with Protein A-gold conjugate (10 nm). Rabbit anti-mouse IgG (Jackson Immunoresearch Europe Ltd, UK) was used as a secondary antibody. Sections were embedded in methylcellulose and examined in a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, the Netherlands) equipped with a Quemesa digital camera (EMSIS GmbH, Münster, Germany). The antibodies used were the same as for IHC.

Gene-expression profiling

The RNA isolation was performed using the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer’s instructions. A GeneChip protocol
was used and the experimental procedures were performed in accordance with the Affymetrix GeneChip Expression Analysis Technical Manual. Using 5 µg of total RNA as template, a double-stranded DNA was synthesized by means of the One-cycle cDNA synthesis kit (Affymetrix, USA) and T7-(dT)24 primer. The GeneChip Sample Cleanup Module (Affymetrix, USA) was used to purify the DNA. By using an in vitro transcription (IVT) labelling kit (Affymetrix, USA) according to the manufacturer’s instructions, the IVT was performed to produce biotin labelled complementary RNA. Biotinylated complementary RNA was cleaned using the GeneChip Sample Cleanup Module (Qiagen), fragmented to 35–200 nucleotides, and hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays that contain ~55 000 human transcripts. After washing, the array was stained with streptavidin-phycocerythrin (Molecular Probes). The staining signal was amplified by biotinylated anti-streptavidin (Vector Laboratories) followed by second staining with streptavidin-phycocerythrin, and then scanned on the GeneChip Scanner 3000.

Analysis

In the analysis of IHC staining, the location of positivity in the malignant B-cells was determined separately in the nucleus, cytoplasm and/ or cell membrane. Then the percentage of positive cells was estimated separately for each location, from 0 to 100% with 5% accuracy. Micrographs from IHC staining patterns were obtained using an Olympus DP11 digital microscope camera (Olympus, Center Valley) and an Olympus BX41 microscope. To import micrographs an HP Photo and Imaging software package ( Hewlett-Packard Company, Palo Alto) was used.

The expression data gained from GEP was analysed using dChip software [26].

For statistical analysis, IBM SPSS Statistics for Windows 20.0 software (IBM Corp. Released 2011. Armonk, NY) was used. In order to analyse the associations between diagnosis (sDLBCL versus sCNSL) and the IHC protein expressions, the cut-off points for the latter were chosen using the receiver operating characteristic curve. The area under the curve (AUC) shows sensitivity versus 1-specificity. Only values greater than 0.5 were taken into account, values closer to 1.0 possessing a better discriminatory power. Due to the small study material, AUCs were relatively low. Cut-off points were chosen by estimating a good balance between sensitivity and specificity. AUCs and cut-off points for proteins with statistically significant associations with diagnosis are presented in the results section.

Immunohistochemical expression in lymphoma samples

E-cadherin did not show any immunoreactivity. For ITGA10, CD44, PTEN, cadherin-11, N-cadherin, P-cadherin and lactoferrin, cytoplasmic immunostaining was seen with all the antibodies used. Nuclear immunostaining was seen with all the antibodies used; except for CD44 there was no nuclear immunoreactivity. Membranous immunostaining was seen for ITGA10, CD44, PTEN and CDH12. The staining percentages are presented in Supplementary Table 2, available at Carcinogenesis Online.

Figure 1 presents IHC staining patterns.

Immunoelectron microscopy expression in lymphoma samples

In PCNSL samples, the strongest expressions were seen for CD44, PTEN, cadherin-11 and N-cadherin. ITGA10 membranous expression was substantial in sCNSL. CD44 membranous and CDH12 nuclear expressions were substantial in sDLBCL. Strong nuclear expression of CDH12 was also detected in lymph node hyperplasia.

The proteins studied were also detected outside of lymphoma cells. Particularly lactoferrin was seen outside of lymphoma cells in all samples, most strongly in PCNSL. In sDLBCL and lymph node hyperplasia CDH12 was similarly expressed in normal lymphocytes as in lymphoma cells. CDH12 staining was also seen outside of lymphoma cells in PCNSL and sCNSL samples. In PCNSL, CD44 seemed to be expressed on the plasma membranes of the cells that came into contact with lymphoma cells with cell protrusions. PTEN was similarly expressed in the membranes of these cells. The precise cell type could not be determined. Possibly these were antigen-presenting microglial cells. In PCNSL samples ITGA10 was also detected in cells that were not lymphoma cells. In addition it was seen in myelinated and non-myelinated neurons. Low expression of N-cadherin was detected in PCNSL in cells surrounding lymphoma cells. In sCNSL, the expression in surrounding cells was higher. High expression of cadherin-11 was seen in the plasma membranes of cells surrounding the lymphoma cells in the sCNSL sample. These cells could possibly represent antigen-presenting microglial cells. Supplementary Figure 1, available at Carcinogenesis Online, presents electronmicroscopic expression in these cells.

Supplementary Table 3, available at Carcinogenesis Online, represents immunoelectron microscopy expression in all samples. Figure 2 presents electronmicroscopic expression.

Differences in gene-expression

The molecules studied were found in GEP data but only CD44, cadherin-11, lactoferrin and E-cadherin presented differences in the gene expression levels, being underexpressed in sCNSL compared to sDLBCL (Table 1).

Associations with central nervous system involvement

There were statistically significant differences between the expression of ITGA10, CD44, PTEN, cadherin-11 and lactoferrin stainings in different diagnostic groups. The differences gained from the Kruskal–Wallis test for continuous variables with all three diagnoses are presented here, mean and median values representing the staining percentages. Table 2 represents the differences gained from the Mann–Whitney U test comparing two diagnoses; sCNSL versus PCNSL and Table 3 the results of the chi-square test with chosen cut-off points (Tables 2 and 3).

ITGA10 cytoplasmic expression was lowest in sDLBCL (mean rank = 22.62, median = 0.00), higher in sCNSL (mean rank = 33.20, median = 50.00) and highest in PCNSL (mean rank = 46.85, median = 100.00) (P < 0.0005) (Figure 3A).
ITGA 10 membranous expression was lowest in sDLBCL (mean rank = 25.40, median 0.00), higher in PCNSL (mean rank = 26.08, median = 0.00) and highest in sCNSL (mean rank = 40.25, median = 100) (P = 0.002) (Figure 3B).

CD44 membranous expression was lowest in sCNSL (mean rank = 34.82, median = 50.00), higher in sDLBCL (mean rank = 46.22, median = 70.00) and highest in PCNSL (mean rank = 67.54, median = 100.00) (P = 0.001) (Figure 3D).

PTEN nuclear expression was lowest in sDLBCL (mean rank = 25.72, median = 0.00), higher in PCNSL (mean rank = 36.04, median = 0.00) and highest in sCNSL (mean rank = 38.37, median = 5.00) (P = 0.024) (Figure 3C).

Cadherin-11 nuclear expression was lowest in sCNSL (mean rank = 34.02, median = 30.00), higher in sDLBCL (mean rank = 51.80, median = 100.00) and highest in PCNSL (mean rank = 52.23, median = 100.00) (P = 0.006) (Figure 3E).

Cadherin-11 cytoplasmic expression was lowest in sCNSL (mean rank = 28.50, median = 0.00), higher in sDLBCL (mean rank = 41.52, median = 0.00) and highest in PCNSL (mean rank = 52.85, median = 100.00) (P < 0.0005) (Figure 3F). When comparing the expression between just two groups, sCNSL and sDLBCL, the result was non-significant.

Lactoferrin cytoplasmic expression was lowest in sDLBCL (mean rank = 28.50, median = 0.00), higher in sCNSL (mean rank = 30.18, median = 0.00) and highest in PCNSL (mean rank = 52.85, median = 100.00) (P < 0.0005) (Figure 3F). When comparing the expression between just two groups, sCNSL and sDLBCL, the result was non-significant.

Associations with clinical parameters
CDH12 nuclear and ITGA10 membranous expressions had a positive association with the number of extranodal lesions.
(P = 0.026 and P = 0.006, respectively). CD44 membranous expression had an inverse association with the number of extranodal lesions (P = 0.037). CD12 nuclear, lactoferrin cytoplasmic, ITGA10 cytoplasmic and ITGA membranous expressions had a positive association with the IPI score (P = 0.010, P = 0.029, P = 0.022 and P = 0.007, respectively). PTEN nuclear expression had a positive association with lactate dehydrogenase (P = 0.002). Higher CD44 membranous expression was associated with the non-GC phenotype (P = 0.049). CD44 membranous expression also had a positive association with age over 60 (P = 0.034).

Discussion

In this work, we studied the biology of DLBCL's CNS tropism with IHC, IEM and GEP with the aim of finding biological markers that would define DLBCL patients at high risk of CNS recurrence. We found that elevated levels of ITGA10 and PTEN were associated with CNS tropism, while CD44 and cadherin-11 expressions seemed to be protective of secondary CNS disease. Interestingly though, CD44, cadherin-11 and lactoferrin expressions were highest in PCNSL samples.

CNS recurrence occurs in about 5% of systemic DLBCL patients (2,3). It is a difficult complication with a very poor prognosis. Abramson et al. conducted a retrospective study with results that were later confirmed in a prospective CRY study by Nordic Lymphoma Group, showing that CNS relapse risk can be reduced with high-dose methotrexate containing prophylactic chemotherapy (6,27). This conclusion was further supported by a retrospective study by Ferreri et al (28). However, because high-dose methotrexate is a resource-demanding therapy with considerable toxicity, it should be limited to patients with a high risk of CNS recurrence. Currently identification of these high-risk patients is performed based on clinical factors, which unfortunately have a poor specificity (8). Thus there is a need to define biological markers with a higher specificity for separating patients in need of CNS prophylactic treatment. Currently there are suggestions that double-hit genotype, non-GC phenotype, as well as CD5 positivity may have some prognostic relevance in this matter (29–31).

Understanding the pathophysiology of CNS lymphomas has been a challenge for years. An attractive hypothesis is that these malignant cells originate from extracranial sites but end up homing into the CNS due to highly selective CNS tropism. Results from recent studies have supported this hypothesis (32). In studies with GEP, differences in transcription of several proteins have been observed when comparing normal lymphatic tissue and nodal or extranodal DLBCL with PCNSL. Our results are in line with these previous findings because it seems that adhesion-, migration- and ECM-related molecules play a pivotal role in CNS involvement of DLBCL (33,34).

In our previous study of CNS lymphomas, we assumed that CXCR4/CXCL12 and CXCR5/CXCL13 axis play an important role...
in CNS tropism of DLBCL (9). Concordantly Rubenstein et al. (35) have shown that CXCL12 and CXCL13 act as mediators of CNS lymphoma cell chemotaxis. In line with these findings, CXCR4/CXCL12 signalling has been shown to induce EMT or an EMT-like process that enables cell migration towards the expression of chemokine ligands and invasive abilities (36–39). CXCR4/CXCL12 signalling has also been shown to induce integrin and CD44 activation and also CD44 localization to the leading edge during migration in human CD34+ stem/progenitor cells. These events together with integrin expression facilitate cell attachment to the extracellular space as well as the invasion and mobilization of cells (10,12). CD44 affects human haematopoietic progenitor cell trafficking to the BM via interactions with hyaluronic acid (10). It is noteworthy that hyaluronic acid is also the main component of brain extracellular matrix (40). He et al. (41) found that perivascular tumour cells in PCNSL showed a strong CD44 expression explaining the perivascular staining pattern. Our results are in line with this data describing the highest CD44 expression in PCNSLs.

Our findings of integrin expression were in line with the hypothesis of cell migration, as ITGA10 showed a strong CNS tropism favouring function (42,43). The expression of membranous ITGA10 had a positive association with two of the known clinical risk factors for CNS tropism: the number of extranodal lesions and higher IPI score.

PTEN is generally regarded as a tumour suppressor gene and in cancers its loss is associated with increased proliferation rate (44). Loss of PTEN expression has also been shown to occur in some B-cell lymphomas (45,46). We found its nuclear location to be associated with enhanced CNS tropism. In a work by Goh et al. nuclear trafficking of PTEN after an injury to the neurons was shown to lead to survival and thus to protect these cells from cell death, probably due to the depletion of PTEN from its functional place, the cytoplasm and cell membrane (47). Loss of PTEN has been shown to result in increased CXCR4 and CXCL12 expression and invasion in prostate cancer cells (11). If nuclear localization is able to inhibit the cell cycle regulatory functions of PTEN, it could be that it can also lead to increased CXCR4 expression as seen when PTEN expression is lost, and enhance the CNS tropism in this way. In our study nuclear PTEN was the only protein associated with increased lactate dehydrogenase levels.

Cadherin-11 cytoplasmic and nuclear expressions were highest in PCNSL, yet the latter was associated inversely with sCNSL. In contrast to this, in epithelial cancers, cadherin-11 is often associated with mesenchymal features gained through EMT (17).

In our results, lactoferrin expression was highest in PCNSL and second highest in sCNSL when studied with IHC and IEM. When comparing sDLBCL and sCNSL, no statistically significant differences were seen. In GEP, higher expression level was seen in sDLBCL samples but GEP did not include PCNSL samples that had the most abundant expression in IHC and IEM. Lactoferrin has been shown to be produced in the CNS by activated microglial cells and its expression has been associated with neurological disorders affecting the CNS, such as Alzheimer’s and Parkinson’s diseases. It seems that lactoferrin expression is upregulated as a consequence of inflammatory conditions and under oxidative stress (48). Most likely this could also be the case in PCNSL.

Taken together, our findings support the hypothesis that CXCR4 signalling might participate in the initiation of lymphoma cells homing into the CNS. This again may enable the morphological and expression changes seen in this study. In a work by Ricciardi et al. (49) mesenchymal stromal cell-like features gained through EMT conferred cancer cells with immune-modulatory properties which again possibly attenuate cancer cell immune escape and progression.

When the IHC, IEM and GEP results were compared they were mostly in concordance with each other. All of the molecules studied were found in GEP and four presented with gene-expression changes. The expression changes were similar to IHC and IEM results, except that E-cadherin was not seen to be expressed at a protein level. Lactoferrin expression also presented with minor differences. The small number of GEP samples used in this study limits the reliability of the results. However, concordant results seen in larger IHC and IEM materials indicate that these findings seem to present a relevant event

| Protein, location | Mean rank, sCNSL | Median sCNSL | Mean rank, PCNSL | Median PCNSL | P value |
|-------------------|-----------------|--------------|-----------------|--------------|---------|
| CD44, M           | 18.12           | 50           | 30.96           | 100          | 0.002   |
| Lactoferrin, C    | 18.00           | 0            | 33.23           | 100          | <0.001  |
| P cadherin, N     | 19.84           | 5            | 28.85           | 20           | 0.030   |
| ITGA10, C         | 18.70           | 50           | 29.62           | 100          | 0.006   |
| ITGA10, M         | 24.73           | 100          | 15.69           | 0            | 0.014   |

C, cytoplasm; M, membrane; N, nucleus.

| Protein, location | AUC   | CI            | Cut-off | Association | P value |
|-------------------|-------|---------------|---------|-------------|---------|
| CD44, M           | 0.668 | 0.514–0.821   | 70.00   | ↓           | 0.015   |
| PTEN, N           | 0.694 | 0.547–0.840   | 0.00    | ↑           | 0.011   |
| ITGA10, N         | 0.657 | 0.500–0.815   | 10.00   | ↓           | 0.044   |
| ITGA10, C         | 0.679 | 0.519–0.838   | 10.00   | ↑           | 0.002   |
| ITGA10, M         | 0.745 | 0.606–0.884   | 0.00    | ↑           | 0.007   |
| Cadherin-11, N    | 0.673 | 0.514–0.831   | 70.00   | ↓           | <0.001  |

Diagnoses studied, sDLBCL and sCNSL. AUC, area under curve; CI, confidence interval; C, cytoplasm; M, membrane; N, nucleus. Cut-off signifies the chosen cut-off point. ↓ = inverse association and ↑ = positive association with CNS involvement.
in DLBCL CNS tropism. The few discrepancies may be explained by the methods used; IHC measures the expression of the studied proteins within a sample tissue whereas GEP measures the expression of specific messenger RNAs in the sample. IHC and IEM results were also mostly in concordance with each other, with only a couple of differences. IHC results represent

Figure 2. Associations of protein immunohistochemical expressions and diagnoses. A Kruskal–Wallis test was used to determine the associations between protein expression and different diagnoses for (A) ITGA10 cytoplasmic expression \( (P < 0.0005) \), (B) ITGA10 membranous expression \( (P = 0.002) \), (C) PTEN nuclear expression \( (P = 0.024) \), (D) CD44 membranous expression \( (P = 0.001) \), (E) cadherin-11 nuclear expression \( (P = 0.006) \) and (F) lactoferrin cytoplasmic expression \( (P < 0.0005) \). The boxes represent the interquartile range, the whiskers represent the 10th and 90th percentiles and the highlighted lines across the boxes indicate the median values. Circles represent extremes and asterisks represent outliers.
a collection of multiple samples and a large number of cells are evaluated. IEM expression on the other hand represents one or two samples, and from those a single cell per view. This might cause a selection bias if the expression in this specific sample is below average. Using light microscopy, it is harder to tell cytoplasmic and membranous staining apart than it is in electron microscopy. This might represent another bias to the results.

Our findings imply that some biological events are common in both primary and secondary CNS lymphomas. Yet the fact that PCNSL samples are brain biopsies and sCNSL samples from lymph nodes from a time of the primary diagnosis, the differences in the microenvironment might also lead to the seemingly different results observed between these two groups, and this impact could not be excluded in this study. In addition, PCNSL and sCNSL are two different entities and there might also be differences due to this.

In this work, we have shown that lymphoma CNS tropism is linked to enhanced expression of integrins (ITGA10 in this study) and PTEN, when again sCNSL is linked to decreased CD44 and cadherin-11 expressions. In PCNSL, lactoferrin seems to be upregulated. This data is in line with current knowledge of morphological and protein expressional changes occurring during cell migration and invasion, and with the current GEP data of CNS lymphomas where differences in the expression of adhesion and ECM-related genes have been seen (33,34). These EMT-like changes seen in our study might also attenuate lymphoma cell immune escape (49). To our knowledge, this is the first study exploring the role of ITGA10, CD44, PTEN, cadherin-11, CDH12, N-cadherin, P-cadherin, lactoferrin and E-cadherin in DLBCLs and especially in CNS lymphomas. The present study was a retrospective one and the size was too small to calculate the positive and negative prognostic values of these markers as well as to perform multivaritate analyses comparing independent values of these markers with clinical factors. Currently the most powerful markers for prediction of CNS tropism seem to be non-GC phenotype, double-hit genotype and CDS5 positivity (28–31). From our candidate markers, only CD44 expression was associated with non-GC phenotype, implying that the rest of the proteins studied may give new prognostic value for CNS relapse prediction in both GC and non-GC phenotypes. Unfortunately translocation data was not available from our cases. However the findings are able to support a hypothesis that cytoplasmic and membranous ITGA10, nuclear PTEN and membranous CD44 expressions should be studied as promising candidates to be used as biological markers for screening of systemic DLBCL patients at elevated CNS relapse risk. These results are highly preliminary and need to be validated in a larger prospective trial.

Supplementary material
Supplementary material is available at Carcinogenesis online.

Funding
This study was supported by grants from the Cancer Society of Northern Finland, Orion-Farmos Research Foundation, the Finnish Medical Society Duodecim, Finnish Cultural Foundation and the Väisänen Fund in Terttu Foundation.

Acknowledgements
The authors would like to thank Anne Bisi for her skilful technical assistance in the preparation and immunohistochemical staining of tissue samples in this study, and the staff of Biocenter Oulu EM laboratory for the preparation of IEM samples.

Conflict of Interest Statement: None declared.

References
1. Hochberg, F.H. et al. (2007) Primary CNS lymphoma. Nat. Clin. Pract. Neurol., 3, 24–35.
2. Bos, G.M. et al. (1998) For which patients with aggressive non-Hodgkin’s lymphoma is prophylaxis for central nervous system disease mandatory? Dutch HOVON Group. Ann. Oncol., 9, 191–194.
3. van Besien, K. et al. (1998) Risk factors, treatment, and outcome of central nervous system recurrence in adults with intermediate-grade and immunoblastic lymphoma. Blood, 91, 1178–1184.
4. Tai, W.M. et al. (2011) Central nervous system (CNS) relapse in diffuse large B cell lymphoma (DLBCL): pre- and post-rituximab. Ann. Hematol., 90, 809–818.
5. Yamamoto, W. et al. (2010) Central nervous system involvement in diffuse large B-cell lymphoma. Eur. J. Haematol., 85, 6–10.
6. Holte, H. et al. (2013) Dose-densified chemoimmunotherapy followed by systemic central nervous system prophylaxis for younger high-risk diffuse large B-cell/follicular grade 3 lymphoma patients: results of a phase II Nordic Lymphoma Group study. Ann. Oncol., 24, 1385–1392.
7. Tilly, H. et al.; Groupe d’Etude des Lymphomes de l’Adulte. (2003) Intensive conventional chemotherapy (ACVBP regimen) compared with standard CHOP for poor-prognosis aggressive non-Hodgkin lymphoma. Blood, 102, 4284–4289.
8. Siegal, T. et al. (2012) CNS prophylaxis in diffuse large B-cell lymphoma: if, when, how and for whom? Blood Rev., 26, 97–106.
9. Lemma, S.A. et al. (2016) Similar chemokine receptor profiles in lymphomas with central nervous system involvement - possible biomarkers for patient selection for central nervous system prophylaxis, a retrospective study. Eur. J. Haematol., 96, 492–501.
10. Avigdor, A. et al. (2009) CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. Blood, 103, 2981–2989.
11. Conley-LaComb, M.K. et al. (2013) PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling. Mol. Cancer, 12, 85.
12. Peled, A. et al. (2000) The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34+ cells: role in transendothelial/stromal migration and engraftment of NGD/SCID mice. Blood, 95, 3289–3296.
13. Sobolik, T. et al. (2014) CXCR4 drives the metastatic phenotype in breast cancer through induction of CXCR2 and activation of MEK and PI3K pathways. Mol. Biol. Cell, 25, 566–582.
14. Zhu, Y. et al. (2013) The effect of CXCR4 silencing on epithelial-mesenchymal transition related genes in glioma U87 cells. Anat. Rec. (Hoboken), 296, 1850–1856.
15. Ma, J. et al. (2016) Cadherin-12 enhances proliferation in colorectal cancer cells and increases progression by promoting EMT. Tumour Biol., 37, 9077–9088.
16. Nieto, M.A. (2011) The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu. Rev. Cell Dev. Biol., 27, 347–376.
17. Nam, E.H. et al. (2014) ZEB2-Sp1 cooperation induces invasion by upregulating cadherin-11 and integrin α5 expression. Carcinogenesis, 35, 302–314.
18. Song, M.S. et al. (2012) The functions and regulation of the PTEN tumour suppressor. Nat. Rev. Mol. Cell Biol., 13, 283–296.
19. Vieira, A.F. et al. (2015) P-cadherin and the journey to cancer metastasis. Mol. Cancer, 14, 178.
20. Xu, H. et al. (2015) The role of CD44 in epithelial-mesenchymal transition and cancer development. Onc. Targets. Ther., 8, 3783–3792.
21. Moehmann, L.H. et al. (2014) ERG induces a mesenchymal-like state associated with chemoresistance in leukemia cells. Oncotarget, 5, 351–362.
22. Oberg, G. et al. (1987) Beta 2-microglobulin, lysozyme and lactoferrin in cerebrospinal fluid in patients with lymphoma or leukaemia: relationship to CNS involvement and the effect of prophylactic intrathecal treatment with methotrexate. Br. J. Haematol., 66, 315–322.
23. Kim, C.W. et al. (2012) Human lactoferrin suppresses TNF-α-induced intercellular adhesion molecule-1 expression via competition with NF-κB in endothelial cells. FEBS Lett., 586, 229–234.

24. Pasanen, A.K. et al. (2012) Expression and prognostic evaluation of oxidative stress markers in an immunohistochemical study of B-cell derived lymphomas. Leuk. Lymphoma, 53, 624–631.

25. Hans, C.P. et al. (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood, 103, 275–282.

26. Li, C. et al. (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. USA, 98, 31–36.

27. Abramson, J.S. et al. (2010) Intravenous methotrexate as central nervous system (CNS) prophylaxis is associated with a low risk of CNS recurrence in high-risk patients with diffuse large B-cell lymphoma. Cancer, 116, 4283–4290.

28. Ferreri, A.J. et al. (2015) Risk-tailored CNS prophylaxis in a monoinstitutional series of 200 patients with diffuse large B-cell lymphoma treated in the rituximab era. Br. J. Haematol., 168, 654–662.

29. Jain, P. et al. (2013) Recent advances in de novo CD5+ diffuse large B cell lymphoma. Am. J. Hematol., 88, 798–802.

30. Oki, Y. et al. (2014) Double hit lymphoma: the MD Anderson Cancer Center clinical experience. Br. J. Haematol., 166, 891–901.

31. Savage, K.J. et al. (2016) Impact of dual expression of MYC and BCL2 by immunohistochemistry on the risk of CNS relapse in DLBCL. Blood, 127, 2182–2188.

32. Jiang, L. et al. (2010) Selective central nervous system tropism of primary central nervous system lymphoma. Int. J. Clin. Exp. Pathol., 3, 763–767.

33. Sung, C.O. et al. (2011) Genomic profiling combined with gene expression profiling in primary central nervous system lymphoma. Blood, 117, 1291–1300.

34. Tun, H.W. et al. (2008) Pathway analysis of primary central nervous system lymphoma. Blood, 111, 3200–3210.

35. Rubenstein, J.L. et al. (2012) CXCL13 plus interleukin 10 is highly specific for the diagnosis of CNS lymphoma. Blood, 121, 4740–4748.

36. Li, X. et al. (2012) SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial-mesenchymal transition in vitro through non-canonical activation of Hedgehog pathway. Cancer Lett., 322, 169–176.

37. Roccaro, A.M. et al. (2015) CXCR4 regulates extra-medullary myeloma through epithelial-mesenchymal-transition-like transcriptional activation. Cell Rep., 12, 622–635.

38. Hu, T.H. et al. (2014) SDF-1/CXCR4 promotes epithelial-mesenchymal transition and progression of colorectal cancer by activation of the Wnt/β-catenin signaling pathway. Cancer Lett., 354, 417–426.

39. Yang, P. et al. (2015) SDF-1/CXCR4 signaling up-regulates Survivin to regulate human sacral chondrosarcoma cell cycle and epithelial-mesenchymal transition via ERK and PI3K/AKT pathway. Med. Oncol., 32, 377.

40. Bignami, A. et al. (1993) Hyaluronic acid and hyaluronic acid-binding proteins in brain extracellular matrix. Anat. Embryol. (Berl.), 188, 419–433.

41. He, M. et al. (2013) Prognostic significance of the aggregative perivascular growth pattern of tumor cells in primary central nervous system diffuse large B-cell lymphoma. Neuro. Oncol., 15, 727–734.

42. Hanahan, D. et al. (2000) The hallmarks of cancer. Cell, 100, 57–70.

43. Guo, W. et al. (2004) Integrin signalling during tumour progression. Nat. Rev. Mol. Cell Biol., 5, 816–826.

44. Song, M.S. et al. (2012) The functions and regulation of the PTEN tumour suppressor. Nat. Rev. Mol. Cell Biol., 13, 283–296.

45. Battistella, M. et al. (2015) The high expression of the microRNA 17-92 cluster and its paralogs, and the downregulation of the target gene PTEN, is associated with primary cutaneous B-cell lymphoma progression. J. Invest. Dermatol., 135, 1659–1667.

46. Ma, Y. et al. (2015) Evaluation of AKT phosphorylation and PTEN loss and their correlation with the resistance of rituximab in DLBCL. Int. J. Clin. Exp. Pathol., 8, 14875–14884.

47. Goh, C.P. et al. (2014) Nuclear trafficking of Pten after brain injury leads to neuron survival not death. Exp. Neurol., 252, 37–46.

48. Fillebeen, C. et al. (2001) Lactoferrin is synthesized by activated microglia in the human substantia nigra and its synthesis by the human microglial CHME cell line is upregulated by tumor necrosis factor alpha or 1-methyl-4-phenylpyridinium treatment. Brain Res. Mol. Brain Res., 96, 103–113.

49. Ricciardi, M. et al. (2015) Epithelial-to-mesenchymal transition (EMT) induced by inflammatory priming elicits mesenchymal stromal cell-like immune-modulatory properties in cancer cells. Br. J. Cancer, 112, 1067–1075.