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IDENTIFICATION OF MOLECULAR TARGETS ASSOCIATED WITH TRANSFORMED DIFFUSE LARGE B CELL LYMPHOMA USING HIGHLY PURIFIED TUMOUR CELLS

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**IDENTIFICATION OF MOLECULAR TARGETS ASSOCIATED
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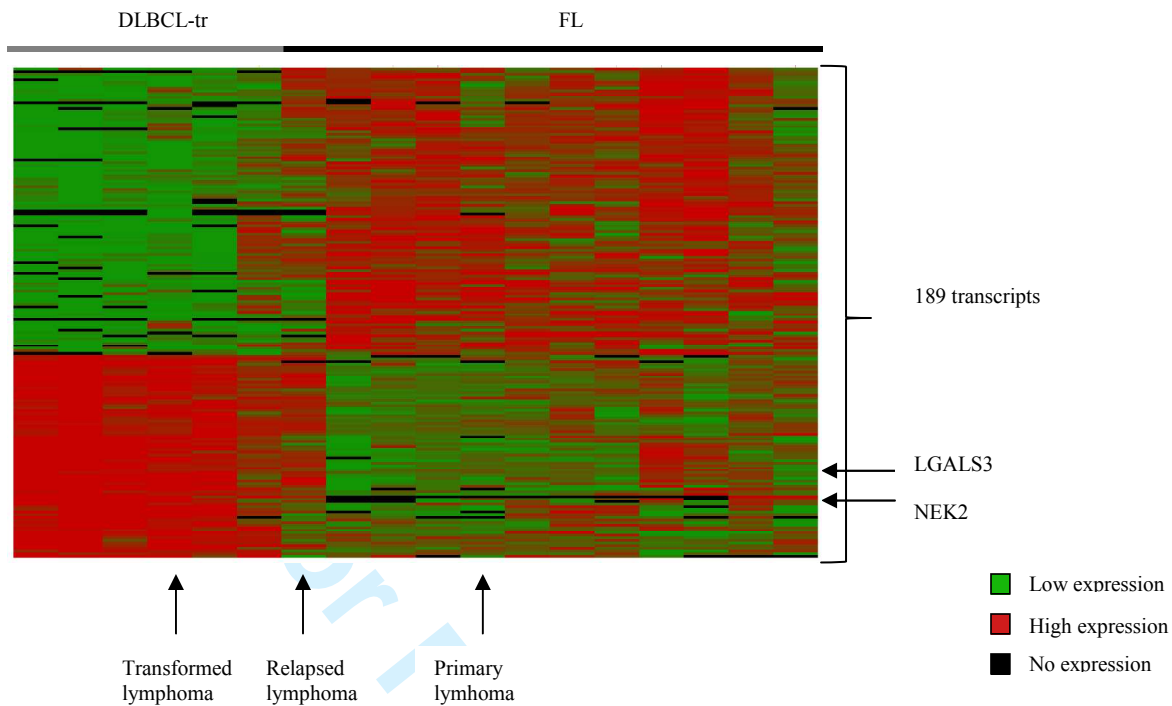


Figure 1

Transformation of FL into DLBCL

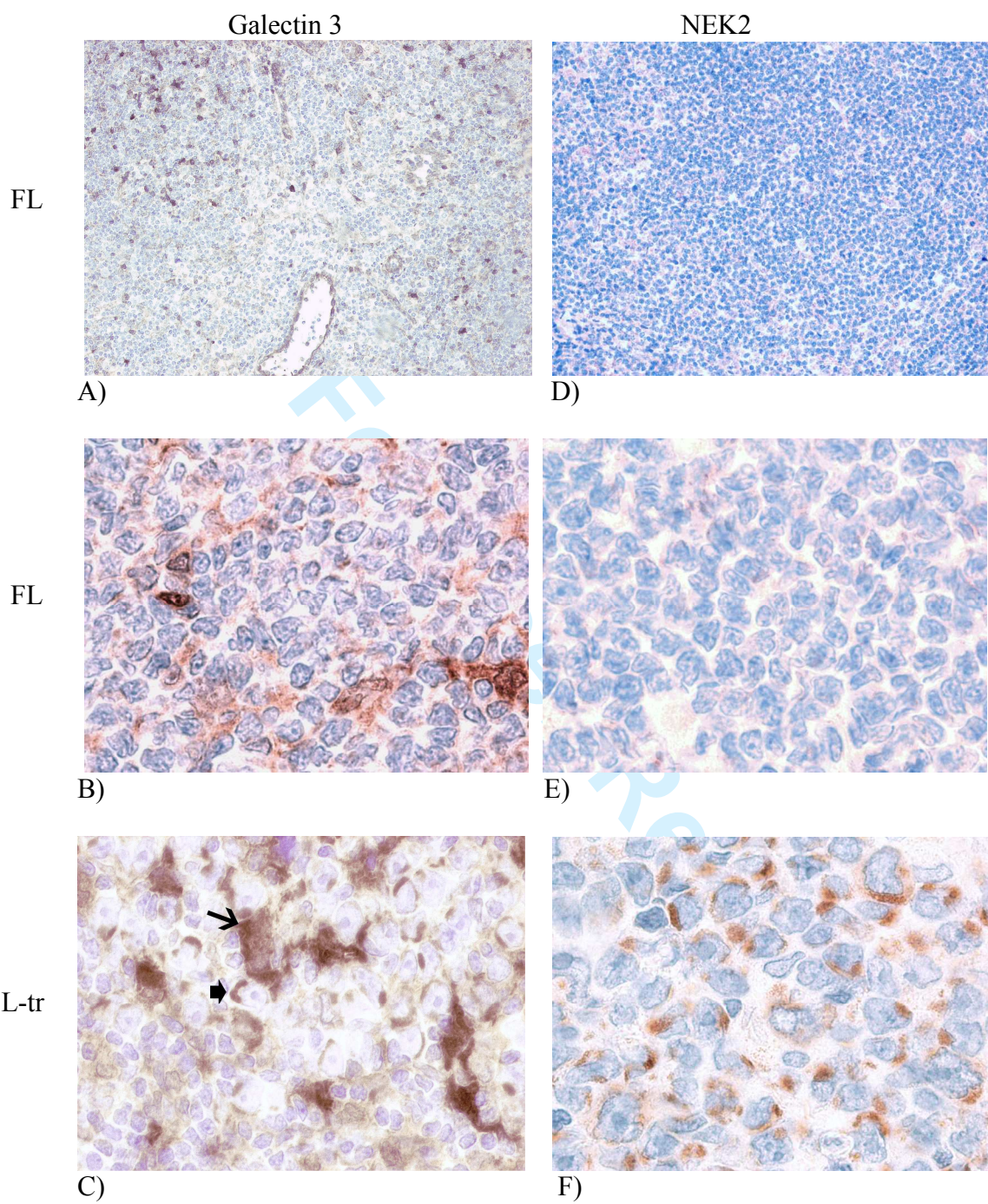


Figure 2

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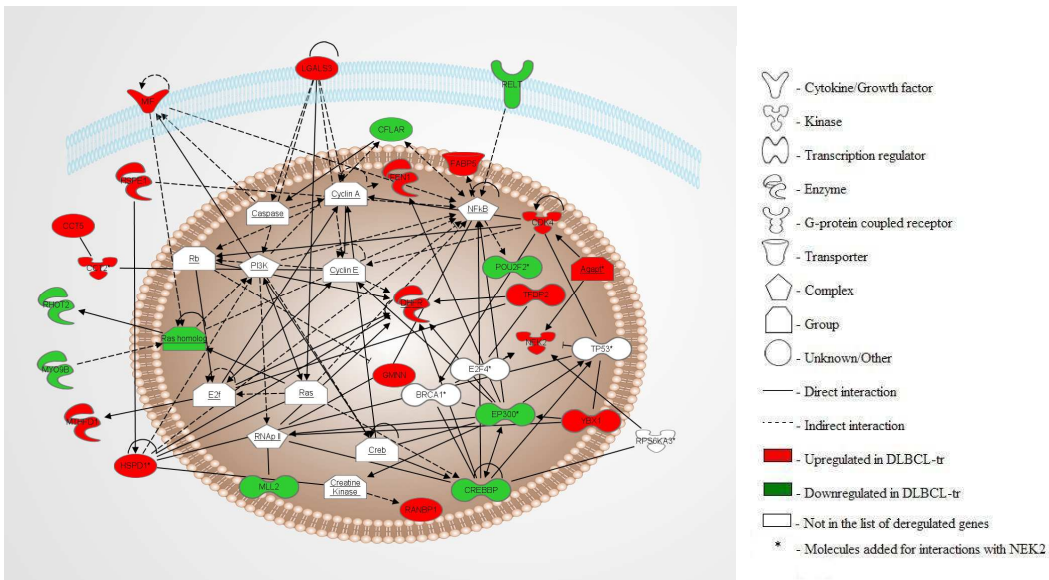


Figure 3

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Transformation of FL into DLBCL

Table 1. Patient material used for mRNA analysis and immunohistochemistry

Non transformed samples	Grade ¹	Stage ² I-II (%)	Stage ² III-IV (%)	Transformed samples	Stage ² I-II (%)	Stage ² III-IV (%)	Time pre transformation (years)		Time post diagnosis without transformation (years)	
							Median	Range	Median	Range
Gene Expression										
Paired lymphoma samples										
mRNA-FL ³ (n=1)	II	NA ⁴		mRNA-DLBCL-tr (n=1)		100	12	-	-	-
mRNA-FL-re ⁵ (n=1)	II	NA ⁴					5	-	-	-
Unpaired lymphoma samples										
mRNA-FL (n=10)	I-II	20	60			-	-	-	10.5	8-21
	-	-	-	mRNA-DLBCL-tr (n=5)		80	1.5	0-3.5	-	-
Immunohistochemistry										
Paired lymphoma samples										
IHC-FL (n=7)	I-II	29	71	IHC- DLBCL-tr (n=7)	29	71	2	1-10	-	-
Unpaired lymphoma samples										
IHC- FL (n = 3)	I-III	33	67		-	-	-	1-2 ⁶	3 ⁶	-
	-	-	-	IHC- DLBCL-tr (n=3)	20	60	0	0-2	-	-
De novo DLBCL										
	-	-	-	IHC-de novo DLBCL (n=104)	25 ⁷	79 ⁸	-	-	-	-

¹ Grade at diagnosis, when available² Stage, when available³ Series of samples from a single patient with an initial primary FL (mRNA-FL), later relapsed FL (mRNA-FL-re) and finally transformed DLBCL (mRNA-DLBCL-tr)⁴ NA - not available⁵ mRNA-FL-re = relapsed FL⁶ One sample never transformed⁷ Stage I⁸ Stage II-IV

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**IDENTIFICATION OF MOLECULAR TARGETS ASSOCIATED WITH TRANSFORMED
DIFFUSE LARGE B CELL LYMPHOMA USING HIGHLY PURIFIED TUMOUR CELLS[#]**

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Running title : **Transformation of FL into DLBCL**

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Keywords: Follicular lymphoma, Diffuse large B cell lymphoma, NEK2, Transformation, Galectin-3, Gene expression, Cell sorting.

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ABSTRACT

Introduction: Follicular lymphoma (FL) frequently transforms into the more aggressive diffuse large B cell lymphoma (DLBCL-tr) but no protein biomarkers have been identified for predictive or early diagnosis. Gene expression analyses have identified genes changing upon transformation but have failed to be reproducible in different studies, reflecting the heterogeneity within the tumor tissue and between tumor samples. **Material and Methods:** Gene expression analyses on Affymetrix Human Genome U133 Plus 2.0 arrays were performed, using flow cytometry sorted tumor cells derived from FL and transformed DLBCL. To identify molecular targets associated with the transformation, subsequent immunohistochemistry (IHC) analyses of the corresponding proteins were performed. **Results:** Using highly purified cells, this study identified 163 genes, which were significantly deregulated during the transformation in a majority of cases. Among the upregulated transcripts, 13 genes were selected for validation using IHC, based on the availability of commercial antibodies, and galectin-3 and NEK2 proteins specifically identify DLBCL-tr, as compared to FL. **Conclusion:** We demonstrate that by purifying tumor cells through cell sorting, thereby reducing the heterogeneity due to infiltrating cells, it was possible to identify distinct differences between tumor entities rather than variations due to cellular

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composition. Galectin-3 and NEK2 both identified a subgroup of DLBCL-tr, and the function of these protein markers also suggests a biological role in the transformation process.

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INTRODUCTION

Follicular lymphoma (FL) is one of the most common lymphomas in the Western World [1] and is associated with an indolent clinical course and a relatively long overall survival [2]. The t(14;18)(q32;q21) translocation, found in the majority of FL [3], results in over-expression of the anti-apoptotic gene *BCL-2*, which augments the ability of tumor cells to resist apoptosis. Many patients with FL remain untreated for years, whereas others are treated with chemotherapy and/or rituximab [4-6].

FLs respond well to treatment, although complete remissions are rare. However, transformation into the more aggressive diffuse large B cell lymphoma (DLBCL) occurs in 20-60%, with increased probability of transformation over time [7-9]. Transformation results in reduced response to therapy and decreased overall survival [9, 10]. FL progresses and in many cases transforms regardless of the treatment, suggesting that this outcome may be determined by the lymphoma's genetic profile.

The mechanism for the transformation has not been fully elucidated. Even though several genes and genetic abnormalities involved in the transformation have been described, such as alterations in *TP53* [11], *c-myc* [12] and *BCL6* [13, 14], as well as inactivation of p15 and p16 [15, 16] and *REL* amplification [17], these changes are observed only in a fraction of transformed lymphomas. Several studies have analyzed the differences in gene expression pre- and post-transformation, but have produced lists of genes with low concordance [13, 18-21]. This may in part be explained by the complex microenvironment of the analyzed tissue [18], resulting in significant intra-tumoral heterogeneity. The diverse genetic features of the primary FL cells may also result in substantial inter-tumoral heterogeneity [13, 22-24]. Biopsy specimens

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are the common source of material and analysis of total, unfractionated tissue mainly identifies differences in cell composition rather than changes in gene expression of tumor cells [13].

Given both the current lack of biomarkers for the transformation of FL into DLBCL (DLBCL-tr) and the tumoral heterogeneity, we used flow cytometry-based cell sorting to purify FL and DLBCL-tr. We here show that the use of purified tumor cells enable tumor-specific gene expression analysis of FL and DLBCL-tr, thereby enabling identification of deregulated genes associated with transformation and offering novel protein biomarkers.

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MATERIAL AND METHODS

Collection and isolation of tumor cells for gene expression analysis.

The study was conducted according to the Helsinki declaration and with approval of the Uppsala University Ethics Committee (approval 01-399). All specimens (FL, n = 12 and DLBCL-tr, n = 6) in this study were diagnosed according to the World Health Organization (WHO) criteria [25]. Freshly frozen tumour cell suspensions were collected from the biobank at the Department of Pathology, Uppsala University Hospital (Uppsala, Sweden). All patients are represented by a single sample except one case where the primary FL, the relapsed FL and the DLBCL-tr are derived from the same patient at three different time-points. After diagnosis, the cells were sorted using a FACSAria (Becton Dickinson, Franklin Lakes, New Jersey, USA), based on the expression of CD19-PB (Dako, Glostrup, Denmark), kappa-APC (Becton Dickinson) or lambda-PE (Dako) and lack of CD3-FITC (Becton Dickinson) expression, enabling identification of a clonal tumour cell population. The purified cells were washed once in PBS and lysed in Trizol (Invitrogen Corporation, California, USA), as previously described [26]. Patient material details are shown in Table 1.

Isolation of mRNA and sample preparation

Briefly, from the sorted cells that were lysed in Trizol (Invitrogen), total RNA was isolated and the quality verified on a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, California, USA). In *vitro* transcription was then performed, according to the Two-cycle Eukaryotic Targeting Labeling assay protocol (Affymetrix, Inc., Santa Clara, CA, USA), as previously described [26]. The labelled cRNA was hybridized to the Human Genome U133 Plus 2.0 arrays containing ~

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50,000 transcripts (Affymetrix) [26]. Different parameters (scaling factor, background, noise and GAPDH expression) were controlled and confirmed arrays of high quality.

Gene expression analysis

The expression level for each probe set is given as a signal value in the GeneChip Operating software 1.4 (Affymetrix) and to enable comparison between different arrays, signal values were normalized by scaling the values against a median target value of 100 on each array (MAS5). The Signal values were then imported into Gene Spring 7.3 GX (Agilent) and deregulated genes, specific for the transformation from FL to DLBCL-tr were identified. First, two different Student's t-tests were performed on FL versus DLBCL-tr (one on transcript present in 2 of 6 DLBCL-tr with an expression above 200 in at least one DLBCL-tr, and one on transcript present in 3 of 12 FL with an expression above 200 in at least one FL to select for significantly up and down-regulated genes with an expression above twice the median value in at least one point. The false discovery rate (FDR) was controlled for by the Benjamini and Hochberg method and the q -value cut off was set to 0.05. Secondly, a quantitative analysis was performed, where selected transcripts had to be deregulated at least 1.5 times in 80% of the DLBCL-tr (80% was chosen as cut-off to target differences occurring in the majority of samples) compared to an average value of the FL samples. Furthermore, the average value of the DLBCL-tr samples had to be deregulated at least 1.5 times, compared to 80% of the FL. Finally, for a transcript to pass, it had to be identified through both the qualitative and the quantitative analyses. This resulted in 189 deregulated transcripts, corresponding to 163 genes (Table 2 in Supplementary materials).

Ingenuity pathway analysis for interaction between genes

The significantly deregulated genes were analyzed, using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, Mountain view, CA, USA www.ingenuity.com) for network analysis. A

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3 core analysis was performed on all transcripts (n = 189) deregulated during transformation. To
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5 determine the probability of each data set belonging to a network, a *p*-value was calculated,
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7 using a right-tailed Fisher's exact test. IPA (Ingenuity Systems) shows interactions between
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9 genes (maximum of 35 genes) as a network. In the network with the best *p*-value, *NEK2* and five
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11 intermittent molecules were added to show the interactions between the deregulated genes
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13 included in the network (maximum of 35 genes) and *NEK2*.
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18 **Immunohistochemistry: patient samples, staining procedure and read out**

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20 Samples used for whole tissue sections were collected from the biobank at the Department of
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22 Pathology, Lund University Hospital. Matched immunohistochemistry (IHC) section pairs from
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24 seven patients with synchronous or metachronous FL (grade I to III) and DLBCL-tr (diagnosed
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26 according to the WHO [25] were selected from the departmental database. Three additional
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28 patients diagnosed with FL and three unrelated patients with DLBCL-tr were included (see Table
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32 1 for details).
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35 Samples used for tissue micro array (TMA) were collected from the Department of
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37 Pathology at the University hospitals in Lund, Uppsala and Umeå. All patients (n=104) were
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39 diagnosed with de novo DLBCL, stage I-IV during the period of 1990-2002. The whole tissue
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41 sections were stained with Mayers hematoxylin (SigmaAldrich Corporation, St. Louis, MO,
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43 USA) and sections of representative, non-necrotic sites were marked. From each tumor tissue,
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45 three 0.6 mm core biopsies were punched and positioned in a recipient paraffin block using
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47 custom made precision instrument. Using three cores per patient was considered to provide a
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49 representative sample for the tissue (Table 1). All above samples were approved by the Lund
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60 University Ethics Committee (Approval 242/2006).

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Briefly, for IHC with antigen retrieval, 2-4 µm-thick paraffin tissue sections were deparaffinized and rehydrated in an electric decloaking chamber. The sections were then incubated with primary antibody at room temperature and the signal was detected either with, (i) REAL detection system (Dako), containing the secondary biotinylated, goat anti-rabbit/mouse antibody and the streptavidin/horseradish peroxidase complex (Labeled StreptAvidin Biotin (LSAB)) or (ii) REAL EnVision detection system (Dako), containing the secondary goat anti-rabbit/mouse antibody conjugated to a peroxidase conjugated polymer. In both cases, the reaction was visualized by 3,3'-diaminobenzidine (Dako). The sections were then counterstained with Mayers hematoxylin (SigmaAldrich Corporation). Primary antibody concentrations, target retrieval buffer and detection methods are summarized in Table 3 in Supplementary materials.

RESULTS

Purification of tumor cells

The fraction of tumor cells in a biopsy can vary between patients, and different tumor entities often have major differences in tumor cell content. In this study, the fraction of tumor cells in the archival material used ranged from as low as 35% up to 80%. The FLs had a tumor cell content ranging from 35% to 80%, while the DLBCL-trs showed higher tumor cell content, ranging between 60 to 80%. So far, most studies use complex tissue for gene expression analysis and will not be able to separate the expression in tumor cells from infiltrating cells. We set out to identify genes specifically expressed by tumor cells and not by cells in the microenvironment. Thus, viable frozen cells from FL (n = 12) and DLBCL-tr (n = 6) were thawed and purified using flow-cytometry based cell sorting. After sorting, the tumor cell content was assessed by re-analysis in flow cytometry and was shown to routinely be > 97%, based on CD19 and clonality for kappa or lambda immunoglobulin light chain. Using such purified cells, we were able to for the first time analyze the gene expression changes associated with the transformation of FL to DLBCL-tr. This method enabled tumor-specific gene expression analysis, and allowed us to identify genes associated with the malignant cells.

Identification of transformation-associated genes

Genes were filtered to identify significantly ($q < 0.05$) deregulated transcripts, associated with the transformation (see Material and Methods for details). In total, 189 transcripts (Figure 1 and Table 2 in Supplementary materials), corresponding to 163 unique genes were significantly changed in the majority of cases. Of note, the material also included samples derived from one patient from three consecutive time points during the transformation process, i.e. the primary FL,

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3 the relapsed FL and the transformed DLBCL. As shown in Figure 1, even though shown for a
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5 single patient, it is evident that the gene expression profile gradually changes as the lymphoma
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7 progresses from FL to DLBCL-tr.
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11 **Protein biomarkers identifying DLBCL-tr vs FL**

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15 Currently, there are no biomarkers used for identifying DLBCL-tr and in an attempt to identify
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17 relevant candidates we focused on the transcripts found to be upregulated in DLBCL-tr, as
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19 compared to FL. Among the 79 upregulated transcripts, 13 genes were selected for a validation
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21 study, using IHC. The selection was based on the availability of commercial antibodies (Table 3
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23 in Supplementary materials). The material used for IHC analyses was collected from a separate
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25 hospital and represents an independent cohort of patients with specimens from the same patients
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27 pre- and post transformation, as compared to the material used for gene expression analyses.
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29 Importantly, the majority of samples used for IHC are paired i.e. both FL and DLBCL-tr derived
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31 from the same patient (Table 1). We were able to confirm the protein content in DLBCL-tr cells
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33 for most of the identified gene products, although the majority of these protein biomarkers did
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35 not display any significant quantitative differences between FL and DLBCL-tr (data not shown).
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37 Interestingly, we were able to demonstrate that two antigens, NEK2 ((NEver in mitosis gene a)-
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39 related Kinase 2) and LGALS3/galectin-3 (Lectin GALactoside-binding Soluble 3), did identify
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41 a subgroup of DLBCL-tr, both quantitatively and qualitatively. Neither of these two proteins
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43 gave an immunohistochemical signal in the malignant cells in any FL samples, whereas 4/10 of
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45 the DLBCL-tr samples showed tumor cells with a clear-cut polar cytoplasmic signal for both
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47 proteins (Figure 2). Thus these two proteins associated with DLBCL-tr were identified using the
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49 above strategy.
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Protein expression in *de novo* DLBCL

The protein content of galectin-3 and NEK2 was also analyzed using a TMA of 104 samples of *de novo* DLBCL. We were able to confirm the protein content in subgroups of *de novo* DLBCL, but with a lower frequency compared to DLBCL-tr. Thus 9% of *de novo* DLBCL show a clear-cut polar cytoplasmic signal for galectin-3 whereas 36% show the same staining pattern for NEK2. Furthermore, we could not see any significant difference in protein content comparing DLBCL-GC with DLBCL-nonGC, nor did the protein content correlate to the stage of the tumor (data not shown). Nevertheless, the correlation of the content of these two proteins was not as high (9%) as in DLBCL-tr, where both proteins were present in consecutive sections in all cases.

Molecular interactions between deregulated genes

Furthermore, pathway analysis was also performed to identify interactions between any of the proteins encoded by the deregulated genes (n = 163). Networks of interacting molecules were created and ranked by significance, using the IPA software. In this study, we focused on the most significant network and also included the interactions with NEK2 (Figure 3). The network display a large number of interacting transcriptional regulators, supporting the fact that DLBCL-tr have a significantly higher proliferation rate as compared to FL. The network also included four of the seven proteins, where protein content could be confirmed in the tumor cells (HSPD1, galectin-3, MIF, TFDP2), although HSPD1, MIF and TFDP2 did not display a quantitative difference between FL and DLBCL-tr. NF- κ B is also included in the identified network, and based on the above data it is suggested that NF- κ B is a key mediator, and potentially coordinates the actions of the deregulated genes. Consequently, the functional association between these deregulated genes further emphasizes their coordinated biological role during the transformation of FL to DLBCL-tr.

DISCUSSION

DLBCL is a heterogeneous group of tumors consisting of different subgroups of *de novo* DLBCL and DLBCL-tr. The microenvironment of DLBCL and FL [27, 28] and the complex origin of DLBCL [13, 22-24] are two main features adding to the heterogeneity of these tumors. The variation in cell composition in FLs may account for the varying results in IHC studies [29-33] where markers expressed by the non-malignant cells in the microenvironment are targeted. Even though recent studies [18, 32, 34] suggest that infiltrating cells determine the clinical behavior in FL, it has been previously shown that the opposite is true for DLBCL where the protein or mRNA expressed by the individual tumor cells correlates with prognosis [22, 23, 35, 36]. The cell of origin concept has been intensely discussed [22, 27, 37], and is considered important for the interpretation of the gene expression profiles. For example, the current classification of *de novo* DLBCL has been defined by molecular profiling based on the cell of origin [22, 37], which divided DLBCL into tumors with a GC phenotype (DLBCL-GC) or a non-GC phenotype. Others have identified subsets of *de novo* DLBCL based on their expression of genes involved in certain functions, as for example BCR signaling and host response [38]. Furthermore, several previous gene expression studies have aimed at defining the mechanisms and proteins involved in the transformation of FL to DLBCL-tr (11-21). However, identified genes have had low concordance between different studies, again reflecting the heterogeneity of these tumors and emphasizing the importance of using purified tumor cells.

Consequently, to eliminate the influence of infiltrating cells from the microenvironment, we performed flow cytometry-based cell sorting of DLBCL-tr and FL. Tumor cells were sorted to high purity and analyzed, allowing determination of changes in transcript levels only in the malignant cells. This approach precludes the possibility of

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2 identifying genes derived from the microenvironment, involved in supporting tumor growth.
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4 Thus, the analytical resolution of the tumor cell-associated genes is much improved.
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7 Filtering of the gene expression data identified genes that are deregulated during
8 the process when FL transforms to DLBCL-tr. An initial hierarchical clustering based on these
9 genes, where multiple samples from a single patient could be followed, exemplified how the
10 gene expression changed not only upon transformation but already during the phase when FL
11 relapsed while maintaining the same histologic grade. With the large heterogeneity in mind, we
12 set out to find protein biomarkers that can be useful for identifying tumors at the onset of
13 transformation. Based on gene expression analysis, we identified the two proteins NEK2 and
14 galectin-3 and showed their significant potential to identify a sub-group of DLBCL-tr. None of
15 the FL expressed these markers in the malignant cells, but there were a single, distinct dot-like
16 cytoplasmic staining pattern in a large subgroup of DLBCL-tr. Interestingly, we could also
17 demonstrate that the mRNA expression for galectin-3 increased already in the relapsed case of
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35 Previous identified alternations associated with the transformation, as for example,
36 mutations in the gene *TP53* are observed only in 26-60% of cases [11, 39, 40], and *C-MYC* is
37 affected in only 0-42% of cases [11, 12, 20, 41] why the markers galectin-3 and NEK2
38 identifying 40% of cases are a major advantage confirming the importance of analyzing pure cell
39 populations. Consequently, immunohistochemical detection of these two proteins confirms their
40 use for identifying DLBCL-tr.
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50 Despite the low number of samples used in this study, we were able to find two
51 potential biomarkers for DLBCL-tr in two completely separate materials. This reinforces the
52 need to analyse pure cell populations. However, further evaluation of these results will require a
53 larger number of specimens.
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Although, both mRNA expression [37] and protein content [42, 43] of *galectin-3* have been shown previously in *de novo* DLBCL and is here confirmed by us, this is the first time *galectin-3* is identified in DLBCL-tr. NEK2 protein can be detected in *de novo* DLBCL, and has previously been identified in various types of cell lines established from either solid or hematological tumors [44]. Further, a global transcription analysis of the transformation process previously reported *NEK2* mRNA as deregulated [21], this correlate well with our finding that for the first time NEK2 is shown to identify DLBCL-tr using routine IHC. The latter point is of major importance, since IHC is the golden standard for lymphoma diagnosis. Moreover, although NEK2 and *galectin-3* are found by us and others in *de novo* DLBCL, our data show that the combination of NEK2 and *galectin-3* is predictive of DLBCL-tr, not only over FL but also over DLBCL *de novo* as the correlation between these two proteins were 100% in DLBCL-tr but only 9% in DLBCL *de novo*. Thus if used in combination NEK2 and *galectin-3* strongly predict DLBCL-tr over FL and DLBCL-*de novo*. It remains to be seen in a larger cohort if this can be used to detect transformation of FL in an earlier stage compared to assessment of morphology and growth pattern.

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Both NEK2 and *galectin-3* are believed to have functional roles in tumor cells. NEK2 can be found both in the nucleus and in cytoplasm [45, 46] and is involved in the centrosomal regulation of DNA damage checkpoint control. Over expression of NEK2 is reported to lead to premature chromosome splitting with an ensuing higher proliferation rate [47]. *Galectin-3* can be seen in a variety of compartments, including the cytoplasm, cell surface, and nucleus as well as in the extra cellular space, depending on cell type and specific conditions. *Galectin-3* on the cell surface is suggestive of a role in cellular recognition and adhesion during metastasis [48]. and is, among other functions, involved in regulating apoptosis and tumor

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2 progression [49-52]. It has also been shown that *galectin-3* transfected prostate tumor cells have
3 a reduced response to chemotherapy [53].
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7 In addition to galectin-3 and NEK2, the corresponding protein of five other
8 deregulated genes were found in both FL and DLBCL-tr (HSPD1, TFDP2, CACYBP, MIF and
9 NME1), as determined by IHC. One reason for the protein content of these antigens in FL might
10 be due to differences in the samples. FLs used for gene expression analyses have after 8 to 21
11 years still not transformed and it has been reported that FLs not transforming within seven years
12 after diagnosis have a different gene profile, as compared to FLs transforming within three years
13 [32]. In contrast, most of tumors used for IHC have transformed within a couple of years and all
14 of them within ten years after diagnosis. Thus, the antigens that are seen in both FL and DLBCL-
15 tr might be markers identifying samples already pre-destined to transform, which is of major
16 interest and needs to be investigated further.
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31 The physical associations of the proteins encoded by the deregulated genes (n =
32 163) were analyzed, using pathway analysis. In the network, with the highest score, interactions
33 with NEK2 were included, and the NF- κ B complex was shown to have a central position
34 although it is not deregulated. It has been shown that a subgroup of *de novo* DLBCL, called
35 activated B cells-like DLBCL, depends on constitutive NF- κ B activation for survival, in contrast
36 to DLBCL-GC [54, 55]. This suggests a different role for NF- κ B in DLBCL-GC and the related
37 DLBCL-tr. Furthermore, the anti-apoptotic signaling pathway of NF- κ B is strongly connected to
38 galectin-3 in glioblastoma cells [56], which indicates that NF- κ B and galectin-3 in concert may
39 promote anti-apoptotic features in DLBCL-tr, adding to the aggressiveness of this lymphoma
40 entity.
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54 In summary, protein biomarkers for DLBCL-tr are needed for the early
55 identification of the transformation process and is so far lacking. Using highly purified tumor
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3 cell samples, we were able to identify galectin-3 and NEK2, two proteins identifying a sub-group
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5 of DLBCL-tr over FL. Thus, we propose the use of a combination of these protein biomarkers
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7 for identification of DLBCL-tr through routine IHC, and suggest further validation of these
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9 markers in a larger case series.
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ABBREVIATIONS:

Agapt: Alcohol group acceptor phosphotransferase; RNAPII: RNA polymerase I; DLBCL: Diffuse large B cell lymphoma; DLBCL-GC: *De novo* germinal center-derived DLBCL; DLBCL-tr: Transformed DLBCL; FDR: False Discovery rate; FL: Follicular lymphoma; FL-re: Relapsed FL; IHC: immunohistochemistry; IPA: Ingenuity Pathway Analysis; LGALS3/Galectin-3: Lectin GALactoside-binding Soluble 3; LSAB: Labeled StreptAvidin Biotin; NEK2: NEver in mitosis gene a-related Kinase 2; TRS: Target retrieval buffer; WHO: World Health Organization.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

UA - Contributed to the design of the study, was responsible for the analysis of the microarray data, performed statistical and IHC analysis and wrote the manuscript.

MD - Responsible for evaluation of material used for IHC analysis and IHC image analysis.

MJ - Contributed to the design of the study and collection of clinical data for IHC analysis.

MB – Provided clinical data, identified and collected samples used for gene expression analysis.

CS – Responsible for diagnosis and selection of material used for gene expression analysis.

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2 JL – Responsible for identifying and collecting samples used for the TMA of *de novo* DLBCL.

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4 RR - Responsible for collection of samples used for gene expression analysis and corresponding
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7 clinical data.

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10 C.A.K B - Contributed to the design of the study and revision of the manuscript.

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12 SE - Responsible for the design of the study, evaluation of data and writing of the manuscript.

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14 All authors read and approved the final manuscript.

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Figure 1. Transcripts deregulated upon transformation.

189 transcripts corresponding to 163 genes were significantly deregulated ($q = 0.05$) with a fold change of 1.5 in the transformation of FL ($n = 12$) to DLBCL-tr ($n = 6$) and are here shown in a hierarchical cluster. Vertical arrows mark the samples from one patient collected at three different time points (primary FL, relapsed FL and transformed DLBCL). Two horizontal arrows marks the two identified DLBCL-tr proteins LGALS3 and NEK2.

Figure 2. IHC of galectin-3 and NEK2 on lymphomas pre- and post- transformation.

Primary FL (A, B, D, E) and DLBCL-tr (C, F) are stained for galectin-3 (A-C) and NEK2 (D-F). Nuclear and cytoplasmic galectin-3 signal is seen in follicular dendritic cells together with endothelium and macrophages but not malignant cells in low-grade FL (A, 20x and B, 100x). However, in DLBCL-tr (C, 400x) malignant B cell-blasts display strong polar cytoplasmic signal (thick arrow), while nuclear and cytoplasmic signal is retained in follicular dendritic cells (thin arrow). NEK2 is not detected in FL (D, 20x and E, 100x) but exhibits polar cytoplasmic staining in DLBCL-tr tumour cells (F, 400x) (colors corrected in Adobe Photoshop (Adobe Systems Incorporated, San Jose, California, USA)).

Figure 3. Interactions between deregulated genes.

The most significant network among the 163 deregulated genes is shown with the addition of NEK2 and five intermittent molecules that connect NEK2 to the network. ^{1, 2, 3,4.}

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¹ Underlined = IPA classified the location as unknown

² Agapt = Alcohol group acceptor phosphotransferase

³ RNAPII = RNA polymerase I

⁴ RPS6KA3 = RPS6KA3 (includes EG:6197)

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