Non-IG Aberrations of FOXP1 in B-Cell Malignancies Lead to an Aberrant Expression of N-Truncated Isoforms of FOXP1

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Abstract
The transcription factor FOXP1 is implicated in the pathogenesis of B-cell lymphomas through chromosomal translocations involving either immunoglobulin heavy chain (IGH) locus or non-IG sequences. The former translocation, t(3;14)(p13;q32), results in dysregulated expression of FOXP1 juxtaposed with strong regulatory elements of IGH. Thus far, molecular consequences of rare non-IG aberrations of FOXP1 remain undetermined. Here, using molecular cytogenetics and molecular biology studies, we comprehensively analyzed four lymphoma cases with non-IG rearrangements of FOXP1 and compared these with cases harboring t(3;14)(p13;q32)/IGH-FOXP1 and FOXP1-expressing lymphomas with no apparent structural aberrations of the gene. Our study revealed that non-IG rearrangements of FOXP1 are usually acquired during clinical course of various lymphoma subtypes, including diffuse large B cell lymphoma, marginal zone lymphoma and chronic lymphocytic leukemia, and correlate with a poor prognosis. Importantly, these aberrations constantly target the coding region of FOXP1, promiscuously fusing with coding and non-coding gene sequences at various reciprocal breakpoints (2q36, 10q24 and 3q11). The non-IG rearrangements of FOXP1, however, do not generate functional chimeric genes but commonly disrupt the full-length FOXP1 transcript leading to an aberrant expression of N-truncated FOXP1 isoforms (FOXP1NT), as shown by QRT-PCR and Western blot analysis. In contrast, t(3;14)(p13;q32)/IGH-FOXP1 affects the 5′ untranslated region of FOXP1 and results in overexpress the full-length FOXP1 protein (FOXP1FL). RNA-sequencing of a few lymphoma cases expressing FOXP1NT and FOXP1FL detected neither FOXP1-related fusions nor FOXP1 mutations. Further bioinformatic analysis of RNA-sequencing data retrieved a set of genes, which may comprise direct or non-direct targets of FOXP1 NT, potentially implicated in disease progression. In summary, our findings point to a dual mechanism through which FOXP1 is implicated in B-cell lymphomagenesis. We hypothesize that the primary t(3;14)(p13;q32)/IGH-FOXP1 activates expression of the FOXP1FL protein with potent oncogenic activity, whereas the secondary non-IG rearrangements of FOXP1 promote expression of the FOXP1NT proteins, likely driving progression of disease.

Introduction
The FOXP1 (Forkhead box P1) gene located at 3p13 (previously assigned to 3p14.1) codes for a transcriptional regulator belonging to the FOX transcription factor family which is implicated in a wide range of biological processes [1,2]. Multiple alternative splicing variants of FOXP1 have been annotated (www.ensemble.org). The FOXP1 protein is widely expressed in human tissues. It harbors an N-terminal poly-Gln region, C2H2-type zinc finger and leucine zipper motifs, and a C-terminal DNA binding forkhead or winged helix domain [3]. FOXP1 is an essential factor in cardiac, lung, neural, monocyte and lymphocyte development and maturation, stem cell biology and in immune responses [4–14]. Multiple lines of evidence indicate that FOXP1 plays an important role in tumorigenesis [15]. Initial studies of Banham et al. [16] suggested that FOXP1 acts as a tumor suppressor in epithelial malignancies recurrently characterized by Δ3p13p14/FOXP1 and loss or decreased expression of the FOXP1 protein. Very recent work of Krohn et al. [17] on prostate cancers supports this concept. Interestingly, subsequent studies postulated an oncogenic role of FOXP1 in lymphoma, particularly in an activated B-cell subtype of diffuse large B-cell lymphoma (ABC-DLBC) with a poor clinical outcome [18–23], and extranodal marginal zone lymphoma (MZL), where a strong expression of FOXP1 protein correlates with a risk of a high grade transformation [24–27]. Further investigations showed that FOXP1-positive
ABC-DLBCL [29], as well as follicular lymphoma [29] and primary central nervous system lymphoma [30], preferentially express shorter FOXP1 isoforms, which in non-malignant conditions, may be induced by B-cell activation [29]. It has been hypothesized that the role of FOXP1 as oncogene is due to expression of short FOXP1 isoforms, while the full length FOXP1 (FOXPI) acts as tumor suppressor [15,28].

Noteworthy, FOXP1 is targeted by rare but recurrent chromosomal translocations in lymphoma, particularly MZL and DLBCL [31–35]. The most frequent is t(3;14)(p13;q32), which brings the gene under the regulatory control of the immunoglobulin heavy chain (IGH) locus at 14q32 [31,34]. Other FOXP1 translocations involve non-IGH sequences; the molecular consequences of these aberrations, however, remain undetermined [32,33,35].

In the reported study, we performed genetic and molecular analysis of four lymphoma cases with non-IGH translocations of FOXP1 and compared these with cases harboring t(3;14)(p13;q32)/IGH-FOXPI and DLBCLs with a strong expression of FOXPI with and with no apparent structural aberrations of the gene (FOXPI-positive DLBCL). Our study demonstrates that non-IG rearrangements of FOXPI do not generate chimeric transcripts but activate an aberrant expression of transcriptional variants of FOXPI and N-terminally truncated FOXPI isoforms (FOXPI\textsubscript{NT}). In addition, our data suggest that non-IG translocations of FOXPI are implicated in progression of various B-cell neoplasms, including chronic lymphocytic leukemia (CLL).

Materials and Methods

Patients

Lymphoma cases with and without FOXPI rearrangements were selected from the database of the Center for Human Genetics and Department of Pathology, KU Leuven, Leuven, Belgium. One case with t(non-IG/FOXPI) was provided by Dr. E. Haralambieva (Institute of Pathology, University of Wu¨rzburg, Wu¨rzburg, Germany). Morphology, immunophenotype and clinical records of the studied cases were reviewed. DLBCL subtyping followed immunohistochemical (IHC) algorithm of Hans et al. [36]. The study was approved by the institutional review board “Commissie Medische Ethiek” of the University Hospital. For this retrospective study the “Commissie Medische Ethiek” waived the need for written informed consent from the participants.

Cytogenetic and Fluorescence in situ Hybridization Analysis

G-banding chromosomal analysis and fluorescence in situ hybridization (FISH) followed routine methods. Probes applied for FISH are listed in Table S1. Non-commercial probes were labeled with SpectrumOrange- and SpectrumGreen-d-UTP (Abbott Molecular, Ottoigne, Belgium) using random priming. FISH images were acquired with a fluorescence microscope equipped with an Axioskop 2 camera (Zeiss Microscopy, Jena, Germany) and a MetaSystems ISIS imaging system (MetaSystems, Altlussheim, Germany). One to ten abnormal metaphases and/or 200 interphase cells were evaluated in each experiment.

Immunohistochemistry

For routine IHC ready-to-use antibodies against CD20, CD10, BCL6 and MUM1 from DAKO (Glostrup, Denmark) were used and stainings were performed using the automated DAKO stainer Anti-FOXPI antibody (ab16645) was purchased from Abcam (Cambridge, UK) and used at dilution 1:200. We additionally used anti-FOXP1 serum from Roche Diagnostics (SP133, Roche Diagnostics, Vilvoorde, Belgium) and staining followed the manufacturer’s recommendations. IHC results were visualized using the OptiView DAB IHC Detection Kit (Ventana, Oro Valley, Tucson, Arizona). Image acquisition was done through a Leica microscope at 200\times and 100\times magnification. Images were assembled using Adobe Photoshop CS3.

RNA Extraction and cDNA Preparation

TRizol LS Reagent (Invitrogen, Carlsbad, CA, USA) was used for a total RNA extraction. cDNA was synthesized from 1 \(\mu\)g of total RNA using reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA, USA) and random primer (Invitrogen, Carlsbad, CA, USA).

Quantitative RT-PCR

QRT-PCR was performed using LC480 SYBR Green I Master and the LightCycler 480 Real-Time PCR System. Data were analyzed with the LC480 software (Roche Diagnostics, Indianapolis, IN, USA). Primers representative for exons 3–18 (Table S2) were designed using Light Cycler Probe Design Software 2.0. C\textsubscript{T} values were averaged for triplicate reactions and used to calculate \(\Delta C\textsubscript{T}\) values for each sample. HPRT1 was used as a reference gene for normalization. RPMI-8402, a T-cell leukemia cell line (www.dsmz.de), was used to normalize the relative expression of FOXPI between the samples.

5’ Rapid Amplification of cDNA Ends PCR (5’RACE-PCR)

First strand cDNA was synthesized from 3 \(\mu\)g of total RNA as previously described but using the oligonucleotide FOXPI/ex8R-Race1 designed on exon 8 of FOXPI. The first strand cDNA was tagged with deoxyadenosine triphosphate (dTTP). Second strand was synthesized using Klenow DNA polymerase (Promega, Fitchburg, WI, USA) and the primers mix TV8. Anchored PCR was performed for 35 cycles with primers FOXPI/ex7R/race-1 and 467, and for nested PCR we used the primers FOXPI/ex7R/race-2 and 468. PCR products were cloned to pGEM-T Easy (pGEM-T-easy Vector system I, Promega, Fitchburg, WI, USA) and sequenced.

RT-PCR

First strand cDNA was synthesized as described above. PCR was performed on the cDNA with the primers FOXPI/ex7R/race-2 and PLEKHG1/forward primers to confirm the FOXPI/PLEKHG1 fusion in case 7 (Table S2).

Western Blotting

Sections from frozen lymph node samples were lysed and processed for Western blotting according to standard procedure using the following antibodies: anti-FOXPI (1:100 dilutions; JC12/ab32010; Abcam, Cambridge, UK) and anti-beta-actine (1:300 dilution; AC-15; Sigma Aldrich, St. Louis, MO, USA). Protein detection was performed with Image Quant Las4000. The AIDA software (Advanced Image Data Analyzer, version 4.15.025, Raytest GmbH, Straubenhardt, Germany) was used for a densitometric analysis of Western blots.

Library Preparation for Paired-end RNA-sequencing

The Illumina standard kit (Illumina® TruSeq™ RNA Sample Preparation Kit, San Diego, CA, USA) was used for the mRNA-sequencing sample preparation according to the manufacturer’s protocol. The quality of the libraries was checked by Agilent Technologies 2100 Bioanalyzer, using the Chip (Agilent DNA 1000 Kit).
Figure 1. Partial karyotypes and examples of FISH analysis performed in the index cases. The applied probes included RP11-79P21-SG and RP11-905F6-SO (a, h), RP11-183N07-SG and RP11-56107-SO (b), FOXP1 BA (c, e, g), RP11-2F13-SO and RP11-346A7-SG (d) and CTD-2234G15-SG and RP11-778P17-SO (f). Note split/separated FOXP1 signals in all index cases (a, c, e, g, h), split of RP11-183N07 spanning AP1S3/2q36.1 in case 1 (b), separation of signals flanking the 10q24 breakpoint in case 2 (d) and cohybridization of CTD-2234G15/3p11 and RP11-778P17/3q11 on 3p of inv(3) in case 3 (f).

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Processing of Illumina RNA-sequencing Reads

Prepared libraries were sequenced using HiSeq 2000 (Illumina) operated in paired-end 2×100 bp mode. Reads were quality-filtered using standard Illumina process.

Analysis of RNA-sequencing Data

The fastq files were mapped to the reference human genome (Human.B37.3) using the Ensembl gene model (Homo_sapiens.GRCh37.67). The mapping and downstream analysis were performed with the software ArrayStudio, version 6.0 [37]. The mapped reads were used to calculate read counts per gene as well as fragments per kilobase of exon per million fragments mapped (FPKM). The read counts were used as input for the application DEseq [38], specially designed to find differentially expressed genes in RNA-sequencing data and for comparisons of single cases. DEseq returns a fold change in expression of every gene as well as the associated p-value and false discovery rate (FDR). We performed a pairwise comparison between every sample expressing FOXP1NT and the sample expressing FOXP1FL. Additionally, we compared all possible combinations of two samples expressing FOXP1NT versus the sample expressing FOXP1FL. The three samples expressing FOXP1NT were grouped and also compared against the sample expressing FOXP1FL. In each comparison, we selected the genes with FDR lower than 0.2 and uploaded them to the Ingenuity Pathways Analysis software (IPA, Ingenuity Systems Inc., Redwood City, CA). In IPA, we retrieved genes that were investigated by BAC-walking FISH using sets of probes for 2q36, 10q24 and 3p11-3q13, respectively. Detailed results of FISH analysis are shown in Table S1. Briefly, the 2q36.1 breakpoint of t(2;3)(q36;p13) (case 1) was mapped in the region spanned by RP11-713J07 and RP11-905F6. Of note, two out of 16 cases with t(FOXPI) studied by Goatly et al. also showed the 3p13 breakpoints within a coding region of FOXPI [32].

The reciprocal partner breakpoints of cases 1, 2 and 3 were investigated by BAC-walking FISH using sets of probes for 2q36, 10q24 and 3p11-3q13, respectively. Detailed results of FISH analysis are shown in Table S1. Briefly, the 2q36.1 breakpoint of t(2;3)(q36;p13) (case 1) was mapped in the region spanned by RP11-135M05 and -79P21 spanning exon 6 and exon 8 onwards, respectively. In contrast, two cases with t(3;14)(p13q32)/IGH-FOXPI (cases 5 and 6) included in this study, revealed the 3p13 breakpoints within the 5’ untranscribed region of FOXPI flanked by RP11-713J07 and -905F6. Of note, two out of 16 cases with t(FOXPI) studied by Goatly et al. also showed the 3p13 breakpoints within a coding region of FOXPI [32].

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| Case | Diagnosis | Cytogenetic analysis | FISH | Expression of FOXP1 by | RNA-seq |
|------|-----------|----------------------|------|------------------------|--------|
|      |           |                      |      |                        |        |
|      |           | Karyotype/chromosome 3 abnormalities | FOXP1 BA | BACs flanking the 3p13/FOXP1 bkpt | partner bkpt/split or flanking BACs/candidate gene | QRT-PCR | WB (kDa) | IHC |
|      |           |                      | sample/status |                      |        |
|      |           |                      | FOXP1 BA | RPM11-135M05 ≤−−−−−→RP11-79P21 | 2q36.1/RP11-183N07/ AP153 |        |        |    |
| 1   | MZL       | BM/D                 | 46,XY,t(2;3)(q36.1;p13), del(7)(3q13;3q36)[2] | rearranged | RPM11-135M05 ≤−−−−−→RP11-79P21 | 2q36.1/RP11-183N07/ AP153 | NA | NA | positive |
| 2a  | CLL       | PBL/D                | 46,XY[15],sh (R81/D13S319x1) | not rearranged | RPM11-135M05 ≤−−−−−→RP11-79P21 | 10q24/RP11-346A7 ≤−−−−→RP11-213/TMEM180, C10orf95, ACTR11 | NA | NA | positive |
| 2b  | CLL in Richter transformation | BM/P | 46,XY,t(3;10)(p13;q24)[5],sh (R81/D13S319x1)/46,XY, t(10;14)(p1q32)[2],sh (R81/D13S319x1) | rearranged | RPM11-135M05 ≤−−−−−→RP11-79P21 | 3q11/CTD-2234G15 ≤−−−−→RP11-778P17/golden path gap (www.ensemble.org) | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 3a  | MZL       | BM/P                 | 45,X,-X,t(3;3)(p21;q26), add(6)(q23)[7] | not rearranged | RPM11-135M05 ≤−−−−−→RP11-79P21 | 14q32/LSI IGH-splt/IGH | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 3b  | Progressive MZL | LN/P | 39–46,XX(X7)[de(3)(3;3)(p21;q26), der(3)(13)(7;26p22) inv(3) (p13q11, del(3)(q22)[2],17q11[6]], 2q32, +2p,mar(p11)] | rearranged | RPM11-135M05 ≤−−−−−→RP11-79P21 | 14q32/LSI IGH-splt/IGH | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 4   | gastric non-GCB-DLBCL | ST/D | 47–48,XX, +1(del)(1)p11, +3[11], del(6)d(2q22)[19] | gain/rearranged (subclone) | RPM11-135M05 ≤−−−−−→RP11-79P21 | 14q32/LSI IGH-splt/IGH | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 5   | non-GCB-DLBCL | LN/D | 46,XX,t(12;p11;p13)(11p33q44, t(3;14)(p13;q32)[add(4)(q31)],12p9[10] | rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 6   | MALT lymphoma, MALT/D | LN/D | 46,XX,t(3;14)(p13;q32)[2/14] | rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 7** | non-GCB-DLBCL | LN/D | 47,XX,complex/+del(3)(q25s27) | not rearranged/gain | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 8   | non-GCB-DLBCL | S/P | 44–44,XX, complex | not rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–6 | ↑ 75/↑ 64/↑ 60 | positive done |
| 9   | non-GCB-DLBCL | LN/D | 58,XY,complex/add(3)(q27) | not rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 10  | non-GCB-DLBCL | LN/D | 49,XX,complex/+del(3)(q27) | not rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 11  | non-GCB-DLBCL | LN/D | 47,XX,complex/t(3;10;14)(q27;p15;q32) | not rearranged | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 12  | non-GCB-DLBCL | LN/D | 54,XY,complex/+3 | not rearranged/gain | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
| 13  | non-GCB-DLBCL | LN/D | 48,XX,complex/+3 | not rearranged/gain | RPM11-713/07 <−−−−−→RP11-905F6 | 14q32/LSI IGH-splt/IGH | ↓ ex3–5 | ↑ 75/↑ 64/↑ 60 | positive done |
Table 1. Cont.

| Case | Diagnoses | Cytogenetic analysis | RNA-seq | Expression of FOXP1 by FISH | Expression of FOXP1 by qRT-PCR* |
|------|-----------|----------------------|---------|---------------------------|---------------------------------|
| 14   | non-GCB -DLBCL | NA                   | NA      | not rearranged             | not rearranged                  |
| 15   | FOXP1-negative DLBCL | NA | NA  | not rearranged             | not rearranged                  |
| 16   | non-GCB -DLBCL | NA                   | NA      | not rearranged             | not rearranged                  |

*Previously published cases; **Case with a non-rearranged FOXP1 by FISH but with the PLEKHLG1-FOXP1 fusion identified by 5' RACE-PCR.

FOXP1 Expression by IHC

All four index cases were subjected to IHC with two monoclonal anti-FOXp1 antibodies, ab 16645 from Abcam and SP133 from Roche Diagnostics, both recognizing epitopes located in the C-terminus of FOXp1 protein. We also analyzed selected positive controls (cases 5–14), negative controls (cases 15–16) and three non-malignant (reactive) LN (NL1–3). Examples of IHC are shown in Figure S1. Neoplastic cells of index cases and positive controls revealed a nuclear expression of FOXp1 (Table 1). Of note, gain of FOXp1 tumoral protein expression in case 3 correlated with the acquisition of FOXp1 rearrangement (sample b). Neoplastic cells of FOXp1-negative DLBCL did not express FOXp1, but a proportion of non-malignant cells displayed positive staining. NL1–3 showed FOXp1 positivity in both T- and B-cells in paracortex and lymphocytic corona, and in a small proportion of the germinal center B-cells, as previously described [5,6].

FOXP1 Expression by qRT-PCR

To determine expression pattern of FOXp1 transcripts in cases with non-IG aberrations of FOXp1, we performed qRT-PCR analysis of two available index cases (cases 3 and 4) using six primer pairs representing exons 3/4, 5/6, 7/8, 11/12, 14/15 and 17/18 (Table S2). We also included cases with t(3;14)(p13;q32) (cases 5 and 6), FOXp1-positive lymphomas with no apparent structural aberrations of the gene (cases 7–14), FOXp1-negative lymphomas (cases 15 and 16) (Table 1), non-malignant lymph nodes (NL1–3) and the sorted CD19+ B-cells. RPMI-8402, a T-cell leukemia cell line expressing FOXp1 transcript on low level, was used as control. The results are summarized in Table 1 and illustrated in Figure 3. Both index cases and FOXp1-positive lymphomas (except of case 7) revealed a low expression of exons 5/4 and 5/6 and an increased expression of exons 7/8 and onwards compared to exons 5/6. Lymphomas with t(3;14)(IGH-FOXp1) (cases 5 and 6) and case 7 displayed an enhanced expression of exons 5/6 onwards when compared with exons 3/4. FOXp1-negative lymphomas showed an enhanced expression of exons 7/8–17/18 (cases 15 and 16), which likely reflected expression of FOXp1 by residual non-malignant cells. Among the non-malignant samples analyzed, the highest expression of FOXp1 mRNA was detected in the sorted CD19+ B-cells. In these specimens, all FOXp1 exons analyzed showed enhanced expression.

5’RACE-PCR

To identify putative partner genes of FOXp1 in the index cases, two available cases (cases 3 and 4) were subjected to 5’RACE-PCR. Following QRT-PCR data, we applied primers for exon 7,
which was the first commonly upregulated coding exon of FOXP1 in these lymphomas (Figure 3), and expected to identify flanking upstream sequences. The analysis was also performed on eight cases of FOXP1-positive DLBCL/MZL (cases 7–14). After 5’RACE-PCR and cloning, up to 44 colonies per case were randomly sequenced. This analysis did not detect any FOXP1 fusion in the index cases, but unexpectedly, identified PLEKHG1 (6q25.1) as a fusion partner of FOXP1 in case 7 (Figure 4). The fusion, however, occurred out of the reading frame of FOXP1 indicating that the PLEKHG1-FOXP1 rearrangement did not result in a functional chimeric product. As rearrangements of both genes were not demonstrated by FISH (Table S1), the fusion was likely generated by either e.g., a cryptic insertion, or was present in a minor clone.

Of note, all cases analyzed by 5’RACE-PCR revealed expression of different isoforms of FOXP1 containing exon 7 and various upstream exons. Particularly frequently expressed was transcript or transcripts expressing an alternative exon 6b, which according to Ensembl (Homo_sapiens.GRCh37.67) is shared by FOXP1-009 and -011. This observation was further confirmed by QRT-PCR, which detected a common expression of exon 6b-positive transcripts in the cases analyzed (data not shown).

**Western Blot Analysis**

Eight cases with available material (cases 3, 5–7, 12, 13, 15 and 16) and three non-malignant LNs were subjected to Western blot analysis with the monoclonal JC12 antibody. As shown in Figure 5a, at least four bands with a differential expression were detected in the samples analyzed: the 75 kDa band corresponding to the FOXP1 protein and bands of 64, 60 and approximately 45 kDa. The 75 kDa band was predominantly expressed in both cases with t(3;14)(p13;q32) (cases 5 and 6) and in three non-malignant LNs. The index case and three FOXP1-positive DLBCLs (cases 7, 12 and 13) showed a marked expression of 64 and 60 kDa isoforms, and a various expression of band of 75 kDa. Case 7 revealed additional bands of 45 kDa and 70–73 kDa, not
whether the latter band represents a processed full-length protein or another isoform is unknown. FOXP1-negative DLBCLs (cases 15 and 16) displayed a weak expression of 75 and/or 64/60 kDa proteins.

To evaluate a relative abundance of the full length protein and shorter isoforms in the cases analyzed, we performed a densitometric analysis of expressed isoforms (Table S4). Then, we merged densitometric values of all three shorter proteins (64/60/45) and compared them with expression of the 75 kDa protein. Results presented in Figure 5b confirmed a predominant expression of the full length protein in both cases with t(3;14)(p13q32) (cases 5 and 6) and in all three non-malignant LNs, and pointed a significantly higher expression of shorter isoforms in case 3 and two of three FOXP1-positive DLBCL cases (cases 12 and 13). The exceptional case 7 displayed an abundant expression of the 75 (70–75?) kDa protein.

RNA-sequencing Analysis

RNA-sequencing of six FOXP1-expressing lymphomas, which included three cases with FOXP1 rearrangements (case 3 with inv(3), case 5 with t(3;14)(p13q32) and case 7 with PLEKHG1-FOXP1), and three cases with no apparent structural aberrations of FOXP1 (cases 8, 11 and 12) was performed. In addition, two FOXP1-negative DLBCLs (cases 15 and 16) were also included. The total number of reads produced by sequencing for each sample ranged from 87134076 to 115622676 with a median of 94596970. The percentage of uniquely mapped paired reads ranged from 81 to 90% with a median of 89%. RNA-sequencing detected FOXP1 transcripts in all cases analyzed, but two FOXP1-negative lymphoma cases showed a significantly lower overall expression of FOXP1 transcripts than FOXP1-positive tumors (on average 486.3 FPKM vs 1190.9 FPKM) (Figure S2).

RNA-sequencing data were initially used to verify IHC subtyping of seven DLBCL cases. We analyzed expression values of the predictor genes reported by Wright et al. [39] to classify DLBCLs into ABC/non-GCB and GCB subtypes. Principal component analysis and hierarchical clustering showed that all six non-GCB-DLBCL cases clustered together and were distinct from the GCB-DLBCL case 15 (Figure S3). Further analysis of potential chimeric transcripts did not detect any fusion of FOXP1, including

![Figure 4. Characterization of the PLEKHG1/FOXPI fusion. (a) Schematic representation of the PLEKHG1-FOXPI fusion identified by 5′-RACE PCR in case of FOXP1-positive DLBCL (case 7). Sequence analysis showed a fusion between an approximately 270 bp 5′ fragment of PLEKHG1 (breakpoint in the intronic region between exon 1 and 2) and exon 7 of FOXPI. (b) Fusion transcript was confirmed by RT-PCR using reverse primer on exon 7 of FOXPI and two forward primers (P1 and P2) on PLEKHG1.](https://doi.org/10.1371/journal.pone.0085851.g004)

![Figure 5. Proteosomic analysis of FOXP1. (A) Results of Western blotting with a monoclonal JC12 antibody performed in the index case 3 with inv(3), two cases with t(3;14)(p13q32) (cases 5 and 6), case 7 with PLEKHG1-FOXPI, two cases of FOXP1-positive DLBCL without FOXPI rearrangements (cases 12 and 13), two cases of FOXP1-negative DLBCL (cases 15–16) and three non-malignant LNs (NL1–3). Anti-beta-actine antibody was used for loading control. (B) Relative abundance of the full-length FOXP1 protein (75 kDa) and three shorter proteins with molecular weight of 64/60/45 kDa, based on merged protein densitometric values.](https://doi.org/10.1371/journal.pone.0085851.g005)
PLEKHG1-FOXP1 previously identified in case 7 by 5’RACE-PCR. This finding suggests that the fusion occurred in a minor clone. Mutation analysis identified a wide range of mutations in all analyzed lymphomas, but none targeted FOXP1. Of interest, non-synonymous mutations of two known DLBCL-related genes, MYD88 and CARD11 [40,41], were detected in 3 cases (no. 3, 7 and 12) and one case (no. 12), respectively (data not shown). Looking for unique mutations in case 5 expressing FOXP1FL, we found eight genes (excluding IGLV) affected by non-synonymous mutations and 32 genes with Del39UTR (Table S5). Notably, KLF5 was affected by two mutations, S236C and S237, both predicted as deleterious by Polyphen 2 algorithm. IPA analysis of the genes uniquely mutated in case 5 revealed a network of interactions (mostly direct interactions) with several cancer genes including MYC, TP53, BCL2, PI3K and NFkB1 (Figure S4).

To get insights into transcriptional networks regulated by FOXP1NT, we ran inference analyses comparing transcriptomes of cases 8, 11 and 12 expressing FOXP1NT with the transcriptome of case 5 expressing FOXP1FL, all diagnosed as non-GCB-DLBCL (see Material and Methods). Cases 3 and 7 were excluded from the inference analysis due to lack of an equivalent control sample in the former case, and complex proteomic pattern of the latter case (Figure 5a). Given a weak expression of short FOXP1 isoforms in case 16 (Figure 5c), this sample was not used as a negative control. IPA analysis of the datasets obtained identified 112 genes commonly dysregulated (downregulated) in all seven inference analysis performed (Table S6). The IPA core analysis of these genes identified three top networks comprising 36, 23 and 21 genes, respectively (Table S7). Top diseases and functions of the second most significantly dysregulated network are “Cell Death and Survival, Cellular Movement, Cancer”. This network, including FOXP1, is shown in Figure 6. It comprises at least three key cancer genes, TP53, CDKN1A and MYC, recently shown to repress microRNA-34a which regulates expression of FOXP1 [42]. Of note, SERPINB5 (maspin) found to be significantly downregulated in cases 8, 11 and 12 versus case 5, is a candidate tumor suppressor in prostate cancer [43]. HIP1R, a postulated target of FOXP1 in ABC-DLBCL was not consistently downregulated in FOXP1NT-positive DLBCLs. We cannot exclude, however, that other accessory proteins also contribute to FOXP1 repression of HIP1R, as previously argued [44].

Discussion

The work presented here was inspired by our previous finding of the novel FOXP1-related t(2;3)(q36;p13) in case of MZL [35]. In contrast to t(3;14)(p13;q32)/IGH-FOXP1 affecting the 5’ untranslated region of FOXP1 [31,34], the p13 breakpoint of t(2;3)(q36;p13) was mapped within the coding domain of the gene. Given that MALTI, another oncogene implicated in the
pathogenesis of MZL, is involved in two types of translocations, t(14;18)(q32;q21)/IGH-MALT1 [45,46] and t(11;16)(q21;q21) generating the API2/BIRC3-MALT1 chimeric gene [47,48], we initially hypothesized that (2;3)(q36;p13) encodes a FOXPI-related fusion transcript [55]. However, the subsequent discovery of a predominant expression of shorter FOXPI isoforms by FOXPI-positive ABC-DLBCL led to the hypothesis that 3p13 translocations targeting the coding region of FOXPI, like (2;3)(q36;p13), may activate expression of N-terminally truncated isoform(s) of FOXPI [28,32]. To validate this interesting concept and to decipher the molecular consequences of non-IG aberrations of FOXPI in general, we performed comprehensive genetic and molecular investigations of four lymphoma cases with non-IG rearrangements of FOXPI and compared these with cases harboring t(3;14)(p13;q32)/IGH-FOXPI and FOXPI-positive DLBCL with no apparent structural aberrations of the gene. Our study demonstrated that FOXPI breakpoints in all index cases fall within the coding region of FOXPI and unlike the t(3;14)(p13;q32) do not locate to the 5’ untranslated region of the gene. We also found that non-IG aberrations of FOXPI promiscuously affect partner sequences at various chromosomal regions (2q26, 3p11, 10q24 and other) targeting either a gene-poor region or gene that is in the incorrect transcriptional orientation to be fused with FOXPI. Therefore these aberrations do not generate functional chimeric products. Even the PLEKHC1-FOXPI fusion identified in case 7 was not functional because it occurred out of the reading frame of FOXPI. To prove that the disruption of the FOXPI coding sequences by these aberrations activates expression of short FOXPI isoforms, we performed preliminary transcriptomic and proteomic investigations of a few available cases. Using QRT-PCR, we showed that the index cases detected a set of dysregulated genes in three cases expressing FOXPI FL, and (ii) non-IG aberrations, which result in ectopic expression of FOXPI FL. The former translocation is regarded as the primary genetic event, because like other well-known IGH-mediated translocations in B-cell lymphoma, it occurs as a sole karyotypic aberration and is present in diagnostic samples. Consequently, the aberrantly expressed FOXPI FL seems to play an oncogenic role in lymphoma. In contrast, non-IG rearrangements of FOXPI are found as secondary genetic hits acquired during clinical course of various B-cell neoplasms (DLBCL, MZL and CLL), frequently heralding inferior outcome. Therefore, the overexpressed FOXPI NT isoforms seem to be implicated in disease progression. Our new finding of the FOXPI NT involvement in high grade transformation of CLL remains in line with the data recently published by Quesada et al. [54]. This group identified a novel C-terminally truncated FOXPI protein aberrantly expressed in CLL and showed that this isoform is generated by mutated SF3B1 [splicing factor 3b, subunit 1]. Mutations of SF3B1 were found in 9.7% of CLLs analyzed by whole-exome sequencing and were associated with an advanced disease at diagnosis and poor overall survival of affected individuals.

Although our study postulates the oncogenic role of FOXPI FL in lymphomagenesis, constitutive expression of FOXPI FL in
transgenic mice seems to be insufficient to drive tumorigenesis [5]. Therefore, we presume that FOXP1, like BCL2 [55,56], may require additional genetic hits to initiate lymphoma. To identify such hit(s), we searched for unique mutations in case 5 with t(3;14)/IGH-FOXP1. Among approximately 40 mutated genes, one gene, KLF5, was targeted by two potentially deleterious mutations. Thus far, the implication of KLF5 in lymphomagenesis is unknown, but this transcription factor is involved in several important biological processes including cell proliferation, transformation, hematopoietic stem cell homing and carcinogenesis [57–59].

Altogether, our data support the important role of FOXP1-associated rearrangements in development and progression of B-cell lymphoma. Further studies, however, are needed to decipher complexity of FOXP1 and presumably opposing roles of the gene in the pathogenesis of lymphoid and epithelial tumors.

Supporting Information

Figure S1 Morphology and FOXP1 expression detected by IHC with SP133 antibody in index and control cases. (A–B) Case 3 with inv(3): polymorphic marginal zone lymphoma showing a strong nuclear FOXP1 expression in the neoplastic cells. (C–D) Case 5 with t(3;14)/p13q32/IGH-FOXP1: non-GCB-DLBC showing a strong nuclear FOXP1 expression in the neoplastic cells. (E–F) Case 8: non-GCB-DLBC without FOXP1 rearrangement showing a strong nuclear FOXP1 expression in the neoplastic cells. (G–H) Case 15: GCB-DLBC showing a strong nuclear FOXP1 expression in the neoplastic cells. (I–J) NL1: reactive follicular hyperplasia with selective FOXP1 expression in both T- and B- cells in paracortex and lymphocytic corona, and in a small fraction of GC cells. Scale bar: 50 um.

Figure S2 Expression of FOXP1 determined by RNA-sequencing.

Figure S3 Performance of GC and non-GCB subgrouping of DLBCL cases subjected to RNA-seq using expression values of predictor genes described by Wright et al. [39].

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Figure S4 Interaction network of genes exclusively mutated in t(3;14)-positive case 5 (in grey) with the well know cancer genes specified by IPA. Continuous and discontinues lines indicate direct and indirect interactions, respectively. Red arrows mark genes found to be upregulated in FOXP1NT-expressing non-GCB-DLBCs when compared with case 5 expressing FOXP1FL.

Table S1 Results of FISH analysis.

Table S2 List of the primers.

Table S3 Relevant clinical features of the index cases.

Table S4 Densitometric measurements of WB bands.

Table S5 List of mutated genes in cases 5 with t(3;14)/IGH-FOXP1.

Table S6 List of commonly dysregulated genes in FOXP1NT-expressing non-GCB-DLBCs when compared with case 5 expressing FOXP1FL specified by IPA. The dysregulated (downregulated) genes are in bold type.

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Author Contributions

Conceived and designed the experiments: LR JFF IW. Performed the experiments: LR JFF TT J-AvdK CV IW. Analyzed the data: LR JFF TT J-AvdK CV PV JC IW. Contributed reagents/materials/analysis tools: NP EH CG BM. Wrote the paper: LR IW.
