Translocations activating *IRF4* identify a subtype of germinal center-derived B-cell lymphoma affecting predominantly children and young adults

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The prognosis of germinal center–derived B-cell (GCB) lymphomas, including follicular lymphoma and diffuse large-B-cell lymphoma (DLBCL), strongly depends on age. Children have a more favorable outcome than adults. It is not known whether this is because of differences in host characteristics, treatment protocols, or tumor biology, including the presence of chromosomal alterations. By screening for novel *IGH* translocation partners in pediatric and adult lymphomas, we identified chromosomal translocations juxtaposing the *IRF4* oncogene next to one of the immunoglobulin (*IG*) loci as a novel recurrent aberration in mature B-cell lymphoma. FISH revealed 20 of 427 lymphomas to carry an *IG/IRF4*-fusion. Those were predominantly GCB-type DLBCL or follicular lymphoma grade 3, shared strong expression of IRF4/MUM1 and BCL6, and lacked PRDM1/BLIMP1 expression and t(14;18)/*BCL2* breaks. *BCL6* aberrations were common. The gene expression profile of *IG/IRF4*-positive lymphomas differed from other subtypes of DLBCL. A classifier for *IG/IRF4* positivity containing 27 genes allowed accurate prediction. *IG/IRF4* positivity was associated with young age and a favorable outcome. Our results suggest *IRF4* translocations to be primary alterations in a molecularly defined subset of GCB-derived lymphomas. The probability for this subtype of lymphoma significantly decreases with age, suggesting that diversity in tumor biology might contribute to the age-dependent differences in prognosis of lymphoma. (*Blood*. 2011;118(1):139-147)

Introduction

The 2 most common lymphoma subtypes in Western countries are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL), accounting for up to 36% and 32% of all lymphomas in adults, respectively.[1] Both subtypes are thought to originate from germinal center (GC)–derived B cells.

Approximately 85% of FL carry the chromosomal translocation t(14;18)(q32;q21) as a primary oncogenic event. This translocation juxtaposes the *BCL2* oncogene from 18q21 next to the immunoglobulin heavy chain (*IGH*) locus in 14q32.[2] The t(14;18) has a lower incidence in FL grade 3 than in FL grade 1 or 2 and, remarkably, is almost completely absent in FL below the age of 18.[3,4] In contrast to adult cases, pediatric FLs are also more frequently grade 3 or composite FL/DLBCL and have a significantly better 5-year event-free survival.[4,5] Therefore, "pediatric FL" has been considered as distinct variant of FL by the updated World Health Organization classification.[2]

DLBCL may derive from transformation of low-grade lymphoma, such as FL, or occur as a de novo malignancy. By gene expression profiling applying "cell-of-origin" signatures, DLBCLs have been divided in biologic subgroups, with the GC B-cell-like (GCB) and the activated B-cell-like (ABC) subtypes being the most prominent.[6] Compared with ABC-DLBCL, the GCB subtype is characterized by a better prognosis and a different pattern of genetic aberrations, including the presence of the t(14;18) in approximately 20% to 30% of cases.[7,8] Whereas in adults GCB- and ABC-DLBCLs account for 48% and 30% of all de novo DLBCLs,[7] respectively, pediatric DLBCLs are predominantly GCB type.[9] Despite this predominance, DLBCLs in children again

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almost completely lack t(14;18). They have a better prognosis than adult DLBCLs.[10,11]

There is an ongoing debate whether age-related differences of tumor biology, host characteristics, or treatment-associated factors, alone or in combination, contribute to the better outcome of pediatric compared with adult B-cell lymphomas. Age is a continuous variable with well-established prognostic impact within the adult lymphoma population. Nevertheless, few studies have investigated biologic variables across all lymphoma age groups.[8,12]

Translocations involving the immunoglobulin (*IG*) loci are the hallmarks of several subtypes of B-cell lymphoma.[13] To determine whether hitherto unidentified recurrent *IG* translocations occur in B-cell lymphomas, we have initiated systematic FISH screening for *IGH* translocations in the lymphomas characterized in the network project Molecular Mechanisms in Malignant Lymphomas (MMML). This has allowed the identification of chromosomal translocation juxtaposing the *IRF4/MUM1* oncogene next to the *IGH* locus, which is cytogenetically cryptic and thus probably has been missed in conventional cytogenetic studies. We have shown that *IG/IRF4* fusions are recurrent genetic changes in GC-derived lymphomas and identify a previously unrecognized subset of B-cell lymphomas with characteristic clinical, morphologic, immunophenotypic, and gene expression profiles. These lymphomas are significantly associated with disease onset in childhood and young adulthood and have a favorable prognosis.

Methods

Lymphoma samples

A total of 720 lymphomas were studied herein. First, a core group of 427 cases was screened by FISH for *IRF4* breaks. This series contained 183 cases from network project MMML,[8,14] 74 cases from the Berlin-Frankfurt-Münster-NHL trials (www.uniklinikum-giessen.de/nhlbfm),[4,9] 161 cases from the NHL-B trials of the German High-Grade NHL Study Group (www.lymphome.de/Gruppen/DSHNHL),[15] and 9 cases with *IG* break and unknown partner from the routine cytogenetic diagnostics. A complete description of the population screened by FISH is available in supplemental Table 1 and supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Array-based data of 438 lymphomas were mined, including 145 cases from the core group studied by FISH. For 167 of those 438 lymphomas, gene expression and molecular cytogenetic data have been released previously (GEO accession nos. GSE4475 and GSE10172),[8,14] whereas 271 lymphomas (GEO accession no. GSE22470) were newly characterized using the published protocols.[8,14]

The protocols of the clinical trials and the MMML have been approved by central and local review boards. Part of the study has been registered at www.Clinicaltrials.gov as NCT00324779.

Immunohistochemistry and FISH

Immunohistochemistry and FISH analyses were performed using standard protocols.[9] Lymphomas were scored positive for CD5, CD10, MUM1, BCL6, BCL2, and BLIMP1 if $> 25\%$ of the tumor cells stained positive as described in previous publications.[4,9] Staining for Ki-67 was assessed in percentage of positive tumor cells. The algorithm published by Hans et al was applied to classify B-cell lymphomas into GCB and non-GCB subtypes.[16] Complete methods and probes used for the detection of breakpoints or gene fusions affecting the *IGH*, *IGL, IGK, BCL2*, *BCL6*, *MYC*, and *IRF4* loci are described in supplemental Table 2.

Long-distance inverse PCR

Long-distance inverse PCR for the *IGH* switch regions was performed as described previously[17] with modifications. Experimental procedures are described in supplemental Table 3.

Mutation analyses

Sequencing of *IRF4* binding sites of *BCL6* gene[18] and *PRDM1/BLIMP1* gene and detection of *EZH2* Tyr641 mutation were performed (supplemental Tables 4-5). Genotypes of the SNP rs872071 in the *IRF4* locus on genomic and cDNA level were determined by high-resolution melting analysis (supplemental Methods). IGHV mutational status was determined by multiplex polymerase chain reaction using the BIOMED2 protocol followed by direct sequencing or after subcloning and comparison with published germline sequences (www.imgt.org).

Bioinformatics and statistical analyses

Gene expression and copy number profiling data from 143 MMML cases analyzed by FISH for *IRF4* aberrations (for sample selection, see supplemental Methods) were investigated.[14]

Differential gene expression was assessed using the LIMMA software Version 3.2.1 in the context of a linear model that included the ABC/GCB status as a confounding factor.[19] A linear classifier of *IG/IRF4*-positive cases was trained on 143 samples using the shrunken centroid method and subsequently applied to 295 independent MMML cases.[20]

Association between age of onset and incidence of *IG/IRF4* translocations was analyzed by logistic regression. Survival curves were estimated by the Kaplan-Meier method. Survival differences were analyzed with the log-rank test. In addition, Cox regression analyses were performed adjusting for age > 60 years. P values $\leq .05$ were considered to indicate statistical significance (supplemental Methods).

Results

Identification and molecular characterization of *IGH/IRF4* translocations

B-cell lymphomas entering the MMML study were screened by FISH for *IGH* translocations with a hitherto unknown partner. From the cases with FISH pattern indicating an *IGH* break but lacking known common partners, 28 cases were subjected to long-distance inverse PCR for cloning the *IGH* partner. In 2 lymphomas, Sμ-long-distance inverse PCR detected a switch μ-associated translocation t(6;14)(p25;q32) (Figure 1A). Both translocations disrupt the coding region of *EXOC2,* which encodes a component (Sec5) of the exocyst complex, a multiple protein complex essential for targeting exocytic vesicles to specific docking sites on the plasma membrane.[21] Sec5 has been linked to cancer as it is required to mediate RalB-dependent survival signals in transformed cells.[22] Although we cannot at present exclude a role of *EXOC2* in lymphomagenesis, its disruption through the translocation along with the typical mechanism of *IG* translocations (ie, activation of intact oncogenes) made us search for further candidates. Remarkably, immediately telomeric of *EXOC2* maps the *IRF4* gene, which through the translocation is juxtaposed to the *IGH* locus on the der(14)t(6;14) in the same transcriptional direction. Because deregulation of *IRF4* expression by a t(6;14)(p25;q32) has already been described as a recurrent event in multiple myeloma[23] and because of the well-known function of IRF4 as transcription factor in B cells associated with oncogenic addiction,[24] we considered *IRF4* to be the probable target of the translocation.

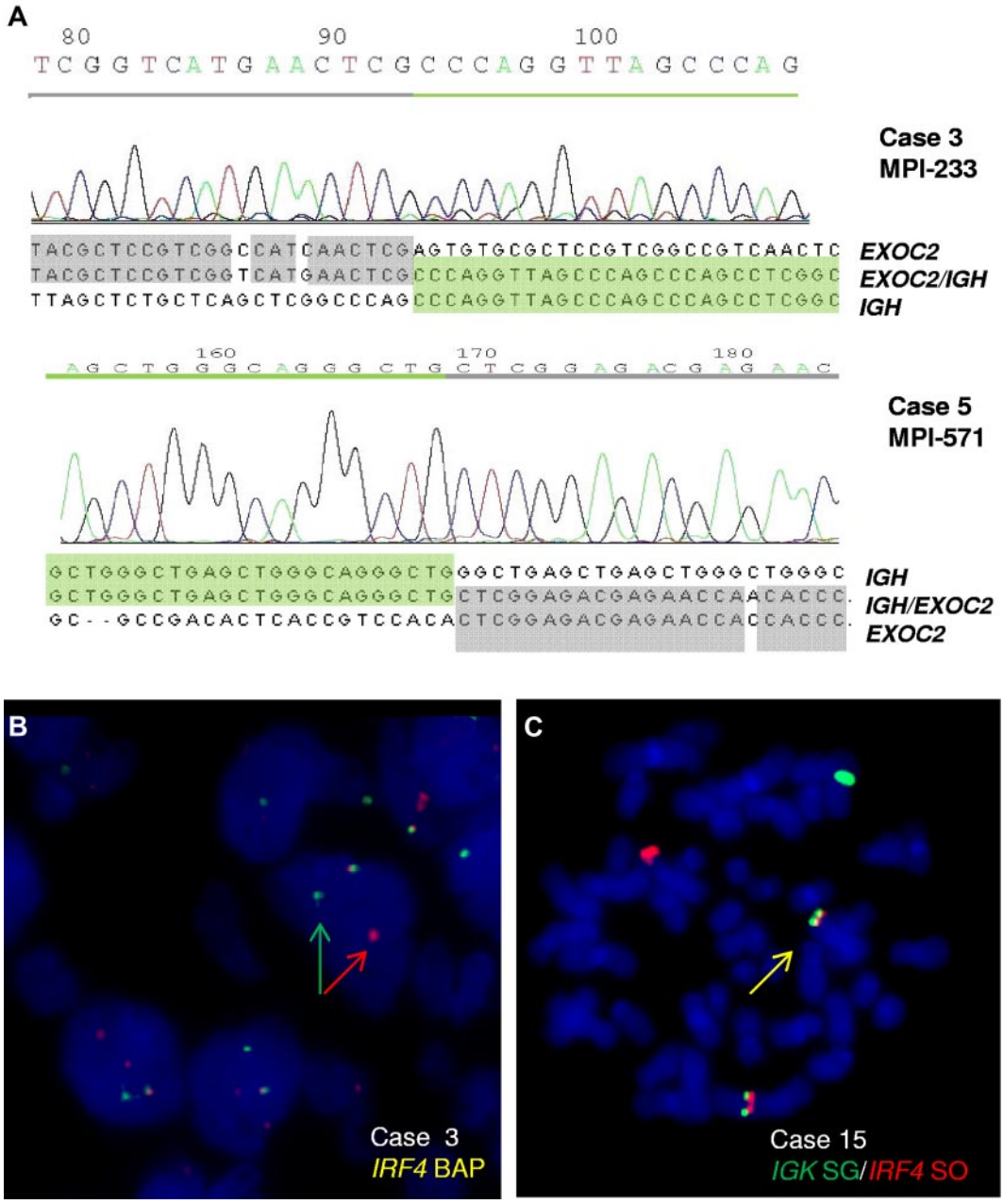

Figure 1. Cloning of the t(6;14)(p25;q32) translocation. (A) Chromatograms and sequences of the different chromosomal junctions of the *IGH*Sμ/*EXOC2* translocations in cases 3 and 5. The sequence of *IGH*Sμ is shown according to GenBank accession no. NG_001019.3 and of *EXOC2* according to GenBank accession no. NT_007592.15. The der(6) genomic breakpoints were located downstream of exon 22 (case 3) and exon 23 (case 5) of *EXOC2* gene at 482 370 and 464 205 bp from 6pter (UCSC, NCBI Build 36.1/hg18). (B) FISH analysis shows *IRF4* breaks in case 3. (C) FISH image on metaphase shows an *IGK/IRF4* fusion in case 15.

FISH screening for *IRF4* translocations

FISH screening of 183 lymphomas of the MMML cohort using probes to *IRF4* on chromosome 6 identified 9 cases with signal patterns indicating *IRF4* breakpoints, including both index cases (Figure 1B). Six cases displayed an *IGH/IRF4* (including both index cases, supplemental Figure 2) and 1 an *IGL/IRF4* fusion (Table 1; supplemental Figure 3B). Two cases showed an *IRF4* break-apart pattern but lacked the typical *IG/IRF4* fusion constellation.

To investigate the frequency of *IG/IRF4* fusion in a population-representative cohort, we next screened 235 B-cell lymphomas from the trials of the German pediatric (Berlin-Frankfurt-Münster-NHL, n = 74) and adult (German High-Grade NHL Study Group, NHL-B trials, n = 161) aggressive lymphoma study groups by FISH for *IRF4* breaks. This led to the identification of 13 additional cases with *IRF4* breakpoints. Finally, 1 of 9 additional lymphomas with unknown *IG* translocation analyzed in a diagnostic setting was shown to have an *IGK/IRF4* translocation (Figure 1C).

In summary, FISH screening identified a total of 23 lymphomas with *IRF4* breakpoints (Table 1): 17 cases with *IGH/IRF4*, 2 with *IGL/IRF4*, and 1 with *IGK/IRF4* fusion (Figure 1C; supplemental Figure 3A-B). In the 3 additional cases, the partner remained unproven or ambiguous. Thus, these cases were classified as "atypical *IRF4*-positive" cases but excluded from further analyses as the nature of the translocation could not be resolved. Among the 20 proven *IG/IRF4*-positive cases, 7 carried a *BCL6* break, 1 carried a *MYC* break, and none a *BCL2* break and/or t(14;18).

Table 1. Diagnosis, FISH, and immunohistochemical data of *IG/IRF4*-positive cases

			FISH					Immunohistochemistry								
Case no.	Study	Diagnosis	Age, y/sex	*IRF4* translocation	*BCL6* break	*MYC* break	t(14;18)/ *BCL2* break	CD5	CD10	MUM1	BCL6	BCL2	BLIMP	ki67	Hans* classifier[16]	GEP
1-MPI093	MMML	DLBCL, NOS	12/female	*IGL/IRF4*	–	–	–	-	NA	++	++	+	NA	+	NA	GCB
2-MPI124	MMML	DLBCL, cb	62/female	*IGH/IRF4*	Split	–	–	++	-	++	++	++	-	++	Non-GC	Unclassified
3-MPI233	MMML	DLBCL, cb	8/female	*IGH/IRF4*†	–	–	–	++	-	++	++	++	NA	++	Non-GC	GCB
4-MPI276	MMML	DLBCL, cb	79/female	*IGH/IRF4*	Split	–	–	-	-	++	+	-	NA	++	Non-GC	GCB
5-MPI571	MMML	DLBCL, cb	7/female	*IGH/IRF4*†	–	–	–	-	++	++	+	++	NA	+	GC	GCB
6-MPI581	MMML	DLBCL, NOS	6/female	*IGH/IRF4*	–	–	–	-	++	++	++	++	-	+	GC	GCB
7-MPI584	MMML	DLBCL, cb	15/male	*IGH/IRF4*	–	–	–	-	++	++	++	-	NA	++	GC	GCB
8	BFM	FL3B	7/male	*IGH/IRF4*	–	–	–	-	+	+	++	++	NA	+	GC	NA
9	BFM	DLBCL, cb	15/male	*IGH/IRF4*	Split	–	–	-	+	++	++	-	NA	+	GC	NA
10	BFM	DLBCL, cb	4/female	*IGH/IRF4*	–	–	–	-	++	++	+	+	-	++	GC	NA
11	BFM	DLBCL, cb	10/male	*IGL/IRF4*	–	–	–	-	++	+	++	-	-	++	GC	NA
12	BFM	FL3B/DLBCL	5/male	*IGH/IRF4*	–	–	–	-	++	++	++	+	NA	+	GC	NA
13	BFM	DLBCL, cb	9/male	*IGH/IRF4*	Split	–	–	-	++	++	++	-	-	++	GC	NA
14	BFM	FL3A/DLBCL	12/male	*IGH/IRF4*	–	Split	–	+	+	++	+	+	-	++	Non-GC	NA
15	Routine	B-cell lymphoma, NOS‡	72/male	*IGK/IRF4*	–	–	–	+	-	++	NA	NA	NA	NA	Non-GC	NA
16	DSHNHL	FL3B/DLBCL	22/male	*IGH/IRF4*	–	–	–	-	+	++	++	-	-	++	GC	NA
17	DSHNHL	DLBCL, cb	20/male	*IGH/IRF4*	Split	–	–	++	-	++	+	++	-	+	Non-GC	NA
18	DSHNHL	FL3B	42/female	*IGH/IRF4*	Split	–	–	-	-	++	NA	++	NA	++	Non-GC	NA
19	DSHNHL	DLBCL, cb	28/male	*IGH/IRF4*	Split	–	–	-	NA	+	-	-	-	++	NA	NA
20	BFM	FL3B/DLBCL	8/female	*IGH/IRF4*	–	–	–	+	+	++	+	++	-	++	GC	NA
Predicted 1 MPI059	MMML	DLBCL, NOS	31/male	Atypical *IRF4* break (no *IG*)	–	–	–	-	++	++	++	-	NA	+	GC	GCB
Predicted 2 MPI514	MMML	DLBCL, cb	79/female	Atypical *IGH/IRF4*§	–	–	–	++	-	++	+	++	NA	++	Non-GC	GCB
Predicted 3 MPI823	MMML	DLBCL, poly	49/male	*IGH/IRF4*	Split	–	–	-	-	++	++	++	+	++	Non-GC	Unclassified
Extra+	BFM	FL3A	14/male	*IRF4* break, *IGH* break	NA	NA	NA	-	+	NA	+	NA	NA	++	GC	NA

Extra+ indicates additional *IRF4*-break-positive case; DSHNHL, German high-grade Lymphoma Study Group; BFM, Berlin-Frankfurt-Münster NHL trials; FL 3A/B, follicular lymphoma grade 3A or 3B; NOS, not otherwise specified; cb, centroblastic; poly, polymorphic; –: negative; ++, high expression (> 50%); +, moderate expression (25%-50%); -, no expression (< 25%); NA, not available; and GEP, gene expression profiling.

*Hans classifier was applied to lymphomas other that DLBCLs, though not developed originally for those.

†Translocation cloned.

‡Transformation, transition in a secondary malignant B-cell lymphoma.

§Lack of der(6)t(6;14)(p25;q32).

Compared with 118 lymphomas of the MMML cohort with copy number data available lacking *IRF4* breakpoints, the 7 *IG/IRF4*-positive lymphomas had fewer chromosomal imbalances (9.2 vs 4.9 alterations, $P = .049$), suggesting that the *IRF4* translocation is an early event in lymphomagenesis (supplemental Figure 4A-D).

Histopathology and immunohistochemistry of *IG/IRF4*-positive lymphomas

Thirteen of the 20 *IG/IRF4*-positive lymphomas were classified as DLBCL, 2 as FL 3B, 4 as composite FL grade 3/DLBCL, and 1 as B-cell non-Hodgkin lymphoma, not further classified. All 11 subclassifiable DLBCLs were of the centroblastic variant.

The immunophenotypes of the *IG/IRF4*-positive lymphomas were mostly characterized by a MUM1$^+$/BCL6$^+$/BLIMP1$^-$ pattern (Table 1; Figure 2). MUM1 protein encoded by the *IRF4* gene was expressed in all 20 cases with *IG/IRF4* fusion, with mostly strong staining intensity (17 of 20). BCL6 was expressed in 17 of 18 (94%) cases, whereas all 10 cases studied were negative for BLIMP1. CD10 was expressed in 12 of 18 (66%), CD5 in 6 of 20 (30%; including 4 of 6 cases lacking CD10 expression), and BCL2 in 12 of 19 (63%) evaluable cases. The expression of

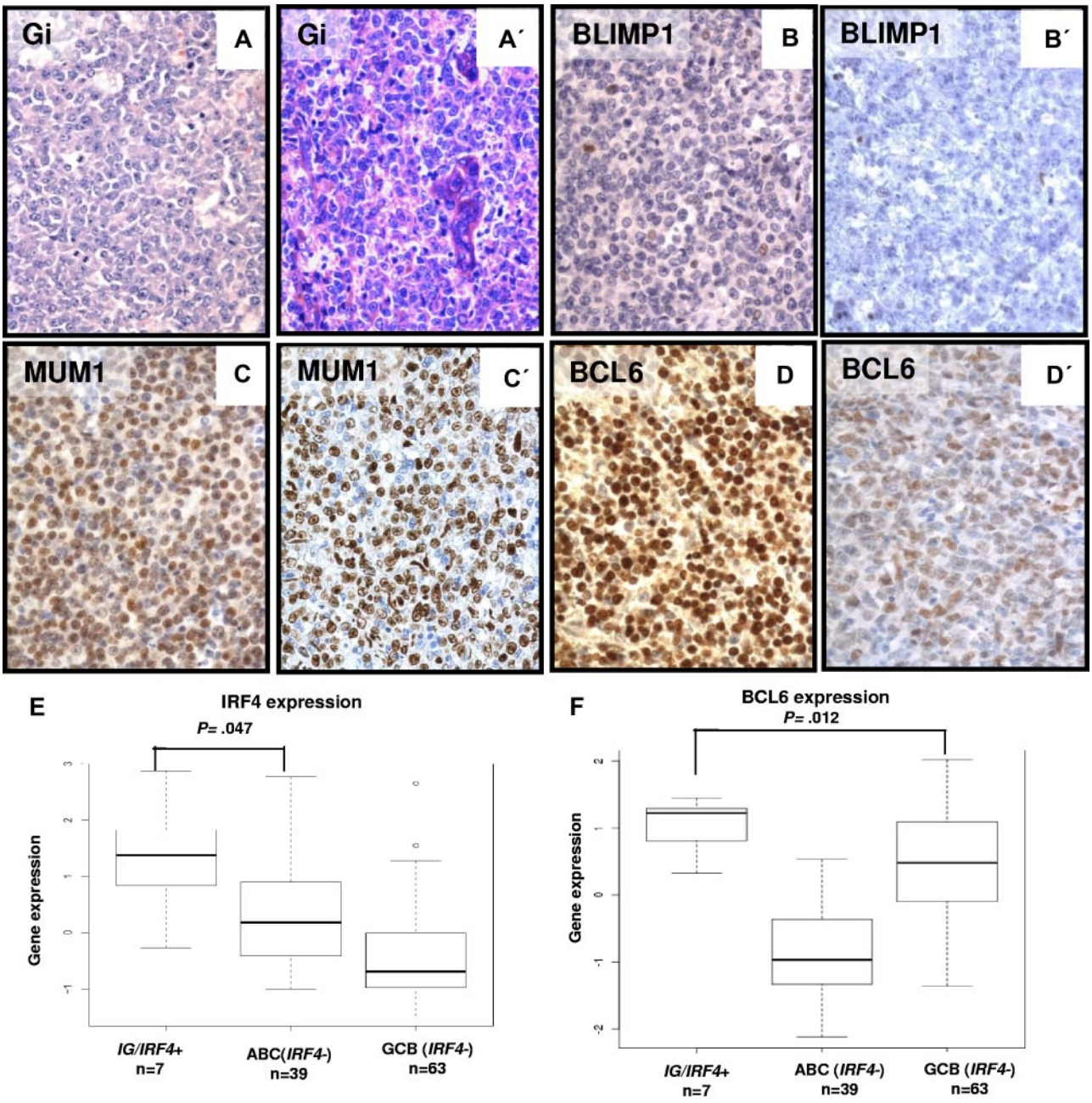

Figure 2. Morphologic and immunohistochemical features of *IG/IRF4*-positive cases. (A-D) Morphology and immunophenotype of 2 *IG/IRF4*-positive cases (A-D, case 14; A′-D′, case 20; original magnification ×400): DLBCL component with GC immunophenotype, strong nuclear staining for MUM1, and negativity for BLIMP1. The follicular component of both cases showed an identical immunophenotype (data not shown). Gi indicates Giemsa staining. (E) Differential expression levels of IRF4 (probe set U133A 216986_s_at) between *IG/IRF4*-positive cases and the other DLBCL subtypes, specifically with the ABC subtype ($P = .047$). (F) Comparison of the expression levels of BCL6 (2 probes encoding *BCL6*, 203140_at and 215990_s_at, summarized to BCL6 index) between *IG/IRF4*-positive cases and the other DLBCL subtypes. Specifically, *IG/IRF4*-positive cases presented higher expression of BCL6 than the GCB-DLBCLs ($P = .012$).

Ki-67 was variable but overall high. According to the Hans algorithm,[16] 11 of 18 (61%) cases were classified as GCB and 7 (39%) as non-GCB.

IG/IRF4-positive lymphomas show characteristics of GCB cells

Gene expression data from 7 lymphomas with typical *IG/IRF4* fusion were available through the MMML consortium. One case could not be classified as either GCB or ABC, and 6 had the GCB signature. All 6 interpretable cases had highly mutated *IGHV* genes. Identity to published germline sequences ranged from 86.1% to 93.9% with ongoing mutation detected in 4 of 4 cases (supplemental Table 6).

Gene expression profiling of *IG/IRF4*-positive lymphomas

IRF4 ($P = .011$) and BCL6 ($P = .013$) were both also expressed at significantly higher transcript levels in *IG/IRF4*-positive than in *IRF4*-negative lymphomas (supplemental Figure 5A-B). Moreover, allelic expression analysis of 3 *IG/IRF4*-positive lymphomas heterozygous for an *IRF4* SNP rs872071 suggested that transcription is heavily skewed toward the translocated allele (supplemental Figure 6). Although *IRF4* and *BCL6* are hallmark genes of the ABC and GCB signatures, respectively, in *IG/IRF4*-positive cases the transcript levels of IRF4 were significantly higher than in ABC-DLBCL ($P = .047$; Figure 2E) and the levels of BCL6 significantly higher than in GCB-DLBCL ($P = .012$; Figure 2F). Therefore, we investigated whether these lymphomas show gene expression features different from both GCB- and ABC-DLBCL. Compared with negative cases (n = 136), lymphomas with *IG/IRF4* fusion (n = 7) showed differential expression of 132 genes (161 probe sets), including 123 up-regulated and 9 down-regulated in *IG/IRF4*-positive cases (supplemental Figure 7; supplemental Table 7).

As these findings suggest *IG/IRF4*-positive lymphomas to be different from GCB and ABC lymphomas, we used a supervised learning algorithm to derive a gene expression classifier allowing prediction of *IG/IRF4*-positive lymphomas. A linear classifier based on 27 genes (32 probe sets), which predicted the 7 *IG/IRF4*-positive cases with 96.5% accuracy in cross-validation, was identified (Figure 3; supplemental Table 8). Application of this classifier to an independent cohort of 293 MMML cases not investigated by FISH as well as the 2 atypical cases identified by FISH (MPI-059 and MPI-514) identified 3 lymphomas with a gene expression profiling typical for *IG/IRF4* fusion. These included 2 "atypical *IRF4*-positive cases" described in the preceding paragraph and 1 additional case, which by FISH subsequently showed a signal pattern indicative of *IG/IRF4* fusion. These 3 predicted cases shared the typical morphologic, immunohistochemical, and somatic hypermutation features with the other *IG/IRF4*-positive cases, except 1 of them that expressed weakly PRDM1/BLIMP1 (Table 1).

Alterations of the BCL6-IRF4-PRDM1/BLIMP1 regulatory pathway

Under physiologic conditions, IRF4 suppresses expression of BCL6 and activates PRDM1/BLIMP1. Because the *IG/IRF4*-positive cases in contrast showed strong expression of BCL6 but lacked PRDM1/BLIMP1 expression, we searched for potential genomic defects in the IRF4-mediated pathway. Sequence and copy number analyses of *PRDM1*/BLIMP1 in 10 *IG/IRF4*-positive lymphomas failed to provide evidence for mutational inactivation of this gene (supplemental Table 9). In contrast, 8 of 23 lymphomas (35%) with proven *IRF4* breaks showed chromosomal breakpoints affecting the *BCL6* locus, and 1 case carried a gain of *BCL6*. In addition, all 7 *IG/IRF4*-positive lymphomas investigated carried somatic *BCL6* mutations, from which one even directly affected a predicted IRF4-binding site (supplemental Table 10).[18] No differential expression of *IRF4* target genes and *BCL6* target genes described by Shaffer et al[24] and Polo et al[25] between cases with and without the translocation was observed (data not shown).

Clinical features of *IG/IRF4*-positive lymphomas

There were 9 female and 11 male patients with *IG/IRF4*-positive lymphomas with a median age of 12 years (range, 4-79 years). Clinical and follow-up data are summarized in supplemental Table 11. Among the 385 cases studied by FISH with available age data, *IG/IRF4*-positive lymphomas were significantly more frequent in children (≤ 18 years) than in adults (> 18 years; 15% vs 2%; $P < .001$). Logistic regression analysis showed a significant decrease in the probability for *IG/IRF4* fusion with age ($P < .001$; Figure 4A). Clinical presentation was significantly skewed toward the involvement of the head and neck region, including Waldeyer ring (80%, $P = .011$) and limited disease stages (84%, $P < .001$). In the whole population analyzed by FISH

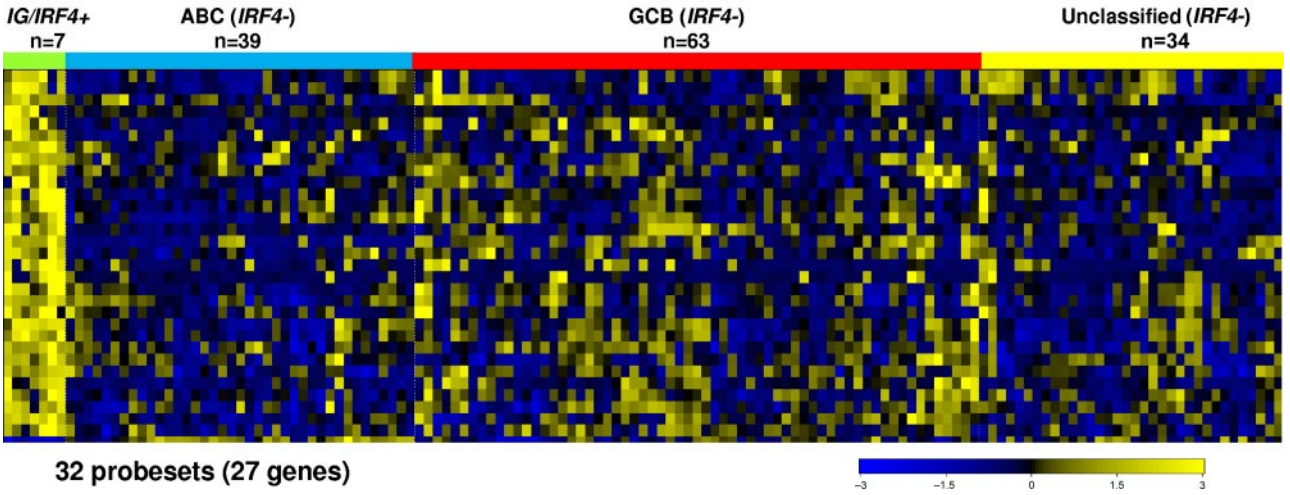

Figure 3. Gene expression profiling-based classifier of *IG/IRF4*-positive cases. This figure represents a heatmap of an *IG/IRF4*-positive classifier consisting of 32 probe sets (27 genes). This classifier distinguishes *IG/IRF4*-positive cases from the rest of DLBCL subtypes.

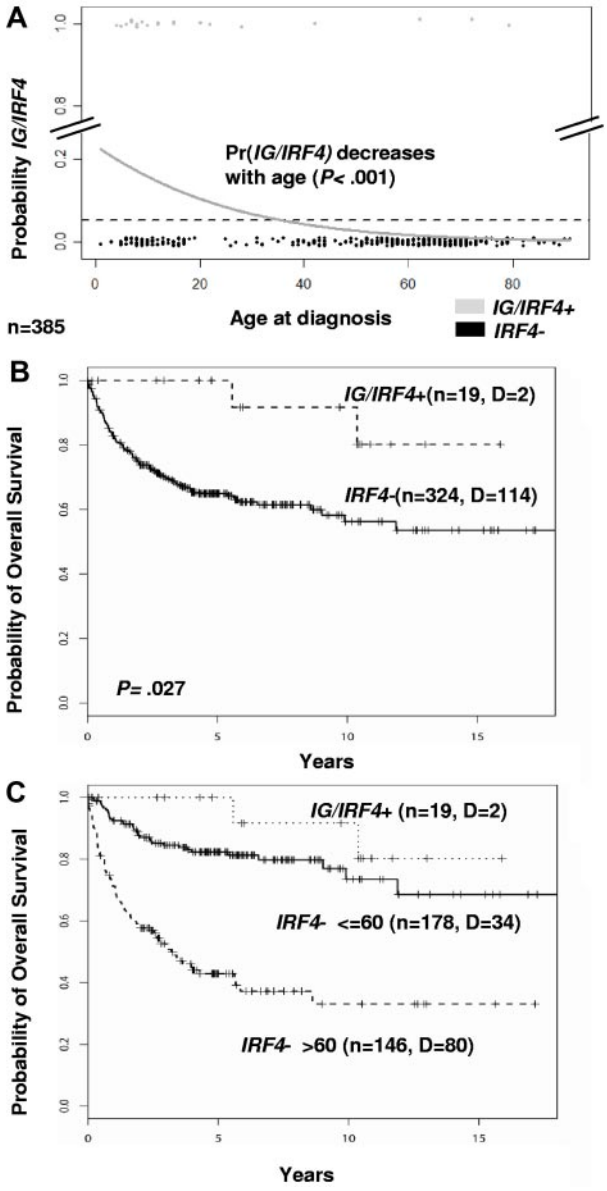

Figure 4. Age and survival analyses. (A) Logistic regression analysis of age distribution. The diagram shows the decreasing probability of having *IG/IRF4* translocations in association with increasing age (n = 385; $P < .001$). Gray dots represent *IG/IRF4*-positive cases; and black dots, *IRF4*-break-negative cases. (B) Kaplan-Meier curves show a better survival of *IG/IRF4*-positive cases ($P = .027$). (C) Kaplan-Meier curves stratifying *IRF4*-break-negative cases by age (≤ 60 years and > 60 years).

in which clinical data were available (n = 343), *IG/IRF4*-positive cases were associated with a significantly better prognosis (5-year overall survival, 100% vs 64.9%; $P = .027$; Figure 4B). Adjusting for age in a COX regression (age, relative risk: 4.26; $P < .001$; *IG/IRF4*, relative risk: 0.39; $P = .19$) showed that this effect was predominantly associated with the low age of the positive cases (Figure 4C; supplemental Figure 8).

Discussion

Recurrent translocations juxtaposing oncogenes and *IG* genes are hallmarks of subtypes of B-cell lymphomas and thus add to their definition and aid their diagnosis.[26] Here we show that recurrent *IG/IRF4* translocations activate the transcription factor *IRF4* in a subtype of mature B-cell lymphomas. *IG/IRF4* fusions are associated with a hitherto unrecognized subgroup of GC B-cell lymphomas composing FL grade 3 or (centroblastic) DLBCL characterized by coexpression of MUM1 and BCL6 in the absence of PRDM1/BLIMP1, a specific gene expression profile, and a disease onset predominantly in childhood or young adulthood.

Remarkably, despite that all 6 classifiable lymphomas with *IG/IRF4* fusion were assigned to the GCB subtype by the gold standard gene expression profiling, 2 of them lacked CD10 expression. In the presence of strong MUM1 positivity, these 2 CD10-negative cases are assigned to the non-GCB subtype according to the immunohistochemistry-based Hans algorithm yielding conflicting results between both classifiers.[16] Interestingly, the expressions of CD10 and CD5 were mutually exclusive, and 5 of 8 CD10-negative cases were positive for CD5, a marker discussed to identify a distinct subgroup of DLBCL.[27] Both features, the lacking correlation of gene expression and immunohistochemical classification and the high rate of CD5 positivity in CD10-negative lymphomas, might indicate that *IG/IRF4* positive lymphomas indeed constitute a defined subgroup of mature B-cell lymphomas.

Translocations affecting the *IRF4* locus have not yet been described as recurrent aberrations in GC B-cell lymphomas, although at least 2 DLBCLs with *IRF4* translocations have been published (supplemental Table 12).[28,29] The reason might be that t(6;14)(p25;q32) is cytogenetically cryptic as shown by Tamura et al.[28] The only case from our series with karyotype available was case 15, which showed der(6)t(2;6)(p12;p25), but light chain variants are obviously rare. In contrast, recurrent aberrations affecting the *IRF4* locus have been described in T-lineage anaplastic large cell lymphomas[30-33] and multiple myeloma.[23,34,35] In the latter, *IRF4* is similarly juxtaposed by an illegitimate *IG* switch recombination to the *IG* loci.[23,34] Moreover, in multiple myeloma, expression of IRF4 is not only driving those cases with *IG/IRF4*-fusion but is also essential for survival in cases lacking this translocation.[24] Whether a subset of GC-derived lymphomas is similarly "addicted" to the expression of the *IRF4* oncogene remains elusive. In contrast to plasma cell neoplasms, the *IG/IRF4*-positive B-cell lymphomas strongly express the GC master regulator BCL6 and lack expression of PRDM1/BLIMP1, which is necessary to drive plasma cell differentiation (supplemental Figure 9).[36] Physiologically, IRF4 suppresses BCL6 expression. The simultaneous high expression of both proteins in *IG/IRF4*-positive cases suggests that this regulatory loop is interrupted by the *IG/IRF4* juxtaposition and the mutations/translocations at the *BCL6* locus. This would explain why attempts to identify a significantly differential expression of *IRF4* target genes and *BCL6* target genes described by Shaffer et al[24] and Polo et al[25] between cases with and without the translocation failed.

The *IG/IRF4*-positive B-cell lymphomas were FL grade 3, DLBCL, or a composite of both. Interestingly, the *IG/IRF4*-positive lymphomas composed 4 FLs grade 3 (with or without additional DLBCL component) in children. Giving the overall rarity of pediatric FL and the overlap in *IG/IRF4* fusion in both FL grade 3 and DLBCL, these findings raise the question of whether pediatric FL might represent part of the spectrum of DLBCL in childhood rather than a distinct FL subtype. Our results also suggest that DLBCL in childhood and young adulthood may be biologically different from older adult DLBCL. Although there is no sharp age border for the presence of *IG/IRF4*-positive lymphomas, the probability of *IG/IRF4* positivity decreases as a function of age. Although age is the strongest predictor of outcome in mature aggressive B-cell

lymphoma, it is possible that the prevalence of favorable versus unfavorable genetic alterations in different age groups accounts for some of this difference, as has been described for B-cell acute lymphoblastic leukemia. Future studies have to show whether, similar to the situation in acute lymphoblastic leukemia, treatment according to genetic rather than age-based risk stratification might improve outcome also in mature B-cell neoplasms.

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Authorship

Contribution: I.S. performed molecular analysis, analyzed data, and wrote the manuscript; I.O. provided samples, performed pathology review, and wrote the manuscript; C. Philipp, S.G., H.T., M.S., M.A., J.R., F.M., C. Pott, I.M.-G., S.P., M.F., L.H., M.H., C.S., and S.W. performed molecular analysis and analyzed data; C.W.K., M.K., H.B., D.H., M.R., M.L., and R. Spang performed statistical analysis; B.B., L.T., and A.R. provided samples, clinical data, and administrative support; M.P., H.S., and W.K. provided samples and performed pathology review; and R.K., W.K., and R. Siebert designed the project, analyzed the data, and wrote the manuscript.

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For a complete list of the members of the Molecular Mechanisms in Malignant Lymphomas Network Project of the Deutsche Krebshilfe, see the online supplemental Appendix.

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References

1. Anderson JR, Armitage JO, Weisenburger DD. Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project. *Ann Oncol*. 1998;9(7):717-720.
2. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008.
3. Lorsbach RB, Shay-Seymore D, Moore J, et al. Clinicopathologic analysis of follicular lymphoma occurring in children. *Blood*. 2002;99(6):1959-1964.
4. Oschlies I, Salaverria I, Mahn F, et al. Pediatric follicular lymphoma: a clinico-pathological study of a population-based series of patients treated within the Non-Hodgkin's Lymphoma–Berlin-Frankfurt-Munster (NHL-BFM) multicenter trials. *Haematologica*. 2010;95(2):253-259.
5. Lenz G, Dreyling M, Schiegnitz E, et al. Myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission prolongs progression-free survival in follicular lymphoma: results of a prospective, randomized trial of the German Low-Grade Lymphoma Study Group. *Blood*. 2004;104(9):2667-2674.
6. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403(6769):503-511.
7. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346(25):1937-1947.
8. Klapper W, Szczepanowski M, Burkhardt B, et al. Molecular profiling of pediatric mature B-cell lymphoma treated in population-based prospective clinical trials. *Blood*. 2008;112(4):1374-1381.
9. Oschlies I, Klapper W, Zimmermann M, et al. Diffuse large B-cell lymphoma in pediatric patients belongs predominantly to the germinal-center type B-cell lymphomas: a clinicopathologic analysis of cases included in the German BFM (Berlin-Frankfurt-Munster) Multicenter Trial. *Blood*. 2006;107(10):4047-4052.
10. Woessmann W, Seidemann K, Mann G, et al. The impact of the methotrexate administration schedule and dose in the treatment of children and adolescents with B-cell neoplasms: a report of the BFM Group Study NHL-BFM95. *Blood*. 2005;105(3):948-958.
11. Fu K, Weisenburger DD, Choi WW, et al. Addition of rituximab to standard chemotherapy improves the survival of both the germinal center B-cell-like and non-germinal center B-cell-like subtypes of diffuse large B-cell lymphoma. *J Clin Oncol*. 2008;26(28):4587-4594.
12. Salaverria I, Zettl A, Bea S, et al. Chromosomal alterations detected by comparative genomic hybridization in subgroups of gene expression-defined Burkitt's lymphoma. *Haematologica*. 2008;93(9):1327-1334.
13. Kuppers R, Dalla-Favera R. Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene*. 2001;20(40):5580-5594.
14. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med*. 2006;354(23):2419-2430.
15. Klapper W, Stoecklein H, Zeynalova S, et al. Structural aberrations affecting the MYC locus indicate a poor prognosis independent of clinical risk factors in diffuse large B-cell lymphomas treated within randomized trials of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). *Leukemia*. 2008;22(12):2226-2229.
16. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood*. 2004;103(1):275-282.
17. Schmitz R, Renne C, Rosenquist R, et al. Insights into the multistep transformation process of lymphomas: IgH-associated translocations and tumor suppressor gene mutations in clonally related composite Hodgkin's and non-Hodgkin's lymphomas. *Leukemia*. 2005;19(8):1452-1458.
18. Saito M, Gao J, Basso K, et al. A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell*. 2007;12(3):280-292.
19. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
20. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A*. 2002;99(10):6567-6572.
21. Moskalenko S, Tong C, Rosse C, et al. Ral GTPases regulate exocyst assembly through dual subunit interactions. *J Biol Chem*. 2003;278(51):51743-51748.
22. Chien Y, White MA. Characterization of RalB-Sec5-TBK1 function in human oncogenesis. *Methods Enzymol*. 2008;438:321-329.
23. Yoshida S, Nakazawa N, Iida S, et al. Detection of MUM1/IRF4-IgH fusion in multiple myeloma. *Leukemia*. 1999;13(11):1812-1816.
24. Shaffer AL, Emre NC, Lamy L, et al. IRF4 addiction in multiple myeloma. *Nature*. 2008;454(7201):226-231.
25. Polo JM, Juszczynski P, Monti S, et al. Transcriptional signature with differential expression of BCL6 target genes accurately identifies BCL6-dependent diffuse large B cell lymphomas. *Proc Natl Acad Sci U S A*. 2007;104(9):3207-3212.
26. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer*. 2005;5(4):251-262.
27. Katzenberger T, Lohr A, Schwarz S, et al. Genetic analysis of de novo CD5+ diffuse large B-cell lymphomas suggests an origin from a somatically mutated CD5+ progenitor B cell. *Blood*. 2003;101(2):699-702.
28. Tamura A, Miura I, Iida S, et al. Interphase detection of immunoglobulin heavy chain gene translocations with specific oncogene loci in 173 patients with B-cell lymphoma. *Cancer Genet Cytogenet*. 2001;129(1):1-9.
29. Hunt KE, Hall B, Reichard KK. Translocations involving MUM1 are rare in diffuse large B-cell

lymphoma. *Appl Immunohistochem Mol Morphol*. 2010;18:109-112.

30. Feldman AL, Law M, Remstein ED, et al. Recurrent translocations involving the IRF4 oncogene locus in peripheral T-cell lymphomas. *Leukemia*. 2009;23(3):574-580.
31. Pham-Ledard A, Prochazkova-Carlotti M, Laharanne E, et al. IRF4 gene rearrangements define a subgroup of CD30-positive cutaneous T-cell lymphoma: a study of 54 cases. *J Invest Dermatol*. 2010;130(3):816-825.
32. Wada DA, Law ME, Hsi ED, et al. Specificity of IRF4 translocations for primary cutaneous anaplastic large cell lymphoma: a multicenter study of 204 skin biopsies. *Mod Pathol*. 2011;24(4):596-605.
33. Feldman AL, Dogan A, Smith DI, et al. Discovery of recurrent t(6;7)(p25.3;q32.3) translocations in ALK-negative anaplastic large cell lymphomas by massively parallel genomic sequencing. *Blood*. 2011;117(3):915-919.
34. Iida S, Rao PH, Butler M, et al. Deregulation of MUM1/IRF4 by chromosomal translocation in multiple myeloma. *Nat Genet*. 1997;17(2):226-230.
35. Chesi M, Kuehl WM, Bergsagel PL. Recurrent immunoglobulin gene translocations identify distinct molecular subtypes of myeloma. *Ann Oncol*. 2000;11(suppl 1):131-135.
36. Schmidlin H, Diehl SA, Blom B. New insights into the regulation of human B-cell differentiation. *Trends Immunol*. 2009;30(6):277-285.