TO THE EDITOR:

CREBBP gene mutations are frequently detected in in situ follicular neoplasia

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Follicular lymphoma (FL) is characterized by the t(14;18)(q32;q21) chromosomal translocation,[1] found in ~85% of manifest FL (mFL) cases. The t(14;18) is also present in early precursor lesions of FL and in a significant fraction of healthy individuals.[2,3] This translocation occurs early in B-cell development resulting from a repair error during the variable diversity joining recombination process.[4] The t(14;18) alone is not sufficient to drive FL lymphomagenesis, indicating the need for additional genetic hits.[5] Another feature of FL cells is the constitutive expression of activation-induced cytidine deaminase, which mediates the processes of somatic hypermutation (SHM) and class switch recombination, leading to genomic instability and accumulation of oncogenic aberrations.[6] Accordingly, secondary hits are frequently identified in mFL, including chromosomal alterations and mutations targeting (eg, acetyl- and methyltransferase genes).[7-10] Such events occur also in t(14;18)$^+$ cells of healthy individuals and in in situ follicular neoplasia (ISFN).[11-13]

These findings suggest a possible model of FL lymphomagenesis in which the t(14;18) is the first hit leading to the accumulation of cells that will then undergo repetitive rounds of germinal center reaction, acquiring additional hits by SHM and class switch recombination, some of which might provide a selective advantage and drive malignant progression.[14] But the particular events needed to transform the ISFN cells into mFL are largely unknown. A hierarchical model recently described *CREBBP* mutations as early events in FL evolution.[15,16] In contrast, *KMT2D* and *TNFRSF14* mutations are reported as late events that function as possible accelerator mutations.[16]

The aim of our study was to identify mutations in ISFN that might represent early driver mutations in the genomic evolution of FL. For this purpose, 5 ISFN-only cases (nonprogressing) and 6 clonally related samples of ISFN and mFL were subjected to analysis (Table 1). The paired ISFN/mFL cases have been the subject of previous studies.[13,17] For this analysis, the ISFN cases were microdissected as previously reported.[13,18] The diagnosis of ISFN and mFL was performed according to the 2016 revised World Health Organization classification.[1] Four mFL cases (mFL-3 to 6) were analyzed by next-generation sequencing (NGS) using an Ion AmpliSeq Custom Panel covering all exons of *TNFRSF14*, *KMT2D*, *FOXO1*, *EP300*, *MEF2B*, *HIST1H1B-E*, and *GNA13*, as well as hot spot regions of *EZH2* (exon 16) and *CREBBP* (exons 24-28 and 30). NGS analysis was done on the Ion Torrent PGM technology, as previously described.[19] mFL cases 1 and 2 were Sanger sequenced for the 2 most recurrently mutated genes *EZH2* (exon 16) and *CREBBP* (exons 26-28 and 30) only.[19] To examine if the mutations found in the 6 mFL cases were present in the paired ISFN cases, we sequenced these regions in the ISFN cases using targeted resequencing and/or Sanger sequencing (supplemental Tables 1 and 2, available on the *Blood* Web site). We extended the analysis to 5 ISFN-only cases (ISFN-7 to 11) by NGS using the same AmpliSeq Custom Panel. Additionally, we performed NGS-based B-cell clonality analysis and determination of *N*-glycosylation sites (supplemental Materials and methods). The study was approved by the local ethics committees of participating institutions (UKT 219/2012/BO2).

The clinical data and genetic analyses are summarized in supplemental Tables 3 and 4. In 4 informative ISFN cases, recurrent *N*-glycosylation motifs (*N*-X-S/T) were identified in the CDR3 and CDR2/FR2 regions. This finding indicates that, already at this early stage, *N*-glycosylation sites occur and substitute conventional antigen binding favoring the generation of long-lived ISFN clones and increasing the chance of secondary hits that will further drive clone fate.[20,21] Sequencing analysis of the 6 mFL cases revealed *CREBBP* as the most frequently mutated gene (6 cases; 100%) (mFL-3 with 2 mutations). *CREBBP* was followed by mutations in *EZH2* (4 cases), *TNFRSF14* (2 cases), *EP300* (1 case), and *KMT2D* (1 case) (Figure 1A). The allelic frequencies of these mutations ranged from 5% to 74.8% (mean, 35.6%) (Table 1). The highest mutant allelic frequencies were detected in *CREBBP* (mFL-4, 74.8%) and *TNFRSF14* (mFL-3, 62.2%; mFL-4, 59.6%), suggesting additional loss or copy number neutral loss of heterozygosity of 16p13 and 1p36 chromosomal regions. Accordingly, comparative genomic hybridization array analysis showed loss of 1p36 region in case mFL-4 (supplemental Figure 1).[13]

In the 6 clonally related ISFN cases, surprisingly, 10 of 15 mutations found in mFL were already present in ISFN, including 6/7 *CREBBP* mutations, 2/4 *EZH2* mutations, 1/2 *TNFRSF14* mutations, and 1/1 *KMT2D* mutation. Only 1 ISFN case (ISFN-1) showed no mutations, despite the presence of a *CREBBP* mutation in the corresponding mFL. Comparison of the allelic frequencies of the mutations in mFL and microdissected ISFN revealed lower frequencies in the ISFN cases (mean, 10.4% vs 35.6%). Sanger sequencing analysis also suggested a lower *CREBBP* mutation allelic frequency in ISFN cases (Figure 1B). Interestingly, 1 ISFN case carried mutations in *CREBBP*, *TNFRSF14*, and *KMT2D* identical to the corresponding mFL, but a different *EZH2* mutation (ISFN-3). This confirms the existence of divergent clonal evolution early in disease progression of FL.[13]

ISFN cases without evidence of mFL (ISFN-7 to 11) carried mutations in 4/5 cases. Mutations were detected in *KMT2D* (case

Table 1. Mutation overview in ISFN/mFL pairs and ISFN-only cases

Case no.	Gene	Protein level	DNA level	Coverage	Frequency, %	Verification*	Method	ISFN no.	Analysis in paired ISFN
ISFN/mFL pairs									
mFL-1	*CREBBP*	p.L1499Q	c.4496T>A	ND	ND	—	Sanger	1	Not present†
mFL-2	*EZH2*	p.Y646N	c.1936T>A	ND	ND	—	Sanger	2	Not present†
	CREBBP	p.I1471T	c.4412T>C	ND	ND	—			Present‡
mFL-3	*TNFRSF14*	p.C185S	c.553T>A	135	62.2	Confirmed	NGS	3	Present (9%)†
	CREBBP	p.L1434P	c.4301T>C	2541	29.7	Confirmed			Present (3%)†
	CREBBP	p.R1446H	c.4337G>A	2515	29.2	Confirmed			Present (6%)†·‡
	EZH2	p.Y646H	c.1936T>C	1190	30.4	Confirmed			Not present but p.Y646N (5%)†
	KMTD2	p.K1840fs	c.5519_5529 delAAGCCGATACA	102	43.6	Confirmed			Present‡
mFL-4	*TNFRSF14*	p.S171C	c.512C>G	777	59.6	Confirmed	NGS	4	Not present†·‡
	EZH2	p.Y646N	c.1936T>A	1482	48.6	Confirmed			Present (33%)†
	CREBBP	p.L1499P	c.4496T>C	3022	74.8	Confirmed			Present (20%)†
mFL-5	*CREBBP*	p.S1382fs	c.4145delA	1663	20.4	Confirmed	NGS	5	Present (15%)†
	EP300	p.D1399Y	c.4195G>T	532	17.5	Confirmed			Not present†
mFL-6	*CREBBP*	p.R1446C	c.4336C>T	1021	6.2	Confirmed	NGS	6	Present (5%)†
	EZH2	p.Y646F	c.1937A>T	498	5.0	Confirmed			Present†·‡
ISFN-only cases									
ISFN-7	*KMT2D*	p.C5227Ter	c.15681C>A	6012	14	Confirmed	NGS		
	KMT2D	p.Q3518Ter	c.10552C>T	1064	15	Confirmed		—	—
ISFN-8	*EP300*	p.T1332P	c.3994A>C	4651	11	Confirmed	NGS		
	TNFRSF14	Splice site	c.179-2A>T	9554	10	Confirmed		—	—
ISFN-9	*TNFRSF14*	p.C121Ter	c.363C>A	6251	8	Confirmed	NGS		
	CREBBP	p.H1487Y	c.4459C>T	6562	12	Confirmed		—	—
ISFN-10	*EZH2*	p.Y646F	c.1937A>T	789	3	Confirmed	NGS	—	—
ISFN-11	—	—	—	—	—	—	NGS	—	—

5% VAF was defined as sensitivity threshold in the analysis of manifest FL samples.

ND, no data available.

*Verification of mutations in mFL was performed either with Sanger sequencing or Illumina MiSeq; verification of mutations in ISFN-only cases was performed with single amplicons on the Ion Torrent PGM.

†Analysis by targeted resequencing on the Illumina MiSeq.

‡Analysis with Sanger sequencing.

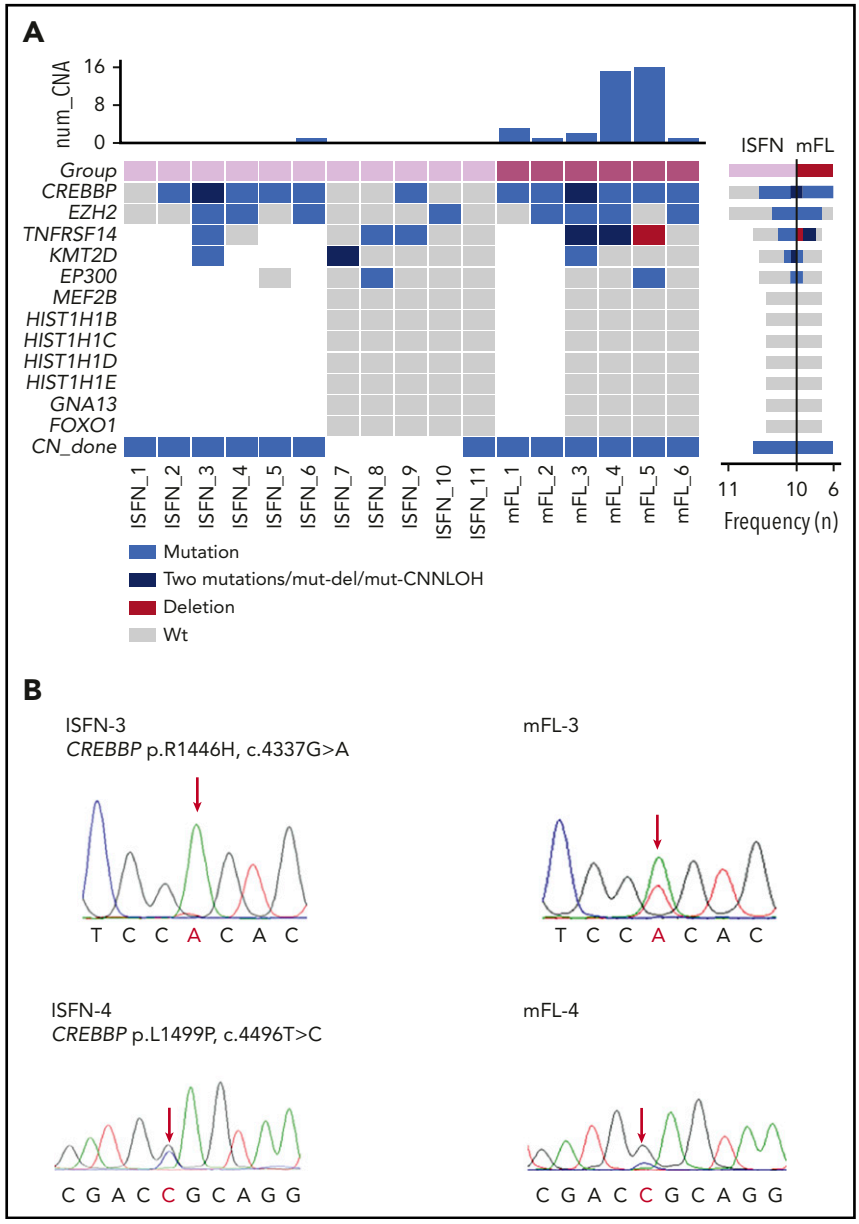

Figure 1. Mutation overview in mFL and ISFN cases. (A) The heat map shows the case-specific pattern of mutations found by Sanger sequencing and NGS. Each column represents a case sorted by ISFN and mFL. Each row represents a gene. (Right) Bar graph illustrates the mutation frequency of each gene in a specific entity. (Top) Bar graph indicates the number of copy number alterations detected by array-comparative genomic hybridization in a previous study.[13] Loss of heterozygosity determination based on polymorphic single-nucleotide polymorphism (data not shown) indicated *TNFRSF14* CNN-LOH alteration in mFL-3. mFL cases 1 and 2 were analyzed for mutations in *CREBBP* and *EZH2* with Sanger sequencing only. (B) Examples of *CREBBP* mutations detected in 2 paired ISFN/mFL samples by Sanger sequencing. The peak of the ancestral base is higher than in the mFL case in the ISFN. The analysis was performed in microdissected ISFN. CNN-LOH, copy number neutral loss of heterozygosity; wt, wild-type.

ISFN-7 with 2 mutations), *TNFRSF14* (2 cases), *CREBBP* (1 case), *EZH2* (1 case), and *EP300* (1 case). The allelic frequencies ranged from 8% to 15% (mean, 11.7%) in microdissected cases. Analyses by sorting intolerant from tolerant, PolyPhen 2, and combined annotation-dependent depletion predicted a damaging effect for all mutations (supplemental Table 5).

Although this is a small cohort and results should be taken with caution, the frequent occurrence of *CREBBP* mutations in 5/6 paired ISFN cases, but in only 1/5 ISFN-only cases (83% vs 20%) suggests that this mutation might be needed for the transforming event. Mutations in *CREBBP* are inactivating events that impair the acetylation activity of CREBBP, favoring the constitutive activity of BCL6 and decreased p53 activity. Recently, it has been shown that loss of *CREBBP* abrogates optimal cellular response to DNA damage because of defective p53 activity, creating a permissive state for the retention and accumulation of subsequent mutations, facilitating transformation.[10,22] Furthermore, FL with *CREBBP* mutations showed significantly higher aberrant SHM-induced immunoglobulin heavy chain variable divergence, highlighting CREBBP as a key protein generating genetic and epigenetic coevolution.[15] Accordingly, our data suggest that mutations in *CREBBP* represent early driver mutations probably facilitating the acquisition of additional mutations and improving the "clonal fitness" of the cells.[15] Whether *CREBBP* mutations alone are sufficient for transformation is not clear; however, a recent study suggests that earlier loss of *CREBBP* is advantageous for lymphoid transformation and final evolution of lymphoid malignancies.[22]

EZH2 Tyr646 mutation was the second most common aberration found in ISFN, indicating that it might represent an early event.[16,23,24] Mutations in *KMT2D* and *TNFRSF14* have been shown to be late events in FL evolution.[16] However, we now show that these mutations also occur in ISFN. Accordingly, a recent mutational analysis on duodenal-type FL, another FL variant considered an early precursor lesion localized to the intestine, demonstrated frequent

mutations in *CREBBP* followed by mutations in *EZH2* and *TNFRSF14* genes.[25] However, *KMT2D* was less commonly mutated than in mFL, further supporting that *KMT2D* mutations are late events in FL pathogenesis.

In conclusion, this study shows for the first time that mutations in *CREBBP*, *EZH2*, *TNFRSF14*, and *KMT2D* are already present in ISFN but with different frequencies.[18] The possible role of *CREBBP* mutations in the transformation of a subclone into overt mFL warrants further analysis.

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Authorship

Contribution: L.Q.-M. conceived and designed the study, supervised the experimental work, and wrote the manuscript; J. Schmidt performed genetic analysis, interpreted the data, and helped write the manuscript; J.E.R.-Z., I.S., I.B., and R.S. interpreted the data and helped write the manuscript; J. Steinhilber, A.H., I.M., S.C.Y., and M.R. performed experimental work; E.S.J., F.F., and L.Q.-M. provided and reviewed the cases; and E.S.J. and F.F. helped write the manuscript.

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Footnote

The online version of this article contains a data supplement.

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