

## TO THE EDITOR:

# CREBBP gene mutations are frequently detected in in situ follicular neoplasia

Janine Schmidt,<sup>1</sup> Joan Enric Ramis-Zaldivar,<sup>2</sup> Irina Bonzheim,<sup>1</sup> Julia Steinhilber,<sup>1</sup> Inga Müller,<sup>1</sup> Andrea Haake,<sup>3</sup> Shan Chi Yu,<sup>4</sup> Mark Raffeld,<sup>4</sup> Falko Fend,<sup>1</sup> Itziar Salaverria,<sup>2,3</sup> Reiner Siebert,<sup>3,5</sup> Elaine S. Jaffe,<sup>4</sup> and Leticia Quintanilla-Martinez<sup>1</sup>

<sup>1</sup>Institute of Pathology and Neuropathology, Eberhard Karls University of Tübingen and Comprehensive Cancer Center, University Hospital Tübingen, Tübingen, Germany; <sup>2</sup>Hematopathology Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Centro de Investigación Biomédica en Red Cáncer, Barcelona, Spain; <sup>3</sup>Institute of Human Genetics, University Hospital Schleswig-Holstein Campus Kiel/Christian-Albrechts University, Kiel, Germany; <sup>4</sup>Hematopathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD; and <sup>5</sup>Institute of Human Genetics, Ulm University and Ulm University Medical Center, Ulm, Germany

Follicular lymphoma (FL) is characterized by the t(14;18)(q32;q21) chromosomal translocation,<sup>1</sup> 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.<sup>2,3</sup> This translocation occurs early in B-cell development resulting from a repair error during the variable diversity joining recombination process.<sup>4</sup> The t(14;18) alone is not sufficient to drive FL lymphomagenesis, indicating the need for additional genetic hits.<sup>5</sup> 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.<sup>6</sup> Accordingly, secondary hits are frequently identified in mFL, including chromosomal alterations and mutations targeting (eg, acetyl- and methyltransferase genes).<sup>7-10</sup> Such events occur also in t(14;18)<sup>+</sup> cells of healthy individuals and in in situ follicular neoplasia (ISFN).<sup>11-13</sup>

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.<sup>14</sup> 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.<sup>15,16</sup> In contrast, KMT2D and TNFRSF14 mutations are reported as late events that function as possible accelerator mutations.<sup>16</sup>

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.<sup>13,17</sup> For this analysis, the ISFN cases were microdissected as previously reported.<sup>13,18</sup> The diagnosis of ISFN and mFL was performed according to the 2016 revised World Health Organization classification.<sup>1</sup> 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.<sup>19</sup> 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.<sup>19</sup> 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.<sup>20,21</sup> 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).<sup>13</sup>

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.<sup>13</sup>

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
<b>ISFN/mFL pairs</b>									
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†
mFL-3	CREBBP	p.I1471T	c.4412T>C	ND	ND	—	NGS	3	Present‡
	TNFRSF14	p.C185S	c.553T>A	135	62.2	Confirmed			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%)†
mFL-4	KMTD2	p.K1840fs	c.5519_5529 delAAGCCGATACA	102	43.6	Confirmed	NGS	4	Present‡
	TNFRSF14	p.S171C	c.512C>G	777	59.6	Confirmed			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†
	CREBBP	p.R1446C	c.4336C>T	1021	6.2	Confirmed			Present (5%)†
mFL-6	EZH2	p.Y646F	c.1937A>T	498	5.0	Confirmed	NGS	6	Present†‡
<b>ISFN-only cases</b>									
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.

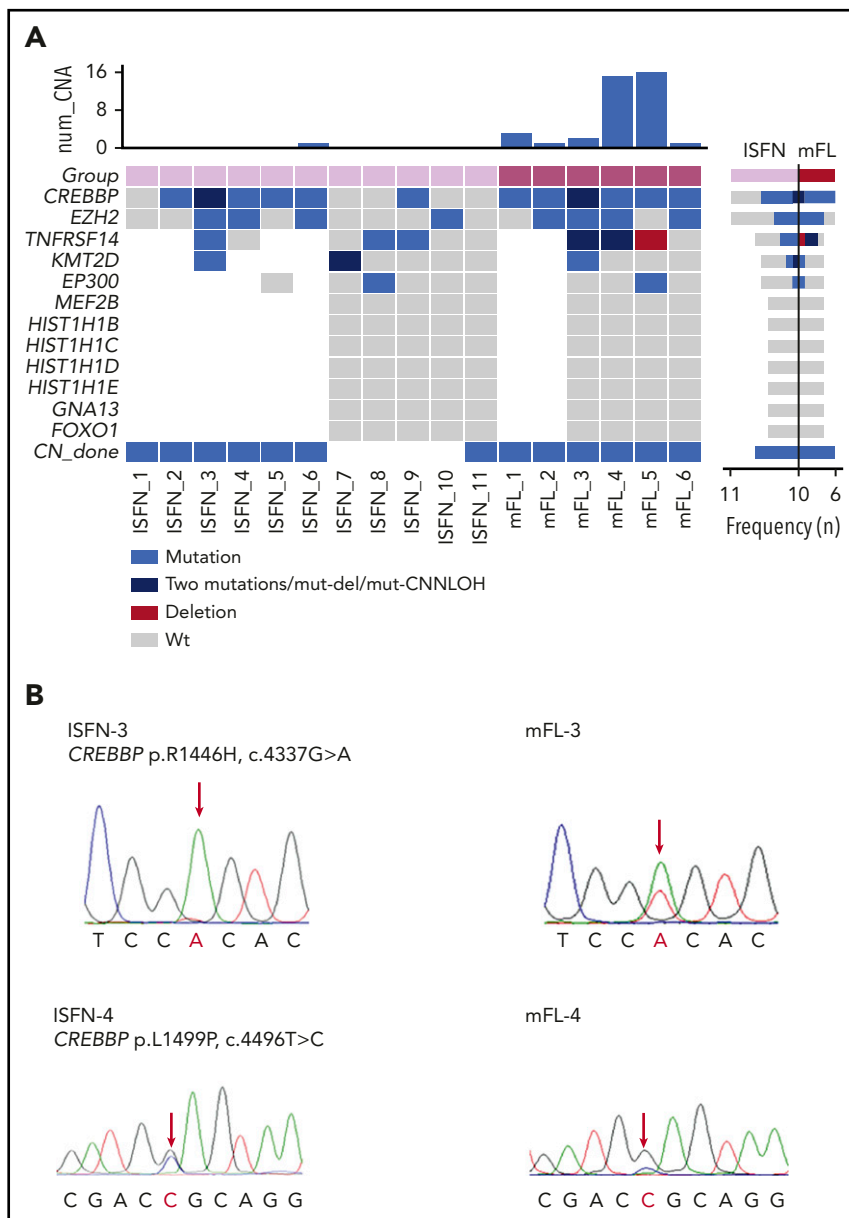
\*Verification of mutations in mFL was performed either with Sanger sequencing or Illumina MiSeq; verification of mutations in ISFN-only cases was performed with single amplicons on the Ion Torrent PGM.

†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.<sup>13</sup> 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.<sup>10,22</sup> 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.<sup>15</sup> 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.<sup>15</sup> 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.<sup>22</sup>

*EZH2* Tyr646 mutation was the second most common aberration found in ISFN, indicating that it might represent an early event.<sup>16,23,24</sup> Mutations in *KMT2D* and *TNFRSF14* have been shown to be late events in FL evolution.<sup>16</sup> 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.<sup>25</sup> 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.<sup>18</sup> The possible role of *CREBBP* mutations in the transformation of a subclone into overt mFL warrants further analysis.

## Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (QU144/1-1 [L.Q.-M.], FE597/4-1 [F.F.], and QUI44/1-1 [I.M.]), and a fellowship from Generalitat de Catalunya AGAUR FI-DGR 2017 (2017 FI\_B01004) (J.E.R.-Z.).

## 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: J.E.R.-Z., 0000-0001-7108-7738; I.B., 0000-0002-7732-0788; J. Steinhilber, 0000-0002-2855-8204; F.F., 0000-0002-5496-293X; I.S., 0000-0002-2427-9822; E.S.J., 0000-0003-4632-0301; L.Q.-M., 0000-0001-7156-5365.

Correspondence: Leticia Quintanilla-Martinez, Institute of Pathology, University Hospital Tübingen, Eberhard-Karls-University of Tübingen and Comprehensive Cancer Center, Liebermeisterstr 8, 72076 Tübingen, Germany; e-mail: leticia.quintanilla-fend@med.uni-tuebingen.de.

## Footnote

The online version of this article contains a data supplement.

## REFERENCES

1. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.
2. Limpens J, Stad R, Vos C, et al. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. *Blood*. 1995;85(9):2528-2536.
3. Cong P, Raffeld M, Teruya-Feldstein J, Sorbara L, Pittaluga S, Jaffe ES. In situ localization of follicular lymphoma: description and analysis by laser capture microdissection. *Blood*. 2002;99(9):3376-3382.
4. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science*. 1985;229(4720):1390-1393.
5. Roulland S, Kelly RS, Morgado E, et al. t(14;18) Translocation: a predictive blood biomarker for follicular lymphoma. *J Clin Oncol*. 2014;32(13):1347-1355.
6. Greeve J, Philipsen A, Krause K, et al. Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. *Blood*. 2003;101(9):3574-3580.
7. Bouska A, McKeithan TW, Deffenbacher KE, et al. Genome-wide copy-number analyses reveal genomic abnormalities involved in transformation of follicular lymphoma. *Blood*. 2014;123(11):1681-1690.
8. Cheung KJ, Shah SP, Steidl C, et al. Genome-wide profiling of follicular lymphoma by array comparative genomic hybridization reveals prognostically significant DNA copy number imbalances. *Blood*. 2009;113(1):137-148.
9. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature*. 2011;476(7360):298-303.
10. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. *Nature*. 2011;471(7337):189-195.
11. Agopian J, Navarro JM, Gac AC, et al. Agricultural pesticide exposure and the molecular connection to lymphomagenesis. *J Exp Med*. 2009;206(7):1473-1483.
12. Mamessier E, Song JY, Eberle FC, et al. Early lesions of follicular lymphoma: a genetic perspective. *Haematologica*. 2014;99(3):481-488.
13. Schmidt J, Salaverria I, Haake A, et al. Increasing genomic and epigenomic complexity in the clonal evolution from in situ to manifest t(14;18)-positive follicular lymphoma. *Leukemia*. 2014;28(5):1103-1112.
14. Roulland S, Faroudi M, Mamessier E, Sungalee S, Salles G, Nadel B. Early steps of follicular lymphoma pathogenesis. *Adv Immunol*. 2011;111:1-46.
15. Loeffler M, Kreuz M, Haake A, et al; HaematoSys-Project. Genomic and epigenomic co-evolution in follicular lymphomas. *Leukemia*. 2015;29(2):456-463.
16. Green MR, Gentles AJ, Nair RV, et al. Hierarchy in somatic mutations arising during genomic evolution and progression of follicular lymphoma. *Blood*. 2013;121(9):1604-1611.
17. Bonzheim I, Salaverria I, Haake A, et al. A unique case of follicular lymphoma provides insights to the clonal evolution from follicular lymphoma in situ to manifest follicular lymphoma. *Blood*. 2011;118(12):3442-3444.
18. Kosmidis P, Bonzheim I, Dufke C, et al. Next generation sequencing of the clonal IGH rearrangement detects ongoing mutations and interfollicular trafficking in in situ follicular neoplasia. *PLoS One*. 2017;12(6):e0178503.
19. Schmidt J, Gong S, Marafioti T, et al. Genome-wide analysis of pediatric-type follicular lymphoma reveals low genetic complexity and recurrent alterations of *TNFRSF14* gene. *Blood*. 2016;128(8):1101-1111.
20. Coelho V, Krysov S, Ghaemmaghami AM, et al. Glycosylation of surface Ig creates a functional bridge between human follicular lymphoma and microenvironmental lectins. *Proc Natl Acad Sci USA*. 2010;107(43):18587-18592.
21. McCann KJ, Johnson PW, Stevenson FK, Ottensmeier CH. Universal N-glycosylation sites introduced into the B-cell receptor of follicular lymphoma by somatic mutation: a second tumorigenic event? *Leukemia*. 2006;20(3):530-534.
22. Horton SJ, Giotopoulos G, Yun H, et al. Early loss of *Crebbp* confers malignant stem cell properties on lymphoid progenitors. *Nat Cell Biol*. 2017;19(9):1093-1104.
23. Bödör C, Grossmann V, Popov N, et al. *EZH2* mutations are frequent and represent an early event in follicular lymphoma. *Blood*. 2013;122(18):3165-3168.
24. Béguelin W, Popovic R, Teater M, et al. *EZH2* is required for germinal center formation and somatic *EZH2* mutations promote lymphoid transformation. *Cancer Cell*. 2013;23(5):677-692.
25. Hellmuth JC, Louissaint A Jr, Szczepanowski M, et al. Duodenal-type and nodal follicular lymphomas differ by their immune microenvironment rather than their mutation profiles. *Blood*. 2018;132(16):1695-1702.

DOI 10.1182/blood-2018-03-837039