

## Article

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# Molecular Cytogenetic Profiling Reveals Similarities and Differences Between Localized Nodal and Systemic Follicular Lymphomas

Heike Horn<sup>1,2</sup>, Vindi Jurinovic<sup>3,4</sup>, Ellen Leich<sup>5</sup>, Sabrina Kalmbach<sup>2</sup>, Julia Bausinger<sup>6</sup>, Annette M. Staiger<sup>1,2</sup>, Katrin S. Kurz<sup>1</sup>, Peter Möller<sup>7</sup> Heinz-Wolfram Bernd<sup>8</sup>, Alfred C. Feller<sup>8</sup>, Karoline Koch<sup>9</sup>, Wolfram Klapper<sup>9</sup>, Harald Stein<sup>10</sup>, Martin-Leo Hansmann<sup>11</sup>, Sylvia Hartmann<sup>11</sup>, Gabriel Scheubeck<sup>3</sup>, Martin Dreyling<sup>3</sup>, Wolfgang Hiddemann<sup>3</sup>, Klaus Herfarth<sup>12</sup>, Marianne Engelhard<sup>13</sup>, Andreas Rosenwald<sup>5</sup>, Eva Hoster<sup>3,4</sup>, German Ott<sup>1</sup>, for the German Low-Grade Lymphoma Study Group (GLSG)

**Correspondence:** German Ott (german.ott@rbk.de).

## ABSTRACT

Recently, we have developed novel highly promising gene expression (GE) classifiers discriminating localized nodal (LFL) from systemic follicular lymphoma (SFL) with prognostic impact. However, few data are available in LFL especially concerning hotspot genetic alterations that are associated with the pathogenesis and prognosis of SFL. A total of 144 LFL and 527 SFL, enrolled in prospective clinical trials of the German Low Grade Lymphoma Study Group, were analyzed by fluorescence in situ hybridization to detect deletions in chromosomes 1p, 6q, and 17p as well as *BCL2* translocations to determine their impact on clinical outcome of LFL patients. The frequency of chromosomal deletions in 1p and 17p was comparable between LFL and SFL, while 6q deletions and *BCL2* translocations more frequently occurred in SFL. A higher proportion of 1p deletions was seen in *BCL2*-translocation-positive LFL, compared with *BCL2*-translocation-negative LFL. Deletions in chromosomes 1p, 6q, and 17p predicted clinical outcome of patients with SFL in the entire cohort, while only deletions in chromosome 1p retained its negative prognostic impact in R-CHOP-treated SFL. In contrast, no deletions in one of the investigated genetic loci predicted clinical outcome in LFL. Likewise, the presence or absence of *BCL2* translocations had no prognostic impact in LFL. Despite representing a genetic portfolio closely resembling SFL, LFL showed some differences in deletion frequencies. *BCL2* translocation and 6q deletion frequency differs between LFL and SFL and might contribute to distinct genetic profiles in LFL and SFL.

<sup>1</sup>Department of Clinical Pathology, Robert-Bosch-Krankenhaus, Stuttgart, Germany

<sup>2</sup>Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, and University of Tuebingen, Germany

<sup>3</sup>Department of Internal Medicine III, University Hospital of the Ludwig-Maximilians-University (LMU), Munich, Germany

<sup>4</sup>Institute for Medical Information Processing, Biometry and Epidemiology, Ludwig-Maximilians-University, Munich, Germany

<sup>5</sup>Institute of Pathology, University of Würzburg and Comprehensive Cancer Center Mainfranken (CCCMF), Würzburg, Germany

<sup>6</sup>Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, and University of Tuebingen, Germany and Hain Lifescience, Nehren, Germany

<sup>7</sup>Institute of Pathology, University Hospital Ulm, Germany

<sup>8</sup>Hämatopathologie Lübeck, Germany

<sup>9</sup>Institute of Pathology, Hematopathology Section and Lymph Node Registry, Universitätsklinikum Schleswig-Holstein, Campus Kiel, Germany

<sup>10</sup>Pathodiagnostik Berlin, Germany

<sup>11</sup>Dr. Senckenberg Institute of Pathology, Goethe University Frankfurt a. Main, Germany

<sup>12</sup>Radiation Oncology, University of Heidelberg, Germany

<sup>13</sup>Department for Radiotherapy, University Hospital of Essen, Germany

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## INTRODUCTION

Follicular lymphoma (FL) is among the most common subtypes of B-cell lymphoma (B-NHL), comprising approximately 25% of all B-NHL in the Western world.<sup>1</sup> Although FL is typically characterized by an indolent clinical course, there is nevertheless marked variability in the outcome of patients: while some succumb to their disease within a few months, others survive up to 20 years.<sup>2</sup> Accordingly, finding the optimal treatment of FL is still challenging and risk stratification of the patients at diagnosis is mandatory for an individualized therapeutic approach. Up to now, assessment of patients' prognosis is mainly based on clinical parameters as defined in the FLIPI.<sup>3</sup> More recent findings, however, indicate that inherent biological features of the tumor cells or the microenvironment contribute to tumor progression and have an impact on the clinical course of patients, as illustrated by gene expression (GE) profiling, or by combined models of clinical and molecular features of tumor and nonmalignant cells.<sup>4-6</sup>

In addition to these more complex models of risk stratification, structural and numerical genetic aberrations have been identified as robust prognostic factors important in the pathogenesis and progression of FL and are identifiable by fluorescence in situ hybridization (FISH).<sup>7</sup> Apart from the founding translocation t(14;18)(q32;q21) detectable in about 85%–90% of FL, copy number alterations, predominantly deletions in chromosomes 1p, 6q, and 17p associated with inferior survival,

have been identified in 15%–30% of FL.<sup>8–12</sup> Candidate genes affected by these deletions are, among others, the tumor suppressor genes *TNFRSF14* located in chromosome 1p36 and *TP53* and/or *HIC1* in chromosome 17p13.<sup>13,14</sup> Systematic screening of deleted regions via siRNA screens allows for the identification of novel target genes and thus emphasizes the impact of genetic alterations in FL as recently shown for the *SESN1* gene in chromosome 6q.<sup>15</sup> Although the presence of these alterations has repeatedly been associated with inferior clinical survival in SFL, systematic studies in larger cohorts of uniformly treated patients in the rituximab era are still missing. Moreover, approximately 20% of patients are diagnosed in early, localized stages (clinical stages I, II, and early III).<sup>1</sup> In the vast majority of nodal FL, these localized stages (LFL) do not exhibit conspicuous histomorphological features. In spite of the fact that many of these LFL do not disseminate and hence, may have particular biological features, a comprehensive molecular characterization of LFL has not yet been performed, particularly not in uniformly treated patient cohorts. The concept that LFL may represent a different disease than systemic FL (SFL) is supported by a number of genetic findings: our group has reported that the t(14;18) occurring in up to 90% of FL is only seen in 50% of LFL,<sup>16</sup> and that FL lacking the *BCL2* translocation are characterized by a particular genetic profile as indicated by discriminative GE, miRNA, and mutational profiles.<sup>17–19</sup> Moreover, we have recently shown that LFL harbor a distinct GE profile that differs from that of SFL, and that can be used to identify patients with inferior clinical outcome also in the group of LFL.<sup>20</sup>

To gain more insight into the molecular diversity of LFL and SFL and the impact of molecular cytogenetic alterations on prognosis, we performed a comprehensive analysis within a large cohort of 684 samples from uniformly treated patients with FL enrolled in prospective clinical trials of the German Low Grade Lymphoma Study Group (GLSG).<sup>21–24</sup> In the present study, we were especially interested in re-assessing the frequency of the t(14;18) in the clinical cohorts of LFL and SFL, in clarifying the deletion status of del1p, del6q, and del17p chromosome regions in FL, the mutational status of *TNFRSF14*, and the prognostic impact of these alterations on prognosis in both LFL and SFL in patients treated with or without rituximab. While it is already known that the t(14;18) status does not affect clinical outcome in SFL,<sup>16</sup> the prognostic impact of *BCL2* rearrangements in LFL was assessed for the first time within the present study.

## MATERIALS AND METHODS

### Patients

All patients (adults older than 18 years) had been enrolled in randomized multicenter clinical trials of the GLSG treating patients with nodal FL grades 1, 2, and 3A. All tumors had been classified as FL grades 1, 2, or 3A FL according to the criteria of the World Health Organization classification of tumors of hematopoietic and lymphoid tissues<sup>1</sup> and had been diagnosed between 1996 and 2006 within a panel review process conducted by expert reference hematopathologists. Altogether, 1223 previously untreated patients with SFL in need of therapy were treated within the GLSG1996 and the GLSG2000 clinical trials and were assigned to either CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) or MCP (mitoxantrone, chlorambucil, prednisone)<sup>20</sup> or to CHOP with or without rituximab (R)<sup>21</sup> treatment arms, respectively. Patients with LFL (clinical stages I, II, and limited stage III: defined as 2–4 affected lymph regions with only 1 on one side of the diaphragm) were all  $\geq 18$  years and presented with nodal manifestations while patients with localized extranodal disease had been excluded from these trials. The patients were recruited from 2000 to 2006 and were either part of a randomized prospective trial comparing different radiotherapy (RT) regimens (n = 321)<sup>23</sup> or a prospective study investigating involved-field radiotherapy with the

addition of rituximab (n = 85) (MIR study).<sup>24</sup> FL specimens with available formalin fixed and paraffin embedded (FFPE) tissue blocks (SFL: n = 527; LFL: n = 144) were used for further analysis. Clinical endpoints for this evaluation were overall survival (OS) for SFL and progression-free survival (PFS) for LFL. PFS was chosen for the LFL cohort because the number of OS events was low within the short follow-up time. All trials were conducted in accordance with the Helsinki declaration and were approved by the local ethics committees.

### FISH and immunohistochemical stainings on tissue microarrays

Assembly of tissue microarrays (TMAs) was performed as described previously.<sup>25</sup> Altogether, 684 FL were represented on TMAs. Interphase-FISH was performed on 4- $\mu$ m thick tissue sections with hybridization conditions as previously described.<sup>25</sup> The slides were hybridized each with 125 ng spectrum orange- and spectrum green-labeled bacterial artificial chromosome (BAC)-DNA probes, following the manufacturer's protocol for Vysis CEP1, CEP6 and CEP17, respectively (Abbott Molecular, Wiesbaden-Delkenheim, Germany). Chromosomal deletions affecting 1p36.1, 1p36.31, 6q21, 6q25.1, 6q25.3, 17p13.1, and 17p13.3 were analyzed by applying locus-specific BAC probes (Suppl. Figure S1 from Source BioScience LifeSciences, Berlin, Germany), as previously described.<sup>14</sup> To evaluate the *BCL2* translocation status of FL in the MIR cohort,<sup>24</sup> a *BCL2* break apart FISH probe was used (Abbott Molecular). Tissue sections from 3 FFPE reactive lymph nodes had been used to determine the cut-off level for each probe (Suppl. Figure S1). The reference range (cut-off) was defined as the mean copy number in lymph nodes plus 3 standard deviations. The cut-off levels for the deletion probes were set at 30% each (with calculated cut-off values ranging from 24% to 28%), in accordance with previously established procedures.<sup>25</sup> At least 100 intact nuclei per case were evaluated by 2 independent, experienced observers using an epifluorescence microscope (Leica Microsystems, Bensheim, Germany). In case of divergent results, a third evaluator was consulted. The identical votes of 2 observers were recorded as the result. Sections from FFPE FL samples with known deletions in 1p, 6q and 17p by OncoScan CNV assays (n = 2, respectively) were used as positive controls. Tumor samples were considered to be deleted for 1p, 6q, or 17p when either 1 of the sub-loci, 2 or all sub-loci showed signal constellations indicative of deletion. Images were captured using the ISIS imaging system (MetaSystems, Altlußheim, Germany). A deletion score was constructed by assessing the number of 1, 2, or 3 deletions (score 3) occurring in SFL and LFL. For immunohistochemical staining of *TNFRSF14* (HVEM), the HVEM antibody (clone D-5, pH 6.0, 1:250) was used (Santa Cruz Biotechnology, Heidelberg, Germany). Cytoplasmic *TNFRSF14* staining in lymphocytes was classified into weak, moderate, and strong expression. *BCL2* staining with clone 124 and E17 and evaluation was performed as previously described.<sup>16</sup>

### Isolation of genomic DNA and sequencing analysis

Genomic DNA was isolated from FFPE tissue samples by using the QIAamp FFPE Kit as described by the manufacturer (Qiagen, Hilden, Germany). The coding sequences covering the *TNFRSF14* gene (exons 1–8) and the DNA-binding domain of TP53 (exons 4–8) were analyzed for the occurrence of mutations by direct sequencing of polymerase chain reaction (PCR) products amplified from genomic DNA using standard PCR conditions and cycling sequencing with the ABI PRISM BigDye Terminator chemistry (ThermoFisher Scientific, Schwerte, Germany), as previously published.<sup>14,26</sup>

### Statistical evaluation

For statistical evaluation of the prognostic value of genetic aberrations in SFL, we additionally stratified according to the respective treatment of SFL patients (either CHOP/MCP

or R-CHOP), since systematic studies in the rituximab era in SFL are still missing. Moreover, the underlying *BCL2* translocation status in LFL and SFL were separately included as stratification factor into statistical analysis. LFL were further separated into LFL with clinical stage I and clinical stage II. For analyses of GE profiles, subgroups were compared according their underlying GE, as assessed previously.<sup>20</sup> GE measurements were taken into account to clarify whether deletions in chromosomes 6q and 17p correlate with reduced expression of target genes in those genetic loci. Furthermore, GE analysis was performed to compare *BCL2* translocation-negative LFL patients with early progression events versus long-term survivors.

Continuous variables were compared with the Mann-Whitney *U* test and the categorical variables with the  $\chi^2$  test. Time to event variables were analyzed with Cox proportional hazards regression, and the Wald test *P* values for regression coefficients were reported. The *P* values indicated in the Kaplan-Meier plots were calculated with the log-rank test. Multivariate testing was performed adjusted for the FLIPI. The *P* values were not adjusted for multiple testing, as the results were interpreted in a purely hypothesis generating and explorative way.

## RESULTS

### Distribution of deletions in chromosomes 1p, 6q, and 17p in SFL and LFL

A total of 144 LFL and 527 SFL samples from the various clinical trial cohorts were available for FISH-based analysis on TMA format and had clinical information. Clinical data of both LFL and SFL cohorts amenable to FISH analyses closely matched the entire study population (Suppl. Table S1A, B). Patients' clinical characteristics are summarized in Suppl. Table S1C–E. Of 144 LFL, inguinal involvement was evident in 54 of 121 LFL with available information (45%), with 33 tumor samples showing inguinal localization exclusively (27%).

Deletions in chromosome arms 1p and 17p were detected in 23 of 114 (20%), and 30 of 110 (27%) LFL, respectively. Similar deletion frequencies were observed in SFL with deletions in chromosomes 1p and 17p detected in 94 of 383 (25%, *P* = 0.25) and 107 of 425 (25%, *P* = 0.39), respectively. In contrast, a significantly lower number of 6q deletions was detected in LFL (31/106, 29%) when compared with SFL (156/400, 39%, *P* = 0.02, Figure 1A, Table 1). Considering the GE<sup>20</sup> of target genes located in the deleted regions, potentially decreased expression of *AKAP12* (6q25.1, *P* = 0.070) and significantly

reduced expression of *TP53* (17p13.1, *P* = 0.007) was detected in deleted compared to non-deleted tumor samples (Suppl. Figure S2).

### Deletions frequencies affecting chromosome 1p differ between *BCL2* translocation-positive and translocation-negative LFL

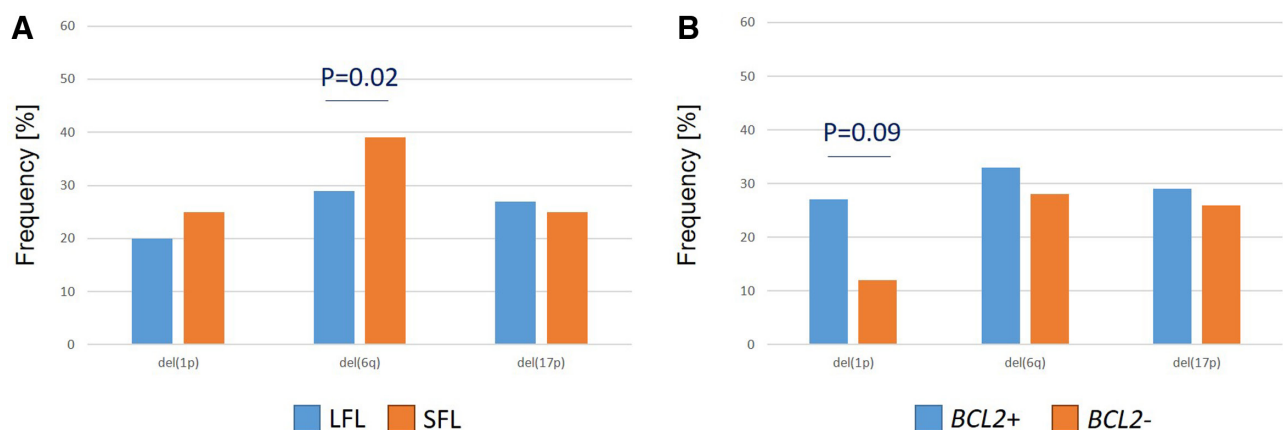
Compared with SFL, a distinctly lower frequency of *BCL2* translocations was observed in the MIR LFL cohort (RT plus rituximab<sup>24</sup>) with only 44% samples (15/34) harboring the translocation, all of which showed high *BCL2* protein expression. In the remaining 19 samples without *BCL2* translocation, *BCL2* protein expression was observed in 15 of 19 (79%), while 4 samples (21%) lacked *BCL2* staining (Table 1).

Deletion status was assessed for chromosomal regions 1p, 6q, and 17p in *BCL2* translocation-positive (*BCL2*-positive) and translocation-negative (*BCL2*-negative) LFL and SFL. Deletions in chromosomes 6q and 17p were evenly distributed between *BCL2*-negative and *BCL2*-positive LFL and SFL (Table S2A and S2B). There was a hypothesis generating trend toward a higher frequency of deletions in 1p for *BCL2*-positive LFL with 27% (16/59) versus 12% (6/50) in *BCL2*-negative LFL (*P* = 0.09; Figure 1B, Suppl. Table S2A). This clearly contrasts the situation in SFL where the distribution of 1p deletions was almost similar in *BCL2*-positive and *BCL2*-negative samples (10/44, 23% vs 71/285, 25%, *P* = 0.90, Suppl. Table S2B).

Since inguinal presentation had been associated with early clinical FL stages and in particular to FL without *BCL2*-translocation,<sup>27,28</sup> LFL samples with and without inguinal involvement were compared. Inguinal presentation was observed in 54 of 121 LFL samples, and exclusive inguinal involvement was present in 33 (27%). No differences were observed concerning their deletion status in the entire LFL cohort (data not shown). Although not significant, in LFL samples with inguinal involvement lacking *BCL2* translocation, the frequency of deletions in 1p and 6q was lower compared with the remaining cases (del1p: 1/22 vs 7/29, *P* = 0.13; del6q: 2/20 vs 8/26, *P* = 0.18). There was no difference in the frequency of 17p deletions.

### The presence or absence of the *BCL2* translocations does not affect prognosis in LFL

We had previously shown that the presence or absence of the t(14;18)/*BCL2* translocation is not associated with prognosis in SFL.<sup>16</sup> Analysis of the clinical data of LFL patients with and without the *BCL2* translocation in the present study equally failed to reveal any difference in PFS. Specifically, no significant difference was observed in the RT cohort (HR = 0.90, 95% CI = 0.51–1.57, *P* = 0.71, Figure 2A) nor in the MIR cohort of



**Figure 1. Frequency of deletions in LFL and SFL.** (A) Frequencies of deletions in chromosomes 1p, 6q, and 17p determined by FISH analysis of distinct genetic loci (1p: 1p36.1 and 1p36.31; 6q: 6q21, 6q25.1 and 6q25.3; 17p: 17p13.1 and 17p13.3) in LFL vs SFL and (B) in *BCL2*-rearranged (*BCL2*+) vs *BCL2*-non-rearranged (*BCL2*-) LFL. FISH = fluorescence in situ hybridization; LFL = localized follicular lymphoma; SFL = systemic follicular lymphoma.

RT plus rituximab (HR = 2.07, 95% CI = 0.49–8.68,  $P = 0.32$ , Figure 2B).

However, a small proportion of *BCL2*-negative LFL ( $n = 11$ ) in the RT only cohort showed very early progression within 2 years. On the other hand, late events ( $\geq 11$  years after diagnosis) were exclusively observed in *BCL2*-positive LFL (Figure 2A), while 12 *BCL2*-negative LFL showed long-term OS without events. There was no difference between *BCL2*-negative patients with early progression and long-term survival regarding *BCL2* protein expression, *TNFRSF14* mutation or deletion status of the samples. When comparing GE data<sup>20</sup> from patients with early progression ( $n = 11$ ) and long-term survival ( $n = 12$ ), the hypothesis of an increased expression of *LGMN* (median GE: 1238 and 2182, respectively), *CD8A* (median GE: 1626 and 2790, respectively), and *CD69* (median GE: 1979 and 2925, respectively) was

generated in patients with long-term survival ( $P = 0.067$ ,  $P = 0.061$ , and  $P = 0.089$ , respectively, Suppl. Figure S3).

#### Prognostic impact of deletions in 1p, 6q, and 17p in SFL and LFL

Deletion status of 1p, 6q, and 17p was correlated with the clinical course of patients with LFL and SFL. No significant association with PFS for RT or MIR patients was observed for chromosomal alterations in 1p, 6q, and 17p in LFL (Suppl. Tables S3A and S3B).

Deletions in chromosomes 1p and 17p were significantly associated with inferior survival in the entire cohort of SFL patients (1p: HR = 1.64, 95% CI = 1.08–2.50,  $P = 0.021$ ; 17p: HR = 1.70, 95% CI = 1.11–2.59,  $P = 0.014$ ), while a trend toward a negative prognostic impact was observed for 6q deletions (HR = 1.43, 95% CI = 0.96–2.13,  $P = 0.076$ , Suppl. Table S4, Suppl. Figure S2).

However, if SFL patient cohorts were separated according to treatment with either CHOP/MCP or R-CHOP, univariate analysis revealed a prognostic impact of 6q and 17p deletions exclusively in the CHOP/MCP-cohort (HR = 1.74, 95% CI = 1.00–3.02,  $P = 0.051$ ), while a hypothesis generating trend toward inferior survival was observed for 6q deletions (HR = 1.64, 95% CI = 0.99–2.73,  $P = 0.06$ , Suppl. Table S4). In multivariate analysis adjusted for the FLIPI in CHOP/MCP-treated patients, deletions in 17p predicted inferior clinical outcome in CHOP/MCP-treated patients, while no significant effect was observed for deletions in 6q (Suppl. Table S5).

In the R-CHOP cohort, only deletions in chromosome 1p were significantly associated with inferior clinical outcome in univariate analysis (HR = 2.32, 95% CI = 1.22–4.42,  $P = 0.02$ , Suppl. Table S4, Figure 3) and in multivariate analysis adjusted for the FLIPI (Suppl. Table S5).

In conclusion, inferior clinical outcome was observed in R-CHOP-treated SFL that harbored chromosome 1p deletions. Deletions in chromosome 6q and 17p were not associated with differences in OS in R-CHOP, but only in CHOP/MCP-treated patients. In LFL none of the deletions showed a prognostic impact.

**Table 1**

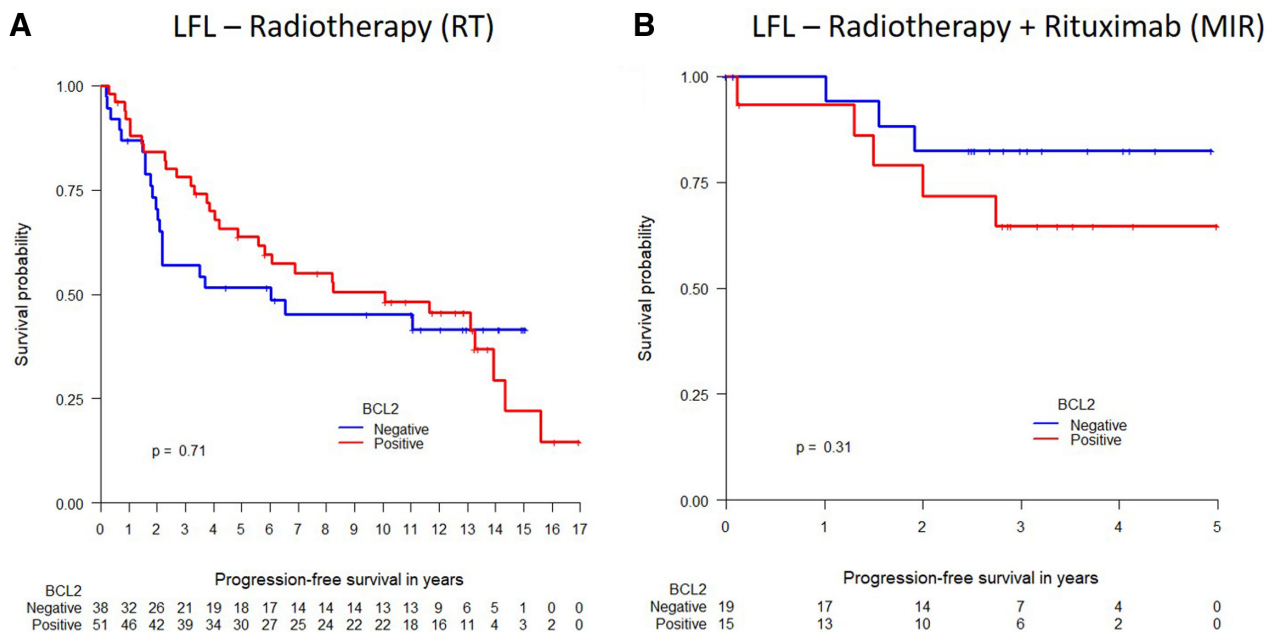
#### Overview About Clinical and Molecular Information of LFL and SFL

	LFL n = 144	SFL n = 527	P Value
Inguinal presentation	54/121 (45%)	Not evaluated	Not evaluated
Inguinal localization exclusively	33/54 (61%)	Not evaluated	Not evaluated
BCL2 translocation	15/34 (44%) <sup>a</sup>	363/422 (86%) <sup>b</sup>	Not evaluated
BCL2 protein expression			
BCL2 translocation positive	15/15 (100%)		Not evaluated
BCL2 translocation negative	15/19 (79%)		Not evaluated
Deletions in 1p	23/114 (20%)	94/383 (25%)	0.25
Deletions in 6q	31/106 (29%)	156/400 (39%)	0.02
Deletions in 17p	30/110 (27%)	107/425 (25%)	0.39
TNFRSF14 mutation	72/107 (67%)	188/352 (53%)	0.02
Exons 1–2 mutated	29/76 (38%)	110/192 (57%)	0.005
Exons 3–5 mutated	32/76 (42%)	52/192 (27%)	0.02

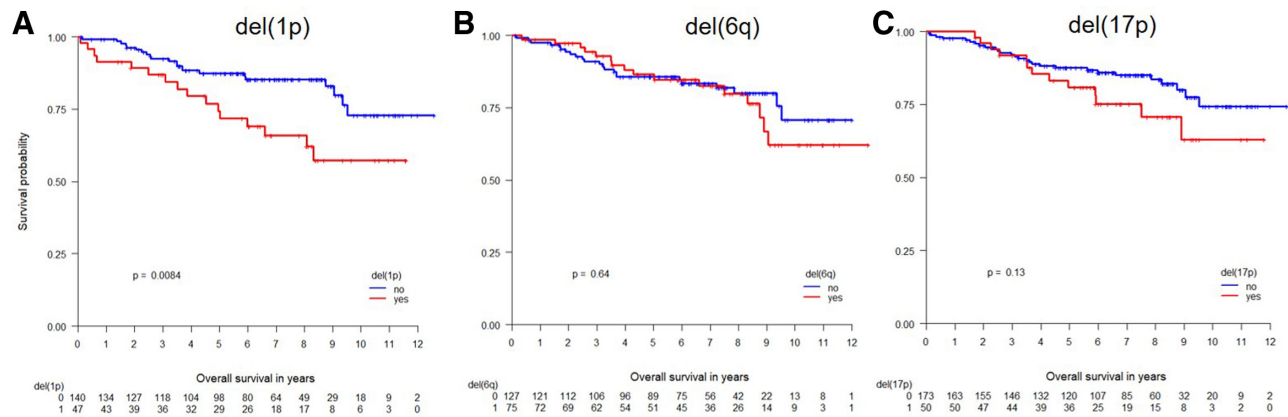
<sup>a</sup>Referring to the data provided in the present study.

<sup>b</sup>Referring to data presented by Leich et al.<sup>16</sup>

LFL = localized follicular lymphoma; SFL = systemic follicular lymphoma.



**Figure 2. Impact of *BCL2* translocation on PFS in LFL and SFL.** Kaplan-Meier plots illustrating PFS in the RT patient cohort of LFL according to *BCL2* translocation status (A) and in the MIR patient cohort treated with RT plus the addition of rituximab (B). LFL = localized follicular lymphoma; PFS = progression-free survival; MIR = mabthera (R) and involved field radiation; RT = radiotherapy; SFL = systemic follicular lymphoma.



**Figure 3. Impact of chromosomal deletions on OS.** Kaplan-Meier plots illustrating OS in univariate analysis in the R-CHOP-treated cohort of SFL: (A) Chromosome 1p [del(1p)]. (B) Chromosome 6q [del(6q)]. (C) Chromosome 17p [del(17p)]. CHOP = cyclophosphamide, doxorubicin, vincristine, prednisone; OS = overall survival.

#### Occurrence of multiple deletions is associated with advanced age and inferior clinical outcome of SFL patients in the pre-rituximab era only

A deletion score was constructed by assessing the number of 1, 2, or 3 deletions in either 1p, 6q, and 17p or combinations of those in 282 SFL. One hundred nineteen of 282 (42%) samples had no deletions in the chromosomal regions analyzed. One, 2, or 3 deletions were found in 105 (37%), 47 (17%), and 11 (4%) SFL, respectively. Compared with patients without deletions, in the MCP/CHOP (=pre-rituximab) cohort patients with any number of deletions had significantly poorer OS in univariate analysis (log-rank  $P = 0.024$ , Figure 4A), but not in the R-CHOP-treated patients ( $P = 0.17$ , Figure 4B, Suppl. Table S6). This finding was confirmed in multivariate analysis adjusted to the FLIPI parameters (Suppl. Table S7). The occurrence of 3 simultaneous deletion events was significantly associated with advanced age in patients with SFL in the entire cohort (Figure 4C,  $P = 0.01$ ), and specifically in the MCP/CHOP cohort ( $P = 0.0016$ ), but not in the R-CHOP-treated patients ( $P = 0.40$ ). In contrast, in the LFL RT cohort, no significant differences in survival were evident for patients with either no ( $n = 28$ , 40%), 1 ( $n = 27$ , 39%), 2 ( $n = 12$ , 17%), or 3 ( $n = 3$ , 4%) deletions ( $P > 0.99$ ) and the number of deletions was not associated with age.

#### Mutations in *TNFRSF14* within a particular mutation hotspot occur more frequently in LFL

Mutations affecting *TNFRSF14* were more frequently observed in LFL (72/107, 67%) compared with SFL (188/352, 53%,  $P = 0.02$ , Table 1), especially with a trend toward higher mutation rates in *BCL2*-positive LFL (43/57, 75%) versus *BCL2*-negative LFL (23/39, 59%,  $P = 0.14$ , Suppl. Tables S2A, B). This observation is well in line with our finding of a higher deletion frequency of 1p in *BCL2*-positive LFL (Figure 1C). When comparing the GE of T-cell markers (CD4 and FOXP3<sup>20</sup>) in samples with and without *TNFRSF14* mutations, no significant difference was detected (data not shown). Mutations in *TNFRSF14* were not associated with OS in the SFL cohort treated with MCP/CHOP (HR = 0.71, 95% CI = 0.41–1.26,  $P = 0.24$ ; Figure 5A), but in the cohort of R-CHOP-treated patients with SFL (HR = 2.01, 95% CI = 1.04–3.89,  $P = 0.035$ ; Figure 5B). There was no significant impact of *TNFRSF14* mutation on PFS in the LFL cohort (Figure 5C). The overall presence of *TNFRSF14* mutations did not correlate with 1p deletions, neither in LFL ( $r = 0.29$ ), nor in SFL ( $r = 0.12$ ). LFL and SFL with both mutation and deletion did not show shorter OS or PFS when compared with wild-type samples or samples

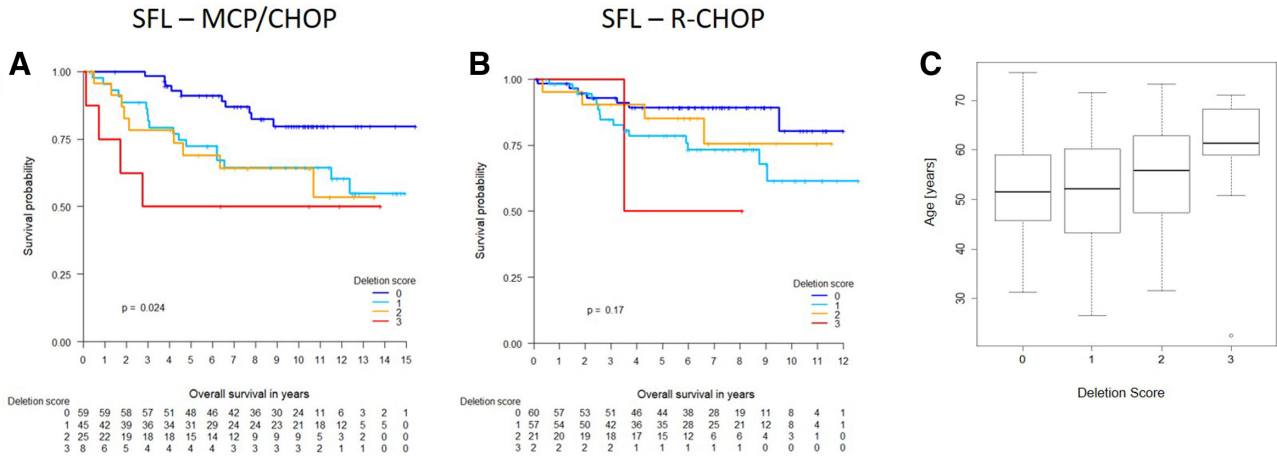
with either mutations or deletions (data not shown). Different types of mutations were equally distributed in LFL and SFL, showing a high percentage of missense mutations (62% each), followed by frameshift mutations (22% in LFL vs 21% in SFL) and premature STOP codons (16% in LFL vs 17% in SFL, Figure 5D). Notwithstanding this, significant differences between LFL and SFL emerged when the distribution of mutations in the different *TNFRSF14* domains were analyzed. Notably, mutations in LFL were more frequently observed in exons 3 to 5 (32/76, 42% vs 52/192, 27% in SFL;  $P = 0.02$ ). In contrast, SFL were more often associated with mutations in exons 1 and 2 (110/192, 57% vs 29/76, 38% in LFL;  $P = 0.005$ ; Figure 5E, Table 1).

To investigate whether underlying *TNFRSF14* mutations might affect expression of the protein, 34 FL samples with differing mutational profiles (*TNFRSF14* mutated:  $n = 18$ ; *TNFRSF14* WT:  $n = 16$ ) were subjected to immunohistochemistry. Overall, protein expression was highly variable showing weak, moderate, or strong cytoplasmic staining. Strong *TNFRSF14* expression was found in both *TNFRSF14* mutated and unmutated cases in similar proportions (10/18, 56% and 9/16, 56%, respectively). Moderate expression was observed in 4 of 18 (22%) mutated samples and in 7 of 16 (43%) unmutated samples. Weak or no expression, however, was only detected in mutated samples (4/18, 22%). Interestingly, *TNFRSF14* mutations more frequently occurring in exons 3–5 in LFL predominantly resulted in reduced protein expression (Figure 5F) when compared with mutations affecting SFL in exons 1 and 2 (or 6–8, Figure 5G). The *TNFRSF14* antibody epitope was not affected by the mutations in those samples. Of the 4 samples with reduced *TNFRSF14* expression, 2 samples harbored 1p deletions in addition to *TNFRSF14* mutations. One sample presented with *TNFRSF14* mutation only, without 1p-deletion. From the remaining sample, FISH results were not available.

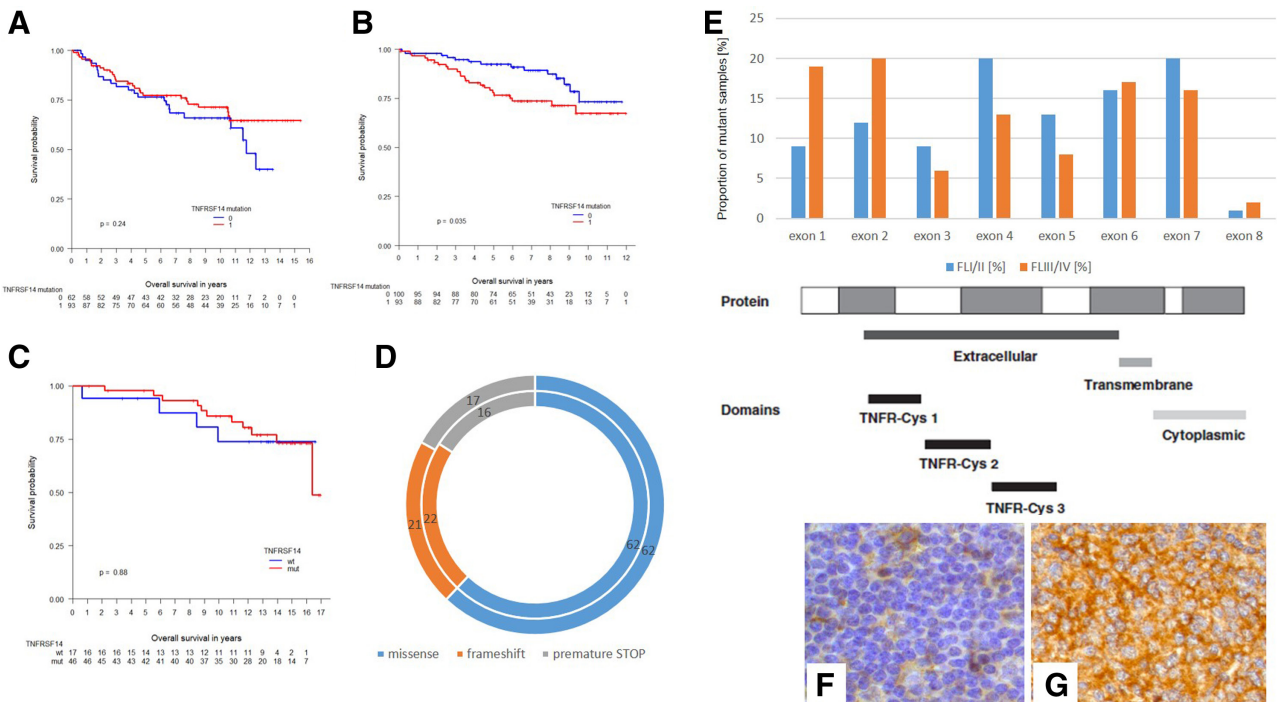
In summary, a higher *TNFRSF14* mutation frequency was observed in *BCL2*-positive LFL. The mutational profile differed between LFL and SFL, affecting different exonic regions in the respective FL subtype. Protein expression was reduced in *TNFRSF14* mutated samples, in particular in samples harboring mutations in exons 3–5 that predominantly occurred in LFL.

## DISCUSSION

SFL, in particular those with early progression and poor outcome, have been in the focus of research for many years.<sup>5,29,30</sup> In contrast, the molecular mechanisms contributing to the development and progression of nodal LFL remained virtually



**Figure 4. Prognostic relevance of the number of deletions in chromosomes 1p, 6q, and 17p.** Kaplan-Meier plots illustrating OS in MCP/CHOP-treated SFL (A) and in R-CHOP-treated SFL (B). An increasing number of deletions split up the survival curves into patient cohorts with clearly differing risk profiles of no deletions (0), 1 or 2 deletions, and 3 deletions in SFL. In MCP/CHOP-treated SFL, a deletion score of 3 was significantly associated with increasing age (C). CHOP = cyclophosphamide, doxorubicin, vincristine, prednisone; MCP = mitoxantrone, chlorambucil, prednisone; OS = overall survival; SFL = systemic follicular lymphoma.



**Figure 5. Prognostic relevance of the mutation status of TNFRSF14 in LFL and SFL.** Kaplan-Meier plot of OS in SFL treated with MCP/CHOP (A) and in patients with SFL treated with R-CHOP (B). Kaplan-Meier plot of PFS in patients with LFL, here shown for LFL of the RT cohort (C). Similar distribution of mutation types in LFL (inner circle) and SFL (outer circle) (D). Frequency of mutations in LFL (blue) and SFL (orange) in different exons revealed an accumulation of *TNFRSF14* mutations in LFL in the extracellular domain TNFR-Cys2/Cys3 (E) (*TNFRSF14* domains according to 13). Mutations in LFL occurred more frequently in exons 3-5 and predominantly resulted in reduced *TNFRSF14* protein expression (F) when compared to mutations affecting SFL in exons 1 and 2 (G). CHOP = cyclophosphamide, doxorubicin, vincristine, prednisone; LFL = localized follicular lymphoma; MCP = mitoxantrone, chlorambucil, prednisone; OS = overall survival; PFS = progression-free survival; RT = radiotherapy; SFL = systemic follicular lymphoma.

unknown. Within previous GLSG studies, we have shown that molecular features between LFL and SFL are different, particularly with regard to GE profiling which enables the identification of a LFL patient subgroup that more closely resembles SFL and displays inferior clinical outcome, indicating the need for improved molecular characterization of LFL.<sup>20</sup> In addition

to differences in GE profiles, LFL and SFL differ also in the make-up of their T-cell content<sup>31</sup> and in the frequency of genetic alterations, especially the *BCL2* translocation: while about 85% of SFL are characterized by this hallmark genetic event, it is found in only about 50% of LFL.<sup>16</sup> A lower *BCL2* translocation frequency of 44% was also observed within an unrelated

LFL cohort analyzed in the current study. The validation of this finding is of particular importance since the translocation status of *BCL2* might indicate—or even influence—mutational profiles distinguishing *BCL2* translocated from *BCL2* non-translocated FL, although the majority of patient samples without *BCL2* translocation (79%) were still associated with an increased expression of *BCL2* protein, as had also been shown previously.<sup>16,18</sup>

It can, therefore, be speculated that LFL and SFL might either represent different diseases or that they at least undergo divergent evolution. A pathway model for FL had been proposed, suggesting poor- and good-prognosis routes of patients that are determined by the sequence of specific genetic alterations, as for example, the occurrence of deletions in chromosomal regions 1p, 6q, and 17p (reviewed in 7). To obtain deeper insights into the tumor biology and prognosis of LFL, we performed a FISH-based screening of genetic alterations that have repeatedly been shown to constitute robust prognostic indicators using different techniques and/or analytical platforms.<sup>8–10,12</sup> Deletions in chromosomes 1p and 17p were detected with similar frequencies in LFL (20% and 27%, respectively) and SFL (25% and 25%, respectively). These findings are well in line with previously published data derived from cohorts of SFL.<sup>8–10,12</sup> In contrast, a significantly higher proportion of 6q deletions and *BCL2* translocations was observed in SFL (39% and 89%<sup>16</sup>) when compared with LFL (29% and 44%, respectively). Very hypothetically, these findings could indicate an oncogenic interaction of 6q deletion and *BCL2* translocation. *PRDM1* (*BLIMP1*) that has been described to be involved in plasma cell differentiation<sup>32</sup> is affected by the 6q deletion. The interplay of a *PRDM1*-deletion in SFL, that more frequently harbor *BCL2* translocations, might support the germinal center (GC) differentiation of B-cells. In contrast, LFL more frequently lacking both 6q deletion and *BCL2* translocation are possibly driven toward plasma cell differentiation, a finding that corroborates our earlier data of late GC phenotypes in *BCL2*-negative FL.<sup>19</sup>

Moreover, considering the underlying *BCL2* translocation status, 1p deletions occurred significantly more often in *BCL2*-positive LFL (27%) versus *BCL2*-negative LFL (12%,  $P = 0.0085$ ). Although the majority of *BCL2*-negative LFL (79%) still showed high expression of *BCL2* protein, these findings suggest that LFL might acquire different genetic repertoires to drive tumor pathogenesis and/or progression possibly driven by the constraints of a pre-existing *BCL2* translocation and/or the lack of 6q deletions. Previous data from Leich et al<sup>16</sup> showed that SFL with or without *BCL2* translocation do not differ in their clinical course (TTF, OS). This finding could be corroborated also for LFL in the present study. However, a striking accumulation of early clinical events was noticeable in *BCL2*-negative LFL treated with RT. Interestingly, retrospective analyses of the data revealed that this phenomenon had already been evident in the SFL cohort previously analyzed by Leich et al<sup>16</sup> regarding TTF and OS. Comparing GE data<sup>20</sup> of *BCL2*-negative LFL with early progression and long-term survival showed an increased expression of genes involved in the recruitment of activated and cytotoxic T-cells in the long-term survivors, possibly providing a microenvironment more favorable to improved outcome (reviewed in 33). The impact of T-cells in FL pathogenesis was recently also shown by Mondello et al,<sup>6</sup> implementing the content of intrafollicular CD4-positive T-cells into the FLIPI and thereby generating a bio-clinical risk model (BioFLIPI). Of particular interest, an increased number of follicular T-helper cells has already been associated with early clinical FL stages, while those cells are less frequently observed in SFL.<sup>31</sup>

Deletions in chromosomes 1p, 6q, and 17p were associated with inferior survival in the entire cohort of SFL, thus reproducing findings described in the literature.<sup>8–10,12</sup> In contrast, none of the deletions were associated with outcome in LFL. Interestingly enough, however, only deletions in 1p retained their prognostic

impact in R-CHOP-treated patients with a diagnosis of SFL in univariate and multivariate analysis. It is well known that some biomarkers are of limited value considering risk assessment in the R-CHOP era (reviewed by 34), as is possibly the fact for deletions in chromosome 6q and 17p. Accordingly, the established deletion score also failed to retain its predictive power in the R-CHOP era, implying that addition of rituximab overcomes the negative effect of both, singular and simultaneously occurring deletions. Intriguingly, none of the analyzed genetic deletions was associated with clinical outcome in LFL, while they predict outcome in advanced stage disease. This suggests that either these deletions are acting in concert with other genetic alterations occurring later, such as mutations or epigenetic alterations, or that the clinical course of the disease can be successfully influenced by appropriate therapy in early evolutionary—yet established—stages of the disease.

To better understand the prognostic impact of deletions in chromosome region 1p36.31, we also analyzed the mutational status of the *TNFRSF14* (also known as herpes virus entry mediator a, *HVEM*) gene localized within this genetic region. *TNFRSF14* mutations are among the most prevalent genetic lesions in GC-derived lymphomas, occurring in approximately 50% of FL. Within the present study, a higher *TNFRSF14* mutation frequency was observed in LFL (67%) when compared with SFL (53%), possibly indicating different *TNFRSF14*-driven microenvironmental interactions in LFL and SFL. Regarding a possible interaction with tumor-adjacent T-cells, the mRNA levels of the T-cells markers CD4 and FOXP3 were compared in *TNFRSF14* mutant and wild-type FL. However, no significant differences were observed.

Mutations in *TNFRSF14* were not correlated with 1p deletions either in LFL nor in SFL, suggesting the occurrence of homozygous mutations possibly resulting in copy number neutral LOH of the *TNFRSF14* gene locus (although not detectable with FISH and Sanger sequencing techniques used in this study). In previous studies, the prognostic impact of *TNFRSF14* mutations has been controversially discussed, showing correlations with superior prognosis<sup>13</sup> but also with poor clinical outcome.<sup>35</sup> Comparable to our results, in pediatric FL that exclusively present in localized clinical stages, the clinical outcome of patients with and without mutations did not differ significantly.<sup>36</sup> In SFL, however, patients with *TNFRSF14* mutations had an inferior clinical course in the cohort treated with R-CHOP, but not in the MCP/CHOP-treated patients. These findings are in line with previous reports identifying *TNFRSF14* mutation as a poor prognostic factor in cohorts of rituximab-treated systemic FL.<sup>35</sup> In contrast, Launay et al<sup>13</sup> described a more favorable outcome for patients harboring *TNFRSF14* mutations. However, it is not clear whether this latter study included LFL patients. Furthermore, only 76% of the patients were treated with rituximab, thus possibly not reflecting the effects observed for SFL patients treated with rituximab only.

In summary, although no differences in the occurrence of deletions in 1p and 17p were observed between LFL and SFL, 6q deletions were shown to be significantly enriched in SFL. Moreover, decreased *BCL2* translocation frequency in LFL and different numbers of 1p deletions in *BCL2* positive and negative LFL indicate that LFL and SFL might harbor a different genetic portfolio. This is paralleled by the fact that different mutational hotspots in *TNFRSF14* were observed between LFL and SFL. Although we were able to corroborate earlier findings of a striking negative prognostic impact of deletions in 1p (R-CHOP-treated patients), 6q, and 17p (MCP/CHOP-treated patients), no such association was seen in LFL. Taken together, this study shed more light on the molecular similarities and differences of LFL and SFL. More detailed investigations, however, are needed to elucidate the similarities and differences of LFL and SFL and to find out whether LFL follow evolutionary paths comparable to SFL or should be viewed as a closely related, yet different disease.

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## AUTHOR CONTRIBUTIONS

HH, GO did conception and design. PM, H-WB, ACF, WK, HS, MLH, AR, GO: pathology reference panel. KSK, PM, H-WB, ACF, KK, WK, HS, MLH, SH, MD, WH, KH, ME, AR, GO: provision of study materials or patients. HH, VJ, EL, SK, JB, AMS, KSK, GS, MD, WH, KH, ME, AR, EH, GO: collection and assembly of data. VJ, EH: biometry. HH, VJ, EH, GO: manuscript writing.

## DISCLOSURES

MD is an editor at HemaSphere. The remaining authors have no conflicts of interest to disclose.

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