Clinical Cancer Research

Genetics and Prognostication in Splenic Marginal Zone Lymphoma: Revelations from Deep Sequencing

Marina Parry[1], Matthew J.J. Rose-Zerilli[1], Viktor Ljungström[2], Jane Gibson[3], Jun Wang[4], Renata Walewska[5], Helen Parker[1], Anton Parker[5], Zadie Davis[5], Anne Gardiner[5], Neil McIver-Brown[5], Christina Kalpadakis[6], Aliki Xochelli[7], Achilles Anagnostopoulos[8], Claudia Fazi[9], David Gonzalez de Castro[10], Claire Dearden[10], Guy Pratt[11], Richard Rosenquist[2], Margaret Ashton-Key[1], Francesco Forconi[1], Andrew Collins[12], Paolo Ghia[9], Estella Matutes[13], Gerassimos Pangalis[14], Kostas Stamatopoulos[7,8], David Oscier[1,5], and Jonathan C. Strefford[1]

Abstract

Purpose: Mounting evidence supports the clinical significance of gene mutations and immunogenetic features in common mature B-cell malignancies.

Experimental Design: We undertook a detailed characterization of the genetic background of splenic marginal zone lymphoma (SMZL), using targeted resequencing and explored potential clinical implications in a multinational cohort of 175 patients with SMZL.

Results: We identified recurrent mutations in *TP53* (16%), *KLF2* (12%), *NOTCH2* (10%), *TNFAIP3* (7%), *MLL2* (11%), *MYD88* (7%), and *ARID1A* (6%), all genes known to be targeted by somatic mutation in SMZL. *KLF2* mutations were early, clonal events, enriched in patients with del(7q) and *IGHV1-2*04* B-cell receptor immunoglobulins, and were associated with a short median time to first treatment (0.12 vs. 1.11 years; $P = 0.01$). In multivariate analysis, mutations in *NOTCH2* [HR, 2.12; 95% confidence interval (CI), 1.02–4.4; $P = 0.044$] and 100% germline *IGHV* gene identity (HR, 2.19; 95% CI, 1.05–4.55; $P = 0.036$) were independent markers of short time to first treatment, whereas *TP53* mutations were an independent marker of short overall survival (HR, 2.36; 95 % CI, 1.08–5.2; $P = 0.03$).

Conclusions: We identify key associations between gene mutations and clinical outcome, demonstrating for the first time that *NOTCH2* and *TP53* gene mutations are independent markers of reduced treatment-free and overall survival, respectively. *Clin Cancer Res; 21(18); 4174–83. ©2015 AACR.*

Introduction

Splenic marginal zone lymphoma (SMZL) is a rare chronic B-cell lymphoproliferative disorder that predominantly affects elderly patients and involves the spleen, bone marrow, and usually the peripheral blood (1). The diagnosis is based on a combination of clinical, morphologic, histopathologic, and immunophenotypic data that serve to distinguish it from other splenic lymphomas (1). Additional distinctive biologic features of SMZL include a remarkable bias to the expression of clonotypic B-cell receptors (BcR) using the *IGHV1-2*04* gene and frequent deletions of chromosome 7q (2, 3). The median survival of SMZL is around 10 years; 70% of patients require treatment for progressive, symptomatic disease and 5% to 10% undergo transformation into large B-cell lymphoma. Response rates to splenectomy, single-agent rituximab and rituximab plus chemotherapy are high, but approximately 40% of patients develop progressive disease within 5 years (4, 5).

[1]Cancer Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. [2]Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden. [3]Centre for Biological Sciences, University of Southampton, Southampton, United Kingdom. [4]Centre for Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom. [5]Department of Pathology, Royal Bournemouth Hospital, Bournemouth, United Kingdom. [6]Department of Hematology, School of Medicine, University of Crete, Heraklion, Greece. [7]Institute of Applied Biosciences, Center for Research and Technology, Thessaloniki, Greece. [8]Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece. [9]Division of Molecular Oncology, Department of Onco-Haematology, IRCCS Istituto Scientifico San Raffaele, Fondazione Centro San Raffaele, Università Vita-Salute San Raffaele, Milan, Italy. [10]Heamato-Oncology Unit, Division of Molecular Pathology, Institute for Cancer Research, Sutton, United Kingdom. [11]School of Cancer Studies, University of Birmingham, Birmingham, United Kingdom; Department of Haematology, Heart of England NHS Foundation Trust, Birmingham, United Kingdom. [12]Genetic Epidemiology and Bioinformatics, Faculty of Medicine, University of Southampton, United Kingdom. [13]Haematopathology Unit, Hospital Clinic, Barcelona University, Villarroel, Barcelona, Spain. [14]Department of Hematology, Athens Medical Center, Athens, Greece.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

M. Parry, M.J.J. Rose-Zerilli, and V. Ljungström contributed equally to this article.

Corresponding Author: Jonathan C. Strefford, Cancer Genomics, Cancer Sciences, Somers Cancer Research Building, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom. Phone: 44-23-80795246; Fax: 023-81205152; E-mail: JCS@soton.ac.uk

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Translational Relevance

This multinational study identifies genomic and immunogenetic factors with prognostic significance in splenic marginal zone lymphoma (SMZL), a rare B-cell non–Hodgkin lymphoma. Genomic mutations in *TP53*, *KLF2*, *NOTCH2*, and *TNFAIP3* were found collectively in more than 40% of cases and 13% used IGHV genes with no somatic hypermutation (SHM). *TNFAIP3* mutations were associated with an increased risk of high-grade transformation. IGHV genes lacking SHM, *KLF2*, and *NOTCH2* mutations were associated with shorter time-to-first treatment, whereas *TP53* and *MYD88* mutations were predictors of short and long overall survival, respectively. In contrast to cytogenetic and FISH data, *NOTCH2* and *TP53* mutations remained independent factors of outcome in multivariate analyses which included the established prognostic markers: anemia and thrombocytopenia. Genomic and immunogenetic data have the potential to aid diagnosis and influence the timing and choice of treatment in SMZL.

Easily measured, non–disease-specific, parameters such as hemoglobin, platelet count, lactate dehydrogenase (LDH), serum albumin, and the presence of extrahilar lymphadenopathy have prognostic significance in multivariate analysis for overall survival and have led to the introduction of scoring systems and a prognostic index (6–8). In contrast, the value of biomarkers to predict outcome is much less clear. Unmutated immunoglobulin heavy variable (*IGHV*) genes, karyotypic complexity, *TP53* loss/mutation alone or in combination with del(8p), and del(14q) have all been suggested to have an adverse prognostic significance in univariate analyses but none have been confirmed in multivariate analyses (3, 9–13). Candidate gene screening and, more recently, whole genomic or whole exomic sequencing (WES) studies in small patient cohorts have identified recurrent mutations of genes involved in NOTCH, BcR, Toll-like receptor (TLR), and NF-κB signaling pathways, chromatin remodeling, and the cytoskeleton (14–17). However, targeted resequencing of larger patient cohorts has resulted in conflicting data on the incidence and prognostic significance of *NOTCH2* mutations, whereas little is known about the clinical significance of other gene mutations (14, 15).

These observations highlight the need for larger studies to determine a more comprehensive picture of the clinical significance of gene mutations in SMZL. Accordingly, using a targeted resequencing approach, we screened for mutations in the largest cohort of well-characterized SMZL cases published to date ($n = 175$) and identified a number of gene mutations that contribute to reduced outcome in SMZL. Most notably, we demonstrate for the first time that previously known gene mutations (*NOTCH2* and *TP53*) are independent markers of poor survival.

Materials and Methods

Patients and samples

Table 1 describes our cohort of 175 patients with SMZL from 8 centers across Europe, all meeting established diagnostic criteria (18). The mean time from diagnosis to sampling was 3.2 years (0–24; SD, 4.7). Mantle cell lymphoma (MCL) was excluded in $CD5^+$ cases using FISH and conventional cytogenetics. Splenic lymphoma/leukemia unclassifiable (SLLU) was precluded either by splenic histopathology or by omission of SLLU-variant cases with distinctive cytology, such as those with splenic diffuse red pulp lymphoma (SDRL). Each transformation event was diagnosed histologically. Informed patient consent was obtained according to the declaration of Helsinki and the study was ethically approved by the local REC.

DNA was extracted from peripheral blood ($n = 135$), bone marrow ($n = 22$), spleen ($n = 17$), or lymph nodes ($n = 1$). Germline DNA was obtained from buccal cells or sorted T cells ($n = 25$). The sequential DNA samples from 9 cases either diagnosed as clonal lymphocytosis of marginal zone origin (ref. 19; CBL-MZ, $n = 1$) or SMZL ($n = 8$; mean, 4.3 years between samples; Supplementary Table S1) were evaluated to investigate the clonal evolution of key gene mutations.

Haloplex resequencing and Sanger validation

A total of 189 DNA samples from 175 SMZL cases were analyzed with Haloplex Target Enrichment system (Agilent Technologies) that enriched 2.39 Mb of gDNA for the coding regions of 49 genes known to be targeted by somatic mutations in SMZL, and an additional 719 genes with a postulated role in the pathophysiology of SMZL or other chronic B-cell lymphoproliferative disorders (Supplementary Methods and Supplementary Table S2). Independent analysis was performed by the University of Southampton (Southampton, UK) and Uppsala University (Uppsala, Sweden) to allow the identification of high-confidence variants in our cohort (Supplementary Methods).

Table 1. Patient characteristics

Variable	Definition	*n* (%)
Number of patients	SMZL diagnosis	175 (100%)
Age at diagnosis, y	Mean (range)	68 (36–90)
IGHV genes	*IGHV1-2*04*	16 (13%)
	not *IGHV1-2*04*	108 (87%)
del(7q) status from karyotype	del(7q)	17 (19%)
	Normal	74 (81%)
Surface CD5 FACS result	$CD5^+$	40 (27%)
	$CD5^-$	108 (73%)
White blood cell	Mean (range) ($\times 10^9$/L)	20 (0.5–158)
Hemoglobin	<12 g/dL	73 (44%)
	≥12 g/dL	93 (56%)
Lymphocyte count	$<4 \times 10^9$/L	41 (27%)
	$\geq 4 \times 10^9$/L	110 (73%)
Platelets $< 100 \times 10^9$/L	$<100 \times 10^9$/L	30 (18%)
	$\geq 100 \times 10^9$/L	134 (82%)
High-risk FISH results	del(17p)	10 (33%)
	Normal	20 (67%)
Splenectomy	Yes	55 (34%)
	No	109 (67%)
Transformation to large cell lymphoma	Yes	19 (17%)
	No	91 (83%)
TTFT status	Treated (inc. splenectomy)	122 (74%)
	Untreated	42 (26%)
EFS status	Event (death, transformation, second treatment)	52 (44%)
	No event	65 (56%)
Status (alive or dead at last follow-up)	Dead	43 (25%)
	Alive	126 (75%)

NOTE: Complex Karyotype was defined as ≥2 cytogenetically visible clonal alterations.

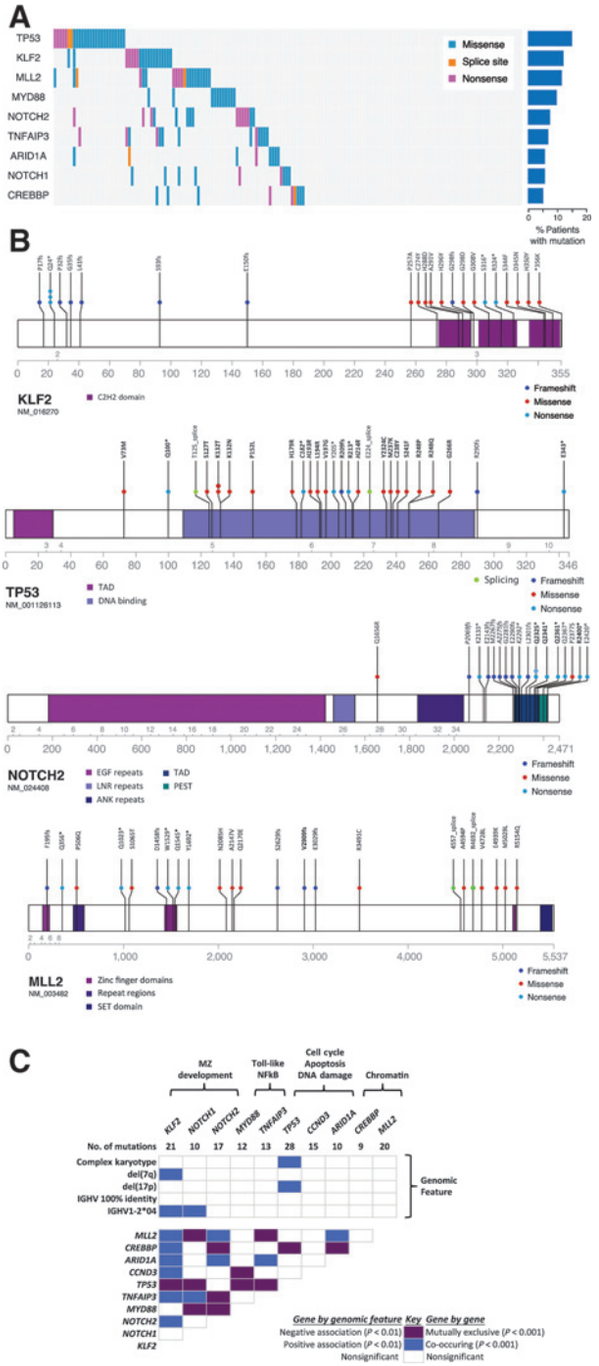

Figure 1.
Distribution of recurrent gene mutations across patients, gene mutation maps, and gene by gene associations for 175 SMZL cases. A, heatmap of the distribution of gene mutation in our cohort. B, schematic diagram of the protein targeted by key mutations in SMZL, with their key functional domains. The symbols and color denote the type of mutation. Mutations annotated in COSMIC v68 database are in bold text. C, associations between genetic and immunogenetic features of our SMZL cohort. Shows the pairwise associations among significantly mutated genes, genetic, and immunogenetic features (labeled as "Genomic Feature") across 175 SMZL cases. Genes are annotated within key pathways known to be important in the pathogenesis of mature B-cell malignancies. The number of mutations (*n*) for each gene mutation in the analysis is shown. An association is shaded on the basis of the significance and only gene by genomic feature (top matrix) and gene by gene (bottom matrix) associations with $P < 0.01$ (χ^2/Fisher exact test) or <0.001 (mutation relation test, Genome MuSiC analysis) are included, respectively.

Using conventional Sanger sequencing, we validated 86 variants identified in a number of genes using the experimental conditions and primers described in Supplementary Table S3. Furthermore, we independently screened *NOTCH2* exon 34 in 145 of 175 SMZL, using primers from Rossi and colleagues (14).

Statistical analysis

Statistical analysis was performed using SPSS (v20). Time to first treatment (TTFT), event-free survival (EFS), and overall survival (OS) as defined in the Supplementary Methods. Our cohort has 81% power to detect an OS of 0.5 HR associated with *NOTCH2* mutations present in 26% of patients (as observed in ref. 14). Results were determined to be statistically significant at the 5% level.

Results

Overview of resequencing data

The mean resequencing depth across our gene panel was 297-fold (range, 129–702). More than 85% of all bases were covered at >50-fold. The analysis described herein focuses on the biologic and clinical importance of key recurrently mutated genes (Fig. 1A) known to be somatically acquired in SMZL based on previously published data (14–17, 20–22). For our data on other gene mutations, while many are annotated in the COSMIC database, they could not be confirmed as somatically acquired because of the lack of patient germline material and were not taken forward for analysis (detailed in Supplementary Table S4). This lack of germline material is not unexpected in an international retrospective cohort of rare tumors such as SMZL.

In our cohort, we identified recurrent mutations, at suitable frequency for accurate clinical correlations, in *TP53* ($n = 26$ cases), *KLF2* ($n = 21$), *MLL2* ($n = 20$), *NOTCH2* ($n = 17$), *TNFAIP3* ($n = 13$), *MYD88* ($n = 12$), *ARID1A* ($n = 10$), *NOTCH1* ($n = 10$), and *CREBBP* ($n = 9$; Fig. 1A and B, Supplementary Fig. S1). For validation, we used Sanger sequencing and confirmed the presence of 86 of 86 selected variants in these genes and we showed 99% concordance between our Haloplex and Sanger sequencing of *NOTCH2* exon 34 (Supplementary Table S5). Furthermore, the Haloplex analysis of paired tumor/normal DNA samples ($n = 14$) showed the presence of somatically acquired mutations in these genes ($n = 77$) and, critically, no germline variants were identified in the genes we focused on herein (Supplementary Table S6). Therefore, our analytical data supports the somatic origin of mutations within these recurrent genes.

Mutation patterns and evolution

To obtain insight into the genomic context of these gene mutations, we submitted our data to 2 analytical packages. First, we searched for pairwise gene correlations and mutually exclusive relationships between our "known somatic" mutated genes using the mutation relation test (MRT, Genome Music; ref. 23). There are 11 and 13 significant MRT co-occurring and mutually exclusive relationships between mutated genes,

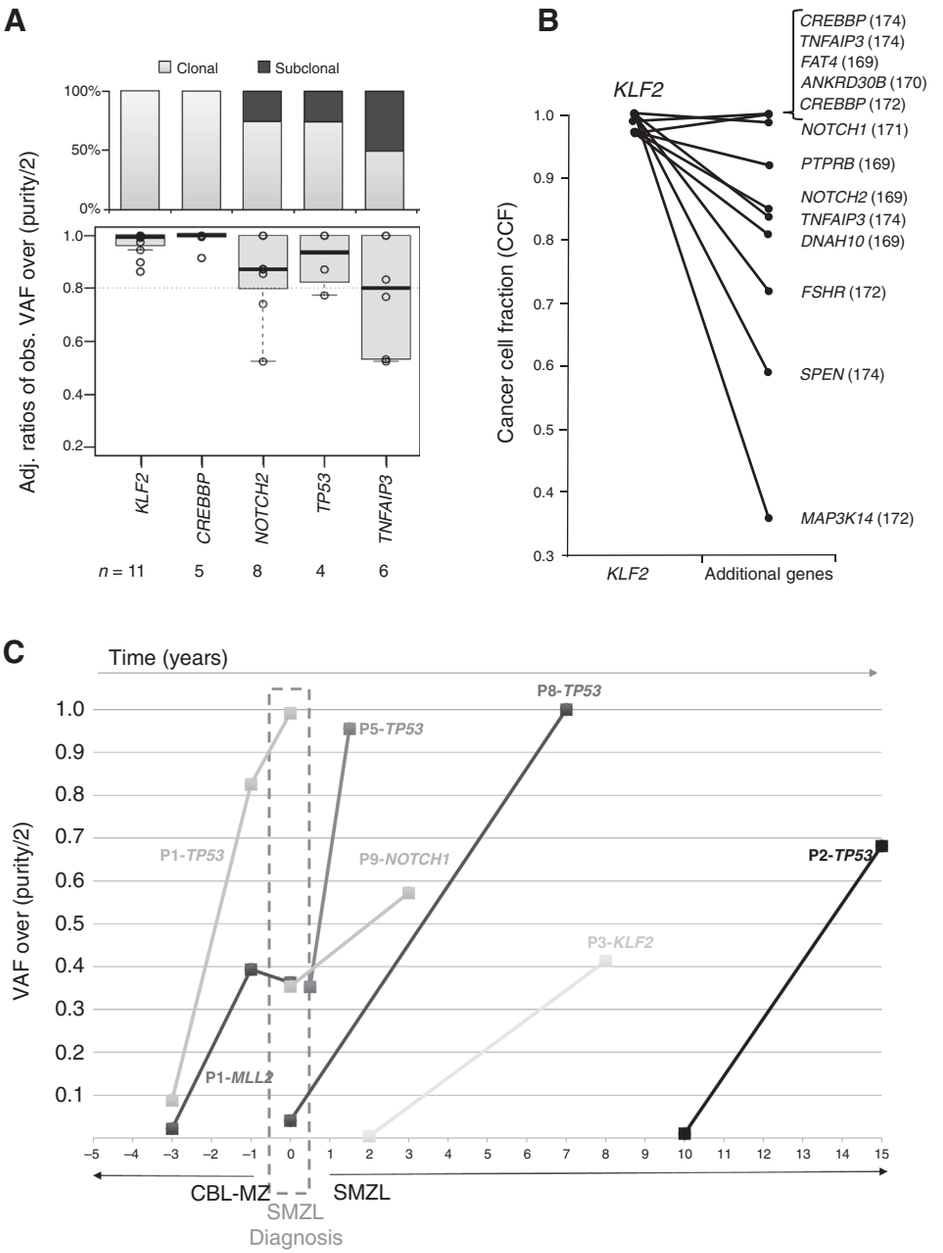

Figure 2.
Clonal distribution and temporal analysis of gene mutations in SMZL. A, the distribution of the estimated proportion of tumor cells harboring a mutation in 45 patients, based on the availability of purity information. Genes are displayed from left to right showing genes displaying more clonal and subclonal mutations, respectively, by using a binomial distribution based on the alternative allele read count, the total read count from cancer cells, and an expected variant allelic fraction (VAF) of 0.45. For each gene, the bone and whisker plots show the adjusted ratio of observed VAF divided by the 50% of the purity estimate derived from $CD19^+$ FACS data. The number of cases (*n*) for each gene mutation in the analysis is shown (bottom). B, the presence of clonal *KLF2* mutations in 5 patients with SMZL with matched deep resequencing and SNP6 data available. No *KLF2* gene deletions were identified by SNP6 copy number data analysis. For each cases, the CCF derived with the ABSOLUTE algorithm is shown for the *KLF2* variant and co-occurring mutations. This approach estimates the CCF harboring a mutation by correcting for sample purity and local copy number changes, where mutations are classified as clonal if the CCF was >0.95 with a probability >0.5, and subclonal otherwise (40). C, temporal resequencing analysis of sequential time points in cases showing clonal expansion of gene mutations (7 of 12 mutations in 9 patients). The *y*-axis shows the VAF for a given mutation after accounting for tumor purity.

respectively (considering only relationships with $P < 0.001$; Fig. 1C), that demonstrated the following classes of gene mutation relationships: (i) a single and distinct independent gene mutation event, such as *MYD88*, where a mutation is invariably observed as an isolated event; (ii) the presence of cancer drivers that have many mutually exclusive relationships, such as *NOTCH2*, *TP53*, and *TNFAIP3*, and (iii) a group of genes such as *KLF2* and *ARID1A* that have more co-occurring relationships, thus suggesting a synergistic function to promote tumorigenesis.

Second, we studied clonal evolution in SMZL, by differentiating between early clonal events and later subclonal mutations. To do this, we initially performed integrative analysis of our Haloplex resequencing and SNP6 copy number data from our 7 published WES SMZL cases (17), using the ABSOLUTE algorithm (ref. 24; Supplementary Fig. S2). Using this approach, all our initial cases harbored a diploid genome, so we extended this analysis to include an additional 38 samples without copy number data but with purity information available from FACS analysis. Using this approach, we were able to classify clonal or subclonal mutations in *KLF2*, *NOTCH2*, *TP53*, *CREBBP*, and *TNFAIP3* (Fig. 2A and B).

To extend this single timepoint bioinformatics analysis, we also analyzed a second DNA sample preceding or subsequent to SMZL diagnosis in 9 patients. For this analysis, we again only focused on variation in genes known to be targeted by somatic mutations in SMZL. We identified 12 variants, and after accounting for tumor purity, these data are outlined in

Supplementary Table S1 and Fig. 2C. While the number of mutations per case was insufficient for comprehensive analysis, the following observations could be made: (i) 3 patients harbored mutations that remained fully clonal over the 2 timepoints [*ARID1A* ($n = 1$) and *CREBBP* ($n = 2$)] which supports the ABSOLUTE data for these genes, (ii) 6 cases contained 7 mutations where the normalized VAF increased over time, supporting the hypothesis that these genes are important in driving the disease, including 4 patients with *TP53* mutations that acquire a deletion of 17p (isochromosomes 17q) at the second timepoint (Fig. 2C), and (iii) 3 patients displayed either a mutation that became undetectable at timepoint 2 (*TNFAIP3* and *NOTCH2*) or one that remained at a low VAF (*NOTCH2*, 0.04% allele frequency), even with a concomitantly emerging *TP53* mutation (Supplementary Table S1).

Biologically significant mutations in SMZL

KLF2, or Krüppel-like factor 2, mutations were detected in 21 of 175 cases (12%, Fig. 1A) and were distributed across the entire protein, with a cluster in the C2H2 domain (C terminus). A Q24X variant was identified in 3 patients, suggesting the presence of mutation hotspots. Mutations were often (43%) stopgains or frameshift variants (Fig. 1B), suggesting an impact on protein function. All mutations tested were somatically acquired ($n = 9$). From our ABSOLUTE analysis, all 11 mutations were defined as clonal (Fig. 2B), and other recurrently mutated genes present in these cases were estimated to have lower cancer cell fractions (CCF) than *KLF2* (e.g., *NOTCH2* and *TNFAIP3*; Fig. 2B). *KLF2* mutations were significantly associated with del(7q) (53% vs. 11%; $P = 0.001$), *IGHV1-2*04* gene usage (50% vs. 7%; $P < 0.001$), and gene mutations including *NOTCH2*, *TNFAIP3*, and *ARID1A* (all $P < 0.001$). Together, these observations suggest that the potential cell survival advantage provided by an early *KLF2* mutation allows the acquisition of additional functionally synergistic gene mutations to promote tumorigenesis (Fig. 1C).

We independently screened *NOTCH2* by Haloplex (mean gene coverage of 572-fold) and direct Sanger sequencing of exon 34 and identified 18 mutations in 17 patients, a frequency of 10% in our cohort (Fig. 1A). We manually examined the *NOTCH2* sequence reads and found no evidence of any additional mutations below the resolution of our variant calling algorithm. As expected, the mutations were nonsense ($n = 9$), frameshift ($n = 7$), and missense ($n = 2$) principally targeting the TAD and PEST domain encoded by exon 34 (Fig. 1B). Several of our mutations (R2360* and R2400*) have been previously reported to result in overexpression of the Notch2 protein and active signaling (14). *NOTCH2* mutations were classified as subclonal or clonal (Fig. 2A). We also identified *NOTCH1* mutations ($n = 10$), several of which were truncating frameshift indels ($n = 2$, P2514fs*4) or stopgain mutations ($n = 2$) in exon 34.

We identified recurrent mutations in *MYD88* (12 of 175 cases, 7%) and *TNFAIP3* (13 of 175 cases, 7%), genes involved in Toll-like receptor and NF-κB signaling. Of the 12 *MYD88* mutations, 7 and 2 were the gain-of-function L265P or S219C variants, respectively (25). Mutations in *MYD88* were single and distinct events, mutually exclusive from mutations in *TP53* and *NOTCH2*. Twenty-one *TNFAIP3* mutations were identified in 13 patients (Fig. 1B), 15 of which would result in truncation of the A20 protein. One of these mutations (E361X) has been shown to abrogate the ability of A20 to negatively regulate NF-κB -signaling (26). Mutations co-existed with *KLF2* ($P < 0.001$) mutations but showed a reverse association with *NOTCH2* ($P < 0.001$) and *TP53* ($P < 0.001$).

Mutations of *TP53* and *ARID1A*, both involved in cell-cycle control and DNA damage response, were identified in 26 of 175 (16%) and 10 of 175 (6%) patients, respectively. We defined 28 missense ($n = 18$), nonsense ($n = 5$), frameshift ($n = 2$), and splicing ($n = 3$) *TP53* mutations, largely annotated in COSMIC (27 of 28, Fig. 1B), in 26 patients who tended to have deletions of 17p ($P = 0.003$) and a complex karyotype ($P < 0.001$, Fig. 1C). Finally, we confirm our previous study by demonstrating recurrent mutations in *CREBBP* ($n = 9$; ref. 17). All our *CREBBP* mutations appear to be early genetic events as they were classified as fully clonal (Fig. 2A) akin to the situation in follicular lymphoma (27); 2 of our mutations were the Y1450C variant previously identified in DLBCL, which has been shown to compromise the protein's ability to acetylate BCL6 and p53 (28).

Clinical significance of mutations in SMZL

Initially, we looked for associations between gene mutations and clinical and laboratory features measured routinely in clinical practice (Fig. 3A). Patients with *KLF2* and *NOTCH2* mutations were at higher risk of receiving treatment including splenectomy [OR, 4.51; 95% confidence interval (CI), 1.68–12.10; $P = 0.002$ and OR, 1.16; 95% CI, 1.08–1.25; $P = 0.007$]. Histologic evidence of transformation to large B-cell lymphoma was reported in 19 of 175 (11%) patients; these patients were more likely to have 100% germline IGHV gene identity (40% vs. 10%, $P = 0.04$) and exhibited a significantly shorter overall survival (9.0 vs. 16.5 years; $P = 0.04$) in comparison to nontransformed cases. The only mutated gene associated with transformation was *TNFAIP3* (32% vs. 4%, $P = 0.002$).

Follow-up outcome data were available for 164, 117, and 169 patients for TTFT, EFS, and OS, respectively. First, we demonstrated the clinical relevance of our cohort by testing for the prognostic significance of previously documented clinical and laboratory features (Table 2). We then performed univariate analysis of the gene mutations against TTFT, EFS, and OS (Table 2). Genes associated with reduced TTFT were: (i) *KLF2* (HR, 1.93; 95% CI, 1.16–3.32; $P = 0.01$) where wild-type and mutant patients exhibited median TTFT of 1.11 and 0.12 years, respectively, and (ii) *NOTCH2* (HR, 2.13; 95% CI, 1.26–3.58; $P = 0.003$) where wild-type and mutant patients exhibited median TTFT of 0.94 and 0.09 years, respectively (Fig. 3B and C). Gene mutations associated with shorter EFS included *TP53* (HR, 2.17; 95% CI, 1–4.74; $P = 0.05$) with median EFS of 3.11 and 0.98 years for wild-type and mutated patients, respectively. Finally, we tested the impact of gene mutations on OS and showed reduced survival for *TP53* (HR, 2.16; 95% CI, 1.05–4.42; $P = 0.032$) mutations with a median OS of 12.21 and 16.03 years for mutant and wild-type cases, respectively, and the reverse for *MYD88* mutated individuals (HR, 0.04; 95% CI, 0.01–2.48; $P = 0.02$; Fig. 3D and E).

***NOTCH2* and *TP53* mutations were independent risk factors for reduced TTFT and OS**

Those gene mutations shown to be associated with reduced outcome in univariate analysis were tested using multivariate Cox proportional hazard analysis. Along with the presence of gene mutations, other variables included in the analysis were age at

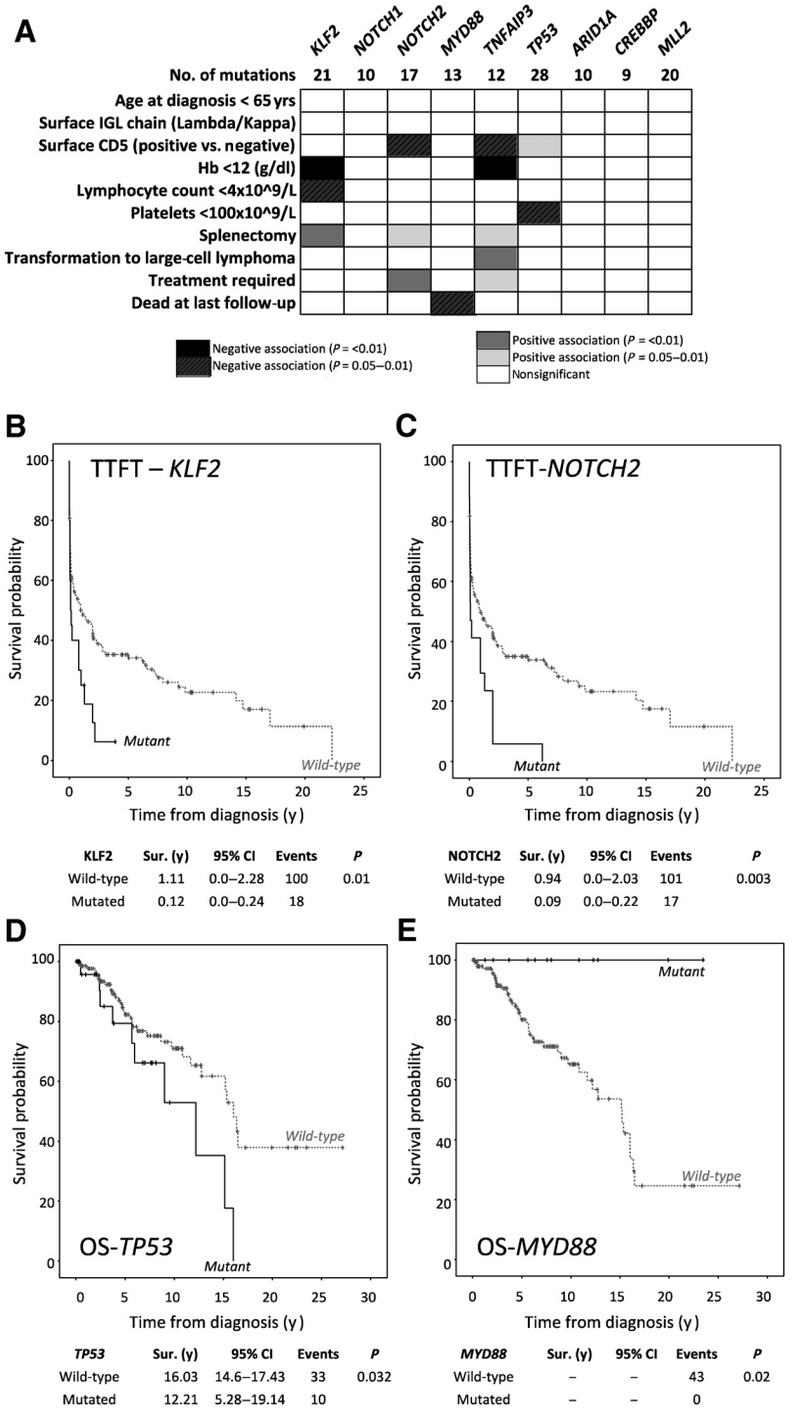

Figure 3.
The clinical significance of gene mutations in SMZL. A, the associations between the presence of gene mutations and clinical features. Where possible genes are annotated within key pathways known to be important in the pathogenesis of mature B-cell malignancies. The number of mutations (*n*) for each gene mutation in the analysis is shown. An association is shaded on the basis of significance and only associations with $P < 0.05$ are included (χ^2/Fisher exact test). B and C, Kaplan–Meier (KM) plots for time to first treatment for patients with *KLF2* and *NOTCH2* mutations, respectively. D and E, overall survival KM plots for patients with *TP53* and *MYD88* mutations, respectively. For each KM plot, the gray and black lines identify the wild-type and mutated patient groups, respectively. The *P* values are derived from KM analysis with a log-rank test and median survival times with 95% CIs.

diagnosis, hemoglobin levels, platelets, and lymphocyte counts. We developed these models for TTFT, EFS, and OS, as they permitted the relative prognostic value of gene mutations to be assessed in a large, informative group of patients in the context of the most available clinical data (Table 3). Our multivariate EFS model identified age at diagnosis, lymphocyte count, and low platelet count as independent risk factors; however, *TP53* became nonsignificant in this analysis. We show that in addition to hemoglobin levels, both *NOTCH2* (HR, 2.12; 95% CI, 1.02–4.4; $P = 0.044$) and 100% germline *IGHV* gene identify (HR, 2.19; 95% CI, 1.05–4.55; $P = 0.036$) are independent risk factors for TTFT. Furthermore, we show that the presence of *TP53* mutation is an independent risk factor for OS (HR, 2.36; 95% CI, 1.08–5.20; $P = 0.03$).

Table 2. Univariate survival analysis of recurrently mutated genes

	Variable	Description	Total	Events	Median, y (95% CI)	HR (95% CI)	*P*
TTFT	*KLF2*	Mutated	20	18	0.12 (0.0–0.24)	1.93 (1.16–3.23)	**0.01**
		Unmutated	140	100	1.11 (0.0–2.28)		
	NOTCH2	Mutated	17	17	0.09 (0.0–0.22)	2.13 (1.26–3.58)	**0.003**
		Unmutated	143	101	0.94 (0.0–2.03)		
	Hb	<12 g/dL	70	63	0.1 (0.04–0.17)	2.75 (1.87–4.02)	<**0.001**
		>12 g/dL	84	51	2.73 (0.0–7.14)		
	Lymphocytes	$<4 \times 10^9$/l	40	33	0.15 (0.07–0.24)	1.76 (1.16–2.68)	**0.007**
		$>4 \times 10^9$/l	101	69	1.43 (0.50–2.37)		
	IGHV identity	100%	12	11	0.14 (0.0–0.38)	2.06 (1.07–3.74)	**0.027**
		<100%	78	50	1.98 (0.98–2.99)		
EFS	*TP53*	Mutated	15	8	0.98 (0.04–12.22)	2.17 (1.00–4.74)	**0.05**
		Unmutated	84	32	3.11 (2.35–6.20)		
	Age	>65 y	53	26	6.82[a] (4.45–9.20)	2.09 (1.07–4.08)	**0.028**
		<65 y	45	14	12.69[a] (9.19–16.18)		
	Platelet count	$<100 \times 10^9$/L	19	11	2.92 (2.03–3.80)	1.99 (0.98–4.02)	**0.052**
		$>100 \times 10^9$/L	78	28	6.91 (4.47–9.34)		
OS	*TP53*	Mutated	26	10	12.21 (5.28–19.14)	2.16 (1.05–4.43)	**0.032**
		Unmutated	134	33	16.03 (14.64–17.43)		
	MYD88	Mutated	12	0	—[b]	—[c]	**0.02**[d]
		Unmutated	148	43	—		
	Age	>65 y	103	37	10.36[a] (9.0–11.76)	6.37 (2.55–15.87)	<**0.001**
		<65 y	56	6	22.65[a] (19.38–25.91)		
	Hb	<12 g/dL	68	24	9.01 (2.90–15.12)	2.69 (1.45–4.99)	**0.001**
		>12 g/dL	87	18	16.35 (14.99–17.70)		

Log-rank *P* values.
[a]Mean survival value as median not reached.
[b]No events in *MYD88* mutated cases and median survival times not presented, follow-up time ranged from 1.25 to 19.9 years.
[c]HR and 95% CI cannot be reliable calculated as there are no events in *MYD88* mutated group.
[d]Log-rank *P* value for χ^2 value reported for the *MYD88* OS Kaplan–Meier analysis (see Fig. 3E).

Discussion

The primary aim of this study was to determine the clinical significance of somatically acquired gene mutations in SMZL, identified in the current and previously reported studies (14–17, 22). Notably, we were able to identify key associations between gene mutations and clinical outcome, demonstrating for the first time that *NOTCH2* and *TP53* gene mutations are independent markers of poor outcome.

Table 3. Multivariate survival analysis of recurrently mutated genes

Variable	HR (95% CI)	*P*
TTFT		
Hb < 12 g/dL	2.28 (1.32–3.96)	0.003
IGHV 100% identity	2.19 (1.05–4.55)	0.036
NOTCH2	2.12 (1.02–4.40)	0.044
EFS		
Platelets $< 100 \times 10^9$ L	3.75 (1.68–8.41)	0.001
Lymphocytes $< 4 \times 10^9$ L	0.41 (0.17–0.96)	0.04
Age at diagnosis < 65 y	0.45 (0.21–0.96)	0.038
OS		
Hb < 12/g/dL	2.18 (1.12–4.23)	0.02
Lymphocytes $< 4 \times 10^9$ L	2.35 (1.11–4.97)	0.03
Age at diagnosis < 65 y	0.09 (0.03–0.27)	<0.001
TP53	2.36 (1.08–5.20)	0.03

NOTE: TTFT multivariate: 83 cases with 56 events; 92 cases with missing data; EFS multivariate: 82 cases with 35 events; 93 cases with missing data; OS multivariate: 134 cases with 38 events; 38 cases with missing data. Backwards-step regression was used, including the following clinical variables (Hb < 12 g/dl, platelets $< 100 \times 10^9$ L, lymphocytes $< 4 \times 10^9$ L, age at diagnosis < 65 years) and the representative gene status variables significantly associated with treatment, event, and survival outcome in univariate analysis (Table 2). The TTFT model also included *IGHV* 100% identity, *KLF2* and *NOTCH2* mutation status. EFS and OS also included *TP53* mutation status. Variables removed from the backwards-step regression are not shown.

The main strengths of the present study were the cost-effective resequencing approach which enabled screening of a large number of candidate genes at high sequencing depth and, most importantly, the size of the cohort in a rare lymphoma, enabling us to overcome limitations befalling previous studies evaluating the clinical significance of clinical and genetic biomarkers in SMZL. Indeed, the lack of a treatment-naïve clinical trial cohort, historical use of splenectomy for diagnosis, inclusion of non-splenectomized cases who might have SLLU and the indolent nature of the disease, where in an elderly population, many patients die from unrelated causes, all underline the need for caution in interpreting outcome data in SMZL. We sought to minimize the effect of these factors in a number of ways: (i) by confining the study to centers with expertise in SMZL, we could ensure expert diagnostic review especially for cases diagnosed before the currently accepted diagnostic criteria, (ii) treatments included a limited range of modalities, predominantly splenectomy, alkylating agents, and rituximab, and splenectomy was considered to be a therapy regardless of the indication (Supplementary Table S7); and (iii) the use of multiple survival endpoints enabled the impact of prognostic markers on disease biology as well as the overall survival of an elderly patient cohort to be assessed.

In additional to confirming the presence of mutations in *TP53*, and in genes involved in NOTCH, BcR, TLR, NF-κB signaling and in chromatin modifiers (14–17, 22), we identified recurrent heterozygous inactivating mutations in *KLF2*, a member of the Krüppel-like family of transcription factors with roles in cell differentiation, proliferation, activation, and trafficking (29), in 12% of analyzed cases. *KLF2* was included in our resequencing experiments due to reanalysis of our published WES data (17) that showed evidence of mutations in 4 of 7 cases despite the low-

sequence read-depth present at this locus. During the preparation and submission of this article, 2 studies independently identified recurrent *KLF2* mutations in SMZL, at a frequency higher than in our study (22, 30), which is likely to be a reflection of the patient cohort analyzed in our current study, as we identified a lower frequency of del(7q) and *IGHV1-2*04* in our cohort, compared with other large studies (3, 22). Interestingly, in mice, KLF2 deficiency is associated with a failure to maintain B-1 B cells, expansion of the marginal zone B-cell pool, and expression of marginal zone characteristics by follicular B cells (31–33). These observations may reasonably be considered as indicating a role of *KLF2* mutations in the natural history of SMZL, an argument also supported by their significant enrichment among SMZL cases with clonotypic BcR using the *IGHV1-2*04* gene. This alludes to acquisition and/or selection of *KLF2* mutations in a context of particular signaling via specific BCRs with distinctive immunogenetic features, similar to what has been observed in other B-cell malignancies, most notably in stereotyped subsets of chronic lymphocytic leukemia (CLL; ref. 34). In 11 cases, we were able to study the clonal architecture of *KLF2* mutations: in each case, the *KLF2* mutations were clonal and were associated with other subclonal mutations, often involving other clinically significant genes such as *NOTCH2* and *TNFAIP3*. The invariable association of clonal *KLF2* mutations with other mutations involving different pathways, and deletions of 7q suggest that the former may have a prosurvival function and additional mutations may be necessary for disease progression. It will be of interest to determine the incidence of *KLF2* mutations and genomic complexity in cases of clonal B-lymphocytosis of marginal-zone origin (19), especially in those cases progressing to SMZL.

Consistent with the role of the NOTCH2–Delta-like 1 ligand pathway in normal marginal zone development (35) and the previously reported finding of *NOTCH2* mutations in SMZL (14, 15), we found recurrent mutations in exon 34 of *NOTCH2* in 10% of cases. This compares to an incidence of 21% (14), 25% (15), and 7% (16) in previously reported series, probably reflecting differences in sample size and cohort composition. We also detected recurrent mutations in other NOTCH pathway genes including *NOTCH3* and *4* and *SPEN*. However, their role in the pathogenesis of SMZL is unclear so we have not focused on them specifically.

We evaluated the prognostic significance of somatically acquired mutations on TTFT, EFS, and OS. Short TTFT was associated with mutations in *KLF2*, *NOTCH2*, and *ARID1A*; short EFS with mutations in *TP53*; and, short OS with mutations in *TP53*, whereas *MYD88* mutations were associated with a longer OS. Although the study by Rossi and colleagues indicated that *NOTCH2* mutations were associated with a prolonged 5-year OS and progression-free survival following first-line treatment (14), our data based on a substantially larger cohort shows that *NOTCH2* mutation are linked to reduced outcome, an observation corroborated by Kiel and colleagues who also noted an association with shorter time from diagnosis to either relapse, transformation or death, albeit in a much smaller cohort of SMZL cases ($n = 46$; ref. 15). Additional studies of larger patient cohorts will be required to validate the clinical importance of *NOTCH2* mutations. Cases with an *MYD88* mutation exhibited longer OS and comparable clinical and laboratory features to other cases with SMZL apart from a higher incidence of low-level IgM paraproteins, detected in 8 of 9 cases with available data. The poor prognostic significance of *TP53* mutations is consistent with previously reported data on *TP53* abnormalities.

Our multivariate analysis demonstrated for the first time in SMZL that both genetic and immunogenetic parameters retained prognostic significance in a model that included age, hemoglobin, platelet count, and lymphocyte count. We chose to base our multivariate analysis on the study of Salido and colleagues, as this is the only large study to include baseline clinical variables with chromosomal features (3). While the independent prognostic significance of age, anemia, and thrombocytopenia were expected and consistent with many previous studies, the biologic basis for the impact of a lymphocyte count of $<4 \times 10^9$/L, noted in an early (36) but not in more recent studies (3, 37), requires further investigation. Interestingly, our study suggests that gene mutations, such as those targeting *NOTCH2* and *TP53*, have more clinical use than cytogenetic features, such as karyotypic complexity, 14q aberrations, and *TP53* deletions, that did not retain prognostic significance in previous reports (3). Specifically, *NOTCH2* and truly unmutated *IGHV* genes (but not unmutated *IGHV* genes using a 98% cutoff) were independent markers of TTFT and *TP53* of OS. Given the historical use of splenectomy to both diagnose and treat patients with SMZL, our associations with TTFT should be considered with a note of caution.

Because transformation to a high-grade lymphoma is usually associated with resistance to treatment and very poor survival, we were also interested to see whether any genomic abnormalities were associated with an increased risk of transformation, as noted for *NOTCH1* mutations in CLL (39). In our study, *TNFAIP3* mutations together with truly unmutated clonotypic *IGHV* genes were all found at a higher frequency in cases that subsequently transformed. Further studies comparing the genomic landscape of paired chronic phase and clonally related transformed samples, as performed in CLL and follicular lymphoma (27, 39), will be required to determine the drivers of transformation.

In summary, we show that gene mutations and immunogenetic features have prognostic significance in a large and well-characterized cohort of patients with SMZL. Additional studies will be required to confirm our findings and to determine the functional consequences of these mutations, the incidence and importance of copy number and epigenetic abnormalities in gene silencing, and the clinical value of mutation screening in the differential diagnosis and management of SMZL.

Disclosure of Potential Conflicts of Interest

R. Walewska reports receiving travel grants from Gilead. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: G. Pangalis, D. Oscier, J.C. Strefford

Development of methodology: V. Ljungström, D.G. de Castro, G. Pangalis, J.C. Strefford

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J.J. Rose-Zerilli, R. Walewska, A. Parker, Z. Davis, A. Xochelli, A. Anagnostopoulos, C. Fazi, D.G. de Castro, C. Dearden, G. Pratt, M. Ashton-Key, F. Forconi, P. Ghia, G. Pangalis, K. Stamatopoulos, D. Oscier, J.C. Strefford

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Parry, M.J.J. Rose-Zerilli, V. Ljungström, J. Gibson, J. Wang, H. Parker, C. Fazi, R. Rosenquist, A. Collins, E. Matutes, K. Stamatopoulos, J.C. Strefford

Writing, review, and/or revision of the manuscript: M. Parry, M.J.J. Rose-Zerilli, V. Ljungström, J. Gibson, J. Wang, R. Walewska, D.G. de Castro,

C. Dearden, G. Pratt, R. Rosenquist, M. Ashton-Key, F. Forconi, A. Collins, P. Ghia, E. Matutes, G. Pangalis, D. Oscier, J.C. Strefford
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Parker, A. Gardiner, N. McIver-Brown, C. Kalpadakis, A. Anagnostopoulos, G. Pangalis, J.C. Strefford
Study supervision: G. Pangalis, J.C. Strefford
Other (performed experiments): M. Parry

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