KLF2 mutation is the most frequent somatic change in splenic marginal zone lymphoma and identifies a subset with distinct genotype

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ABSTRACT

To characterise the genetics of splenic marginal zone lymphoma (SMZL), we performed whole exome sequencing of 16 cases and identified novel recurrent inactivating mutations in KLF2, a gene whose deficiency was previously shown to cause splenic marginal zone hyperplasia in mice. KLF2 mutation was found in 40 (42%) of 96 SMZLs, but rarely in other B-cell lymphomas. The majority of KLF2 mutations were frameshift indels or nonsense changes, with missense mutations clustered in the C-terminal zinc finger domains. Functional assays showed that these mutations inactivated the ability of KLF2 to suppress NF-κB activation by TLR, BCR, BAFFR and TNFR signalling. Further extensive investigations revealed common and distinct genetic changes between SMZL with and without KLF2 mutation. IGHV1-2 rearrangement and 7q deletion were primarily seen in SMZL with KLF2 mutation, while MYD88 and TP53 mutations were nearly exclusively found in those without KLF2 mutation. NOTCH2, TRAF3, TNFAIP3 and CARD11 mutations were observed in SMZL both with and without KLF2 mutation. Taken together, KLF2 mutation is the most common genetic change in SMZL and identifies a subset with a distinct genotype characterised by multi-genetic changes. These different genetic changes may deregulate various signalling pathways and generate cooperative oncogenic properties, thereby contributing to lymphomagenesis.
INTRODUCTION

Splenic marginal zone lymphoma (SMZL), a low grade B-cell lymphoma, is difficult to diagnose accurately due to a lack of specific histological, immunophenotypic and genetic markers. Patients with SMZL present with a highly variable clinical course with the majority showing a median survival of 10 years, ~25% of cases die of the disease within 5 years and a further ~5% of cases display high-grade transformation. Despite the advances in treatment of other lymphomas, the survival of patients with SMZL has not been improved over the last decade. These dilemmas in diagnosis and clinical management are largely due to poor understanding of its genetics and molecular mechanism.

There is mounting evidence suggesting a role for antigenic stimulation in the pathogenesis of SMZL. Approximately 20% of patients with SMZL present with autoimmune phenomena. A small proportion of cases are associated with HCV infection and can be effectively treated by antiviral therapy. Importantly, >30% of SMZL has biased usage of IG heavy chain variable gene, IGHV1-2. Most of the IGHV1-2 rearrangements are characterised by minimal somatic mutations and longer complementarity determining region-3 (CDR3) sequence with common motifs, suggesting a possible selection by superantigens. Together, these findings indicate a critical role of active BCR signalling in the pathogenesis of SMZL.

SMZL lacks recurrent chromosome translocations. Approximately 30% of SMZLs show hemizygous 7q deletion, which is also seen frequently in splenic B-cell lymphoma/leukaemia unclassifiable (SBCLU), but rarely in other lymphoma subtypes. The gene(s) targeted by the 7q deletion remain obscure despite the combined investigation of genomic and transcriptomic profiles and mutation analysis of a number of candidate genes.
Recent studies by whole exome sequencing (WES) identified a plethora of somatic mutations in SMZL. These studies together with candidate gene sequencing showed a diverse spectrum of mutations in the NOTCH, NF-κB, BCR and TLR pathways, and in histone modifiers and transcriptional regulators. Most of these mutations were found in <10% of cases, with only NOTCH2 mutations occurring more frequently, but variably among different studies (6.5-25%). Importantly, a significant proportion of SMZL lack any of these candidate pathogenic mutations. As the number of cases investigated by WES in each of the above studies was small (6-15 cases), the mutation landscape in SMZL is likely not yet fully characterised. In this study, we identified further novel recurrent mutations in SMZL by WES and showed that KLF2 (Kruppel-like factor 2) was inactivated by mutations in 42% of SMZL. Mechanistically, KLF2 mutations abrogated KLF2-mediated suppression of NF-κB activation by TLR, BCR, BAFFR and TNFR signaling. KLF2 mutation is significantly associated with 7q deletion, IGHV1-2 usage, NOTCH2, TNFAIP3 and TRAF3 mutation, and may potentially cooperate with these genetic changes in oncogenesis.

MATERIALS AND METHODS

Patients samples: Tumour DNA was extracted from 105 cases of SMZL (77 from fresh frozen [FF] lymphoma tissues, 3 from leukemic peripheral blood samples, 25 from formalin-fixed paraffin-embedded [FFPE] lymphoma tissue), SBCLU (n=3), chronic lymphocytic leukaemia (CLL, n=39, all from bone marrow aspirate), hairy cell leukaemia (HCL, n=30, all from bone marrow aspirate), extranodal marginal zone lymphoma of mucosa-associated lymphoma tissue (MALT lymphoma, n=47, all from FFPE diagnostic tissue biopsies), mantle cell lymphoma (MCL, 7 from FF and 4 from FFPE diagnostic tissue biopsies), follicular lymphoma (FL, 5 from FF and 6 from FFPE diagnostic tissue biopsies), and diffuse large B-cell lymphoma (DLBCL, n=28, all FFPE diagnostic tissue biopsies). The lymphoma diagnosis was made according to the 2008 WHO classification of tumours of haematopoietic and lymphoid tissues. Where indicated, germline DNA was prepared from non-
neoplastic cells of tissues. The DNA quality was assessed by PCR of variably sized genomic fragments, and samples with successful amplifications of >300bp were used for genetic analyses.

Partial data on 7q deletion and IGHV usage in SMZL were available from previous studies, with all other genetic data collected in this study. Local ethical guidelines were followed for the use of archival tissues for research with the approval of the ethics committees of the involved institutions.

**Exome sequencing and somatic variant calling:** These were carried out by the Wellcome Trust Sanger Institute. High molecular weight (HMW) tumour DNA samples from 16 cases of SMZL and matched germline DNA samples from 3 of these cases (non-neoplastic FFPE tissues in 1, buccal swap or non-involved peripheral blood sample in 2) were used to generate genomic libraries with the Illumina Paired End Sample Prep Kit (Table S1). Enrichment was performed using the Agilent SureSelect Human All Exon 50Mb kit. Each exome was sequenced using a 75bp paired-end protocol on an Illumina HiSeq platform. Sequencing reads were aligned to the hg19 reference genome using the BWA algorithm on default settings.

Novel variants were called by comparison of tumour and germline sequence reads. CaVEMan (Cancer Variants through Expectation Maximisation) was used to call single nucleotide substitution, while Pindel was used to call insertions and deletions. Post processing filters were applied to increase the specificity of the output, remove variants reported in poor quality sequences and remove known SNPs in databases and unmatched normals from this study and the 10,000 genomes project.

**Somatic variant validation by PCR and Sanger sequencing:** Where indicated, novel variants identified by WES and their potential somatic origin were first confirmed by PCR and Sanger sequencing. Depending on the nature of gene sequences, different approaches were employed for mutation screening. Mutations in KLF2 were screened by PCR and Sanger sequencing as the gene
has a high GC content (Table S2). In each case, sequence change was confirmed by at least two independent PCR and sequencing experiments. The somatic mutation was ascertained by excluding germline changes through SNP database search and analysis of germline DNA samples where possible.

**Somatic variant validation by Fluidigm Access Array PCR and Illumina MiSeq sequencing:**

Mutations in NOTCH2, TNFAIP3, TRAF3, MYD88, IKBKB, CARD11, BCL10, CD79A, CD79B and TP53 were screened by massive parallel Fluidigm Access Array PCR and Illumina MiSeq sequencing using our established protocol from a parallel investigation (manuscript in preparation). The in house variant calling algorithm was developed and optimised against a large number of various known somatic mutations by Sanger sequencing. Please refer to Supplementary Methods for experimental details and variant calling algorithms (Table S3). Each sample was investigated in duplicate to eliminate any potential false positives. Any novel variants seen in both replicates of the same sample were further ascertained by an independent Fluidigm PCR and MiSeq sequencing or Sanger sequencing. Where indicated, their somatic nature was confirmed by PCR and Sanger sequencing of the paired non-tumour DNA sample, or by search of COSMIC somatic mutation database.

**NF-κB reporter assay:** The full-length coding sequence and various truncated forms of KLF2 were amplified from pCMV6-AC-GFP (OriGene, USA) by PCR and cloned into the pIRES-puro2-HA vector at the EcoRI and BamHI sites. The KLF2 mutant containing a single point mutation was generated from the wild type using the QuickChange Site-directed mutagenesis kit (Stratagene, USA). PCR and sequencing were performed to verify the KLF2 sequence and reading frame. The effect of KLF2 and its mutants on suppression of NF-κB activation by various stimuli (TNFα, BAFF, mutant MYD88 or mutant CARD11) was investigated where appropriate in both HEK293T and OCI-LY19 human B-cell lymphoma cell lines using a Dual-Luciferase reporter assay (Promega, UK). Please refer to Supplementary Methods for experimental details.
Analysis of rearranged IGH genes by PCR and Sanger sequencing: The rearranged IGH genes were amplified using BIOMED-2 FR1 and consensus JH primer sets. The PCR product was purified and sequenced as previously described. The VH sequence was identified using the IMGT/V-QUEST database (http://www.imgt.org). Cases harbouring < 97% homology were considered significantly mutated, while those with 97-99.9% identity were regarded minimally mutated.

Statistical analyses: The student's t-test was performed using GraphPad Prism version 5.00 software (GraphPad Software, San Diego, USA). The correlation among categorical variables was evaluated by Fisher's exact probability test. Overall survival (OS) was measured from the date of diagnosis to death from any cause. Probabilities of OS were calculated by the Kaplan–Meier method, and the comparison between subgroups was performed via the log-rank test. Kaplan-Meier analysis, log-rank test and Fisher’s exact test were carried out using SPSS, version 13.

RESULTS

Identification of KLF2 mutation by WES

WES was successful for all 16 tumour and 3 matched germline DNA samples (Table S4). Based on the 3 cases with matched germline DNA, a total of 174 variants in 163 genes (average 58/case; range 45-82/case) were seen, with variants in 135 genes being novel, not reported previously in SMZL (Table S5). The number of variants in the remaining cases was much higher due to a lack of WES data from matched germline DNA, thus preventing the filter of all SNPs. Nonetheless, a total of 223 variants were observed in 159 genes known to be mutated in SMZL by previous studies, including those described in the NOTCH2 signalling pathway (NOTCH2, NOTCH4, SPEN), NF-κB pathway (TNFAIP3, TRAF3, BIRC3), BCR pathway (CARD11) and TLR pathway (MYD88) (Figure S1).
Comparative analyses of the exome sequencing data from the 4 published WES studies also revealed little overlap among the variants identified in these studies (Figure S2). Together, these findings suggest the presence of a remarkable heterogeneity or incomplete discovery of the somatic mutation profile in SMZL, or both.

Among the variants not reported previously, there were several recurrent changes not seen in the matched control DNA. Based on the frequency, possible functional impact and a comprehensive literature search, we identified KLF2 mutation as a potentially significant genetic abnormality in SMZL (Figure S1). Among the 16 SMZLs investigated by WES in this study, KLF2 mutation was seen in 5 cases, all being deleterious changes (frameshift insertion/deletion in 2, nonsense mutation in 2, and substitution change at an essential splice site in 1 case). Further PCR and Sanger sequencing confirmed these mutations, and their somatic origin in all 5 cases. In support of the pathogenic importance of these mutations in SMZL, Klf2 deficient mice were previously shown to have a marked increase in marginal zone B-cells and splenic marginal zone hyperplasia.  

**KLF2 is frequently targeted by mutation in SMZL.**

Next, we investigated KLF2 mutation in 96 cases of SMZL including 13 of the 16 cases investigated by WES, and 7 other B-cell lymphoma entities to determine its frequencies and mutation spectrum. As the KLF2 gene has a high GC content that may have accounted for the failure of detection of its mutation by previous exome sequencing studies, PCR and Sanger sequencing were used for mutation screening. A total of 47 KLF2 mutations were seen in 40 (42%) of the 96 cases of SMZL with double mutations in 7 cases, and their somatic nature was confirmed in each of the 8 cases (including 3 indels and 7 substitutions), for which non-tumour DNA was available (Figure 1A, Figure S3, Table S6). Of note, Sanger sequencing identified 4 additional mutations in 3 cases missed by WES, which included 1 frameshift deletion, 1 in-frame deletion and 2 missense substitutions. In contrast,
KLF2 mutation was not or rarely seen in SBCLU (0/3), CLL (0/39), HCL (3/30), FL (1/11), MCL (1/11), MALT lymphoma (2/47) and DLBCL (0/28) (Figure 1B).

Among the 47 KLF2 mutations identified in SMZL, 27 were frameshift insertions / deletions, nonsense mutations, or substitutions affecting the essential splice site, thus resulting in a potentially truncated protein product (Figure 1A). Importantly, a high proportion of these deleterious mutations were localised toward the N-terminal activation domain (AD) and middle inhibitory domain (ID). The remaining 20 mutations seen in SMZL were 19 missense substitutions and 1 in-frame deletion, with 12 clustered in the C-terminal zinc finger (ZF) 1, mainly at conserved amino acid residues (Figure 1A). Fourteen missense mutations were predicted to be damaging by the PolyPhen-2 program, and a further 2 missense mutations abolished the stop codon with potential extension of a further 62 amino acids. Among the 7 KLF2 mutations found in other lymphoma entities, 2 were frameshift deletions in the ID and ZF3 domains respectively, and 5 were missense mutations with only 1 in the C-terminal ZF1 domain (Figure 1A).

**Functional characterisation of KLF2 mutations**

The nature and distribution of the KLF2 mutations suggest that these genetic changes are likely to inactivate KLF2 function. We thus generated a series of KLF2 expression constructs, representing various C-terminal truncated products that were lacking 1 or more ZF domains, and recurrent missense or in-frame deletion mutants that affected conserved amino acid residues in the ZF1 domain, and tested their impact on KLF2 function using in vitro reporter assays in both HEK293T and OCI-LY19 B-lymphoma cells. Previous studies have shown that KLF2 inhibits the transcriptional activity of NF-κB. We therefore tested the ability of various KLF2 mutants to suppress NF-κB activation by TNFα, MYD88(S219C), CARD11(F130V) and BAFF, which were used to activate TNFR, TLR, canonical and non-canonical NF-κB signalling pathways respectively.
As expected, wild type KLF2 was highly potent in the suppression of NF-κB activation by different signalling pathways including stimulation by TNFα, MYD88(S219C), CARD11(F130V) and BAFF (Figure 2B). With the exception of KLF2-A291V mutant, all other 5 mutants including KLF2-C274Y and KLF2-ΔTY mutants showed a total or major loss in NF-κB suppression, although to varying extents depending on stimuli used to activate NF-κB. Interestingly, both cases with KLF2-A291V had a second mutation, one with P70S in the activation domain, the other with C274S, at which a C274Y change was shown to impair KLF2 function as described above (Table S6).

**KLF2 mutation identifies a subset of SMZL with distinct genotypes**

To further characterise the genetics of SMZL and understand their potential cooperation in lymphomagenesis, we comprehensively investigated somatic mutations in NOTCH2, TNFAIP3, TRAF3, MYD88, CD79A, CD79B, CARD11, BCL10, IKBKB and TP53 (Figure 3, Table S6), 7q deletion and IGHV usage in the entire cohort of SMZL and correlated their changes with KLF2 mutation using the Fisher’s exact probability test. The analyses revealed several significant associations.

First, KLF2 mutation identified a subset of SMZL with distinct genetic changes. The mutation was significantly associated with both 7q deletion ($P=7.33\times10^{-7}$) and IGHV1-2 usage ($P=1.02\times10^{-7}$), seen in 77% cases with 7q deletion and 83% of those with IGHV1-2 (Figure 4). As with previous studies, the rearranged IGHV1-2 was characterised by minimal somatic mutations (Figure 4A).

Second, mutations in the NOTCH2, TRAF3, TNFAIP3 and CARD11 genes were found in SMZL both with and without KLF2 mutation, with NOTCH2, TRAF3 and TNFAIP3 mutations being significantly associated with KLF2 mutations ($P=0.007$, $P=0.012$ and $P=0.015$ respectively). In line with
the recent studies, NOTCH2 mutations were characterised by frameshift insertion/deletion, and nonsense mutations, which were clustered at the C-terminus and predicted to eliminate the C-terminal PEST, a domain critical for NOTCH2 proteasomal degradation (Figure 3). TRAF3 and TNFAIP3 mutations were featured by frameshift insertions/deletion and nonsense mutations, while CARD11 mutations were typically activating changes reported elsewhere (Figure 3). Third, most SMZL without KLF2 mutation showed heterogeneous usage of IGHV in their rearranged IGH genes, and the majority of these rearranged IGHV harboured high loads of somatic mutations. Interestingly, MYD88 mutations were exclusively seen in cases without KLF2 mutation (Figure 4A&C, P=0.021). MYD88 mutations were typically those of activating changes, while TP53 mutations were characteristic inactivating changes reported elsewhere. Mutation in CD79A and CD79B (both seen in a single case), BCL10 [2 cases, both mutations predicted a C-terminal truncated BCL10 with a potential gain of function\textsuperscript{34}], and IKBKB (1 case) was found to be low in SMZL. Interestingly, these mutations were mutually exclusive from CARD11 mutations. Correlation among genetic abnormalities and clinicopathological parameters

Follow-up data were available for 60 cases of SMZL, ranging from 12 to 288 months (median = 55 months). Kaplan-Meier univariate analysis of the genetic and clinical variables showed that only TP53 mutation was significantly associated with poor 5-year overall survival (P =0.002) (Table S7). However, the number of cases and death events were not sufficient for reliable multivariate analysis.

DISCUSSION
By WES and validation of the mutations identified, we have made several novel and significant discoveries in the present study. First, KLF2 is frequently mutated in SMZL (42%), but not or rarely in other lymphomas; second, KLF2 mutations are characterised by frameshift insertion/deletion, nonsense mutations, and a cluster of missense mutations in the ZF1 domain, which impair KLF2 function; third, there are distinct genetic changes according to KLF2 mutation status. IGHV1-2 rearrangement and 7q deletion are essentially seen in SMZL with KLF2 mutation, while MYD88 and TP53 mutations are nearly exclusively seen in those without KLF2 mutation. Mutations in NOTCH2, TRAF3, TNFAIP3 and CARD11 genes were found in SMZL both with and without KLF2 mutation. These distinct mutation patterns indicate overlapping molecular mechanisms between SMZL with and without KLF2 mutation, and also suggest the presence of different oncogenic cooperation between the two subgroups.

KLF2 mutation and its distinctively associated genetic changes

Among the diverse spectrum of mutations identified in SMZL, KLF2 mutation (42%) is the most frequent genetic change, much higher than the recently identified NOTCH2 mutation (6.5-25%).13-16 The nature of KLF2 mutations and our in vitro functional studies of KLF2 mutants indicate that these mutations inactivate KLF2 function.

A pathogenic role of KLF2 inactivating mutations in SMZL is strongly supported by recent findings, particularly those by studies of Klf2 knockout mice. Remarkably, B-cell specific Klf2-deficient mice show a dramatic increase of marginal zone B-cells.27-29 Klf2 deficiency appears to promote follicular B-cells to gain a marginal zone like phenotype and migrate to the splenic marginal zone, but have little impact on their proliferation.27-29 The molecular mechanism underlying the altered B-cell homeostasis and trafficking in Klf2-deficient mice is unclear although Klf2 most likely exerts such effects through transcriptional regulation of its target genes.
KLF2 is a member of the KLF family of transcription factors, and has been recently shown to be a negative regulator of inflammation and NF-κB activities. KLF2 appears to regulate NF-κB activities by modulating recruitment of critical NF-κB coactivators. Using an in vitro reporter assay, we showed that wild type KLF2 was a potent inhibitor of NF-κB activation by several signalling pathways including BCR (CARD11 mutant), TLR (MYD88 mutant), TNFR (TNFα) and BAFFR (BAFF). In contrast, KLF2 mutants had a total or major loss in suppression of NF-κB activation triggered by these signals. Given the importance of TLR, canonical and non-canonical NF-κB pathways in the development of marginal zone B-cells, KLF2 inactivation by mutation may exert its oncogenic activities at least in part by deregulation of NF-κB activities triggered by these signals, leading to altered gene expression favouring B-cells homing to the marginal zone. However, KLF2 inactivation alone is insufficient for malignant transformation, and requires cooperating genetic and cellular events in SMZL development.

The majority of SMZL with KLF2 mutation have both 7q deletion and IGHV1-2 rearrangement. The genes targeted by 7q deletion are unclear. IGHV1-2 usage is over-represented in SMZL, accounting for 30% of cases. Although the epitope recognised by IGHV1-2 expressing BCR and its potential impact on clinicopathological presentation are unknown, the features of IGHV1-2 rearrangements, including minimal somatic mutations and longer CDR3 sequence with common motifs, suggest a possible selection of T-cell independent marginal zone B-cells by superantigens, thus a role of antigenic drive in the lymphomagenesis (Figure 5). Such active BCR signalling may cooperate with KLF2 inactivation in SMZL development. Apart from IGHV1-2 BCR stereotype, there are further genetic changes that potentially cooperate with KLF2 inactivation although these genetic changes occur in SMZL both with and without KLF2 mutation.

Genetic changes common to SMZL both with and without KLF2 mutation
 NOTCH2, TRAF3, CARD11 and TNFAIP3 mutations were found in SMZL both with and without KLF2 mutation. NOTCH2, TRAF3 and CARD11 mutations are most likely to enhance the NOTCH2, non-canonical NF-κB and BCR signalling respectively,13,14,16,41,42 while TNFAIP3 mutation may augment several molecular pathways including TNFR, TLR/IL1-R and BCR signalling.43 All these innate signals are critical for the development of marginal zone B-cells although their precise role remains to be dissected. NOTCH2 signalling is critical for generation of marginal zone B-cells and their retention in the splenic marginal zone.44-47 Active NOTCH2 signalling alone appears to have little impact on cell proliferation and survival, but sensitises B-cells to stimulation of surface TLR and CD40.46,47 Non-canonical NF-κB signalling, typically triggered by stimulation of surface BAFFR and CD40, is also pivotal in development of marginal zone B-cells and formation of the splenic marginal zone.48 Chronic active BCR signalling promotes cellular proliferation and survival, and TLR signalling may contribute to both the development and survival of marginal zone B-cells.40 Marginal zone B cells express NOTCH2, BAFFR, CD40, and high levels of TLRs, while the splenic innate lymphoid cells (ILCs) express surface DLL1 (Notch ligand Delta-like 1), BAFF, CD40L and provide contact-dependent help to marginal zone B-cells by stimulation of the respective receptors in a cooperative manner.39,40,49 Thus, NOTCH2, TRAF3, CARD11 and TNFAIP3 mutation may cooperate with the aforementioned surface receptor stimulation and cause constitutive activation of the corresponding signalling pathway.

The respective signalling enhanced by NOTCH2, TRAF3, TNFAIP3 and CARD11 mutation likely complements the molecular mechanism deregulated by KLF2 mutation, IGHV1-2 expressing BCR and genes targeted by 7q deletion, thus cooperating in SMZL development (Figure 5). However, the genetic events that cooperate with these mutations in SMZL without KLF2 mutation are unclear. Genetic changes preferentially associated with SMZL without KLF2 mutation
There are also several interesting features in SMZL without KLF2 mutation, including infrequent 7q deletion and IGHV1-2 rearrangement, and nearly exclusive association with MYD88 and TP53 mutations (Figure 5). Among SMZL without KLF2 mutation, there is a heterogeneous usage of IGHV and the majority of these rearranged IGHV genes show high levels of somatic mutations, suggesting origin from T-cell dependent marginal zone B-cells.\textsuperscript{50}

The MYD88 mutations seen in SMZL are typically those of gain-of-function change, capable of spontaneously assembling a signalling complex to activate NF-κB, STAT3 and AP1 transcription factors.\textsuperscript{51} MYD88 activation by mutation may lead to biological consequences similar to that by TLR activation implicated by KLF2 and/or TNFAIP3 inactivation.

The TP53 mutations seen in SMZL were typically those reported elsewhere, and these mutations likely inactivate TP53 function. TP53 mutation in SMZL, like in other lymphomas, is likely to be a secondary genetic event. In line with this, TP53 inactivation is associated with progression and poor prognosis in SMZL.\textsuperscript{12,52,53}

In summary, there are common and distinct genetic changes between SMZL with and without KLF2 mutation and these different genetic changes most likely deregulate several signalling pathways important for the generation of marginal zone B-cells, their migration and retention in the splenic marginal zone. Each of these genetic changes may have a predominant impact on a particular biological process and contribute to the lymphoma development through oncogenic cooperation with other concurrent changes (Figure 5). It is pertinent to tentatively speculate that 1) KLF2 inactivation may deregulate gene expression through the modulation of NF-κB activities and other unknown mechanisms, thereby promoting B-cells homing to the splenic marginal zone; 2) NOTCH2 activation, TRAF3 and TNFAIP3 inactivation and MYD88 activation by mutations may contribute to
the generation of marginal zone B-cells and their retention in the splenic marginal zone by augmenting the NOTCH2, non-canonical NF-κB pathway and TLR signalling respectively; 3) IGHV1-2 rearrangement and CARD11 activation by mutations may lead to chronic active BCR signalling, consequently enhancing cell proliferation and survival. A simultaneous deregulation of the above signalling pathways in SMZL with KLF2 mutation may generate complementary properties in oncogenic cooperation, leading to lymphoma development.

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Supplementary information is available at Leukemia's website.
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FIGURE LEGENDS:

**Figure 1:** Nature and incidence of *KLF2* mutations in SMZL and other B-cell lymphomas.

A) Nature and distribution of *KLF2* mutations in lymphoma. The majority of *KLF2* mutations seen in SMZL are frameshift deletion / insertion or nonsense mutations. Missense mutations are largely clustered in ZF1 (zinc finger), particularly at conserved amino acid residues. Mutations were confirmed by two independent PCR and sequencing experiments. Where possible the somatic nature of the mutation identified was confirmed by PCR and sequencing analysis of the paired non-tumour DNA and indicated by red symbols. Mutations identical to those confirmed to be somatic are highlighted in blue. Concurrent mutations seen in the same cases are indicated by their case number.

B) Frequencies of *KLF2* mutations in SMZL and various other B-cell lymphomas.

ZF: zinc finger; NLS: putative nuclear localisation signal.

**Figure 2:** Functional characterisation of *KLF2* mutations.

A) The representative *KLF2* mutants investigated by *in vitro* reporter assays, which include three truncation, two recurrent missense and one in-frame deletion mutants. The missense change and in-frame deletion affect the conserved amino acid residues.

B) NF-κB reporter assay shows that wild type KLF2 is a potent inhibitor of NF-κB activation by TNFα, BAFF, MYD88 and CARD11 mutants in both HEK293T and OCI-LY19 B-lymphoma cells. With the exception of KLF2-A291V mutant, all other 5 mutants including KLF2-C274Y and KLF2-ΔTY mutants showed a total or major loss in NF-κB suppression. Interestingly, both the cases with KLF2-A291V
had a second mutation, one with P70S in the activation domain, the other with C274S. The data is from at least three independent experiments and presented as a mean ± standard deviation, and the difference between KLF2 and its mutants is analysed by the Student t-test. As indicated by an arrowhead, the KLF2-AD-ZF1 mutant consistently shows an additional band, ~7 kDa larger than the expected size (32 kDa). We have performed a series of experiments to confirm the correct sequence and also rule out any cross-contamination of an additional clone. The band shift is most likely caused by post-translational modifications, but its nature remains to be established. * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 3:** Nature and distribution of mutations in NOTCH2, TNFAIP3, TRAF3, CARD11, MYD88 and TP53 in SMZL. NOTCH2 mutations are characterised by frameshift insertion/deletion, and nonsense mutations that are clustered at the C-terminus and predicted to eliminate the C-terminal PEST, a domain critical for NOTCH2 proteasomal degradation. TNFAIP3 and TRAF3 mutations are featured by frameshift insertion/deletion and nonsense mutations, which inactivate their protein functions. CARD11 and MYD88 mutations are typically those of activating changes reported elsewhere. TP53 mutations are also characteristic inactivating changes extensively reported in the literature. The mutations identified by Fluidigm PCR and MiSeq sequencing are confirmed either by an independent Fluidigm PCR and MiSeq sequencing or Sanger sequencing. Where possible, the somatic nature of mutation was determined: those confirmed by PCR and sequencing of the paired non-tumour DNA are shown by red symbols, while those identified in a search of the COSMIC somatic mutation database are shown by blue symbols. Concurrent mutations seen in the same cases are indicated by their case number.

LNR: LIN-12/NOTCH repeats; HD: heterodimerisation; TM: transmembrane; RAM: regulation of amino acid metabolism; TAD: transactivation domain; PEST: Proline, glutamic acid, serine and
threonine rich domain; ZF: zinc finger; CARD: caspase recruitment domain; PDZ: (PSD95, DLG and ZO1 homology) domain; SH3: Src homology motif; GUK: guanylate kinase domain

**Figure 4:** Correlation of *KLF2* mutation with other genetic changes in SMZL.

A) Heatmap shows *KLF2* mutation and other genetic changes in 101 cases of SMZL. Rows correspond to genetic change, while columns indicate individual cases. Positive genetic changes are shown in green. Genes included in the BCR pathway mutations are *CARD11, BCL10, CD79A* and *CD79B.*

B) Frequencies of *KLF2* mutation and other genetic changes in SMZL;

C) Correlation among *KLF2* mutation and other genetic changes in SMZL. *KLF2* mutation is significantly and positively associated with 7q deletion, *IGHV1*-2 usage, *NOTCH2, TRAF3* and *TNFAIP3* mutations, but negatively correlated with *MYD88* mutation. BCR: B-cell receptor; Mut: mutation; Del: deletion.

**Figure 5:** A summary of the proposed molecular mechanism of SMZL.

The majority of SMZL with *KLF2* mutation have the rearranged *IGHV1*-2 that carries minimal levels of somatic mutations, suggesting derivation of these lymphoma cells from T-cell independent marginal zone B-cells. The biased usage of *IGHV1*-2 indicates possible antigenic drive by superantigen, hence chronic BCR signalling. *KLF2* inactivation by mutation may facilitate marginal zone B-cell differentiation and their homing to the splenic marginal zone. 7q deletion is predominately seen in cases with *KLF2* mutation and its role in the lymphoma pathogenesis is unknown. *CARD11, NOTCH2, TRAF3* and *TNFAIP3* mutations are found in cases with and without *KLF2* mutation. Mutations in *CARD11* and others (*CD79A/B, BCL10*) may lead to active BCR signalling, thereby
promoting cell proliferation and survival. Activation of NOTCH2, BAFFR/CD40 and TLR signalling by
\textit{NOTCH2}, \textit{TRAF3} and \textit{TNFAIP3} mutation may primarily contribute to marginal zone B-cell generation
and their retention in the splenic marginal zone.

The majority of SMZL without \textit{KLF2} mutation have heterogeneous usage of \textit{IGHV} that carries high
loads of somatic mutation, suggesting origination of these lymphoma cells from T-cell dependent
marginal zone B-cells. \textit{MYD88} and \textit{TP53} mutations are nearly exclusively seen in cases without \textit{KLF2}
mutation. \textit{MYD88} mutation most likely causes constitutive TLR signalling, while \textit{TP53} mutation
inactivates its tumour suppressor function and may promote disease progression and high-grade
transformation.
SUPPLEMENTARY MATERIALS:

Supplementary methods

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**Figure 1:** Nature and incidence of *KLF2* mutations in SMZL and other B-cell lymphomas.
**Figure 2:** KLF2 mutations and their functional characterisation.

**A**

<table>
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<th>Representative KLF2 mutants</th>
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**B**

**NF-κB reporter assay**

**HEK293T**

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**OCI-LY19 B-lymphoma cells**

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