BCR and TLR signaling pathways are recurrently targeted by genetic changes in splenic marginal zone lymphomas

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ABSTRACT

The genetics and pathogenesis of splenic marginal zone lymphoma are poorly understood. The lymphoma lacks chromosome translocation, and approximately 30% of cases are featured by 7q deletion, but the gene targeted by the deletion is unknown. A recent study showed inactivation of A20, a "global" NF-κB negative regulator, in 1 of 12 splenic marginal zone lymphomas. To investigate further whether deregulation of the NF-κB pathway plays a role in the pathogenesis of splenic marginal zone lymphoma, we screened several NF-κB regulators for genetic changes by PCR and sequencing. Somatic mutations were found in *A20* (6/46=13%), *MYD88* (6/46=13%), *CARD11* (3/34=8.8%), but not in *CD79A*, *CD79B* and *ABIN1*. Interestingly, these genetic changes are largely mutually exclusive from each other and MYD88 mutation was also mutually exclusive from 7q deletion. These results strongly suggest that deregulation of the TLR (toll like receptor) and BCR (B-cell receptor) signaling pathway may play an important role in the pathogenesis of splenic marginal zone lymphoma.

Key words: *CARD11*, *MYD88*, *A20*, somatic mutation, SMZL

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Introduction

The genetics and pathogenesis of splenic marginal zone lymphoma (SMZL) are poorly understood. The lymphoma lacks recurrent chromosome translocation, a common feature in other low grade B-cell lymphomas. Approximately 30% of cases have 7q deletion, but the gene targeted by the deletion is unknown. Although 7q deletion is a characteristic feature of SMZL, clinicopathological investigation and gene expression microarray analyses to date show that the cases with 7q deletion do not constitute a distinct subgroup. Thus, it is likely that there is a common molecular mechanism between SMZL with and without 7q deletion.

A recent study showed inactivating mutation of *A20*, also known as *TNFAIP3*, in 1 of 12 (8%) SMZLs.[1] *A20* encodes for a "global" NF-κB negative regulator and is frequently inactivated by mutation/deletion in several B-cell lymphomas characterized by constitutive NF-κB activation including MALT lymphoma, activated B-cell like diffuse large B-cell lymphoma (ABC-DLBCL), Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma.[1-7] In addition to A20, several NF-κB positive regulators, including CARD11, MYD88 and CD79 are frequently activated by mutation in ABC-DLBCL.[8-10] Our recent study also showed frequent inactivation of ABIN1, a critical adaptor molecule of the A20 inhibitory complex, in gastrointestinal DLBCL.[7] In the light of these findings and the biological connection of these NF-κB regulators, we investigated whether they are also targeted by genetic changes in SMZL.

Design amd Methods

Patients' materials

A total of 57 cases of well-characterized SMZL were investigated in the present study. The diagnosis of these lymphomas was made based on histological assessment of the spleen according to the latest WHO classification of tumors of hematopoietic and lymphoid tissues.[11] The DNA samples extracted from the lymphoma involved spleen were available from a previous study and these included 23 cases from frozen tissues and 34 cases from formalin fixed paraffin embedded tissues.[12] Due to a lack of sufficient DNA for some samples, mutation screening could not be comprehensively performed for

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each case. Genetic data on 7q deletion, *TP53* mutation and *IGH* mutation were available from a previous study.[12] Local ethical guidelines were followed for the use of archival tissues for research with the approval of the ethics committees of the institutions involved.

PCR and DNA sequencing

CARD11, *CD79A*, *CD79B*, *MYD88*, *A20* and *ABIN1* were investigated for mutation by PCR and DNA sequencing. For *MYD88*, *A20* and *ABIN1*, all coding exons were studied. For *CARD11*, exons 5-9, which encode the coiled-coil domain, were investigated as mutation was exclusively found in this region,[8] while for *CD79A* and *CD79B*, only coding exons (5 and 6) containing the mutation hot spots were examined. The primer sequences and PCR conditions are shown in the *Online Supplementary Table S1*. PCR products were routinely purified and sequenced from both orientations using the BigDye terminator chemistry 3.1 system (Applied Biosystems, Foster City, CA, USA). In each case, mutation was confirmed by at least two independent PCR and sequencing experiments. The somatic mutation was excluded from polymorphisms and germline mutation by database search and analysis of the DNA samples extracted from the microdissected normal cells as described previously.[7]

In cases where double mutations were found in the same gene, we also investigated whether the double mutations affected one or both alleles. The region spanning the double mutations was amplified and the PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen, Paisley, UK). The plasmid DNA was extracted from multiple clones using the QIAprep Spin Miniprep Kit (QIAGEN, Crawley, UK) and then sequenced using the BigDye terminator chemistry 3.1 with vector primers.

Statistical analysis

Correlation among categorical variables was evaluated by Fisher's exact probability test. Probabilities of overall survival and event free survival were calculated by the Kaplan-Meier method and the comparison between subgroups was carried out by using the log rank test. *P* values <0.05 were considered statistically significant.

Results and Discussion

A total of 4 *CARD11* mutations were identified in 3 (8.8%) of the 34 SMZLs investigated, with a single case (C18) harboring two genetic alterations, one missense mutation and one in-frame deletion in exon 5 (Figure 1 and

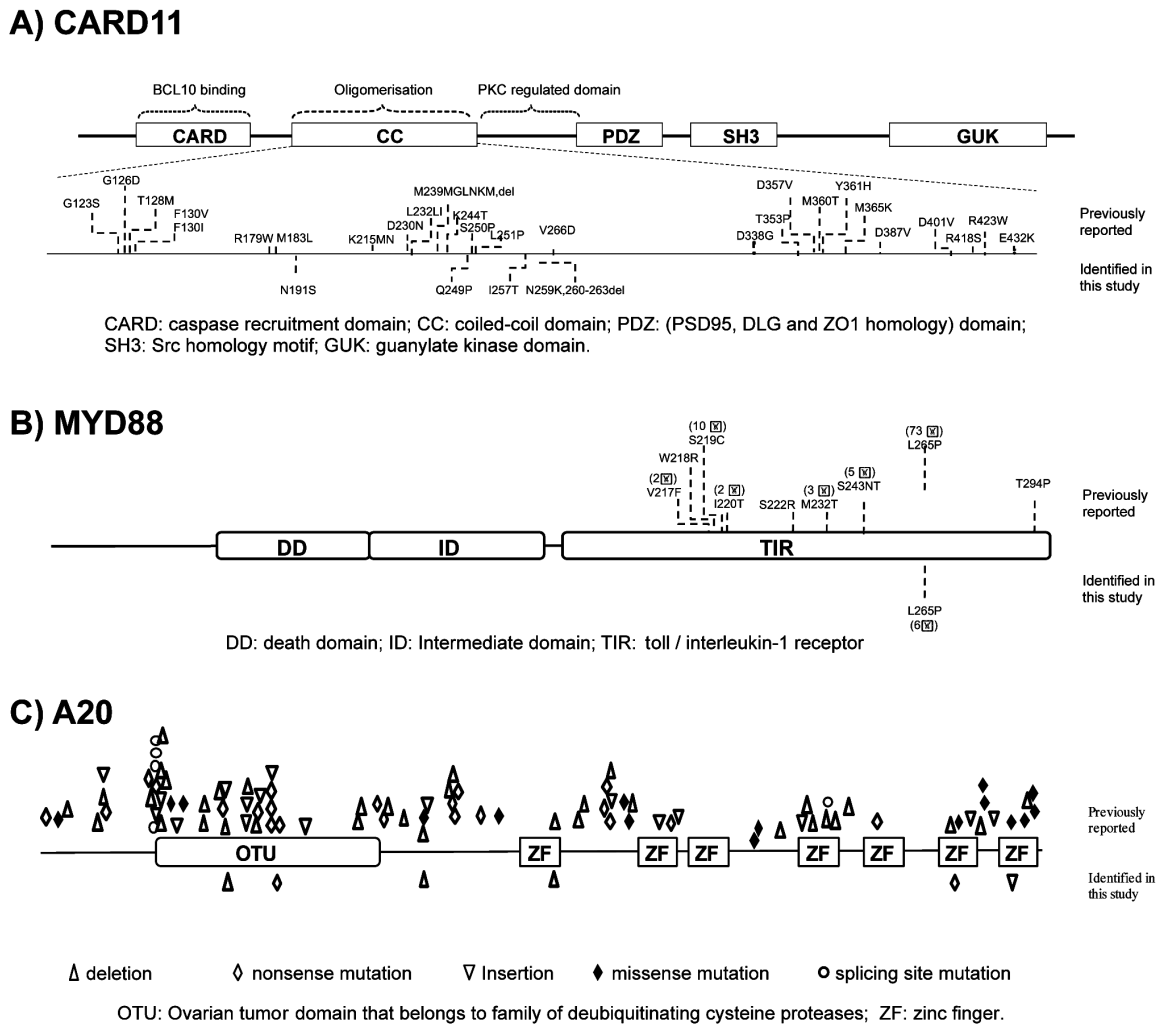

Figure 1. ***CARD11*, *MYD88* and *A20* mutations and their distribution.**

Online Supplementary Table S1). All 4 mutations were novel. Among the 3 cases with *CARD11* mutation, sufficient normal cells could be microdissected for DNA preparation only in case C18 and analysis of the normal DNA extracted confirmed the somatic origin of the double mutation (*Online Supplementary Figure S1*). To further investigate whether the two mutations occurred on the same allele, we cloned and sequenced the PCR product and the results showed that the two mutations affected the same allele. Although the functional impact of the novel *CARD11* mutations reported here is unknown, all the 20 *CARD11* mutants, including an in-frame deletion which has been investigated in previous studies, show gain of function, being more potent in NF-κB activation than the wild type as demonstrated by *in vitro* reporter assay.[7-8]

MYD88 mutation was seen in 6 (13%) of the 46 SMZLs investigated and in each of these cases, the mutation was a recurrent missense change (L265P) in the TIR domain, which was the most frequent alteration in DLBCL (Figure 1 and *Online Supplementary Table S2*).[9] In the 2 cases with the *MYD88* mutation, DNA was successfully extracted from microdissected normal cells and analyses of the normal DNA confirmed the somatic origin of the mutation. The function of the MYD88 L265P mutation has been investigated and the mutant promotes cell survival by enhancing the NF-κB and JAK-STAT3 signaling.[9]

A total of 6 *A20* mutations were seen in 6 (13%) of the 46 cases investigated and all of them were potentially functionally disruptive changes including 4 out-frame deletion / insertion and 2 nonsense mutations (Figure 1 and *Online Supplementary Table S2*). In 3 cases with *A20* mutation, DNA was successfully extracted from microdissected normal cells and analyses of the normal DNA confirmed the somatic origin of the mutation. The nature and distribution of the A20 mutations seen in the present study were similar to those reported previously.[1,3-7] These mutations most likely inactivate the NF-κB inhibitory function of A20.

PCR and sequencing of all the coding exons of ABIN1 showed no evidence of mutation in 34 cases of SMZL. Similarly, analyses of *CD79A* exon 5 and *CD79B* exons 5 and 6, in which mutation hotspots were seen in DLBCL,[10] did not demonstrate any mutation in 34 cases of SMZL investigated.

Of the 57 cases investigated, 31 had complete data on *CARD11*, *MYD88*, *A20* and *TP53* mutation as well as 7q deletion, thus allowing comprehensive correlation analyses. Interestingly, *CARD11*, *MYD88* and *A20* mutations were largely mutually exclusive with only a single case showing concurrent *CARD11* and *MYD88* mutation (Figure 2). These mutations were also largely mutually exclusive from the *TP53* mutations (Figure 2). In addition, *MYD88* mutation was mutually exclusive from 7q deletion (P=0.065), while *A20* mutation was often detected in cases with 7q deletion (P=0.068). There was no association between *CARD11*, *MYD88* and *A20* mutations, and *IGH* hyper-somatic mutation. Overall, 38% of SMZLs investigated showed somatic mutations in either *CARD11*, *MYD88* or *A20* genes. None of the above genetic abnormalities correlated with overall or event free survival (*Online Supplementary Table S3*).

During the writing of this manuscript, Rossi and colleagues also reported frequent but mutually exclusive genetic abnormalities in multiple NF-κB regulators involved in both the canonical (*A20* and *IKBkB*) and non-canonical (*BIRC3*, *TRAF3*, *MAP3K14*) activation pathways.[13] Of note, both the present study and that by Rossi and colleagues reported an identical incidence of *A20* somatic mutation in SMZL, but Rossi and colleagues found no evidence of *CARD11* and *MYD88* somatic mutation in their pilot screening of a discovery panel of 18 SMZL. Conventional PCR and Sanger sequencing were used in both studies, and the reason underlying the discrepancies in the *CARD11* and *MYD88* mutation between the two studies is not clear.

CARD11 is a scaffolding molecule that links the BCR signaling to the canonical NF-κB activation pathway (*Online Supplementary Figure S2*). IKBkB encodes IKKβ, a critical component of the IKK complex that is responsible for the phosphorylation and subsequent degradation of IKB, thus activation of the canonical NF-κB pathway. MYD88 is a universal adaptor for IL1-R and TLRs with the exception of TLR3, and also couples the receptor signaling to the canonical NF-κB, AP1 and IRF activation pathway (*Online Supplementary Figure S2*). A20, a ubiquitin modification enzyme, can specifically remove the K63-linked ubiquitin chain that is crucial for protein function, and catalyses the K48-linked polyubiquitination that targets proteins for proteasome degradation.[14-15] Through such ubiquitin modification, A20 inactivates several proteins including RIP-1, TRAF6, NEMO and TAK1, and thus negatively regulates the TLR/IL1-R and BCR signaling (*Online Supplementary Figure S2*).[16-18] *MAP3K14* encodes NIK, an essential positive regulator of the non-canonical NF-κB activation pathway. NIK is negatively regulated by TRAF3 and BIRC3 (also known as API2). The finding of somatic mutation in the above diverse regulators involving multiple cellular signaling pathways, particularly the BCR and TLR signaling, strongly suggests that a constitutive activation of these pathways may play an important role in the pathogenesis of SMZL.

The above hypothesis is also supported by several strands of evidence from previous studies, which also indicate a role for chronic antigenic stimulation in the development of SMZL. Firstly, around 12% of SMZL have a stereotyped *IGH* CDR3,[19] strongly suggesting the importance of antigen receptor signaling in the lymphoma development. Secondly, at least in Italy, 19% of SMZL are associated with HCV infection and a high proportion of these lym-

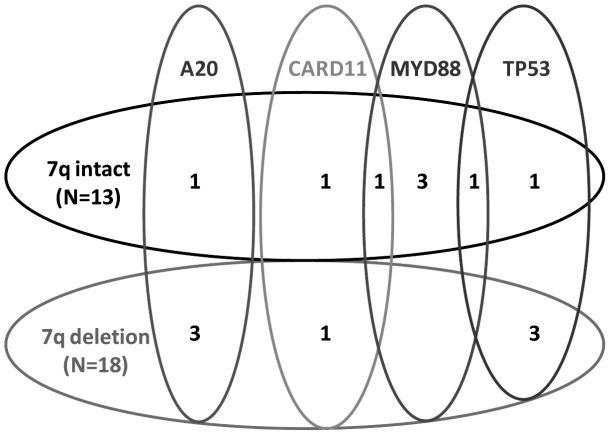

Figure 2. Correlation between 7q deletion, *TP53*, *CARD11*, *MYD88* and *A20* mutation.

phomas respond to antiviral treatment alone.[20] Nonetheless, the nature and molecular mechanisms of the implicated antigenic stimulation in SMZL have yet to be clarified. Identification of such antigenic stimuli and comprehensive investigation of genetic abnormalities in the NF-κB pathway will unravel the molecular pathogenesis of SMZL and provide critical insights into a strategy for targeted therapy.

Authorship and Disclosures

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