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**Statement of Translational Relevance:**

Diffuse large B cell lymphoma (DLBCL) shows variable clinical outcome with the activated B-cell subtype (ABC-DLBCL) characterised by constitutive NF-kB activation and poor prognosis. The genetic bases for the constitutive NF-kB activation in DLBCL remain largely unknown, nor the value of such genetic abnormalities in DLBCL diagnosis and treatment prediction. We report several important novel observations on genetic abnormalities of NF-kB regulators in DLBCL and specifically we demonstrated that:

1) *ABIN-1* and *ABIN-2*, the adaptors of the A20 inhibitory complex of the NF-kB activation pathway, were also recurrently targeted by inactivating somatic mutations;
2) *A20, ABIN-1, ABIN-2* and *CARD11* somatic mutation were almost mutually exclusive;
3) *A20* somatic mutation was significantly and independently associated with both poor overall survival and event free survival.

These findings provide further evidence of NF-kB pathway genetic abnormalities in DLBCL, which are potentially valuable in prognosis and design of future therapeutic strategy.
Abstract

Purpose: Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of aggressive lymphomas with the activated B-cell-like subtype characterised by constitutive NF-kB activation. Activating mutations of CARD11 and inactivating mutations of A20 are frequent events in DLBCL. However, the full extent of genetic alterations in the NF-kB pathway regulators and their potential prognostic value in DLBCL remain to be investigated. We investigated the genetic abnormalities of CARD11, A20 and ABIN-1/2/3 (the A20 binding inhibitor of NF-kB) and their clinicopathological correlation in gastrointestinal DLBCL.

Experimental design: The somatic mutation and copy number changes of CARD11, A20, and ABIN-1/2/3 were investigated in 71 gastrointestinal DLBCLs by PCR / sequencing, and interphase fluorescence in situ hybridisation / array comparative genomic hybridisation respectively. The mutations identified were functionally characterised by NF-kB report assays and immunoprecipitation experiments.

Results: Recurrent somatic mutations were found in CARD11 (10%), A20 (17%), ABIN-1 (4%), and ABIN-2 (3%), but not in ABIN-3. In comparison to the wild-type, all CARD11 mutants were potent NF-kB activators in vitro. Based on the destructive nature of the observed mutations, and the findings by reporter assays and immunoprecipitation studies, most if not all of the somatic mutations that were seen in A20, ABIN-1, and ABIN-2 could impair their normal functions. Among these genetic abnormalities, A20 somatic mutation was significantly associated with both poor overall survival and event-free survival.

Conclusions: We demonstrate further evidence of NF-kB pathway genetic abnormalities in DLBCL, which are potentially valuable in the prognosis and design of future therapeutic strategies.

Keywords: DLBCL; A20; ABIN-1; ABIN-2; CARD11; NF-kB
Introduction

NF-κB is a master transcription factor that is critical to a number of biological processes that are involved in both innate and adaptive immunity. There is growing evidence that NF-κB is constitutively activated in several lymphoma subtypes, including MALT lymphoma (1), activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL) (2, 3), primary mediastinal large B-cell lymphoma (PMBL) (4, 5), and multiple myeloma (MM) (6), and NF-κB activity is essential to the survival of these lymphoma cells (7). The genetic bases that underlie the constitutive NF-κB activation in these lymphomas have received extensive investigation (8-11). The two most recent exciting advances in this field of research concern the findings of CARD11 activation and A20 inactivation in several lymphoma subtypes.

CARD11, which is also known as CARMA1 (CARD-MAGUK protein 1), is a scaffolding molecule that links antigen receptor signalling to the BCL10/MALT1-mediated NF-κB activation (12). Using an unbiased loss-of-function RNA interference screen, Ngo et al. have shown that CARD11 was critical for the proliferation and survival of ABC-DLBCL cells but not GCB-DLBCL cells (13). Subsequent mutational analysis by Lenz et al. identified missense mutations in 7/73 (9.6%) ABC-DLBCL and 3/79 (3.8%) GCB-DLBCL, and all of the CARD11 mutations were within the coiled-coil domain and were capable of activating the NF-κB reporter gene in vitro (14). Two recent studies confirmed similar frequencies of CARD11 mutation in nodal DLBCL (15, 16).

A20, which is also known as TNFα-induced protein 3 (TNFAIP3), is a “global” essential negative regulator of the NF-κB activation pathway and can attenuate the NF-κB activity that is triggered by signalling from TNF and Toll-like receptors (17-19). By array comparative genomic hybridisation (CGH), we and others have identified A20 as the target of a 6q23 deletion in ocular adnexal MALT lymphoma (8, 20, 21). By interphase fluorescence in situ hybridisation (FISH), we have demonstrated that A20 deletion, which frequently involves both alleles, preferentially occurs in translocation-negative MALT lymphoma of the ocular adnexa (19%), salivary gland (8%), and thyroid (11%), but not in those of the lung and stomach (8). Several independent studies have further demonstrated the frequent inactivation of A20 by mutation and/or deletion in MALT lymphoma (18-22%), DLBCL (7.8-54%), Hodgkin’s lymphoma (33-44%), and PMBL lymphoma (36%) (15, 16, 22-24). In a recent study, we found that a A20 hemizygous deletion was commonly associated with its promoter methylation and that complete inactivation of the A20 gene by
deletion/promoter methylation or biallelic deletion is significantly associated with a poor lymphoma-free survival in ocular adnexal MALT lymphoma (9).

Despite the aforementioned exciting advances, several imperative questions remain to be answered. Are A20 inactivation and CARD11 activation mutually exclusive? A20 requires its binding partner, such as ABIN-1/2/3 (the A20 binding inhibitor of NF-kB), to function as a negative regulator of the NF-kB activation pathway (25). Are these A20 adaptor molecules also targeted by genetic abnormalities in lymphoma? What are the clinical impacts of these NF-kB regulator abnormalities, and can these abnormalities be used as a prognostic marker? To address these questions, we have comprehensively investigated the genetic abnormalities of CARD11, A20, and ABIN-1/2/3 (the A20 binding inhibitor of NF-kB) in gastrointestinal DLBCL, which is poorly understood at the genetic level although the majority of gastric DLBCL are associated with H. pylori infection and the response of gastrointestinal DLBCL to treatment is much favourable than nodal DLBCL (26-28). Comprehensive correlations among the genetic abnormalities identified and clinicopathological parameters were performed.

Materials and methods

Tissue materials
A total of 71 cases of primary gastrointestinal DLBCL (43 gastric, 28 intestinal) from the authors’ institutions were successfully investigated in this study. Among these cases, a low grade MALT lymphoma component was identified in 13 cases (18%). Thirty-nine cases had both frozen and formalin-fixed paraffin-embedded (FFPE) tissue specimens, whereas the remaining 32 cases had only FFPE tissues. The clinicopathological features of the majority of these cases have been described elsewhere (29, 30). Local ethical guidelines were followed for the use of archival tissues for research with the approval of the ethics committees of the involved institutions.

Immunohistochemistry
This was carried out routinely on formalin-fixed paraffin-embedded tissue sections with streptavidin-biotin peroxidase method using mouse monoclonal antibodies. Classification of DLBCL into GC and non-GC subgroups was based on immunohistochemical analysis of CD10 (Novocastra), BCL6 and MUM1 (Dako) according to the algorithm of Han et al (31). For each antibody, cases were considered positive if 30% of DLBCL cells showed positive staining.
Microdissection and DNA preparation
Crude microdissection was performed in each case to enrich tumour cells (32). DNA was extracted using standard proteinase K digestion, followed by phenol/chloroform/isoamyl-alcohol extraction or using the QIAamp DNA Micro Kit (QIAGEN, Crawley, UK). The quality of the DNA samples was assessed by the PCR amplification of variably sized gene fragments (33), and those with successful amplifications of genomic fragments in excess of 300bp, in addition to the DNA samples from the frozen tissues, were used for mutational screening.

High-resolution melting analysis
High-resolution melting analysis (HRM) was used initially for the CARD11 mutation screening. Fourteen primer sets were designed to amplify the coiled-coil domain of CARD11, which is encoded by exons 5-10, within which activating mutations were found in a previous study (14). The primer sequences and PCR conditions are detailed in supplementary Table S1. HRM analysis was performed immediately after PCR using the Rotor-Gene 6000 analyser (Corbett Life Science, Sydney, Australia). PCR samples with possible mutations were further investigated by direct DNA sequencing.

PCR and sequencing
In subsequent study, CARD11 mutations were screened by PCR and direct sequencing since this was much efficient. In all cases, mutations in the A20, ABIN-1, ABIN-2, and ABIN-3 genes were screened by PCR followed by sequencing. The primer sequence and PCR conditions are detailed in Table S1. PCR products were routinely purified and directly sequenced in both orientations using the BigDye terminator chemistry 3.1 system (Applied Biosystems, Foster City, CA). In each case, the presence of a mutation was confirmed by at least two independent PCR and sequencing experiments, and each mutation was verified as not being a polymorphism by a search of online NCBI and Ensemble databases and from a germline mutation by an analysis of DNA samples that had been prepared from normal tissues or microdissected normal cells.

Interphase fluorescence in situ hybridisation (FISH)
A20 (6q23) and TNFA/B/C (6p21) loci copy number changes were investigated using a 3-colour FISH assay as described in our previous study (8). Chromosome translocations involving the BCL2, BCL6, CCND1, MYC, and IGH loci were investigated using respective dual-colour break-
apart probes and appropriate dual-colour dual-fusion probes, where indicated (Vysis/Abbott Laboratories, UK) (34).

**A20 promoter methylation analysis**

DNA samples (600 ng) were bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, USA). Two separate bisulfite treatments were performed in each case so as to facilitate independent pyrosequencing experiments. One-fifth of the converted DNA was PCR-amplified for a 222-bp fragment of the A20 promoter that is upstream from the first exon (9). The PCR products were checked for specificity on 3% agarose gels and subjected to pyrosequencing using Pyro Gold SQA reagents (Biotage, Uppsala, Sweden) on a PyroMark MD pyrosequencer (Q-CPG software™ version 1.0.9, Biotage) as previously described (9).

**NF-κB reporter assay**

The full-length coding sequence of CARD11, ABIN-1, and ABIN-2 was cloned into a modified pIRESpuro2 expression vector (Clontech, Saint-German-en-Laye, France), and various mutants of CARD11, ABIN-1, and ABIN-2 were generated using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla). The capacity of these mutants to induce or suppress NF-κB activation was measured in Jurkat T-cells and HEK293 cells using a dual-luciferase reporter assay system (Promega, Southampton, UK) (35). Briefly, Jurkat T-cells (5×10⁶) were transfected with 2μg of expression vector, 0.8μg of pNFκB-luc (a firefly luciferase reporter for NF-κB activity), and 0.6μg of pRL-TK (a Renilla luciferase reporter as a control) using Amaxa nucleofector system (Amaza, Cologne, Germany). The transfected cells were cultured for 24 hours and then harvested for a luciferase assay. Similarly, HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen), cultured for 22 hours, stimulated with TNFα (300 IU/ml) for 2 hours, and then harvested for luciferase activity measurement. For each experiment, at least three independent transfections and duplicate reporter assays were performed, and the data were normalised to appropriate controls and presented as a mean ± standard deviation.

**Immunoprecipitation**

HEK293 cells were co-transfected with HA-A20 and Flag-ABIN-2 (wild-type or mutant) using Lipofectamine 2000 and then collected for immunoprecipitation 24 hours later. Cells that were similarly transfected with an empty vector were used as a control. Immunoprecipitation of HA-
A20 and Flag-ABIN-2 were performed using the anti-HA immunoprecipitation kit and the Flag-tagged protein immunoprecipitation kit (Sigma-Aldrich, UK), respectively, according to the manufacturer’s instructions. Total cell lysate (input), flow-through, and immunoprecipitation eluate were analysed by Western blot. The A20-binding capacity was calculated by quantifying the chemiluminescent signals of ABIN-2 and A20 proteins that were detected in the immunoprecipitation eluate using the Quantity One software package (BioRad). For immunoprecipitation with HA-A20, the difference between the wild-type and mutant ABIN-2 that co-immunoprecipitated with A20 were compared after normalisation by the amount of A20 in the eluate and vice-versa for immunoprecipitation with Flag-ABIN-2. The immunoprecipitation experiments were conducted twice.

Statistical analysis
Overall survival (OS) was measured from the date of diagnosis to death from any cause. Event-free survival (EFS) was measured from the date of diagnosis to disease progression, relapse, or death from any cause. Probabilities of OS and EFS were calculated by the Kaplan-Meier method, and the comparison between subgroups was carried out via the log-rank test, wherein any variable that demonstrated a P value of ≤ 0.1 was further tested by a multivariate analysis using the Cox proportional hazard regression model. The correlation among variables was evaluated by Fisher’s exact probability test. All statistical analyses were carried out using SPSS, version 13 (Surrey, UK).

Results

CARD11, A20, and ABIN-1/2/3 genetic abnormalities in gastrointestinal DLBCL
Of the 60 cases of gastrointestinal DLBCL that were analysed by 1Mb resolution CGH, 9 (15%) exhibited an extra copy of 7p22 that contained CARD11, whereas none exhibited an amplification of this genomic locus (data not shown). A total of 71 cases of gastrointestinal DLBCL were investigated for mutations in the CARD11 coiled-coil domain by a combination of HRM and sequencing (39 cases), direct sequencing (32 cases), or both methods (7 cases). A total of nine mutations excluding known polymorphisms were identified in seven cases, and a single case was observed to harbour three mutations (G126D, V266D, and T353P) (Figures 1A and 2A, Table S2). Because there was no frozen tissue available, it was not possible to amplify the genomic or cDNA sequence that included all three of the mutation sites to investigate whether these different
mutations occurred in one or both alleles of the \textit{CARD11} gene. The somatic origin of these mutations was confirmed in each of the six cases where DNA samples had been successfully extracted from the microdissected normal cells. In the remaining case, it was not possible to microdissect enough normal cells; however, the mutant was much more potent than the wild-type in NF-kB activation (please see the section below), and, therefore, it was most likely pathogenic. The distribution of \textit{CARD11} mutations that was observed in this study is similar to those that have been recently reported. These mutations appear to be clustered into four regions, and the only recurrent mutation has been observed in codon 126 ($2/27=7\%$) (Figure 2A) (14, 15).

Interphase FISH demonstrated a heterozygous deletion of \textit{A20} and a gain of the \textit{TNFA/B/C} locus in 13/71 (18.3\%) and 11/71 (15.5\%) gastrointestinal DLBCL, respectively (Figure 1A). There was no association between \textit{A20} deletion and \textit{TNFA/B/C} gain. PCR analysis of all \textit{A20}-coding exons followed by DNA sequencing identified a total of 15 mutations, excluding known polymorphisms, in 13/69 (19\%) cases, wherein two cases were each observed to harbour two mutations (one case exhibited a 77-bp deletion and a M476I mutation in exon 7, whereas the other displayed a 6-bp deletion in exon 2 and a 1-bp deletion in exon 6) (Figure 1A, Table S2). Because there was no frozen tissue, it was not possible to investigate whether these mutations occurred in one or both alleles of the \textit{A20} gene. PCR and sequence analyses of the DNA samples that had been extracted from microdissected normal cells confirmed the somatic origin of the detected mutations in 12/13 cases, whereas the remaining case exhibited a germline missense mutation (Table S2). There was no association between \textit{A20} deletion and mutation. Pyrosequencing showed evidence of promoter methylation in 1/69 (1.4\%) cases (Figures S1A and S2), and this single case did not display an \textit{A20} deletion or mutation.

Among the 14 \textit{A20} somatic mutations that were identified in this study, the majority (79\%) were predicted to produce truncated proteins due to out-frame insertion (two cases) or deletion (six cases), nonsense mutation (one case), or mutation in the splicing site (two cases), whereas the remaining 3 mutations (21\%) were missense mutations (Figure 2B). These mutations are similar in nature to those that have been recently reported (Figure 2B), and they would most likely impair A20 function (15, 16, 22-24). Nonetheless, unlike these reported mutations, the mutations that were observed in gastrointestinal DLBCL were biased towards the OTU domain (ovarian tumour domain, which belongs to the family of deubiquitinating cysteine proteases) (Figure 2B).
A20 requires its binding partner, i.e., adaptor proteins, to function as a negative regulator of the NF-kB activation pathway, and ABIN-1/2/3 are its major adaptor molecules. We next investigated ABIN genetic abnormalities. None of the 60 cases of gastrointestinal DLBCL that were analysed by 1Mb resolution array CGH exhibited evidence of deletion at the ABIN-1 (5q32-33.1), ABIN-2 (4p16.3), or ABIN-3 (4q27) loci (data not shown). PCR and sequencing of all of the ABIN-1-coding exons identified a total of nine mutations, excluding known polymorphisms, in 7/68 (10.3%) cases (Figure 2C, Table S2). Two cases each harboured two mutations (one showed somatic R263W and E476K in exons 8 and 14, respectively, whereas the other displayed germline T286M and I374T in exons 9 and 11, respectively). Another two cases exhibited somatic insertions or deletions that caused a reading frame shift, and the predicted truncated products lack important functional domains, including AHD1 (ABIN homology domain), AHD2, UBAN (ubiquitin-binding domain in ABIN and NEMO), and NBD (NEMO binding domain). The remaining three cases each displayed a recurrent missense mutation (R263W) that was downstream of the second coiled-coil domain, which was confirmed to be a germline alteration in two cases where normal DNA was available for analysis (Figure 2C).

Similarly, PCR and sequencing of all ABIN-2-coding exons identified a total of five mutations, excluding known polymorphisms, in 5/68 (7.4%) cases (Figure 1A). Two cases exhibited a recurrent somatic missense mutation at the conserved residue (E255) of AHD1, whereas the remaining three cases displayed a recurrent germline missense mutation at amino acid 249, which is immediately upstream of AHD1 (Figure 2D). The AHD1 in ABIN-2 is critical to the binding of both A20 and NEMO (36), whereas the region (amino acids 194-250) that is upstream of AHD1 is responsible for the binding of TPL2 (37). Thus, these mutations may affect these protein-protein interactions and impair ABIN-2 function.

ABIN-3 contains 14 coding exons (including alternative spliced exons) and exons 8-10, which encode AHD1, 2, and 4 and the UBAN domain. The PCR analysis and sequencing of these three coding exons in the 40 cases of gastrointestinal DLBCL did not show any evidence of somatic mutation.

Interphase FISH was performed to investigate the chromosome translocations that are frequently seen in DLBCL and exhibited evidence of translocations involving the BCL2 (2/51 cases), BCL6 (10/48), and MYC (2/49) genes but not the CCND1 gene (0/50) (Table S2).
Comprehensive correlation analyses demonstrated that there was no association among the above somatic mutations and chromosomal structural and numerical changes. A20, ABIN-1, ABIN-2, and CARD11 somatic mutations were almost mutually exclusive. Among the 21 cases with somatic mutation in any of these four genes, only one case exhibited concurrent mutations (Table S2).

**The functional impact of ABIN-1, ABIN-2, and CARD11 mutations**

To examine the functional consequence of CARD11, ABIN-1, and ABIN-2 mutations, we first investigated whether the CARD11 mutants gained the ability to activate NF-kB and whether ABIN-1/2 mutants lose the ability to inhibit NF-kB activation via the use of a reporter assay.

In comparison to the wild-type, all nine CARD11 mutants that were identified in the present study were capable of activating the NF-kB reporter in Jurkat T-cells in the absence of any immune receptor stimulation, and interestingly, these CARD11 mutants were much more potent than the BCL10, MALT1, and API2-MALT1 fusion products (Figure 3A).

For ABIN-1 mutation, we did not include the two frameshift mutations in the *in vitro* functional investigations. These mutations predicted truncated proteins that lacked the critical functional domains, including AHD1, UBAN, and NBD, and would certainly impair ABIN-1 function. All of the remaining ABIN-1 and ABIN-2 mutations were subjected to the NF-kB reporter assay. As expected, both wild-type ABIN-1 and ABIN-2 were capable of inhibiting CARD11- and TNFα-mediated NF-kB activation (Figure 3B). Among the four *ABIN-1* missense mutations that were investigated, the E476K somatic mutant totally lost the ability to inhibit NF-kB activation, and the remaining three germline mutants exhibited no apparent difference from the wild-type. Among the two ABIN-2 missense mutations, both the E255K somatic mutant and the Q249H germline mutant were less efficient than the wild-type ABIN-2 in the inhibition of CARD11-mediated NF-kB activation in Jurkat T-cells (Figure 3C). Intriguingly, such inhibition was not seen in TNFα-mediated NF-kB activation in HEK293 cells (Figure 3C).

To further investigate the ABIN-2 E255K mutant, we investigated its binding to A20 by co-immunoprecipitation. In comparison to the wild-type ABIN-2, the E255K mutant lost 70% of its A20 binding capacity (Figure 3D).

*A20 mutation was significantly associated with poor survival*
Comprehensive correlation between the aforementioned genetic abnormalities and clinicopathological parameters was performed. 

A20 somatic mutation was significantly associated with the non-GC subtype of DLBCL (Figure 1C, P=0.038), as defined by the immunohistochemistry algorithm of Hans et al.(38). Such an association was not seen for ABIN-1/2 and CARD11 somatic mutations (Figures 1B and 1C). Conversely, CARD11 somatic mutations exhibited a strong trend of association with more advanced stages (>IIE or above) of DLBCL (Figure 1B, P=0.066).

Clinical follow-up data were available for 55 cases. The majority (32) of these cases were first treated with an anthracycline containing chemotherapy, for example CEOP (cyclophosphamide, epirubicin, vincristine, and prednisone), CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) and R-CHOP (rituximab-CHOP), with the remaining cases being treated with other therapy regimens, surgery alone or surgery in combination with chemotherapy (Table S2).

In light of the heterogeneous treatments, we focused our analyses on the cases treated with an anthracycline containing therapy. The clinical follow up period in this group of patients ranged from 0 to 150.5 months (median 51.5 months) and the 5-year OS and EFS were 48.1% and 40.7%, respectively. Comprehensive correlation between the observed somatic genetic abnormalities and clinicopathological parameters was carried out. The results of a univariate analysis of the prognostic variables are summarised in Table 1. Patients with advanced stages of disease exhibited a strong trend of association with both poor OS (P=0.063) and EFS (P=0.043). Among the A20, ABIN-1, and CARD11 genetic abnormalities, the A20 somatic mutations showed a significant association with both poor OS (P=0.001) and EFS (P=0.002) (Table 1). CARD11 mutation also displayed a trend of association with poor EFS albeit not statistically significant (P=0.088). There was no association between A20 deletion, ABIN-1 and ABIN-2 somatic mutations and patient survival. However, the number of cases with these genetic abnormalities was small for a reliable survival analysis. Multivariate analyses of the parameters that initially exhibited a trend of association with survival (P ≤ 0.1) by univariate analysis demonstrated that only the A20 somatic mutation was an independent prognostic marker for both OS and EFS (Table 2).

**Discussion**
By screening the genetic abnormalities of NF-kB regulators in gastrointestinal DLBCL and correlating these abnormalities to clinicopathological parameters, we have made several important and novel observations in the present study. First, we have shown that $A20$-inactivating mutations and $CARD11$-activating mutations were frequent events in gastrointestinal DLBCL. Second, we have demonstrated, for the first time, that ABIN-1 and ABIN-2, which are the adaptors of the $A20$ inhibitory complex of the NF-kB activation pathway, are also recurrently targeted by inactivating mutations, and that $A20$, $ABIN-1$, $ABIN-2$, and $CARD11$ somatic mutations are almost mutually exclusive. Finally, among $A20$, $ABIN-1$, $ABIN-2$, and $CARD11$ genetic abnormalities, the $A20$ somatic mutation was significantly and independently associated with both OS and EFS.

In line with previous findings (14, 15), we have also found frequent $CARD11$ somatic mutations in gastrointestinal DLBCL, and all of these mutations were observed to cause missense changes. Intriguingly, among the 26 mutations that have been reported so far, only 1 mutation was recurrent, which was seen in two cases (Figure 1A). Nonetheless, all CARD11 mutants were much more potent in NF-kB activation than the wild-type in the absence of any immune receptor stimulation, suggesting that these mutations may cause constitutive NF-kB activation.

$A20$, which is a target of the NF-kB transcription factor, attenuates NF-κB activities by inactivating several proteins that are critical to NF-kB signalling, such as RIP1/2, TRAF6, NEMO, and TAK1 (17-19). $A20$ can specifically remove the K63-linked ubiquitin chain that is crucial to protein function, and this is essentially mediated by the N-terminal OTU domain that belongs to the family of deubiquitinating cysteine proteases (25, 39). Additionally, $A20$ catalyses the K48-linked polyubiquitin that targets proteins for proteasome degradation, and this is mediated by the C-terminal ZF domains that possess E3 ligase activity (25, 39). In line with previous findings (15, 16, 22-24), the majority (79%) of the somatic mutations that have been seen in gastrointestinal DLBCL are insertion, deletion, nonsense, or splicing site mutations, of which all are predicted to produce truncated proteins that impair $A20$ function (Figure 2B). Of the remaining three somatic missense mutations, two occurred within the known functional domain (one in OUT and one in ZF) and the third between the third and fourth ZF domains. These mutations may also impair $A20$ function, although they have yet to be tested.

$A20$ does not directly recognise its substrates; this is mediated by $A20$ adaptor molecules, such as ABIN-1/2/3, TAX1BP1, Itch, and RNF11 (25). In the present study, we have shown, for the first
time, that \textit{ABIN-1} and \textit{ABIN-2} are also targeted by somatic mutations and that all of the identified somatic mutations could impair their respective functions. Of the three cases with \textit{ABIN-1} somatic mutation, two cases exhibited frameshift mutations that predicted truncated products that lacked AHD1, UBAN and NBD, which are critical to the binding of A20, ubiquitin, and NEMO, respectively (36). The deletion of these critical protein interaction domains is known to abolish the NF-kB inhibitory capacity of ABIN-1 (40-42). The remaining ABIN-1 E476K somatic mutation occurred within the highly conserved DFxxER motif of AHD2 (42). As shown in our NF-kB reporter assay, the single amino acid substitution (E476K) caused by a somatic point mutation was sufficient to completely abolish the NF-kB inhibitory function of the wild-type ABIN-1. The importance of this amino acid was also demonstrated by the functional characterisation of a double mutant (ER476-7AA) in a previous study (42). Similarly, the recurrent ABIN-2 E255K somatic mutation was also observed to occur at a highly conserved residue of AHD1, which is critical to the binding of A20 (43). Although this mutant did not exhibit unequivocal evidence of impaired NF-kB inhibition by reporter assays, immunoprecipitation clearly demonstrates its defect in binding to A20. Thus, in essence, the somatic mutations that are seen in \textit{ABIN-1} and \textit{ABIN-2} could impair, if not completely abolish, their NF-kB inhibitory function.

Interestingly, \textit{CARD11}, \textit{A20}, \textit{ABIN-1}, and \textit{ABIN-2} somatic mutations were almost mutually exclusive, wherein only 1 of the 21 positive cases exhibited concurrent mutations that involved two or more of these genes. Given that all of these protein products commonly regulate the NF-kB activation pathway, it is conceivable that it might be unnecessary for tumour cells to acquire genetic abnormalities that affect more than one molecule in the same molecular pathway. Interestingly, among these abnormalities, the \textit{A20} somatic mutation was significantly and independently associated with poor OS and EFS. In view of the fact that ABC-DLBCLs are associated with both \textit{A20} mutations and poor clinical outcome (15, 16), further study is required to address whether the prognostic value of \textit{A20} somatic mutation is totally independent of its association with the ABC subtype. There was no association between \textit{A20} deletion, \textit{CARD11}, \textit{ABIN-1}, and \textit{ABIN-2} somatic mutation and patients’ survival. However, it should be noted that the number of cases with these abnormalities, particularly \textit{ABIN-1} and \textit{ABIN-2} somatic mutation, is small, not permitting a reliable survival analysis. In addition, the capacity of ABIN-1, most likely ABIN-2, in NF-κB inhibition is not as strong as A20 (44).
Germline mutation in the $A20$, $ABIN-1$, and $ABIN-2$ genes is another interesting finding in this study. All of these germline mutations cause amino acid changes and have not been reported in the current SNP databases. It remains to be investigated whether these germline mutations are functional. By NF-kB reporter assay, the ABIN-1 and ABIN-2 germline mutants did not show apparent abnormalities in comparison to their wild-types. However, the reporter assay may not be an efficient approach to detecting the potential functional abnormalities of these missense mutations. For example, the ABIN-2 E255K somatic mutant, which had a major defect in its A20 binding, but showed no apparent defect by the reporter assay. The function of A20, ABIN-1, and ABIN-2 and their interacting proteins are not yet fully characterised. At this time, it is not possible to further test these germline mutants using conventional immunoprecipitation experiments. Nonetheless, the recent finding of the association of the $A20$ and $ABIN-2$ gene polymorphisms to several chronic inflammatory disorders, including rheumatoid arthritis and systemic lupus erythematosus (17, 45-52), highlights the importance of the functional characterisation of these germline mutations in the future.

The NF-kB activation pathway is governed and regulated by many positive and negative regulators. In addition to the aforementioned $CARD11$, $A20$, $ABIN-1$, and $ABIN-2$ gene mutations, mutations in $TNFSF11A$ ($RANK$), $TRAF5$, $TRAF2$, and $MAP3K7$ ($TAK1$), although generally at a low frequency, have also been reported in nodal DLBCL (15). Recently, Davis et al. reported the frequent activating mutation (16%) of CD79B in DLBCL (53). Studies of multiple myeloma have shown inactivating mutations in the NF-kB negative regulators, including $TRAF2$, $TRAF3$, $CYLD$, and $API1/API2$, and activating mutations in the NF-kB positive regulators, including $NFkB1$, $NFkB2$, $CD40$, $LTBR$, $TACI$, and $NIK$ (10, 11). The full scale of NF-kB regulator genetic abnormality in DLBCL and in reference to disease prognosis and treatment prediction remains to be investigated.

Nonetheless, the finding of a significant association between NF-kB pathway gene abnormalities and poor prognosis in gastrointestinal DLBCL may have important implications to patient management. Previous studies have shown that the NF-kB pathway gene mutations that are seen in DLBCL are more frequent in the ABC- than the GCB-subtype (14-16). In line with this, we have also found that $A20$, $ABIN-1$, and $ABIN-2$, but not $CARD11$ somatic mutation, were more frequent in non-GC than in GC-DLBCL, as defined by the immunohistochemistry algorithm of Hans et al (38). ABC-DLBCL is characterised by constitutive NF-kB activation (13) and exhibits a much poorer survival than GCB-DLBCL, regardless of being treated with CHOP or R-CHOP.
In light of the recent advances in the development of NF-kB inhibitors and their potential application in cancer therapies, a comprehensive characterisation of NF-kB regulator gene abnormalities and their prognostic value in DLBCL will not only benefit current patient care, but also provide a basis for the design of future therapeutic strategies that incorporate NF-kB inhibitors.

Supplementary information that accompanies the paper is available at the Clinical Cancer Research website.

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References:


Figure legends

Figure 1. The frequencies of genetic abnormalities in gastrointestinal DLBCL and their correlation with clinical stage and immunophenotype. Gain of the TNF locus, deletion of the A20 locus and somatic mutation of the A20 and CARD11 gene are a frequent event, while A20 promoter methylation and somatic mutation of the ABIN-1 and ABIN-2 gene are relatively infrequent (Panel A). CARD11 somatic mutation is more frequently seen in cases with advanced stages, while A20 and ABIN-1 somatic mutations show no association with clinical stage (Panel B). Of the 4 genes investigated, only A20 somatic mutation is significantly associated with non-germinal centre immunophenotype (Panel C). Germline mutation was also found in A20, ABIN-1 and ABIN2, but not included in this figure.

* P < 0.05; SM: somatic mutation; pro-methyl: promoter methylation; GC: germinal centre immunophenotype; non-GC: non-germinal centre immunophenotype.

Figure 2. The distribution of mutations in CARD11, A20, ABIN-1, and ABIN-2 in gastrointestinal DLBCL. Multiple mutations that occur in the same gene in the same case are indicated by * or # in bold. CARD11 mutations are exclusively somatic missense changes that gain capacity to activate NF-κB. Majority of A20 somatic mutations seen in gastrointestinal DLBCL are destructive changes (frameshift and nonsense mutations), similar to those reported in other lymphoma subtypes. ABIN-1 somatic mutations are composed of frameshift changes and missense mutations, and the latter often occurs at the conserved amino acid residuals. Two recurrent ABIN-2 mutations are seen in gastrointestinal DLBCL and the somatic mutation (E255K) occurs at the conserved amino acid residual.

Figure 3. The functional characterisation of CARD11, ABIN-1, and ABIN-2 mutations.

A) All nine CARD11 mutants that are seen in gastrointestinal DLBCL are much more potent than the wild-type CARD11 in NF-κB activation, as shown by the luciferase reporter assay in Jurkat T-cells.

B) In comparison to the wild-type ABIN-1, the E476K somatic mutant does not inhibit NF-κB activation by CARD11 in Jurkat T-cells and TNFα in HEK293 cells. In contrast, the three
germline mutants (R263W, T286M, and I374T) show no apparent difference from the wild-type.

C) In comparison to the wild-type ABIN-2, the E255K somatic mutant and the Q249H germline mutant are less efficient in inhibiting NF-kB activation by CARD11 in Jurkat T-cells; however, such an effect is not seen in TNFα-mediated NF-kB activation in HEK293 cells.

D) Co-immunoprecipitation shows that the ABIN-2 E255K mutant is impaired in its binding to A20 in comparison to its wild-type.

The data that correspond to the NF-kB reporter assays represent three (Jurkat T-cells) or four (HEK293 cells) independent experiments, whereas the co-immunoprecipitation (IP) experiment was performed twice. * P < 0.01.

Vector control; WT: wild-type; mut: mutant; AM: API2-MALT1.

**Figure 4:** The impact of A20 somatic mutation on the survival of patients with gastrointestinal DLBCL. Only 32 cases of gastrointestinal DLBCL treated with an anthracycline containing therapy were included in survival analysis. Among these cases, A20 somatic mutations are significantly associated with both poor overall and event free survival. A single germline mutation was also found in A20, but not included in this survival analysis.
Table 1. The results of the univariate analysis for prognosis, as evaluated by the Kaplan-Meier method.

<table>
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<th>Factors</th>
<th>OS 5y</th>
<th>P'</th>
<th>EFS 5y</th>
<th>P'</th>
</tr>
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<td>0.750</td>
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\(^1\)Log-rank test; \(^2\)Cases with A20 somatic mutations were excluded from the analysis so as to avoid confounding; \(^3\)Chromosome translocation involving the BCL2, BCL6, or MYC gene loci.
Germline mutation was also found in A20, ABIN-1 and ABIN2, but not included in this Table.
Table 2: The multivariate analysis of p2. Multivariate analysis of prognostic factors by Cox proportional hazards regression.

<table>
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<th>Prognostic variable*</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>Relative risk</th>
<th>P value</th>
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<td>5.697</td>
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<tr>
<td>Event free survival</td>
<td>$A20$ somatic mutation</td>
<td>1.414</td>
<td>0.613</td>
<td>4.112</td>
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*The prognostic variables that were included in the multivariate analysis are those with a $P$ value of $\leq 0.1$ by univariate analysis.
Figure 1

A

Frequency (%)

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<th>Event</th>
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<td>Trisomy ch6</td>
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<td>A20 deletion</td>
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<td>A20 SM</td>
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<td>A20 pro-methy</td>
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<td>ABIN-1 SM</td>
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<td>CARD11 SM</td>
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B

Frequency (%)

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<td>A20 SM</td>
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<tr>
<td>CARD11 SM</td>
<td>20</td>
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C

Frequency (%)

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<td>TNF gain</td>
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<td>ABIN-1 SM</td>
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<tr>
<td>CARD11 gain</td>
<td>15</td>
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</tbody>
</table>
Figure 2

A) CARD11

BCL10 binding  
Oligomerisation  
PKC regulated domain

CARD  
CC  
PDZ  
SH3  
GUk

[All mutations found in this study and included in the above figure are somatic.]

CARD: caspase recruitment domain; CC: coiled-coil domain; PDZ: (PSD95, DLG and ZO1 homology) domain; SH3: Src homology motif; GUK: guanylate kinase domain.

B) A20

K63 deubiquitinase  
E3 ubiquitin ligase (K48)

OTU: Ovarian tumour domain that belong to family of deubiquitinating cysteine proteases; ZF: zinc finger

[All other mutations found in this study are somatic unless indicated.]

Δ deletion (35.7%)  ◇ nonsense mutation (24.5%)  ▼ insertion (18.4%)  ◆ missense mutation (16.3%)  ○ splicing site mutation (5.1%) 

aa: amino acid; AHD: ABIN homology domain; CC: coiled coil; LZ: leucine zipper; NBD: NEMO-binding domain; NES: nuclear export signal; NLS: nuclear localization signal; UBAN: ubiquitin-binding domain in ABIN proteins and NEMO; YPPM: Src kinase phosphorylation motif

C) ABIN-1

D) ABIN-2

AHD: ABIN homology domain; UBAN: ubiquitin-binding domain in ABIN proteins and NEMO;

Identical or homologous amino acids residues that are present in the AHD1 of different ABINs are indicated in bold or underlined.

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Figure 3

A

Fold increase of NF-κB activity

B

Jurkat T cells (activated by CARD11)

HEK293 cells (stimulated by TNFα)

C

Fold change of NF-κB activity

D

Input

Elu

IP: HA

IP: Flag

A20-binding capacity
Figure 4

![Graphs showing survival analysis for A20 somatic mutation and no A20 somatic mutation.](image)

- Left graph: Overall survival (months) for A20 somatic mutation (n = 6) vs. No A20 somatic mutation (n = 26). The cumulative probability is shown with significant difference indicated by P = 0.001.
- Right graph: Event free survival (months) for A20 somatic mutation (n = 6) vs. No A20 somatic mutation (n = 26). The cumulative probability is shown with significant difference indicated by P = 0.002.