A20, ABIN-1/2, and CARD11 Mutations and Their Prognostic Value in Gastrointestinal Diffuse Large B-Cell Lymphoma

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Abstract

Purpose: Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of aggressive lymphomas with the activated B-cell–like subtype characterized 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 clinicopathologic 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 FISH/array comparative genomic hybridization, respectively. The mutations identified were functionally characterized by NF-kB reporter 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 with the wild-type, all CARD11 mutants were potent NF-kB activators in vitro. On the basis of 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 show further evidence of NF-kB pathway genetic abnormalities in DLBCL, which are potentially valuable in the prognosis and design of future therapeutic strategies. Clin Cancer Res; 17(6); 1440–51. ©2011 AACR.

Introduction

NF-kB 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-kB is constitutively activated in several lymphoma subtypes, including MALT lymphoma (1), activated B-cell–like diffuse large B-cell lymphoma (ABC-DLBCL; refs. 2, 3), primary mediastinal large B-cell lymphoma (PMBL; refs. 4, 5), and multiple myeloma (MM; ref. 6), and NF-kB activity is essential to the survival of these lymphoma cells (7). The genetic bases that underlie the constitutive NF-kB 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 signaling to the BCL10/MALT1-mediated NF-kB activation (12). Using an unbiased loss-of-function RNA interference screen, Ngo and colleagues have shown that CARD11 was critical for the proliferation and survival of ABC-DLBCL cells but not germinal center B-cell like (GCB) DLBCL cells (13). Subsequent mutational analysis by Lenz and colleagues identified missense mutations in 7 of 73 (9.6%) ABC-DLBCL and 3 of 79 (3.8%) GCB-DLBCL and all of the CARD11 mutations were within the coiled-coil domain and were capable of activating the NF-kB 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" negative regulator of the NF-κB activation pathway and can attenuate the NF-κB activity that is triggered by signaling from TNF and Toll-like receptors (17–19). By array comparative genomic hybridization (aCGH), we and others have identified A20 as the target of a 6q23 deletion in ocular adnexal MALT lymphoma (8, 20, 21). By interphase FISH, we have shown 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 shown 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%; refs. 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 Helicobacter pylori infection and the response of gastrointestinal DLBCL to treatment is much favorable than nodal DLBCL (26–28). Comprehensive correlations among the genetic abnormalities were identified and clinicopathologic parameters were performed.

Materials and Methods

Tissue materials

A total of 71 cases of primary gastrointestinal DLBCL (43 gastrointestinal, 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 clinicopathologic 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 FFPE tissue sections with streptavidin-biotin peroxidase method by using mouse monoclonal antibodies. Classification of DLBCL into germinal center (GC) and non-GC subgroups was based on immunohistochemical analysis of CD10 (Novocastra), and BCL6 and MUM1 (Dako) according to the algorithm of Hans and colleagues (31). For each antibody, cases were considered positive if 30% of DLBCL cells showed positive staining.

Microdissection and DNA preparation

Crude microdissection was done in each case to enrich tumor cells (32). DNA was extracted by using standard proteinase K digestion, followed by phenol/chloroform/isoamyl-alcohol extraction or by using the QIAamp DNA Micro Kit (QIAGEN). 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 300 bp, 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 to 10, within which activating mutations were found in gastrointestinal DLBCL to treatment is much favorable than nodal DLBCL (26–28). Comprehensive correlations among the genetic abnormalities were identified and clinicopathologic parameters were performed.
a previous study (14). The primer sequences and PCR conditions are detailed in Supplementary Table S1. HRM was performed immediately after PCR by the Rotor-Gene 6000 analyzer (Corbett Life Science). 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 because 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 Supplementary Table S1. PCR products were routinely purified and directly sequenced in both orientations by using the BigDye terminator chemistry 3.1 system (Applied Biosystems). In each case, the presence of a mutation was confirmed by at least 2 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 hybridization**

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

**A20 promoter methylation analysis**

DNA samples (600 ng) were converted by bisulfite treatment by using the EZ DNA methylation kit (Zymo Research). 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 by using Pyro Gold SQA reagents (Biotage) 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), and various mutants of CARD11, ABIN-1, and ABIN-2 were generated by using the QuickChange II XL site-directed mutagenesis kit (Stratagene). The capacity of these mutants to induce or suppress NF-κB activation was measured in Jurkat T cells and HEK293 cells by using a dual-luciferase reporter assay system (Promega; ref. 35). Briefly, Jurkat T cells (5 x 10⁶) were transfected with 2 μg of expression vector, 0.8 μg of pNF-κB-luc (a firefly luciferase reporter for NF-κB activity), and 0.6 μg of pRL-1K (a Renilla luciferase reporter as a control) by using Amaxa nucleofector system (Amaxa). The transfected cells were cultured for 24 hours and then harvested for a luciferase assay. Similarly, HEK293 cells were transfected by 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 3 independent transfections and duplicate reporter assays were performed, and the data were normalized to appropriate controls and presented as a mean ± SD.

**Immunoprecipitation**

HEK293 cells were cotransfected with HA-A20 and Flag-ABIN-2 (wild-type or mutant) by 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 by using the anti-HA immunoprecipitation kit and the Flag-tagged protein immunoprecipitation kit (Sigma-Aldrich), respectively, according to the manufacturer’s instructions. Total cell lysate (input), flow-through, and immunoprecipitation eluate were analyzed 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 by using the Quantity One software package (BioRad). For immunoprecipitation with HA-A20, the difference between the wild-type and mutant ABIN-2 that coimmunoprecipitated with A20 were compared after normalization 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 showed P < 0.1 was further tested by a multivariate analysis by 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 by SPSS, version 13.

**Results**

CARD11, A20, and ABIN-1/2/3 genetic abnormalities in gastrointestinal DLBCL

Of the 60 cases of gastrointestinal DLBCL that were analyzed by 1 Mb 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 \textit{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 9 mutations excluding known polymorphisms were identified in 7 cases, and a single case was observed to harbor 3 mutations (G126D, V266D, and T353P; Figs. 1A and 2A; Supplementary Table S2). Because there was no frozen tissue available, it was not possible to amplify the genomic or cDNA sequence that included all the 3 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 6 cases where DNA samples had been successfully extracted from 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-\textit{kB} activation (please see the following section), 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. 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 (2 cases) or deletion (6 cases), nonsense mutation (1 case), or mutation in the splicing site (2 cases), whereas the remaining 3 mutations (21%) were missense mutations (Fig. 2B). These mutations are similar in nature to those that have been recently reported (Fig. 2B), and they would most likely impair \textit{A20} function (15, 16, 22–24). Nonetheless, unlike these reported mutations, the mutations that were observed in gastrointestinal DLBCL were biased toward the ovarian tumor (OTU) domain, which belongs to the family of deubiquitinating cysteine proteases (Fig. 2B).
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 residues. Two recurrent ABIN-2 mutations are seen in gastrointestinal DLBCL and the somatic mutation (E255K) occurs at the conserved amino acid residue.
A20 requires its binding partner, that is, adaptor proteins, to function as a negative regulator of the NF-κB 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 analyzed by 1 Mb 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 9 mutations, excluding known polymorphisms, in 7 of 68 (10.3%) cases (Fig. 2C; Supplementary Table S2). Two cases each harbored 2 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 2 cases exhibited somatic insertions or deletions that caused a reading frameshift, and the predicted truncated products lack important functional domains, including AHD1 (ABIN homology domain), AHHD2, UBAN (ubiquitin binding domain in ABIN and NEMO), and NBD (NEMO binding domain). The remaining 3 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 2 cases, in which normal DNA was available for analysis (Fig. 2C).

Similarly, PCR and sequencing of all of ABIN-2–coding exons identified a total of 5 mutations, excluding known polymorphisms, in 5 of 68 (7.4%) cases (Fig. 1A). Two cases exhibited a recurrent somatic missense mutation at the conserved residue (E255) of AHD1, whereas the remaining 3 cases displayed a recurrent germline missense mutation at amino acid 249, which is immediately upstream of AHD1 (Fig. 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 to 10, which encode AHD1, 2, and 4 and the UBAN domain. The PCR analysis and sequencing of these 3 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 of 51 cases), BCL6 (10 of 48), and MYC (2 of 49) genes but not the CCND1 gene (0 of 50; Supplementary Table S2).

Comprehensive correlation analyses showed that there was no association among these 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 4 genes, only 1 case exhibited concurrent mutations (Supplementary 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-κB and whether ABIN-1/2 mutants lose the ability to inhibit NF-κB activation via the use of a reporter assay.

In comparison with the wild-type, all 9 CARD11 mutants that were identified in this study were capable of activating the NF-κB 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 (Fig. 3A).

For ABIN-1 mutation, we did not include the 2 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-κB reporter assay. As expected, both wild-type ABIN-1 and ABIN-2 were capable of inhibiting CARD11- and TNFα-mediated NF-κB activation (Fig. 3B). Among the 4 ABIN-1 missense mutations that were investigated, the E476K somatic mutant totally lost the ability to inhibit NF-κB activation, and the remaining 3 germline mutants exhibited no apparent difference from the wild-type. Among the 2 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-κB activation in Jurkat T cells (Fig. 3C). Intriguingly, such inhibition was not seen in TNFα-mediated NF-κB activation in HEK293 cells (Fig. 3C).

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

A20 mutation was significantly associated with poor survival

Comprehensive correlation between the aforementioned genetic abnormalities and clinicopathologic parameters was performed. A20 somatic mutation was significantly associated with the non-GC subtype of DLBCL (Fig. 1C; P = 0.038), as defined by the immunohistochemistry algorithm of Hans and colleagues (31). Such an association was not seen for ABIN-1/2 and CARD11 somatic mutations (Fig. 1B and C). Conversely, CARD11 somatic mutations exhibited a strong trend of association with more advanced stages (>IIE or above) of DLBCL (Fig. 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 (Supplementary 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 clinicopathologic parameters was carried out. The results of a univariate analysis of the prognostic variables are summarized 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 and Figure 4). CARD11 mutation also displayed a trend of association with poor EFS although 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 showed 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-κB regulators in gastrointestinal DLBCL and correlating these abnormalities to clinicopathologic parameters, we have

Figure 3. The functional characterization of CARD11, ABIN-1, and ABIN-2 mutations. (A) all 8 CARD11 mutants (mut) that are seen in gastrointestinal DLBCL are much more potent than the wild-type (wt) CARD11 in NF-κB activation, as shown by the luciferase reporter assay in Jurkat T cells. (B) In comparison with 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 3 germline mutants (R263W, T286M, and I374T) show no apparent difference from the wild-type. (C) In comparison with the wild-type ABIN-2, the E255K somatic mutant and the Q249H germline mutant are less efficient in inhibiting NF-κB activation by CARD11 in Jurkat T cells; however, such an effect is not seen in TNFα-mediated NF-κB activation in HEK293 cells. (D) Coimmunoprecipitation shows that the ABIN-2 E255K mutant is impaired in its binding to A20 in comparison with its wild-type. The data that correspond to the NF-κB reporter assays represent 3 (Jurkat T cells) or 4 (HEK293 cells) independent experiments, whereas the coimmunoprecipitation (IP) experiment was performed twice. * P < 0.01. Pires (+): Vector control; AM: API2-MALT1.
made several important and novel observations in this study. First, we have shown that A20-inactivating mutations and CARD11-activating mutations were frequent events in gastrointestinal DLBCL. Second, we have shown, for the first time, that ABIN-1 and ABIN-2, which are the adaptors of the A20 inhibitory complex of the NF-κB 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 somatic mutation cases with A20 somatic mutations were excluded from the analysis so as to avoid confounding.

Table 1. The results of the univariate analysis for prognosis, as evaluated by the Kaplan–Meier method

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<td>23</td>
<td>0.625</td>
<td>0.609</td>
<td>0.609</td>
<td>0.336</td>
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<td>0.500</td>
<td>0.545</td>
<td>0.545</td>
<td>0.755</td>
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<tr>
<td>Negative</td>
<td>22</td>
<td>0.636</td>
<td>0.600</td>
<td>0.600</td>
<td>0.405</td>
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<tr>
<td>BCL6-involved translocation</td>
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<tr>
<td>Positive</td>
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<td>0.000</td>
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<tr>
<td>Negative</td>
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<td>Any chromosome translocation</td>
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<td>0.625</td>
<td>0.983</td>
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<tr>
<td>Negative</td>
<td>8</td>
<td>0.750</td>
<td>0.684</td>
<td>0.684</td>
<td>0.625</td>
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NOTE: Germline mutation was also found in A20, ABIN-1, and ABIN2, but are not included in this table. Data with significance (P < 0.05) is boldface.

<sup>a</sup>Log-rank test.

<sup>b</sup>Cases with A20 somatic mutations were excluded from the analysis so as to avoid confounding.

<sup>c</sup>Chromosome translocation involving the BCL2, BCL6, or MYC gene loci.
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 2 cases (Fig. 1A). Nonetheless, all CARD11 mutants were much more potent in NF-κB activation than the wild-type in the absence of any immune receptor stimulation, suggesting that these mutations may cause constitutive NF-κB activation.

A20, which is a target of the NF-κB transcription factor, attenuates NF-κB activities by inactivating several proteins that are critical to NF-κB signaling, 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, 38). Additionally, A20 catalyzes the K48-linked polyubiquitin that targets proteins for proteasome degradation, and this is mediated by the C-terminal zinc finger (ZF) domains that possess E3 ligase activity (25, 38). 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, all of which are predicted to produce truncated proteins that impair A20 function (Fig. 2B). Of the remaining 3 somatic missense mutations, 2 occurred within the known functional domain (1 in OTU and 1 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 recognize its substrates; this is mediated by A20 adaptor molecules, such as ABIN-1/2/3, TAX1BP1, Itch, and RNF11 (25). In this study, we have shown, for the first time, that ABIN-1 and ABIN-2 are also targeted by somatic mutations and that all of the identified somatic mutations could impair their respective functions. Of the 3 cases with ABIN-1 somatic mutation, 2 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-κB inhibitory capacity of ABIN-1 (39–41). The remaining ABIN-1 E476K somatic mutation occurred within the highly conserved DExxER motif of AHD2 (41). As shown in our NF-κB reporter assay, the single amino acid substitution (E476K) caused by a somatic point mutation was sufficient to completely abolish the NF-κB inhibitory function of the wild-type ABIN-1. The importance of this amino acid was also shown by the functional characterization of a double mutant (ER476-7AA) in a previous study (41). 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 (42). Although this mutant did not exhibit unequivocal evidence of impaired NF-κB inhibition by reporter assays, immunoprecipitation clearly shows its defect in binding to A20. Thus, in essence, the somatic

### Table 2. The multivariate analysis of prognostic factors by Cox proportional hazards regression

<table>
<thead>
<tr>
<th>Prognostic variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>Relative risk</th>
<th>P</th>
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<tbody>
<tr>
<td>Overall survival</td>
<td>A20 somatic mutation</td>
<td>1.740</td>
<td>0.648</td>
<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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</table>

*The prognostic variables that were included in the multivariate analysis are those with P ≤ 0.1 by univariate analysis.
mutations that are seen in ABIN-1 and ABIN-2 could impair, if not completely abolish, their NF-kB inhibitory function. Interestingly, CARD11, A20, ABIN-1, and ABIN-2 somatic mutations were almost mutually exclusive, wherein only 1 of the 21 positive cases exhibited concurrent mutations that involved 2 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 tumor cells to acquire genetic abnormalities that affect more than one molecule in the same molecular pathway. Interestingly, among these abnormalities, the 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 A20 mutations and poor clinical outcome (15, 16), further study is required to address whether the prognostic value of A20 somatic mutation is totally independent of its association with the ABC subtype. There was no association between A20 deletion, CARD11, ABIN-1, and ABIN-2 somatic mutation and patients' survival. However, it should be noted that the number of cases with these abnormalities, particularly ABIN-1 and 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-kB inhibition is not as strong as A20 (43).

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 with the wild-type. 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 characterized. At this time, it is not possible to further test these germline mutants by 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, 44–51), highlights the importance of the functional characterization 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 and colleagues reported the frequent activating mutation (16%) of CD79B in DLBCL (52). Studies of MM 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 NF-kB1, NF-kB2, 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 subtype than the germinal center B-cell like (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 and colleagues (31). ABC-DLBCL is characterized by constitutive NF-kB activation (13) and exhibits a much poorer survival than GCB-DLBCL, regardless of being treated with CHOP or R-CHOP (53). In light of the recent advances in the development of NF-kB inhibitors and their potential application in cancer therapies, a comprehensive characterization of NF-kB regulator gene abnormalities and their prognostic value in DLBCL will not only benefit current patient care, and also provide a basis for the design of future therapeutic strategies that incorporate NF-kB inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Author contributions

G. Dong, E. Chanudet, N. Zeng, A. Appert performed key experiments, analyzed data, and prepared the figures; R.A. Hamoudi assisted in DNA sequencing and statistical analyses; A.J. Watkins reviewed histology and assisted in microdissection; H. Ye and H. Liu assisted in interphase FISH detection of chromosome translocation; Y.-W. Chen, W.-Y. Au, Z. Gao, S.-S. Chuang, G. Srivastava contributed majority of the cases for the study; M.-Q. Du designed, coordinated the study, and wrote the manuscript.

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No potential conflicts of interest were disclosed.
References


