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Original Article

Mutation analysis of NF-κB signal pathway-related genes in ocular MALT lymphoma

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Abstract: Constitutive nuclear factor-*kappa* B (NF-κB) activation has been reported in ocular adnexal lymphoma (OAL). *TNFAIP3/A20* is a "global" inhibitor of NF-κB pathway. We have shown that OAL has preferential loss of the 6q23.3 region where *TNFAIP3/A20* exist, which is suggested to involve in lymphomagenesis of OAL. The mechanisms causing NF-κB activity in OAL remain elusive. Recently, NF-κB canonical pathway genes including *CARD11, CD79B* and *MYD88* were shown to be frequently mutated in diffuse large B-cell lymphomas. In this study, we analyzed the mutation status of these genes by direct sequencing in 24 OAL cases including 9 cases with loss of 6q23.3 previously identified by array comparative genomic hybridization. We showed that genetic alterations of these genes were not found in OAL, a finding differing from that of most B-cell lymphomas. Genetic or epigenetic alterations in other genes are likely to be relevant in pathogenesis of OAL case without A20 loss.

Keywords: Ocular adnexal lymphoma, TNFAIP3 (A20) deletion, NF-κB related gene mutation

Introduction

Primary ocular adnexal lymphoma accounts for approximately 8% of extranodal lymphomas [1], and of these about 50-70% are ocular adnexal mucosa-associated lymphoid tissue (MALT) lymphomas (OALs) [2]. Several chromosomal translocations resulting in activation of the NF-κB complex have been described in OALs including t(11;18)(q21;q21)/*API2-MALT1*, t(14;18)(q32;q21)/*IGH-MALT1* and t(3;14)(p14.1;q32)/*IGH-FOXP1* [3]. Occurrences of the aforementioned translocations in ocular adnexae are rare compared to those which occur in the stomach and lung [3]. The frequency of the first two translocations in OALs is reported to be 10% and 7%, respectively [4]. The t(3;14)(p14.1;q32) translocation was reported to have been found in 4 out of 20 OAL patients [5].

TNFAIP3/A20 is a "global" inhibitor of NF-κB pathway. We have shown that OAL has preferential loss of the 6q23.3 region which includes *TNFAIP3/A20* [6]. A recent study concerning *A20* inactivation in OAL showed that there was a significant association between *A20* mutation and heterozygous deletion. *A20* mutation/deletion was also significantly relevant in relation to increased expression of the NF-κB target genes *CCR2*, *TLR6* and *BCL2* [7]. Although the above studies suggested that *A20* deletion/mutation is involved in lymphomagenesis of OAL, the mechanisms responsible for NF-κB activity in OAL have yet to be fully delineated.

Recently, much progress has been made in uncovering the root causes of NF-κB activation in non-germinal center (GC) type lymphomas, especially in the activated B-cell-like (ABC) subtype of DLBCL. Mutations of a series of NF-κB pathway genes including *CARD11* [8], *CD79B* [9], and *MYD88* [10] in ABC DLBCL have been found, which result in activation of NF-κB. Antigen specificity of B-cell-receptor (BCR) is determined by surface immunoglobulin, but downstream signaling to NF-κB is mediated by two associated proteins, *CD79A* and *CD79B*. The *CD79* mutants are not loss-of-function mutants,

but it has been shown that the mutations cause the exaggerated response to antigen, leading to tumor development [9]. *CARD11* is a cytoplasmic scaffolding protein that is involved in the antigen receptor-induced activation of NF-κB, and is required for the differentiation and/or survival of the B cell-subpopulation. In the absence of BCR signals, *CARD11* mutations can lead to a gain-of-function by coordinating the activation of IκB kinase β (IKK), a positive regulator of NF-κB [8]. *MYD88*, an adaptor protein that mediates signaling of toll-like receptors (TLR) and IL-1 and IL-18 receptors (ILR), transduces signals to NF-κB that, in turn regulate the production of cytokines and anti-apoptotic molecules. *MYD88 L265P* is also a gain-of-function driver mutation that can promote cell survival by inducing constitutive NF-κB activation [10].

A20 is required for termination of TLR-induced NF-κB activation [11] by targeting *TRAF6*, which is a molecule downstream of the CRAD11-BCL10-MALT1 complex in the NF-κB pathway [12]. Given a recurrent deletion of 6q23.3-24.1 including *TNFAIP3* (*A20*) in OAL (9/ 24; 37.5%) in our previous study [6], we aimed to study if these mutations of NF-κB pathway genes might occur in OAL except *A20* loss in this study.

Materials and methods

Patient's samples

The 24 OAL samples examined for sequencing in the present study were identical to cases previously studied for array CGH [6, 13]. They consisted of 9 cases with *A20* loss and 15 cases without *A20* loss. Lesion sites of these cases consisted of conjunctiva (18 cases), orbit (4 cases), and lacrimal gland (2 cases) [13]. This study was performed in accordance with the ethical guidelines of Aichi Cancer center.

Genomic DNA extraction

Standard proteinase K treatment and phenol-chloroform extraction method was used for genomic DNA extraction for all 24 OAL samples. Briefly, after overnight incubation with proteinase K (20mg/ml) at 37°C, phenol-chloroform (1:1) was added and centrifuged at 3000rpm for 10min (2 times extraction). 3M NaOAc (1/10 volume of the supernatant) and 2.5 volume of cold 100% ethanol were added into the supernatant, swirl gently with hand and the genomic DNA will be observed. The genomic DNA was washed using 70% ethanol for 2 times and dissolved in TE buffer after air dry for 5-10min. DNA concentration was measured by Nanodrop-1000 3.5.1 device (Nanodrop, Inc.).

Polymerase chain reaction (PCR) of CARD11, MYD88 and CD79B

The TLR/ILR domain of *MYD88* (exons 3, 4, 5) and the coiled-coil domain of *CARD11* (exons 5, 6, 7, 8, 9) were amplified using genetic specific PCR, while the immunoreceptor tyrosine-based activation motif/ITAM domain of *CD79B* (exons 5, 6) were amplified using touch-down PCR. Information concerning genomic DNA of the three genes and the coding region is shown in **Figure 1**. Each exon was tested under the best Tm conditions determined by the gradient PCR method, and using 30 cycles in the second round of PCR.

The primers and conditions of all tested exons are described in **Table 1**. Total PCR reaction volume was 50.0μl including 5.0μl DNA template (100ng), 1.0μl 2.5mM dNTP, 5.0μl 10×buffer, 2.0μl forward primer(10mM), 2.0μl reverse primer (10mM), 0.25μl rTaq DNA polymerase (Takara, 5U/μl), 35.0μl ddH2O. DNA templates were excluded in negative controls. PCR products were checked by electrophoresis on 2% agarose gels.

Sequence PCR of CARD11, MYD88 and CD79B

Purified PCR products were subjected to cycle direct sequence reactions for *CARD11, MYD88* and *CD79B* using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). All mutations were confirmed on independent PCR products in both orientations. Total reaction volume of sequence PCR was 20.0μl, including 3.5μl 5×sequence buffer (ABI 4336697); 2.0μl Primer (1.6pmol/μL); 2.0μl sample DNA; 11.5μl ddH2O; 1.0μl BigDye Terminator v3.1 (ABI 4337455). The standard sequence PCR protocol included initial denaturation at 96°C for 5min, and followed by 25 cycles for 96°C 30s, 50°C 30s, 60°C 4min. The final step was performed at 60°C for 5min. The ethanol-precipitated sequencing PCR product was dissolved at 15μl Hi-Di Formamide (ABI 4440753) followed by denaturation at 95°C for 3min, and cooled down on ice before detection by capillary electrophoresis in an ABI 3100 sequencing apparatus (Applied Biosystems).

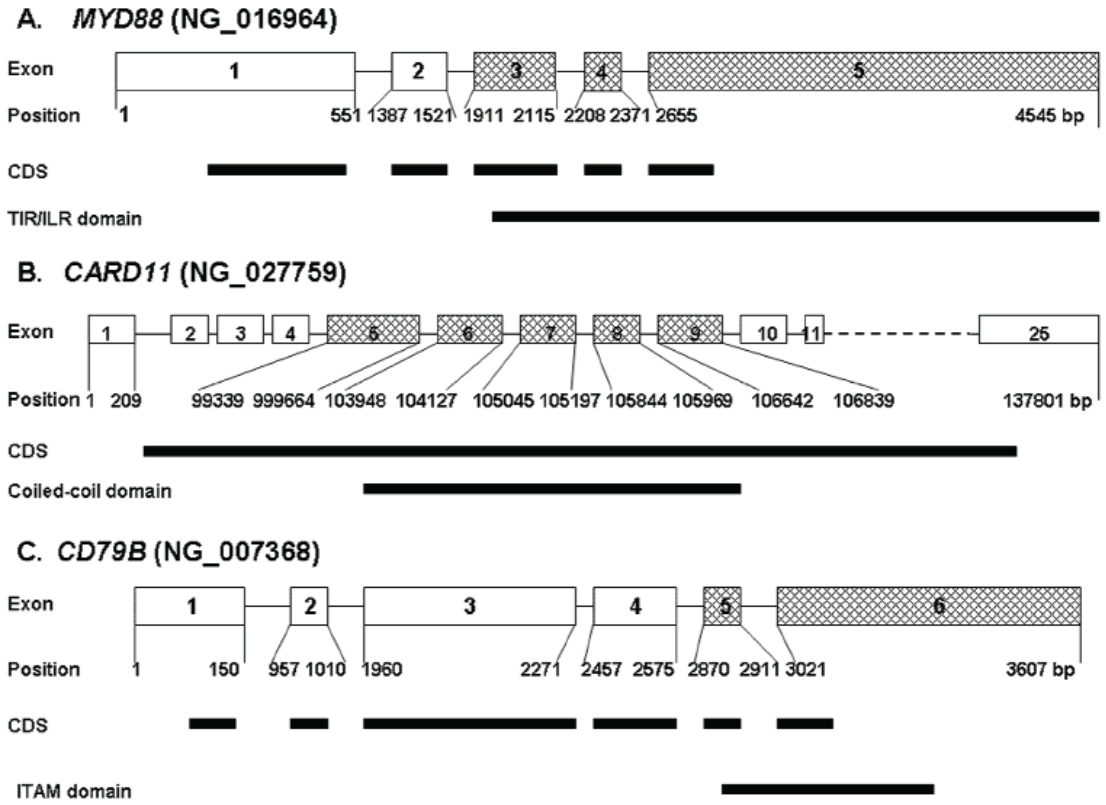

Figure 1. Coding regions and exons information for MYD88, CARD11 and CD79B. The square frame with gridlines indicates tested exons.

Results and discussion

NF-κB activation in MALT lymphoma has been reported by various investigators [7]. The genetic bases for NF-κB activation in OAL may therefore be elucidated by studying the multiple genetic alterations converging on the NF-κB pathway. We first analyzed *MYD88*, which had been shown to be the most frequently altered gene in ABC DLBCL, harboring the highest overall frequency of mutations (39%), and the most common L265P mutation existed in exon 5 (29%, 45/155) [10]. This gene might give us the highest possibility for finding a mutation in OAL, but no mutations in exons 3, 4, and 5 covering the TLR/ILR domain were detected in any of the examined samples with or without *A20* Loss. We next examined the ITAM domain of *CD79B*, the motif that initiates signal transduction following BCR aggregation. The missense mutations affecting the first tyrosine of the ITAM domain were shown to induce the positive signals. The mutation was reported in two ABC DLBCL cell lines and 18% (29/161) of ABC DLBCL cases [9]. Although very specific products could be generated by touch-down PCR, the mutations of *CD79B* were not found. Thus, we next determined the mutation status for the coiled-coil domain of *CARD11*. As a cytoplasmic scaffolding protein, *CARD11* is required in normal antigen receptor-induced NF-κB activation. 9.6% (7/73) of ABC DLBCL was shown to have missense mutations at the coiled-coil domain [8], but no mutation was found in our OAL samples.

Our results are in accordance with previous mutation studies on MALT lymphoma. In these mutation analyses of MALT lymphoma, no mutations were found in tested cases for *CARD11* (19 MALT cases) [8] and *CD79B* (16 gastric MALT lymphoma cases) [9]. Additionally, Adam et al. analyzed genetic aberrations using BAC clones for *CARD11 in extranodal DLBCL and marginal zone B-cell lymphomas of the MALT type including 40 OAL cases, and* reported that *CARD11* is not targeted by chromosomal translocations in these lymphoma entities [14]. It should be noted that only 9% of gastric MALT

Table 1. Primers for PCR and sequences.

Genes	Primers	Length of coding region	PCR conditions
MYD88	TLR/ILR domain		
Exon3	F 5' AAGCCTTCCCATGGAGCTCTGACCAC-3' R 5' GCTAGGAGGAGATGCCCAGTATCTG-3'	204bp	94°C 3 min 94°C 30s **69°C 30s** 72°C 30s } 30 cycles 72°C 7 min
Exon4	F 5' ACTAAGTTGCCACAGGACCTGCAGC-3' R 5' ATCCAGAGGCCCCACCTACACATTC-3'	91bp	94°C 3 min 94°C 30s 72°C 30s 72°C 30s } 30 cycles 72°C 7 min
Exon5	F5' GTTGTTAACCCTGGGGTTGAAG-3' R5' GCAGAAGTACATGGACAGGCAGACAGATAC-3'	154bp	94°C 3 min 94°C 30s 66°C 30s 72°C 30s } 30 cycles 72°C 7 min
CARD11	Coiled-coil domain		
Exon5	F5' TGAGTGAGTGAATGAATGGCACCTG3' R5' AGAATTGAGCCCTGGTGACATTTGT 3'	325bp	94°C 3 min 94°C 30s 64°C 30s 72°C 30s } 30 cycles 72°C 7 min
Exon6	F5' AGAAGGTTTCTTGGAGCCCTTCTCT3' R5' TCACACCCTGGCAGGTTCATCGTTT 3'	189bp	94°C 3 min 94°C 30s 68°C 30s 72°C 30s } 30 cycles 72°C 7 min
Exon7	F5' CCTTCTGCTCTGTCCGTATCTGTTG 3' R5'GAGGTCCTGCTGACACACACCACTG3'	152bp	94°C 3 min 94°C 30s 68°C 30s 72°C 30s } 30 cycles 72°C 7 min
Exon8	F5' CGAGCAGAGAACAGCTTTCAGTCCT 3' R5' AAACACTCTGAAGGAGCCGGTGGTC 3'	125bp	94°C 3 min 94°C 30s 66°C 30s 72°C 30s } 30 cycles 72°C 7 min
Exon9	F5' CCTCAGTGCCCTCATCTGTAAAATG 3' R5' CAAAGGACAAGGAGCCATTCATTG 3'	197bp	94°C 3 min 94°C 30s 66°C 30s 72°C 30s } 30 cycles 72°C 7 min
CD79B	IATM domain		Touch-down PCR
Exon5	F5'- GCCTGGCCCAGCAGGGGATGGGGCT -3' R5'- CAACCACACCAGCAGATAGTGGCCA -3'	32bp	94°C 3 min 94°C 30s 68°C→64 oC 30s 72°C 30s } 25 cycles×5 94°C 30s 64°C 30s 72°C 30s } 25 cycles 72°C 7min
Exon6	F5'- GTCAGTGGCCACTATCTGCTGGTGT -3' R5'- CCATGAGCCAGGCAGCTCCGAAGCA -3'	98bp	94°C 3min 94°C 30s 62°C→58°C 30s 72°C 30s } 25 cycles×5 94°C 30s 58°C 30s 72°C 30s } 25 cycles 72°C 7 min

lymphomas (56 tested cases) were shown to have the *MYD88 L265P mutation [11]. MYD88* and the TLR pathway play a central role in immunity, and deficiency of *MYD88* in humans results in life-threatening pyogenic bacterial infections [15]. It is well known that the vast majority of gastric MALT lymphomas are caused by infection with *Helicobacter pylori*, and most translocation (*API2-MALT1*)-negative gastric MALT lymphomas can be cured by *H. pylori* eradication alone, which indicates that microbe-generated immune responses support the survival of lymphoma cells [16]. This report also showed that 30% of cases were resistant to *H. pylori* eradication therapy and half of the resistant cases were shown to have *API2-MALT1*, but the genetic bases for the resistance in the remaining half were not clear. It is therefore possible for such gastric MALT lymphoma cases to have the *MYD88* mutation. For OAL, the case of microbe-mediated lymphomagenesis is controversial based on the reported research [17], and we speculated that another unknown mechanism which also targets the NF-κB pathway might play a role in OAL.

As an indolent low-grade B-cell lymphoma, OAL might be less complex than DLBCL in genetic abnormality. It is reasonable that *A20* loss cases in OAL have NF-κB activation. Therefore, it became important to determine if cases without *A20* loss had genetic alterations in NF-κB-related genes. The present study failed to find any mutation in *MYD88, CARD11* or *CD79B*, suggesting that other factors, such as genetic abnormality of non-canonical NF-κB pathway genes or epigenetic alterations of some genes, might have an effect on the lymphomagenesis of this group of cases without A20 loss.

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