Intron-derived aberrant splicing of A20 transcript in rheumatoid arthritis

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Abstract

Objective. Aberrant splicing is one of the most significant components generating functional diversity in many pathological conditions. The objective of this study was to analyse the mutations or aberrant splicing of A20 transcript, the region encompassing the ovarian tumour (OTU) domain [which is functionally important as an inhibitor of nuclear factor (NF)-κB activation] in fibroblast-like synoviocytes (FLSs) from RA patients.

Methods. Alterations in A20 transcripts were determined through sequence analysis of 10 clones of A20 cDNA in FLSs from each of the five RA patients. The levels of aberrant A20 transcript were measured by quantitative real-time RT-PCR with primers to specifically recognize the inserted introns. The functional role of A20 and its aberrant variants were examined by analysing NF-κB luciferase reporter activity and NF-κB-dependent target gene expression.

Results. In RA FLSs, we discovered four novel aberrant A20 transcripts, most of which resulted from insertion of partial intron 2, intron 4 and/or deletion of exon 4. In each of these FLSs, sequence analysis revealed that these aberrant insertion sequences were flanked by consensus splice donor and acceptor sequences without nucleotide substitution, suggesting alternative splicing as the likely mutational mechanism. These variants elicited a codon frame shift by creating a premature translational stop codon, and eventually, disruption of the OTU domain (which is functionally important as an inhibitor of NF-κB activation) of A20. The expression level of aberrant A20 transcript was correlated well with persistently enhanced status of NF-κB signalling, as evident by the phosphorylation of inhibitor of NF-κB (IkB)-α and transcription of NF-κB target genes.

Conclusion. The results suggest that A20 inactivation by the novel aberrant splicing may contribute to RA progression by inducing persistent NF-κB activation.

Key words: rheumatoid arthritis, A20, aberrant splicing variants, NF-κB, tumor necrosis factor.

Introduction

RA is a common chronic inflammatory disease that affects the small joints of the hands and feet [1]. The propagation of inflammation is characterized by migration of mononuclear cells into the local inflammatory sites, leading to the proliferation of fibroblast-like synoviocytes (FLSs) and damage to cartilage and bone [2]. The major mediators of chronic inflammation in RA include TNF-α, IL-1β, IL-6, IL-8 and PGE2 [3]. Induction of pro-inflammatory mediators requires activation of the nuclear factor (NF)-κB-inducing kinase- or inhibitor of NF-κB (IkB) kinase (IKK)-mediated NF-κB signal transduction pathways [4, 5]. The transcription factor NF-κB has been well recognized as a key regulator of inflammation in RA [6–8]. NF-κB proteins are rendered inactive by binding to IκBα in normal conditions. The activation of IKK complex by the stimulation of multiple receptors that phosphorylate IκB at...
two specific serine residues, which is followed by its ubiquitination and proteasomal degradation, thereby releases NF-κB proteins and allows for their nuclear translocation [5]. Constitutive activation of NF-κB regulates expression of multiple pro-inflammatory cytokines that play an essential role in the pathogenesis of RA [9-12].

As one of the NF-κB target genes, A20 [also known as TNFAIP3 (TNF-induced protein 3)] has been well established for its negative feedback mechanism to block NF-κB activation through its ubiquitin-editing function in response to various inflammatory signalling, including TNF, IL-1β and lipopolysaccharides [13-16]. In the TNF-mediated NF-κB signalling pathway, A20 eliminates Lys63-linked ubiquitin chains from RIP1 with its N-terminal ovarian tumour (OTU) domain and attaches Lys48-linked polyubiquitins to RIP1 with the C-terminal zinc finger (ZnF)-containing domain, thereby targeting this factor for proteasomal degradation [17]. Mice null for A20 develop severe multiorgan inflammation, including inflammation of synovial joints, due to prolonged NF-κB activation [18]. Multiple variants within chromosome 6q23, which encodes for A20, has been associated with the pathogenesis of a number of diseases, particularly autoimmune diseases and certain types of cancer. Inactivation of A20 by the typical two mechanisms, deletions and inactivation mutations, has been reported in marginal zone lymphomas, Hodgkin’s lymphoma and activated B cell-like diffuse large B cell lymphoma, which may contribute to lymphomagenesis [19, 20]. Also, multiple polymorphisms in the A20 are closely associated with many pathological conditions, including SLE, coronary artery disease in type 2 diabetes, psoriasis and RA [21-24]. Moreover, in our previous study, in vivo experiments in CIA using A20 to block the NF-κB pathway efficiently inhibited inflammatory response and bone destruction [25], indicating the critical role of A20 in RA pathophysiology. However, aberrantly spliced variant forms of A20 have not yet been discovered in RA.

Since the N-terminal OTU domain of A20 plays an essential role as a negative feedback inhibitor of NF-κB activity, we analysed the mutations or aberrant splicing of the A20 transcript region encompassing the OTU domain in FLSs from RA patients. In the present study, we discovered the existence of four novel spliced forms, which were aberrantly encoded into the truncated OTU domain. These aberrantly spliced variant forms of A20 may function to dysregulate NF-κB, leading to the pathogenesis of RA.

Materials and methods
Isolation of primary human RA FLSs and cell culture
RA FLSs were isolated by the enzymatic digestion of synovial tissues obtained from 10 RA patients undergoing total joint replacement surgery or knee synovectomy at Chungnam National University Hospital, Daejeon, Korea. This study was approved by the Chungnam National University Hospital institutional review board (approval number 1012-164) according to the Declaration of Helsinki and written informed consent was obtained from each patient prior to surgery. After discarding fat and fibrous tissue, the synovium was minced into small pieces and treated for 2 h with 2 mg/ml of type II collagenase in Hanks’ balanced salts solution (HBSS) at 37°C in 5% CO2. The tissue was then filtered using fine sterile gauze, washed and resuspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Dissociated cells were then centrifuged at 800g, then at 1000g, and then plated in 10-cm dishes. After overnight culture, the non-adherent cells were removed and the adherent cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were kept at 37°C in 5% CO2 and the medium was replaced every day. When the cells approached confluence, they were passaged into fresh culture dishes after trypsin/EDTA treatment. RA FLSs from passages four to nine were used in each experiment. The cells were morphologically homogeneous and exhibited the appearance of FLSs, with typical bipolar configuration under inverse microscopy. HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2.

RT-PCR analysis
Expression of the OTU domain of A20 in FLSs from 10 RA patients was analysed by semi-quantitative RT-PCR analysis. After the total RNA was prepared using a TRIZOL reagent (Life Technologies, Inc.), oligo(dT)-primed cDNA was synthesized using an RT-PCR kit (Stratagene, La Jolla, CA, USA). PCR amplification was carried out using the primer pair specific to human A20 (5’-TGGCTGAACAGTCCTTCCTC-3’ and 5’-CTTCAAGGTCACCAAGGTA-3’) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5’-GACCCCTTATGACCTC-3’ and 5’-GCCATCCACGCTTCTG-3’). After PCR amplification, the products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining.

A20 transcript sequencing
To sequence A20 transcripts in five representative FLSs, 5 µg of RNA were reverse transcribed into the N-terminal region encompassing the entire OTU domain of cDNA and cloned using a T-Blunt PCR cloning kit (Solgent, Daejeon, Korea), according to the manufacturer’s instructions. To sequence the amplified cDNA bidirectionally, we used universal M13 forward and reverse primers (5’-TAAACGAGCTGCGGACCC-3’ and 5’-CAGGAAACACGCTTGAC-3’). At least 10 clones were randomly selected, and the purified cDNAs were sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). All mutations were confirmed on independent PCR products and analysed by alignment of each cDNA sequence to its respective genomic sequence of A20 (GenBank accession no. NT025741).
Plasmid construction of variant forms of cDNA and transfection

Wild-type and splice-variant forms of A20 cDNA were subcloned into pCMV-Tag2b (Stratagene), generating flag-A20/WT, flag-A20/INS-pI2, flag-A20/INS-I4, flag-A20/Del-E4/INS-I4 and flag-A20/INS-pI2/Del-E4/INS-I4 with an N-terminal flag tag. All constructs were verified to confirm the integrity of the inserts by DNA sequencing.

To detect the protein expression, HEK293 cells were transiently transfected either with the wild type or with the variant forms of A20 using Lipofectamine PLUS reagent by following instructions provided by the manufacturer (Invitrogen, Carlsbad, CA, USA), and immunoblotted with anti-flag antibody.

Immunoblot analysis

After treatment with TNF (15 ng/ml) as described in the figure legends, FLSs were collected and lysed in M2 buffer (20 mM Tris at pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM ethylene glycol tetraacetic acid, 2 mM diithiothreitol 0.5 mM phenylmethylsulphonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin). Fifty micrograms of the cell lysates were subjected to SDS polyacrylamide gel and blotted onto a polyvinyl difluoride membrane. After blocking with 5% skim milk in phosphate-buffered saline–Tween 20, the membrane was probed with the relevant antibody and visualized by enhanced chemiluminescence (ECL), according to the manufacturer’s instructions (Amersham, Piscataway, NJ, USA).

Quantitative real-time PCR

After FLSs were treated with TNF (15 ng/ml) at different time points, cells were collected and RNA was extracted with TRIZOL reagent (Life Technologies, Inc., Carlsbad, CA, USA). Diluted RNA was analysed as described [26], and primers were obtained using the Qiagen QuantiTect Primer Assay kit: TNF (Hs_TNF_3_SG, catalog no. QT01079561), IL-6 (Hs_IL6_1_SG, catalog no. QT00083720) and GAPDH (Hs_Gapd_2_SG, catalog no. QT01192646). Real-time quantitative PCR was carried out using an SYBR Green detection system (Applied Biosystems). The PCR conditions were optimized as follows: 95°C for 10 min, and 40 cycles each of 95°C for 15 s, 60°C for 10 s, and a dissociation stage at the end of the run from 60°C to 95°C. In order to detect aberrantly spliced variants, primers for intron 2 and intron 4 were designed: primer for intron 2, 5′-TGGCCAGTTTTGCTCT CAGTTTC-3′ and 5′-CCCTCAATGTCGTCGTTGCTG-3′; primer for intron 4, 5′-GGAAACAGACACACGCAACTTTA A-3′ and 5′-CAAAGGCATAAGGCTGAAAGCA-3′. All reactions were independently repeated at least three times to ensure reproducibility of the results.

Luciferase reporter assay

HEK293 cells were co-transfected with p2xNF-κB-Luc, pRSV-β-galactosidase and flag-tagged wild-type and splice variant forms of A20 constructs as indicated in the figure legends. Twenty-four hours after transfection, cells were treated with TNF (15 ng/ml) for an additional 10 h and luciferase activities were measured using a luciferase assay kit (Promega, Madison, WI, USA). Luciferase activity was normalized relative to the β-galactosidase activity of each sample.

Results

Expression analysis of A20 mRNAs from FLSs of RA

Since A20 plays an essential role as a negative regulator of NF-κB in the condition of inflammatory disease, the level of A20 expression or the presence of functionally inactive mutant of the A20 gene may have a close relationship with the progression of RA (for instance, hyperplastic growth of FLSs). Therefore the expression profiles of A20 mRNA from 10 RA patients were analysed by RT-PCR using a pair of primers encompassing the sequence of the OTU domain, a functionally important inhibitor of NF-κB activation. As expected, we detected a major RT-PCR product that is predicted to be 791 bp from the published A20 cDNA sequence (GenBank accession no. NM006290). Interestingly, additional larger fragments were observed in 7 of the 10 FLSs compared with that of HEK293 cells, which harbour ectopically expressed wild-type A20 cDNA (Fig. 1). These results suggest the possibility that aberrant transcript of A20 might be widely expressed FLSs of RA.

Identification of four novel aberrant splice variants in the OTU domain of A20 from RA FLSs

To identify any novel splicing in A20 mRNA, N-terminal regions encompassing the entire OTU domain of A20 cDNA in five representative FLSs were individually cloned and at least 10 clones of each cDNA sample were examined for potential alterations at the transcriptional level by clone sequencing. Analysis of A20 cDNA sequence revealed that the expression of A20 mRNA was present in all five RA patients examined and their sequence integrity was well maintained (data not shown).

However, a total of 23 clones of A20 transcripts were detected and categorized into four aberrantly spliced variant forms (Table 1). These aberrant transcripts included insertion and/or deletion of one or more region of the A20 gene.

A summary of these sequence alterations and diagrams of the aberrant splicing are presented in Fig. 2. Specifically, we found a partial insertional mutation (50 bp) of intron 2 at the junction between exon 2 and exon 3 (A20/INS-pI2; Fig. 2A, S1A), and another insertion of intron 4, reserving the whole sequence of intron 4 (160 bp) at the junction between exon 4 and exon 5 (A20/INS-I4; Fig. 2B, supplementary Fig. S1B, available as supplementary data at Rheumatology Online). We also found a mutation at position 553–700, deleting the whole of exon 4 and inserting the whole intron 4 instead (A20/Del-E4/INS-I4; Fig. 2C, supplementary Fig. S1C, available as supplementary data at Rheumatology Online) as well as additional partial insertion of intron 2 with A20/Del-E4/INS-I4 (A20/INS-pI2/Del-E4/INS-I4; Fig. 2D, supplementary Fig. S1D, available as supplementary data at Rheumatology Online).
Online). The result of deduced amino acid sequence alignment was thought to produce various abnormal A20 proteins by frame shift translation (Fig. 2E and F). These aberrant variants generated the premature termination codon at the intron-derived sequence and might produce truncated forms of A20, which disrupts the structure of the N-terminal region of A20, including the OTU domain. A BLAST (National Center for Biotechnology Information) search of the inserted nucleotides revealed 100% identity with an A20 genomic DNA sequence in intron 2 and intron 4 (GenBank accession no. NT025741, nt 42362336/C150 42362385 and nt 42366430/C150 42366589). These four types of alteration were found singly or in combination (i.e. deletion of exon 4 and/or insertion of partial intron 2 and whole intron 4) in five FLSs from RA patients examined.

Based on the sequence alignment of the variant A20 transcript, the mutational mechanism is probably an aberrant RNA splicing event. In particular, the TTTCAG sequences immediately at the end of exon 4 are well matched to consensus splice acceptor sequence (YYNCAG) (Fig. 3B). Collectively these data suggested that the efficiency of mRNA splicing might be affected without nucleotide substitutions, leading to the formation of aberrant A20 transcript in FLSs from RA patients.

Loss of NF-κB inhibitory function by aberrantly spliced variants of A20

As A20 is a key regulator of NF-κB signalling that negatively modulates NF-κB activation through a variety of receptors including TNF receptor [17], we investigated the function of these aberrantly truncated forms of A20 proteins. To confirm the protein expression of aberrant variants of A20 transcripts, HEK293 cells were transiently transfected with flag-tagged wild-type and four novel variants (A20/INS-pI2, A20/INS-I4, A20/Del-E4/INS-I4, A20/INS-pI2/Del-E4/INS-I4) of A20. As shown in Fig. 4A, we detected the expression of various truncated sizes of A20 proteins predicted to be 11.9, 27.5, 21.3 and 11.9 kDa, which correlates well with their corresponding mRNAs. We then further examined whether these truncated forms of A20 affect TNF-induced NF-κB activation using NF-κB luciferase reporter plasmid bearing the NF-κB-binding DNA sequence. As consistent with previous reports [17], ectopic
over-expression of wild-type A20 almost completely suppressed TNF-induced NF-κB activation. In contrast, over-expression of four RA FLS-derived A20 mutants (A20/INS-pI2, A20/INS-I4, A20/Del-E4/INS-I4, A20/INS-pI2/Del-E4/INS-I4) failed to show down-regulation of TNF-induced NF-κB activity (Fig. 4B), indicating that these were actually loss-of-function mutations.

Aberrant A20 transcript enhanced NF-κB signalling in FLSs from RA patients

To detect the variant-specific mRNA transcript in the conditions of FLSs from RA patients, real-time RT-PCR was performed with one primer selected within inserted intron 2 or intron 4 sequence and the other from a
remote exon encompassing the corresponding intron. This approach revealed that the levels of expression of variant forms of A20 varied among 10 distinct FLSs (Fig. 5A). We then proceeded to examine whether the splice variant forms of A20 indeed affect the status of NF-κB activity. To address this issue, we compared the extent of TNF-induced phosphorylation of IkB-α in FLSs that harbour different levels of aberrant A20 transcript (sample FLS #1 vs sample FLS #4). As expected, TNF treatment resulted in rapid and progressive induction of A20 by a monoclonal antibody, which only recognizes the full length of wild-type A20 protein in both FLS #1 and FLS #4 (Fig. 5B, top panel). Notably, the basal and TNF-induced expression level of A20 in FLS #4 carrying the highest variant forms of A20 was much lower than that of FLS #1. Furthermore, when FLS #1 was treated with TNF, the phosphorylation of IkB-α was transiently enhanced by 1h, after which the level continued to decrease gradually. In FLS #4, however, the TNF-induced phosphorylation of IkB-α was not only greatly enhanced but also persistent (Fig. 5B, middle panel). These results suggest that the level of aberrant A20 transcript affects the NF-κB signalling pathway in response to TNF.

Discussion

Eukaryotic genes characteristically produce large amounts of pre-mRNA-containing introns, which are removed by a highly accurate cleavage/ligation reaction known as splicing. Nonetheless, aberrant splicing is one of the most significant components generating functional diversity in many pathological conditions, such as tumorigenesis and autoimmune disease [27, 28]. In this study we provided clear evidence that four types of novel aberrant splicing transcript of A20 occur in FLSs from RA patients. The fact that these aberrant splicing variants result in frame shift, stop translation and eventually OTU domain (which targets RIP1 or TRAF6 de-ubiquitination) disruption of A20 indicates that these novel forms of spliced variants could contribute to the dysregulation of NF-κB activation status in RA FLSs. These data suggest the important role of aberrant splicing of A20 in the aetiology and/or progression of RA.

Although the aetiology of RA is still not understood, accumulating evidence largely indicates that transactivation of NF-κB-dependent gene expression plays a key role in the development of RA and many other autoimmune diseases. Indeed, aberrant dysregulation of NF-κB activity or an increased nuclear level of the NF-κB subunit has been commonly observed in cultured FLSs, human arthritic joints and joints of animals with experimentally induced RA [9–12]. Consequently, selective targeting of the products of NF-κB-driven genes, such as TNF, IL-6 and IL-1, has been emerging as a valuable breakthrough in the treatment of RA patients who do not respond to standard treatment [29]. To achieve tight regulation of
Fig. 3 A schematic representation of aberrant splicing forms of the A20 gene.

(A) The 50 bp of intron 2 sequence that is inserted between exon 2 and exon 3, is shown in a shaded box. Consensus splice donor (ttgcag) and acceptor (gtgatt) sequences flanking this 50-bp insert are depicted as bold. The aberrant cleavage sites within intron 2 are indicated by the arrow. (B) A20 genomic sequence showing the deletion of exon 4 and insertion of entire intron 4. The universally conserved motifs near the nucleotide sequences surrounding the exon/intron borders that act as essential splicing signals are depicted as bold. Arrows indicate aberrant cleavage sites for exon 4 deletion and the nucleotide sequences of the entire intron 4 that are inserted into the mutant A20 are shown in the shaded box.
Fig. 4 Effects of ectopic expression of wild-type and aberrantly spliced variants on TNF-induced NF-κB activation.

(A) Wild-type and various truncated forms of A20 were transfected into HEK293 cells, and the expression was determined by immunoblotting with anti-flag antibody (top panel). The blot was verified using an antibody against β-actin to confirm equal amounts of proteins were loaded (bottom panel). (B) HEK293 cells were transiently transfected with expression vectors of wild-type and various truncated forms of A20 constructs along with p2xNF-κB-Luc and pRSV-β-gal. After 24 h of transfection, cells were treated for 10 h with 15 ng/ml of TNF. Cells were then lysed and the luciferase activities were measured as described in the Materials and methods section. The luciferase activity of each sample was normalized according to β-galactosidase activity. Results show the mean (s.e.) of at least three independent experiments.

NF-κB signalling, a number of cells employ different control mechanisms to keep NF-κB signalling in check [15]. Functioning as a gatekeeper, A20 has a pivotal role in the restriction of the duration of both TNF and Toll-like receptor-mediated NF-κB signals, and thus has been described as a potent anti-inflammatory molecule [13–16]. Based on the recent data using genome-wide association studies (GWASs), variants in the A20 gene have been shown to be associated with multiple inflammatory and autoimmune diseases, including RA, SLE, Crohn’s disease and psoriasis [22–24, 30], which suggests that a defect in A20 expression or activity may be involved in the pathogenesis of these diseases. Moreover, A20 was frequently inactivated by somatic mutation or deletion in several subsets of B-cell lymphomas [19, 20], suggesting that the genetic variants of A20 also function as a tumour promoter in certain types of tumour.

In addition to the mutations and chromosomal aberrations of A20 described previously, we identified here the four types of aberrant splicing transcript of A20 in RA FLSs: (i) 50 nt in the middle of intron 2 inserted out-frame between exon 2 and exon 3 (A20/INS-pI2); (ii) 160 nt of the entire intron 4 inserted between exon 4 and exon 5 (A20/INS-I4); (iii) exon 4 deleted in A20/INS-I4 (A20/Del-E4/INS-I4); (iv) additional partial intron 2 (50 nt) inserted in A20/Del-E4/INS-I4 (A20/INS-pI2/Del-E4/INS-I4). In our study, in the most abundant aberrantly spliced form (A20/Del-E4/INS-I4) exon 4 was skipped and intron 4 inserted, which introduced premature translation termination codon, resulting in 192 amino acids of protein (Table 1, Fig. 2). Three other minor A20 variants—A20/INS-pI2, A20/INS-I4 and A20/INS-pI2/Del-E4/INS-I4—also produced truncated proteins 108, 248 and 108 in amino acid size, in contrast to the 790 amino acids of full-length A20 protein (Fig. 2). Although the precise mechanism of aberrant splicing in pathological conditions such as autoimmune disease and cancer is still unknown, the common reason for splicing defects is a point mutation in the genomic splice site [31, 32]. To access whether the aberrant transcripts of A20 were caused by genomic sequence mutation, PCR was used to amplify genomic DNA across the regions where the aberrant splicings occurred. In FLSs containing the aberrant A20 transcripts, we did not detect any mutation or deletion of the A20 gene at the genomic level, especially within the cis-acting consensus sequence of intron/exon boundaries (data not shown). In contrast, 5’ and 3’ splice sites in A20 aberrant transcripts obeyed the GT/AG rule, and the branch sites matched well with consensus sequence (Fig. 3), indicating that the aberrant splicing of A20 in RA FLSs was not a result of mutations in cis-acting consensus sequence at the potential cryptic donor and acceptor sites. In a recent GWAS, it was reported that several SNPs at 6q23 were associated with RA susceptibility [33, 34]. Given that among these RA-associated SNPs, rs5029937 in particular is located in the intron 2 of A20, the generation of such novel aberrant transcripts may be affected, especially the insertion of partial intron 2 (A20/INS-pI2 and/or A20/INS-pI2/Del-E4/INS-I4) in our study. However, the rs5029937 risk allele is not only located >2.2 kb from the aberrant splicing site within intron 2 but more importantly, it does not contribute to the generation of consensus splice donor/acceptor sequence (GT/AG). Thus it seems that the presence of an allele of rs5029937 and aberrant
splicing transcripts of A20 are independently associated with RA susceptibility. Nonetheless, since it has been proposed that the combination of independent genetic variants at the intergenic region encompassing the RA-associated SNPs at 6q23 can increase the risk estimate for RA [35], future large-scale genetic studies are required to ascertain the association of the identified SNPs and aberrant splicing variants of A20 in the progression of RA.

Although the reason why A20 transcripts are defective in FLSs from RA patients remains unclear, accumulating evidence suggests that certain lineages of cells such as...
FLSs are resistant to apoptotic cell death under the condition of RA progression, as when FLSs are unable to restrict a NF-κB signal and subsequently induce hyperplastic growth through the production of a set of anti-apoptotic molecules including Mcl-1, Bcl-2 family proteins, cellular FLICE inhibitory protein (c-FLIP) and inhibitor of apoptosis proteins (IAPs) [36, 37]. Four of the A20 splicing variants identified in our study comprise a premature translation termination codon (Fig. 2F). We could not detect protein expression of truncated A20 in FLSs due to the low expression level of the protein. If translated, however, these splicing variants would encode a non-functional OTU domain of A20 that has lost its de-ubiquitinating function against RIP1 or TRAF6. Accordingly, overexpression of four of the A20 mutants was not able to inhibit TNF-induced NF-κB activation (Fig. 4), which suggests that the translation of these splicing variants could antagonize the anti-NF-κB activity of wild-type A20, probably in a dominant negative manner. Moreover, the presence of a high level of aberrantly spliced A20 transcript was significantly correlated with the persistent activation of NF-κB signal as well as enhancement of the expression of pro-inflammatory cytokines (Fig. 5), a phenomenon that gave support to a pathological role of these spliced variants in a subset of RA patients.

Collectively, the presence of aberrant splicing of A20 that we discovered and altered functions of the A20 variants indicate that aberrant splicing might be an important mechanism through which A20 loses its anti-NF-κB activity in FLSs from RA patients. The features of the aberrant A20 variants arise from a defect in the mRNA splicing process during the development and/or progression of RA. Nevertheless, further studies will be required to elucidate the mechanism and pathological consequences of aberrant A20 splicing in RA. Moreover, further discovery of such A20 variants under the condition of NF-κB activity dysregulation (i.e. several solid tumours and haematopoietic malignancies) will also help to confirm the significance of aberrant splicing transcript in the development and/or progression of these relevant diseases. Moving forwards, because the aberrant A20 transcripts can be analysed at the mRNA level, it will be important to confirm the potential value of using these variants for diagnostic or therapeutic application with even larger series of cases.

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Supplementary data
Supplementary data are available at Rheumatology Online.

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