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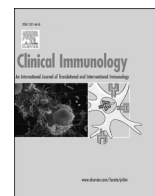
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A large deletion in a non-coding regulatory region leads to NFKB1 haploinsufficiency in two adult siblings

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ABSTRACT

Mutations in NFκB pathway genes can cause inborn errors of immunity (IEI), with NFKB1 haploinsufficiency being a significant etiology for common variable immunodeficiency (CVID). Indeed, mutations in NFKB1 are found in 4 to 5% of in European and United States CVID cohorts, respectively; CVID representing almost ¼ of IEI patients in European countries registries. This case study presents a 49-year-old patient with respiratory infections, chronic diarrhea, immune thrombocytopenia, hypogammaglobulinemia, and secondary lymphoma. Comprehensive genetic analysis, including high-throughput sequencing of 300 IEI-related genes and copy number variation analysis, identified a critical 2.6-kb deletion spanning the first untranslated exon and its upstream region. The region's importance was confirmed through genetic markers indicative of enhancers and promoters. The deletion was also found in the patient's brother, who displayed similar but milder symptoms. Functional analysis supported haploinsufficiency with reduced mRNA and protein expression in both patients. This case underscores the significance of copy number variation (CNV) analysis and targeting noncoding exons within custom gene panels, emphasizing the broader genomic approaches needed in medical genetics.

1. Introduction

The nuclear factor of kappa light polypeptide gene enhancer in B cells or NF-κB pathway is a major regulator of several cellular processes such as immune responses, inflammation, proliferation, apoptosis, and stress response [1]. The NF-κB family comprises five transcription factors (TF): p105/p50, also known as NF-κB1, p100/p52, also known as NF-κB2, RelA, RelB, and c-Rel [2]. The proteasome processes the two precursor proteins p105 and p100 to generate two shorter mature

proteins, p50 and p52, respectively [2]. The mature p50 and p52 and Rel proteins can form various homo- and heterodimers with distinct regulatory properties [3]. Among them, the p50/RelA heterodimer is the main TF of the canonical NF-κB1 pathway [4]. Upon stimulation with cytokines such as TNFα or IL-1β, microbial products, or antigen receptors, the degradation of the inhibitor of NFκB (IκB) allows p50/RelA dimers to enter the nucleus to activate the transcription [5]. To do so, p50 absolutely requires association with RelA which contains a trans-activation domain [6]. Conversely, p50 homodimers behave as

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transcriptional repressors because of the lack of a transactivation domain [3].

Human NF- κ B1 deficiency is caused by heterozygous germline variations in the *NFKB1* gene leading to haploinsufficiency (OMIM # 616576) [7]. Previous studies revealed that this inborn error of immunity (IEI) is the main cause of monogenic common variable immunodeficiency (CVID) with 4% in European countries and 5% in United States [8,9]. In addition, observations from French and European IEI registries established a CVID frequency of 23 to 26% of all IEI patients; we can thus expect that 1% of European IEI patient could suffer from *NFKB1* haploinsufficiency [10,11]. In line with these observations, more than hundred heterozygous variants of *NFKB1* have been described [7,8,12–26]. Antibody deficiency is a prominent feature of *NFKB1* deficiency [19]. However, clinical phenotypes are extremely diverse and autoimmune or auto-inflammatory diseases are also common [17]. In addition, analysis of familial segregations revealed that some subjects are healthy carriers of *NFKB1* pathogenic variation, suggesting an incomplete penetrance and/or late disease onset [19].

Most damaging variant in *NFKB1* are single nucleotide variants or small insertions/deletions; however, two large deletions have also been reported [8]. These two deletions encompass large parts of the *NFKB1* gene, removing several exons, leaving no doubt on their deleteriousness. Here we report two patients carrying a 2,6 kb heterozygous deletion encompassing a noncoding region of *NFKB1* leading to *NFKB1* haploinsufficiency and CVID phenotype. We performed a genetic analysis of the deleted region and confirmed the pathogenic effect of this new deletion.

2. Materials and methods

Human subjects. All patients provided written informed consent. The study protocol was approved by the local investigational review boards and the French Advisory Committee on Medical Research (IRB registration no. 00001072, no. CPP: 2015-03-03/DC 2014–2272).

Flow cytometry. Cell membrane staining and flow cytometry-based phenotypic analyses were performed according to standard flow cytometry methods. All data were collected on Canto II cytometer (BD Biosciences).

Genetic explorations. Next-Generation Sequencing targeting 300 genes involved in IEI was performed in P1 as previously described [16]. Briefly, after capture by hybridisation with biotinylated complementary 120-bp RNA baits designed with SureSelect SureDesign software (Agilent, *H. sapiens*, hg19, GRCh37, February 2009), targeted regions were sequenced on an Illumina HiSeq2500 HT system (Paired-End sequencing 130 × 130 bases). Data analysis was performed using Paris University/Imagine Institute's Bioinformatics core facilities according to documented best practices (<https://software.broadinstitute.org/gatk/best-practices/>).

CNV analysis. We evaluated CNV (*i.e.* large duplications or deletions) by determining the relative read count for each targeted region as the ratio of the read count for that region divided by the total absolute read counts for all the targeted regions. The ratio of the relative read count for a region in a given individual to the mean relative read count in other individuals of the run gave the estimated CNV for the region concerned in that individual (method adapted from [27]). Homozygous deletion was suspected when this ratio was close to zero (no aligned reads). For the detection of monoallelic CNV, a ratio below 0.7 was considered suggestive of a heterozygous deletion, whereas a ratio above 1.3 was considered suggestive of a heterozygous duplication.

Sanger. The *NFKB1* abnormal PCR product induced by the deletion (295 bp) and a control PCR amplification of an irrelevant gene (*PGM3*) were co-amplified using KAPA2G Robust HotStart ReadyMix PCR Kit and the following couple of primers: Forward-ACACAAAGGAAACACGGGGA / Reverse-CACCAACCACCTCCCTCTC. Wild-type *NFKB1* first noncoding exon was amplified separately with the following couple of primers: Forward-CCTAGAAGTGGGGCTT /

Reverse-CTGTCGCTAATCCCATGTGC. PCR products were sequenced with a BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, University Park, Ill) and analysed on a 3500xL Genetic Analyzer (Applied Biosystems).

Immunoblotting. Cells were lysed in lysis buffer (1% NP-40 [NP-40 alternative, Calbiochem], 50 mM Tris pH 8, 150 mM NaCl, 20 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails 2 and 3 (Sigma)). After cell lysates clarification, protein concentrations were determined by Coomassie protein assay reagent (Thermo Fischer Scientific). Next, 80 μ g of proteins were denatured by boiling for 5 min in sample buffer (125 mM Tris pH 6.8, 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue), separated by SDS-PAGE, and transferred on polyvinylidene difluoride membrane (Millipore, Burlington, Mass). Membranes were blocked with milk buffer before incubation with the following antibodies: *NFKB1* (Clone D4P4D, Cell Signaling Technology), GAPDH (Clone 14C10, Cell Signaling Technology). Quantification was done with ImageJ software. Signals of p105 and p50 were normalised on GAPDH signals and then normalised on the mean of controls to allow inter-experiments comparison.

qPCR. Total RNA was extracted from T-cell blasts using Qiagen RNeasy micro kit. RNA was reverse transcribed in the presence of oligo (dT) with Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, United Kingdom). *NFKB1* expression and interferon-stimulated gene signature (ISG) in activated T-cell blasts were assessed by quantitative RT-PCR using TaqMan Gene Expression Assays. The following TaqMan Gene Expression Assays obtained from ThermoFisher Scientific was used- *NFKB1*: Hs00765730_m1. ISG was performed using TaqMan probes for *IFI27* (Hs01086370_m1), *IFI44L* (Hs00199115_m1), *IFIT1* (Hs00356631_g1), *ISG15* (Hs00192713_m1), *RSAD2* (Hs01057264_m1), and *SIGLEC1* (Hs00988063_m1). The relative abundance of each target transcript was normalised to the expression level of *GAPDH* (Hs03929097_g1). Real Time quantitative PCR was performed in duplicate using the LightCycler VIIA7 System (Roche). Relative quantification (RQ) represents the fold change compared to the calibrator (*GAPDH*). The RQ value is equal to $2^{\Delta\Delta Ct}$ where $\Delta\Delta Ct$ is calculated by (CT target-CTGAPDH) test sample minus (CT target-CTGAPDH) calibrator sample. We then normalised each RQ to mean of control's RQ for each experiment in order to allow proper comparison.

3. Results

3.1. Case description

The index case was born from nonconsanguineous parents of Caucasian ethnicity. He had suffered from recurrent ear-nose-throat (ENT) infections during childhood, had been treated for anal condyloma Acuminatum (human papilloma virus (HPV) infection) at the age of 25 and exhibited seasonal eczema. He presented with chronic diarrhea in his late 30's that disclosed lymphocytic colitis and led to find hypogammaglobulinemia (1.5 g/L, with undetectable IgM). Immunophenotyping revealed a decrease in several lymphocyte populations (CD8 T, regulatory T, B and NK cells). Intravenous immunoglobulins (IVIg) substitution was subsequently initiated, with mitigated results on the frequency of infections, and no efficacy on chronic diarrhea. At the age of 43, he developed immune thrombocytopenia although not requiring any treatment, owing to platelets above 50 G/L. In the same year, abnormal liver blood tests and the detection of hepatosplenomegaly conducted to the diagnosis of nodular regenerative hyperplasia with portal hypertension. He also suffered from periodic erythema nodosum of the lower limbs. Six years later, aged 49, he was hospitalised for dizziness and vertigo that revealed a suspicious lesion of the superior cerebellar vermis. A splenectomy was performed to raise platelets above 100 G/L, thus allowing brain biopsy that disclosed an EBV-driven diffuse large B cell lymphoma. He received chemotherapy

with rituximab and methotrexate but unfortunately died 3 years later from decubitus complications in a context of severe post-operative cerebellar syndrome.

3.2. Genetic exploration

Given the clinical and biological phenotype of the patient, we hypothesised that a genetic etiology could explain his disease. We performed high-throughput sequencing targeting 300 IEI-related genes. The first layer of analysis did not evidence any causative single nucleotide variation in exonic or flanking intronic regions of *NFKB1* nor other IEI-related genes included in the targeted panel. We then applied CNV detection algorithm that compute a dup/del score by mean of a double coverage normalisation (see methods). We discovered a dup/del score of 0,62 on the first noncoding exon of *NFKB1*. This score below 0,7 suggested the presence of a heterozygous deletion. However, this deletion affected only one noncoding region of interest and untranslated regions are known to frequently have a high-GC content leading to sequencing issues and increased coverage variability [28]. Moreover, the dup/del score was slightly above the usual dup/del score interval for heterozygous deletions [0,4-0,6] [16]. These cues often imply a false positive CNV detection. Thus, we carefully looked at the coverage data and observed a drop in the mean coverage of the patient compared to the expected gaussian repartition obtained in the other patients of the same run (Fig. 1A). This sudden decrease in coverage depth was reminiscent of a 3' deletion breakpoint. Read alignment on the Integrative Genome Viewer showed that some reads were only partially aligned with the *NFKB1* exon 1 sequence and that the misalignment stopped at the breakpoint of coverage. Moreover, several reads abnormally matched with a sequence located in the 5'UTR region, 2641 bp upstream (Fig. 1B). Given that this region is not covered by the design of the baits and not known as an off-target, we extrapolated that this region could be the 5' deletion breakpoint. We designed two primers to amplify the putative breakpoint in the patient's DNA. PCR amplification revealed the presence of an abnormal product of 285 bp that was not seen for a control DNA. Sanger sequencing of this product confirmed the position of the deletion breakpoint with a 3-base pairs microhomology (GAA) between the flanking sequences of the deletion (Fig. 1C).

3.3. Deletion analysis

To understand the potential impact of this deletion on *NFKB1* transcription, we mapped the deletion (NC_000004.11: g.103420546_103423187del (chr4/hg19)) on the UCSC Genome Browser [29]. As shown in Fig. 1D, the deletion encompasses completely the non-coding exon 1 shared by the transcripts NM_001165412.2, NM_003998.4, and half of the non-coding exon 1 of the NM_001319226.2. Visualisation of histones modifications from ENCODE revealed that H3K27Ac and H3K4Me3 were enriched in similar regions mainly overlapping with the two different exon 1. These two histone marks are known to be found near active regulatory elements and promoters. In addition, the deletion also spans a region upstream enriched in H3K4Me1, a histone mark associated with enhancers. The chromatin state segmentation analysis by Hidden Markov Model predicted the presence of an active enhancer, a weak promoter and an active promoter in the region of interest. DNaseI hypersensitivity and DNA methylation at the same location corroborate the presence of the active promoter. The presence of many transcription factor motifs strengthens the importance of this active promoter. Finally, we investigated the role of repeated elements that could explain the deletion (<http://www.repeatmasker.org/>). We only found that the 5' breakpoint was located within a long terminal repeat (LTR) sequence named MLT1c without the corresponding repeated element on the 3' breakpoint.

3.4. Familial segregation

The patient had one sister aged 46 years and one brother aged 50 years, both initially considered healthy (Fig. 2A). However, the latter had presented with recurrent ENT infections and cyclic aphthous stomatitis since childhood. He had abnormal liver blood tests from the age of 41 (cholestasis and mild hepatic cytolysis), without explanation after a biologic and radiological diagnostic workup. Because *NFKB1* deficiency is known to have an incomplete penetrance and/or very late disease onset, a complete familial segregation was undertaken. For this purpose, we designed a multiplex PCR to amplify the abnormal PCR product of the patient along with a control PCR of an irrelevant gene to confirm the correct amplification. As expected, the patient presented a lower band of 295 bp representing the abnormal PCR product resulting from the deletion (Fig. 2B). Genetic testing of the siblings revealed that the brother carried the same heterozygous deletion as the patient. On the contrary, only the control PCR product was amplified in the sister, which ruled out any issue with the sister's DNA. We were unable to perform a multiplex PCR containing both the deleted and the WT allele so we amplified the wild-type non-coding exon 1 in a separate PCR to confirm the heterozygous state of the deletion (Fig. 2C). This analysis confirmed the presence of a 759-bp band corresponding to the WT allele in all siblings (Fig. 2C).

3.5. Functional validation

Next, we performed an evaluation of the transcription and expression of NF- κ B1 to assess the functional impact of this new non-coding deletion. We assumed that the deletion would affect the correct transcription of the mutated allele, leading to haploinsufficiency. Indeed, qPCR analysis on RNA extracted from the patient PHA-activated T cells evidenced a drastic reduction in *NFKB1* mRNA compared with healthy controls (Fig. 2D). We decided to analyse protein expression *in vitro* on PHA-activated T cell blasts. After normalisation on GAPDH expression, we detected a decrease of the p50 cleaved form of NF κ B1 in the two subjects carrying the deletion, whereas the sister was comparable to the control (Fig. 2E-F). However, we did not see any significant differences in p105 expression. The decrease in p50 expression corroborated the suspicion of NF κ B1 haploinsufficiency. Finally, as NF-KB1 deficiency can lead to an inflammatory phenotype in some patients, we surmised that the patient could present an elevated interferon signature. We evaluated five interferon target genes by qPCR (namely, *IFI44L*, *IFI27*, *IFIT1*, *RSAD2* and *ISG15*) and compared their relative expression to a healthy donor as a negative control or a patient with *STING1* p.V155M activating mutation as a positive control. However, this analysis did not reveal any increase of interferon signature for the patient (Fig. 2G).

4. Discussion

We describe here the two first patients with a large noncoding heterozygous deletion in *NFKB1* gene. Albeit not specific, these two patients' manifestations are similar to the clinical presentation already described in patients with *NFKB1* haploinsufficiency. Indeed, respiratory infections and hypogammaglobulinemia were the most common features of this disease, followed by autoimmunity and lymphoproliferation [19]. *Ex vivo* tests performed directly on patient's T cells confirmed the deleteriousness of the promoter and the first exon deletion on *NFKB1* transcription and protein expression. Nevertheless, a targeted sequencing approach by Sanger sequencing or a broad strategy using Whole Exome Sequencing (WES) would have been unsuccessful to discover this deletion. Indeed, most commercial WES kit do not include probes targeting non-coding exons because they focus on the coding part of the genome. In this study, we took advantage of customised targeted NGS to design probes to cover the non-coding exons present in over 40 % of IEI-related genes [30]. As tools and analysis used in medical genetics mainly focus on the coding part of the genome, we found only a few

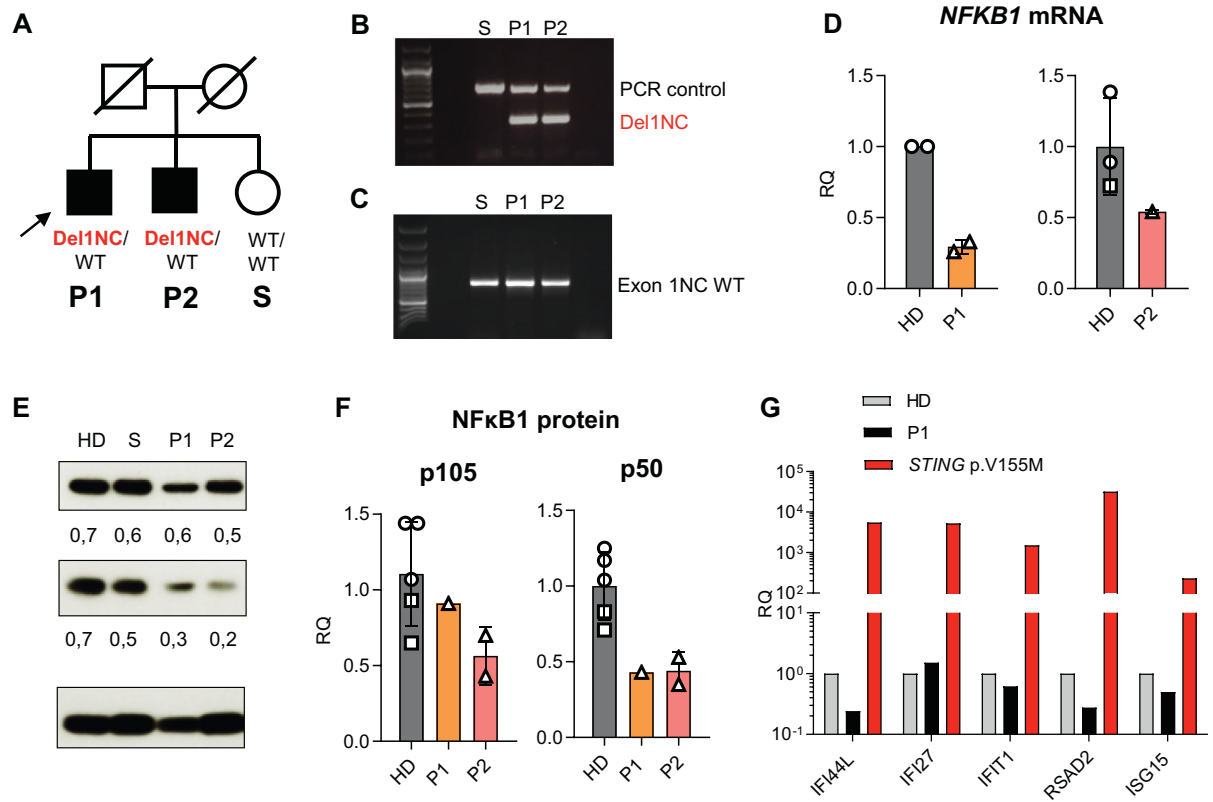


Fig. 2. Familial segregation and functional analysis.

A. Familial segregation of siblings. Arrow indicates the *index case*. S: healthy sister. B. Multiplex PCR for the three siblings. Upper band is a PCR control targeting an irrelevant gene (*PGM3*), lower band is the abnormal PCR product only amplified when the 2.7 kb deletion in *NFKB1* is present. C. PCR targeting the wild type noncoding exon 1 of *NFKB1*. D. Expression of *NFKB1* transcript by qPCR. E. Representative Western blot analysis of p105 and p50 from 2 experiments (except for P1, one experiment), GAPDH was used as a loading control. F. Western blot quantification of p50 and p105 normalised on GAPDH signal and on controls. G. Interferon signature by qPCR of five interferon-regulated genes. *STING* p.V155M-mutated patient was used as a positive control. HD: healthy donors. Healthy sister is depicted as square among HD. RQ: relative quantification (see methods).

studies reporting IEI caused by a deletion restricted to noncoding exons.

A large deletion encompassing the first untranslated exon of *RAB27A* was discovered in a patient with typical Griscelli syndrome [31]. Authors suspected a deletion because of ineffective PCR amplification of this exon with patient's DNA. In this study, Sanger sequencing of the first exon of *NFKB1* from the patient was successful because of the presence of the WT allele. In a recent report, Sbihi et al. found a deletion restricted to the promoter and first untranslated exon of *XIAP* in three unrelated patients [30]. The limits of the three deletions were different but overlapped in a region of 838 bp probably containing the promoter of *XIAP*. Two deletions were driven by *Alu* elements suggesting this zone could represent a hotspot of CNV breakpoint as observed in other genes [32]. As most diagnostic methods do not investigate the first exon of *XIAP*, it is possible that this diagnosis has been missed in several other patients. It is noteworthy that the probes designed for our targeted NGS panel cover this region of *XIAP*. A Whole Genome Sequencing (WGS) analysis in 886 patients with IEI highlights a genetic defect in *ARPC1B* due to a 9-kb heterozygous deletion of the promoter and the first untranslated exon associated to a heterozygous frameshift variant *in trans* [33]. This example demonstrates the utility of WGS to discover pathogenic variations in cis-regulatory elements.

Awaiting for the global spread of WGS in routine genetic exploration, medical geneticists should improve their targeted NGS panel or WES kit by including probes to capture non-coding exons. Indeed, only a systematic approach could help increase the discovery of such cases. In conclusion, our results underline the importance of efficient diagnostic tools for genetic explorations of IEI.

CRediT authorship contribution statement

Mathieu Fusaro: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Cyrille Coustal:** Data curation, Resources, Writing – original draft, Writing – review & editing. **Laura Barnabei:** Investigation, Writing – review & editing. **Quentin Riller:** Investigation, Writing – review & editing. **Marion Heller:** Investigation, Writing – review & editing. **Duong Ho Nhat:** Investigation, Writing – review & editing. **Cécile Fourrage:** Data curation, Software, Writing – review & editing. **Sophie Rivière:** Data curation, Supervision, Writing – review & editing. **Frédéric Rieux-Laucat:** Resources, Supervision, Writing – review & editing. **Alexandre Thibault Jacques Maria:** Data curation, Supervision, Writing – review & editing. **Capucine Picard:** Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare they have no conflicts of interest.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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