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Novel *UNC13D* intronic variant disrupting a NF κ B enhancer in a patient with recurrent macrophage activation syndrome and systemic juvenile idiopathic arthritis

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Abstract

Objective—Macrophage activation syndrome (MAS) is a life-threatening complication of systemic juvenile idiopathic arthritis (SJIA), and has pathologic similarity to hemophagocytic lymphohistiocytosis (HLH). Intronic variants in *UNC13D* are found in patients with familial HLH-3 (FHL3), but the role of non-coding variants in MAS is unknown. The objective of this study was to identify deep intronic *UNC13D* variants in patients with MAS.

Methods—A custom enrichment library was constructed to sequence approximately 1 MB flanking *UNC13D* in 24 patients with SJIA, recurrent MAS, and negative prior genetic (exon/coding) testing. Functional consequences of intronic variants were assessed using quantitative PCR on patient derived peripheral blood mononuclear cells (PBMC), electrophoretic mobility shift assay (EMSA), *in vitro* transcriptional enhancer assays, and NK cell degranulation assays.

Results—We report a patient with SJIA and recurrent MAS, found to have a novel functional intronic variant in *UNC13D*, c.117+143A>G. This variant occurs in a proposed regulatory region that drives lymphocyte specific *UNC13D* expression, and is associated with reduced transcript levels in patient PBMC. The patient's variant also disrupts NF κ B binding to a functional transcriptional enhancer, leading to reduced enhancer activity *in vitro*. Partial knockdown of *UNC13D* expression also led to impaired NK cell degranulation. An additional patient was

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identified with a previously described *UNC13D* intronic variant, for a total non-coding variant hit rate of 8.3% (2/24).

Conclusion—These findings highlight that intronic variants in key regulatory regions may be associated with MAS in patients with SJIA, and support deep sequencing approaches when causative coding variants are not identified.

Keywords

Transcriptional enhancer; non-coding genetic variants; hemophagocytic lymphohistiocytosis; perforin cytolytic pathway

Macrophage activation syndrome (MAS) is a life-threatening episode of hyperinflammation, characterized clinically by cytopenias, liver dysfunction, coagulopathy, and a systemic “cytokine storm”. While MAS has been reported to occur in numerous rheumatic diseases, in pediatric rheumatology it is most often a complication of systemic juvenile idiopathic arthritis (SJIA), a severe and distinct subtype of childhood chronic arthritis (1). MAS bears striking similarity to the rare histiocytic disorders hemophagocytic lymphohistiocytosis (HLH), where genetic defects in the perforin exocytosis pathway lead to profoundly depressed cytolytic activity of natural killer cells and CD8 T cells (2). Increasingly, hypomorphic protein-altering variants in these genes have been reported in patients with MAS, including MUNC13-4 (encoded by *UNC13D*), which mediates fusion of perforin-containing lytic granules with the plasma membrane, and deficiency of which causes familial HLH-3 (FHL3) (2–4). Deep intronic variants in *UNC13D* have also been identified in HLH patients from Europe and North America (5–7). These variants frequently involve the first intron of *UNC13D*, which appears to serve as a key regulatory region, and one variant, c.118-308C>T, has been shown to disrupt transcription factor binding to a cytotoxic lymphocyte-specific alternative promotor, thereby selectively diminishing *UNC13D* expression in cytolytic cells (8). However, the presence of intronic or non-coding variants in MAS has been largely unexplored.

Here, we report a previously healthy 13-month-old Caucasian male diagnosed with SJIA, who later developed recurrent episodes of MAS despite traditionally effective treatment. While testing for coding variants in HLH-associated genes was unrevealing, he was found to have a novel *UNC13D* variant in the lymphocyte-specific intronic promotor. In support of a causative role for this variant, reduced levels of the alternative, lymphocyte-specific transcript were found in patient peripheral blood mononuclear cells (PBMC). Finally, we further show that this variant disrupts an NFκB site that functions as a transcriptional enhancer *in vitro*, potentially resulting in decreased *UNC13D* expression and compromised NK cell degranulation.

Materials and Methods

Patient data and study approval

This study was approved by the Cincinnati Children’s Hospital Institutional Review Board (#2011-1517), and informed consent was obtained from all patients and/or their legal guardians. Data pertaining to this patient’s clinical course, laboratory values, and treatment

were collected from the electronic medical records. Whole blood was obtained using sodium heparin as an anticoagulant. PBMC were separated by Ficoll gradient centrifugation, and RNA extracted using Trizol as described (9). Where indicated, PBMC were treated with recombinant human tumor necrosis factor (TNF) (ThermoFisher, Carlsbad, CA) for 6 hours prior to RNA extraction.

UNC13D sequencing

In order to examine possible intronic variants, a custom enrichment library was constructed using SeqCap EZ from NimbleGen to sequence a genomic region of approximately 1 MB on chromosome 17 (Human genome hg19 start site 73,448,832 and stop site 74,504,525) that flanks *UNC13D*. The design specification shows 98% of the targeted region overlapped with specific probes. Patient DNA was enriched for the target genomic region by solution-based SeqCap EZ library with 2 million probes for the custom target. A cohort of 24 patients with recurrent SJIA-associated MAS or secondary HLH with prior negative genetic exonic testing were sequenced in two runs using Illumina HiSeq2000 with paired end design and large library with average size of 600bps to ensure covering regions of alu sequence. Sequence reads were filtered first by quality measurement, then matched to the reference sequence of human genome (hg 19). Then, variants were annotated using NextGENe Software version 2.3.4 (SoftGenetics LLC). Variants were filtered based on frequency in dbSNP and ExAC database, and then examined with respect to nucleotide conservation PhyloP scores and overlap with transcription factor binding motifs from ChIP-seq data. Additionally, variants were assessed based on manually curating motif score to remove lower similarity sites, and visual inspection of quality sequence was used to remove common artifacts known in repetitive regions. Transcription factors for specific reference and mutant nucleotide sequences were matched via the TRANSFAC database.

Real-time RT-PCR

RNA extraction and cDNA synthesis were as described (9). Real-time PCR was performed using a ViiA 7 real-time PCR instrument with gene-specific primers and SYBR Green Supermix (ThermoFisher). Message copy numbers were normalized against the copy number of the housekeeping gene, *GAPDH*. Primers for *GAPDH* (9) and the conventional and intron 1 transcripts of *UNC13D* (8) were as described previously.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed by mixture of nuclear extracts from activated NK-92 cells and IRDye-700 labeled NF κ B consensus oligonucleotides as previously described (10). The wild type (WT) oligonucleotide probe sequence for the NF κ B-like enhancer (143A) located within *UNC13D* intron 1 was ACCCTGGGGAGAGCCCCCTGGG. The mutant type oligonucleotide probe sequence for the NF κ B-like enhancer (143G) included in *UNC13D* intron 1 was ACCCTGGGGAGGGCCCCCTGGG.

Enhancer assay

The WT or mutant NF κ B-like enhancer (143A/G, 3 $\times$ TGGGGAG(A/G)GCCCCCT) was inserted into the enhancer site of the immediate early CMV promotor or HIV-1 LTR-driven GFP reporter plasmid previously generated (10) to create the CMV-143A/G-GRP or the HIV-143A/G-GFP constructs. These reporter plasmids were transfected into NK-92 cells by nucleofection as previously described (10). The cells were rested for 24 hrs and then stimulated with irradiated K562 cells (dead) for 1 hr (the ratio between NK-92 and K562 cells was 2:1) prior to measuring GFP expression levels by flow cytometry as described (11). To further exclude the potential influence of artificially repeated core sequences, short genomic DNA of 111 nucleotides as shown below, including the NF κ B-like WT or mutant sequence enhancer core DNA, was synthesized and inserted into the HIV-1 LTR-driven GFP reporter plasmid in either forward or reverse orientation to generate HIV-143A/G111nt-F/R-GFP plasmids. NK-92 cells were transfected with the GFP reporter plasmids and activated, and GFP was measured by flow cytometry as above. (Short genomic DNA sequence of 111nts length –

5'CGAAGGCTGGGACTGGGGACCAGCGACCCTCCCCTGCCTACTCCACCCTGGGGAG(A/G)GCCCCCTGGGCCAGCAGAGCAGGGGCTGCCAGATGGACTCTGGGCCACTGTGGC3'.)

UNC13D knockdown and degranulation assays

The TriFECTaRNAi kit was purchased from IDT (Coralville, Iowa). Transfection of NK-92 cells was performed using a 4D-nucleofector (Allendale, NJ). In detail, 4×10^6 cells mixed with siRNA were suspended with 100 μ l of nucleofector solution, and transfection was performed immediately using an optimally pre-set program. Transfected cells were cultured for 24 hrs. For the degranulation assay, transfected cells were stimulated with K562 target cells (effector : target = 1:1) for 1 and 2 hrs in the presence of PE-conjugated anti-CD107a antibody (Biolegend, San Diego, CA). This was quickly followed by flow cytometry and analysis as described above. To detect *UNC13D* mRNA expression levels, total RNA was extracted from transfected NK-92 cells, and reverse transcribed into cDNA using a SuperScriptIII first strand kit from Invitrogen (Carlsbad, CA, USA). Real-time PCR were performed with a Bio-Rad CFX PCR device (Hercules, CA, USA). The housekeeping gene beta-2-microglobulin mRNA was used for normalization, and scramble siRNA was used for the control of *UNC13D* specific siRNA. All oligonucleotides used in these experiments are listed in supplementary material.

Results

We report a previously healthy 13-month-old Caucasian male who developed daily fevers for one month, intermittent rash, and difficulties with crawling. His parents reported that he would be very irritable for about 1-2 hours upon awakening and then would improve over the course of the day. He was a former term infant with no perinatal complications and no significant family medical history or exposure history. His initial exam revealed mild hepatomegaly but no splenomegaly, diffuse salmon-colored rash, and polyarthritis involving the right wrist, bilateral elbows and knees, and left ankle. His initial laboratory evaluation

was notable for elevated white blood cell count of $32.1 \times 10^3/\mu\text{L}$, hemoglobin 11.0 g/dL, erythrocyte sedimentation rate of 56 mm/hr, C-reactive protein of 11.0 mg/dL, and negative autoantibodies. He was diagnosed with SJIA and treated with prednisone, with addition of methotrexate and then intermittent pulse-dose methylprednisolone for refractory polyarthritis.

Approximately 4 months after diagnosis, the patient acutely deteriorated after an Epstein-Barr virus infection, with persistent fevers, rash, active arthritis, and liver and spleen enlargement. His laboratory findings included white blood cell count of $17.5 \times 10^3/\mu\text{L}$, hemoglobin 7.0 g/dL, platelets $62 \times 10^3/\mu\text{L}$, ferritin 1,904 ng/mL, erythrocyte sedimentation rate falling to 6 mm/hr, aspartate transaminase 140 U/L, fibrinogen 246 mg/dL, and d-dimer 10.87 $\mu\text{g/mL}$. In addition, soluble IL-2R alpha (CD25) was elevated to 30,152 pg/mL, and soluble CD163 was elevated to 80,182 ng/mL. No functional testing of cytolytic or NK cells was performed at this time. He also had a bone marrow biopsy performed which showed hemophagocytosis. He was clinically diagnosed with MAS, having met 5/8 HLH-2004 diagnostic criteria (12), and would have satisfied the proposed 2016 MAS classification criteria (13). He was treated with intravenous methylprednisolone and cyclosporine A, as well as the recombinant human interleukin-1 receptor antagonist anakinra (1), leading to a full recovery. However, while weaning steroids he experienced a flare of MAS with elevated transaminases and markedly elevated soluble CD25 levels. This was again controlled with methylprednisolone and increased dosing of cyclosporine A.

Given recurrent episodes of MAS at an early age, he underwent genetic testing for possible HLH gene mutations including *PRF1*, *UNC13D*, *STX11*, and *STXBP2*, but no variants were found in all coding exons and exon/intron boundaries. As mutations in *UNC13D* are relatively common in SJIA patients with MAS in North America (3), a more in depth exploration of *UNC13D* was undertaken. In order to examine possible intronic variants, a custom enrichment library was constructed to sequence a genomic region of approximately 1 MB on chromosome 17 that flanked *UNC13D*. This array was used to sequence DNA from the above patient in a batch of 24 patients with recurrent SJIA-associated MAS or secondary HLH with prior negative exonic genetic testing. For the above patient, thirty-three million reads were generated of which 90% passed sequence quality filtering, and 67% of quality reads were matched to the target region. The average coverage depth was 2,122 and the target region coverage at depths 10 $\times$, 30 $\times$ and 100 $\times$ were 95%, 94%, and 92%, respectively (full coverage statistics for all 24 patients is in supplemental material).

Using this approach, a heterozygous variant was identified in the first intron of *UNC13D*, c. 117+143A>G (Chr17(GRCh37):g.73840159T>C, NM_199242.2) (Figure 1A). Unfortunately, parental DNA samples were not available for analysis to determine whether this variant was *de novo* or inherited. This variant was not present in 1000 genomes, present in only 1 of over 30,000 alleles sequenced through gnomAD, and was not identified in any of the 23 additional sequenced patients with SJIA and MAS, although one did have the previously reported c.118-308C>T intronic variant described below and in Figure 1A. Notably, there is substantial evidence that this intronic region has key roles in regulating *UNC13D* expression. First, there are reported H3K27A marks typical of transcriptional promoters on 7 unique cell lines, *DNaseI* hypersensitivity clusters suggesting transcriptional

activity in 125 cell types, and at least 15 transcription factor binding motif overlaps from ChIP-seq (Figure 1A). Second, the identified novel c.117+143A>G variant specifically overlaps with and alters a predicted consensus NFκB transcription factor binding site (Figure 1A). This variant is not predicted to disrupt additional consensus binding sites. Finally, two other variants from this intronic region, c.118-308C>T and c.118-307G>A, have been previously reported in patients with HLH (6,7). Indeed, the c.118-308C>T variant was also found in the heterozygous state in one of the 24 patients sequenced (patient 10). In addition, patient 10 was also found to have a previously reported 253kb inversion that straddles the last two exons of *UNC13D*, and is linked to FHL-3 in both Northern Europe and North America (5,6). No other rare coding or non-coding variants (MAF<1%) were identified in *UNC13D* in any of the 24 patients.

Interestingly, the previously identified c.118-308C>T variant has been shown to markedly reduce *UNC13D* expression in lymphocytes selectively, including CD8 T cells, by disrupting transcription factor binding to an alternative promoter specifically active in cytolytic cells (6,8). This promoter leads to production of a second, distinct *UNC13D* transcript, which is preferentially expressed in CTL and NK cells. PBMC from the patient described herein were not available to allow for cell-type specific transcript analysis. However, total RNA previously isolated from PBMC during a period of inactive disease was used to determine *UNC13D* mRNA levels by Real-time RT-PCR as described (8). This demonstrated reduced levels of the alternative, lymphocyte-specific intron 1 *UNC13D* transcript compared to healthy age-matched control PBMC, despite normal levels of the conventional *UNC13D* transcript (Figure 1B), suggesting that the novel c.117+143A>G variant may impair expression of lymphocyte-specific *UNC13D* transcript. Of note, this patient sample was obtained during a period of inactive disease; as the alternative promoter contains a putative NFκB binding site it is reasonable to speculate that transcript expression may increase during periods of active systemic inflammation. To examine this, healthy control PBMC were stimulated with TNF for 6 hours, and expression of *UNC13D* transcript levels were quantified. Treatment with TNF lead to a significant and dose-dependent increase in expression of the alternative intron 1 transcript, without significant change in expression of the conventional transcript (Figure 1c).

To further characterize the functional role of this intronic region, we examined the predicted NFκB binding site which is altered by the c.117+143A>G variant. EMSA were performed using a consensus NFκB oligonucleotide probe and nuclear extracts from the human NK cell line, NK-92. Two retarded bands were identified, the slower of which could be supershifted by anti-NFκB p65 antibody (Figure 2). The lower band migrates consistent with NFκB p50 (10). Binding of nuclear extracts to the consensus NFκB oligonucleotide (both p50 and p65 bands) was largely disrupted by either unlabeled self-competitor or by a 22-mer probe corresponding to the WT *UNC13D* intron 1 region containing the predicted NFκB motif (Figure 2). Notably, a competitor oligonucleotide containing the c.117+143A>G variant was less efficient at blocking formation of the NFκB bands, thus confirming that this variant is less efficient at disrupting NFκB binding (Figure 2).

The deep intronic location of this NFκB motif suggested that this could function as a transcriptional enhancer, which can regulate gene expression from a distance independent of

position and orientation. To determine whether this intronic region can serve as a transcriptional enhancer, a trimerized 15-mer containing either the WT intronic sequence or c.117+143A>G variant was cloned into the enhancer site of both the immediate early CMV promoter or the HIV-1/LTR-driven GFP reporter plasmid and transfected into NK-92 cells (Figure 3a). Cloning of the WT (143A) trimerized 15-mer sequence into either vector enhanced GFP expression in activated NK-92 cells (Figure 3b-c). In contrast, the patient variant (143G) showed significantly reduced effect, compared to the WT sequence, on enhancing GFP expression driven by promoters of either CMV or HIV-1 (Figure 3b-c). To confirm this further and eliminate potential influence of artificially repeating the core sequence, a 111 nucleotide genomic DNA region including either the WT or mutant core NFκB enhancer was inserted into the HIV-1 vector. As shown in Figure 3d-e, the short genomic DNA containing the WT enhancer significantly increased HIV-1/LTR-driven GFP expression in activated NK-92 cells, activity which was significantly attenuated with the mutant enhancer. Notably, cloning of the short genomic sequence in either the forward or reverse orientations significantly increased GFP expression driven by the HIV-1/LTR, further demonstrating that this region can serve as a transcriptional enhancer *in vitro* (Figure 3d-e). Collectively, these results suggest that the c.117+143A>G variant alters the ability of NFκB to bind to a transcriptional enhancer element, and supports the finding of reduced alternative transcript levels in this patient's PBMC.

Together, these results support a model where the c.117+143A>G variant leads to reduced lymphocyte *UNC13D* expression, causing diminished target cell killing. In order to examine whether a partial reduction in *UNC13D* could compromise NK cell function, *UNC13D* transcript was knocked down using siRNA in the human NK-92 cell line. NK-92 cells transfected with scrambled sequence or specific *UNC13D* siRNA were incubated with K562 target cells for 1-2 hrs, and NK cell degranulation was determined by CD107a cell surface staining. Compared to cells transfected with scrambled siRNA, NK-92 transfected with low (5nM) or high (10nM) concentrations of specific *UNC13D* siRNA showed significantly reduced, but not absent, CD107a surface expression (Figure 4A-B). Indeed, the degree of impaired NK cell degranulation correlated with residual *UNC13D* expression in a dose-dependent manner (Figure 4C). These findings demonstrate that even partial reductions in *UNC13D* expression can compromise cytolytic cell function, and further support roles for hypomorphic non-coding mutations such as c.117+143A>G in MAS pathogenesis.

Discussion

The genetic basis of MAS in patients with SJIA remains unclear. However, contemporary studies have found that as many as one-third of patients with MAS associated with SJIA have rare, protein-altering variants in genes associated with HLH (3,14). Recent work by us and others has shown that these variants can impair lymphocyte function, and may explain in part the increased risk for MAS in these patients (11,15). In addition, whole exome sequencing (WES) of patients with MAS associated with SJIA has identified novel candidate genes that could be implicated in this process, including several that interact with known components of the cytolytic pathway (3). Together, this suggests that hypomorphic variants in HLH-associated genes involved in the perforin cytolytic pathway may serve as risk factors for MAS, although this remains to be formally tested in a prospective manner.

However, many patients, even those with recurrent MAS, lack any protein-altering variants in known or putative genes linked to cytolytic dysfunction. Non-coding, regulatory genomic regions are often not examined, either in targeted gene sequencing or in WES. However, there is increasing evidence that intronic variants are significant contributors to human disease. Variants in the first intron of *UNC13D*, for example, are frequently identified in patients with FHL3. The first of these described was c.118-308C>T, which causes a lymphocyte-specific loss of *UNC13D* expression due to disrupted transcription factor binding (6,8). In addition, the neighboring c.118-307G>A variant is associated with diminished *UNC13D* expression and impaired cytolytic cell degranulation (7). Altogether, in our large North American cohort of more than 1,700 HLH patients, 79 were found to have FHL3 (biallelic *UNC13D*), and more than 31% carried at least one non-coding mutation (5).

Here, we report a patient with SJIA and recurrent MAS found to have a novel *UNC13D* intronic variant c.117+143A>G that alters an NFκB engagement and transcriptional enhancer element. Several lines of evidence support this variant impacting *UNC13D* expression in cytolytic cells. First, this intronic region includes H3K27A marks, *DNaseI* hypersensitivity clusters, and at least 15 transcription factor binding motif overlaps from ChIP-seq data (Figure 1a). Second, PBMC from this patient showed 40% reduction in levels of the alternative, lymphocyte-specific intron 1 *UNC13D* transcript compared to control samples (Figure 1b). Of note, patient transcript levels were quantified from a sample obtained during a period of inactive disease; more profound reductions in message levels could occur during periods of hyperinflammation. Indeed, we find that TNF stimulation of control PBMC leads to a significant increase in expression of the alternative intron 1 transcript (Figure 1c). These findings are in agreement with previously reported data on other intron 1 variants, most notably c.118-308C>T, shown previously to disrupt binding of the ETS family transcription factor ELF1 (5–7) and leading to lymphocyte-specific loss of *UNC13D* expression and defective cytolytic cell degranulation. We cannot discount that the c.117+143A>G variant may also impact transcript splicing, thereby also impacting conventional *UNC13D* transcript expression. Third, this variant disrupts the consensus sequence of an NFκB binding site (Figure 1a), and indeed we show that the variant sequence is less efficient at blocking NFκB bands in EMSA (Figure 2). Finally, this sequence both serves as a transcriptional enhancer *in vitro*, and the c.117+143A>G variant showed significantly reduced transcriptional enhancement (Figure 3). Taken together, this strongly supports the notion that the novel c.117+143A>G mutation is a functional intronic variant that diminishes lymphocyte-specific *UNC13D* expression.

Substantial evidence supports the notion that even partially reduced lymphocyte *UNC13D* expression would have functional consequences. Patients with previously described intron 1 variants display both reduced lymphocyte-specific *UNC13D* expression and impaired cytolytic function (6–8), and *UNC13D* expression in strongly correlates with degranulation and cytotoxicity (8). In agreement with this, our previous work has shown that even partial defects in the interaction of MUNC13-4 with the effector Rab27a (approximately 50% diminished) resulted in decreased cytolytic activity, delayed granule polarization, and increased IFNγ production from prolonged engagement with target cells (11). Finally, and most importantly, we show here that partial knockdown of *UNC13D* message levels with targeted siRNA lead to corresponding partial defects in NK cell degranulation (Figure 4).

This supports a model where variants such as c.117+143A>G reduce *UNC13D* transcript levels, causing partial defects in cytolytic function and lowering the threshold for MAS development, particularly in those patients with underlying inflammation such as SJIA (4,16).

To the best of our knowledge this is the first report of functional intronic variants associated with MAS in children with SJIA. Of note, we also identified the known c.118-308C>T in heterozygous form in an additional patient with sHLH/MAS (patient 10). This rare variant is present in less than 0.02% of genomes listed in gnomAD, but is reported in up to 9% of alleles in FHL3 patients (5). Extensive functional study of this variant has demonstrated that it disrupts binding of the ETS family transcription factor ELF1, impairing recruitment of STAT4 and diminishing chromatin remodeling (8). Interestingly, ELF1 can cooperate with NFκB to activate transcription (17), which could suggest that the biologic consequences of these two intronic variants are linked. Thus, together with the novel c.117+143A>G variant identified herein, the total hit rate for potentially deleterious *UNC13D* intronic variants in the present study was 8.3% (2/24). Jointly, the findings in these patients suggest that intronic variants in key conserved regulatory regions should be examined in children with SJIA and MAS, and that genetic screening for variants in *UNC13D* should be expanded to include intron 1. Along with hypomorphic coding variants in HLH-associated genes, these intronic variants may serve as further risk factors for MAS in patients with SJIA and could have implications for genetic counseling. This further supports the utility of targeted sequencing approaches or even whole genome sequencing in cases where causative protein-altering mutations are not identified, and provides evidence for cell-specific effects of intronic variants on transcript expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Novel *UNC13D* intronic variant sits in a key regulatory region. (A), Genomic view of novel variant c.117+143A>G (indicated by blue lines) detected in this patient and the reference changes c.118-308C>T and c.118-307G>A (indicated by red lines), showing relative increased histone markers of the sequence of intron 1 of *UNC13D*. Inset shows consensus transcription factor findings sites that are disrupted by these changes. (B), Levels of conventional and lymphocyte-specific alternative *UNC13D* transcripts in the reported patient compared to 4 healthy controls, as determined by quantitative RT-PCR. Total RNA was extracted from unstimulated PBMC obtained during a period of inactive disease. Data are presented as relative expression normalized to *GAPDH* and error bars represent means $\pm$ SEM. (C), Levels of conventional and lymphocyte-specific alternative *UNC13D* transcripts in control PBMC upon treatment with TNF, as determined by quantitative RT-PCR. Data are presented as relative expression normalized to *GAPDH* and error bars represent means $\pm$ SEM. * $p < 0.05$.

Figure 2.

The mutant NFκB-like enhancer located within *UNC13D* intron partially disrupts protein binding to a consensus NFκB probe. EMSA was performed by mixture of nuclear extracts from activated NK-92 cells and IRDye-700 labeled NFκB consensus oligonucleotide probe. The binding band was either super-shifted by anti-p65 antibody or blocked by self, wild type (143A), or mutant (143G) oligonucleotide competitors. The band intensities were analyzed by ImageJ (v1.50I), and reported values are listed below the gel lanes relative to the respective non-competed bands (2nd lane).

Figure 3.

NF κ B-like enhancer located within *UNC13D* intron 1. (A) Schematic representation of HIV-1 and CMV-driven GFP reporter plasmids inserted with either triple core sequence (45nts) or short genomic sequence (111nts) of the enhancer. (B) Comparison of enhancer activity of triple core DNA of the wild-type (WT, 143A) or mutant enhancer (Mut, 143G) sequences in augmenting GFP expression driven by either CMV or HIV-1 promoters within NK-92 cells post activation by irradiated K562 cells. (C) The means $\pm$ SD are shown in bar graph form for 3 independent experiments as shown in (B). (D) Comparison of enhancer activity of short genomic DNA of the wild-type (WT, 143A) or mutant enhancer (Mut, 143G) sequences, inserted in either forward or reverse orientation, in augmenting GFP expression driven by HIV-1 promoter within NK-92 cells post activation by irradiated K562 cells. (E) The means $\pm$ SD are shown in bar graph form for 3 independent experiments as shown in (D).

Figure 4.

Knockdown of *UNC13D* expression with siRNA impairs NK cell cytotoxicity. (A) Flow cytometry analysis of NK-92 cell degranulation as determined by cell surface CD107a expression. Cells were transfected with siRNA as shown and incubated with or without K562 target cells at target:effector ratio of 1:1. (B) Bar graph showing the results (means $\pm$ SD) from 3 independent experiments. CD107a surface expression percentage was calculated with the formula ($UNC13D$: MFI*%/scramble: MFI*%)*100%. (C) Bar graph showing *UNC13D* mRNA expression in NK-92 cells treated with scrambled sequence or *UNC13D*-specific siRNA at the indicated concentrations. The housekeeping gene beta-2-microglobulin mRNA was used for normalization. Ct was calculated by subtracting $Ct_{scramble}$ from Ct_{UNC13D} . The expression percentage was calculated by the formula $(1-1/2^{-Ct}) \times 100\%$.