

Precision Molecular Diagnosis Defines Specific Therapy in Combined Immunodeficiency with Megaloblastic Anemia Secondary to *MTHFD1* Deficiency



Kesava A. Ramakrishnan, MD^{a,*}, Reuben J. Pengelly, PhD^{b,*}, Yifang Gao, PhD^c, Mary Morgan, FRCPATH^d, Sanjay V. Patel, MD^d, E. Graham Davies, MD^e, Sarah Ennis, PhD^b, Saul N. Faust, MD, PhD^{a,d}, and Anthony P. Williams, MD, PhD^{c,f} Southampton and London, United Kingdom

What is already known about this topic? Recessively inherited methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*) deficiency has previously been described to present with a severe combined immunodeficiency.

What does this article add to our knowledge? Exome sequencing demonstrated *MTHFD1* deficiency as a novel cause of a combined immunodeficiency and folinic acid was established as precision therapy to reverse the clinical and laboratory phenotype.

How does this study impact current management guidelines? Early use of new molecular diagnostic techniques can personalize treatment in managing unexplained immunodeficiencies and in some cases prevent the need for bone marrow transplantation.

^aAcademic Unit of Clinical and Experimental Sciences, Faculty of Medicine and Institute for Life Sciences, University of Southampton and National Institute for Health Research (NIHR) Biomedical Respiratory Unit, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom

^bAcademic Unit of Human Development and Health, Human Genetics and Genomic Medicine, Faculty of Medicine, University of Southampton, Southampton, United Kingdom

^cAcademic Unit of Cancer Sciences, Faculty of Medicine and Institute for Life Sciences, University of Southampton, and Cancer Research UK (CRUK) National Institute for Health Research (NIHR) Experimental Cancer Medicine Centre, Southampton, United Kingdom

^dSouthampton National Institute for Health Research (NIHR) Wellcome Trust Clinical Research Facility and Department of Paediatric Medicine, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom

^eDepartment of Paediatric Immunology, Great Ormond Street Hospital for Children, NHS Foundation Trust, London, United Kingdom

^fDepartment of Allergy, Asthma and Clinical Immunology, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom

Conflicts of interest: A. P. Williams has been supported by an Experimental Cancer Medicine Centre Infrastructure grant from the NIHR and CRUK. S. N. Faust has consultancy arrangements with AstraZeneca, Cubist, and Merck (fees for authors' advisory board participation) and has received 1 or more grants from or has 1 or more grants pending with Pfizer, Sanofi, GlaxoSmithKline, Novartis, Alios, and Cubist (clinical trial investigation). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 2, 2016; revised July 6, 2016; accepted for publication July 8, 2016.

Available online October 1, 2016.

Corresponding author: Saul N. Faust, MD, PhD, Southampton NIHR Wellcome Trust Clinical Research Facility, Mailpoint 218, University Hospital Southampton, NHS Foundation Trust, Southampton SO16 6YD, United Kingdom. E-mail: S.Faust@soton.ac.uk.

*These authors contributed equally to this work.

2213-2198

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaip.2016.07.014>

BACKGROUND: Methylenetetrahydrofolate dehydrogenase (*MTHFD1*) deficiency has recently been reported to cause a folate-responsive syndrome displaying a phenotype that includes megaloblastic anemia and severe combined immunodeficiency. **OBJECTIVE:** To describe our investigative approach to the molecular diagnosis and evaluation of immune dysfunction in a family with *MTHFD1* deficiency.

METHODS: The methods used were exome sequencing and analysis of variants in genes involved in the folate metabolic pathway in a family with 2 affected siblings. Routine laboratory and research data were analyzed to gain an in-depth understanding of innate, humoral, and cell-mediated immune function before and after folinic acid supplementation.

RESULTS: Interrogation of exome data for concordant variants between the siblings in the genes involved in folate metabolic pathway identified a heterozygous mutation in exon 3 of the *MTHFD1* gene that was shared with their mother. In view of highly suggestive phenotype, we extended our bioinformatics interrogation for structural variants in the *MTHFD1* gene by manual evaluation of the exome data for sequence depth coverage of all the exons. A deletion involving exon 13 that was shared with their father was identified. Routine laboratory data showed lymphopenia involving all subsets and poor response to vaccines. *In vitro* analysis of dendritic cell and lymphocyte function was comparable to that in healthy volunteers. Treatment with folinic acid led to immune reconstitution, enabling discontinuation of all prophylactic therapies.

CONCLUSIONS: Exome sequencing demonstrated *MTHFD1* deficiency as a novel cause of a combined immunodeficiency. Folinic acid was established as precision therapy to reverse the clinical and laboratory phenotype of this primary

Abbreviations used

MTHFD1-Methylenetetrahydrofolate dehydrogenase 1
PCP-Pneumocystis jirovecii pneumonia
SCID-Severe combined immunodeficiency
SVs-Structural variations

immunodeficiency. © 2016 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2016;4:1160-6)

Key words: Megaloblastic anemia; C-1-Tetrahydrofolate synthase (C1-THF synthase); Folinic acid; Combined immunodeficiency; Lymphopenia

Derivatives of folic acid play a critical role in cellular one-carbon metabolism, acting as donors and recipients of one-carbon units involved in synthesis and breakdown of amino acids as well as in the synthesis of thymidine and purines.¹ C-1-tetrahydrofolate synthase (C1-THF synthase) is a cytoplasmic trifunctional enzyme essential for processing single carbon folate derivatives, encoded by the gene methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*). Mutations in *MTHFD1* have previously been reported to cause a syndrome displaying megaloblastic anemia, severe combined immunodeficiency (SCID), mild mental retardation, and treatment-refractory epilepsy that was responsive to metabolic supplementation.^{2,3} Four further case reports,⁴ 2 of whom died of overwhelming sepsis, have extended the phenotype to include atypical hemolytic uremic syndrome, microangiopathy, and retinopathy. We present the long-term follow-up data of a family with 2 siblings who carry novel compound heterozygous mutations in the *MTHFD1* gene. Specifically, we have characterized the immune function in more detail and documented the response in immune function to folinic acid supplementation.

METHODS

Subjects

The subjects were 2 brothers born to a mother of Southeast Asian and father of white origin managed in our specialist immunology service for more than a decade. Consent for research investigations was obtained (NHS Research Ethics 09/H502/4).

Exome sequencing

Exome enrichment was performed using the SureSelect Human All Exon V4.0 kit (Agilent, Cheshire, UK) before sequencing on the Illumina HiSeq 2000 system. Following sequencing, data processing was performed as previously described.⁵

PCR amplification

PCR was performed according to manufacturer instruction using a Phusion high-fidelity PCR kit from Thermo Scientific (Loughborough, UK). PCR conditions and primer sequences used for amplification exons 3 and 13 are summarized in Table E1 in this article's Online Repository at www.jaci-inpractice.org.

RNA amplification

RNA was extracted from lithium heparin anticoagulated whole blood using QIAamp RNA Blood Mini Kit (Qiagen, Manchester, UK). A total of 200 ng of total RNA was reverse transcribed and cDNA amplified using *MTHFD1* gene-specific primers (cDNAF

and cDNAR) as per manufacturer instructions using SuperScript III One-Step RT-PCR System (Life Technologies, Warrington, UK).

Flow cytometry

A 2-step staining process for surface and intracellular antigens was performed according to manufacturer instructions using BD fix-perm kit. Flow cytometry data were acquired on a BD FACS canto II flow cytometer (BD Biosciences, Oxford, UK). At least 200,000 events were collected for lymphocyte assays and 500,000 for dendritic cell assays. Data were analyzed using FACS Diva software (BD Biosciences), and gating strategy is illustrated in Figures E1 and E2 in this article's Online Repository at www.jaci-inpractice.org.

RESULTS

Clinical manifestations

The elder sibling (patient A) presented at age 4 months with clinical and radiological features consistent with *Pneumocystis jirovecii* pneumonia. SCID was diagnosed on the basis of susceptibility to *Pneumocystis jirovecii* pneumonia and severe lymphopenia involving all lymphoid subsets. Subsequent investigations found no mutations in the genes known to cause SCID at that time. Patient A remained free of significant infections on immunoglobulin replacement, prophylactic cotrimoxazole, and fluconazole. While on follow-up, he presented at age 4 years with severe macrocytic anemia (hemoglobin level, 34 g/L) requiring transfusion that was confirmed to be megaloblastic on bone marrow examination. Although stem cell transplant was considered in infancy, the lack of molecular diagnosis and clinical stability on prophylactic treatment persuaded the clinicians to delay this procedure indefinitely.

The younger sibling (patient B) was investigated at birth and found to have severe lymphopenia similar to his brother. He was given prophylactic cotrimoxazole and fluconazole. Immunoglobulin replacement was delayed to establish a better assessment of humoral immune function by vaccination. B was given the UK immunization schedule primary course of inactivated vaccines between age 2 and 5 months. Assessment of vaccine-specific immunoglobulin response showed that he responded to protein (tetanus toxoid) but not conjugated polysaccharide (*Haemophilus influenzae* type B) vaccines (see Table E5 in this article's Online Repository at www.jaci-inpractice.org). Regular antibacterial (azithromycin) prophylaxis was added to his treatment regimen in view of poor humoral response to vaccines. Despite this, he was hospitalized with pneumococcal septic arthritis at age 9 months after which immunoglobulin replacement was commenced. He remained susceptible to pyogenic bacterial infections, with further hospitalizations at 19 months (pneumonia), 22 months (peri-orbital cellulitis), and 24 months (pneumococcal bacteremia). He also developed severe episodes of megaloblastic anemia during the course of his hospitalization with septic arthritis at 9 months and pneumonia at age 19 months.

Retrospective review of both patients' RBC indices revealed a persistent macrocytosis from young infancy (Figure 1). A detailed chronology of clinical manifestations is presented in this article's Online Repository at www.jaci-inpractice.org (see case description in this article's Online Repository at www.jaci-inpractice.org).

Immunological phenotype

Both patients have had severe persistent lymphopenia involving all lymphoid subsets (see Tables E2 and E3 in this

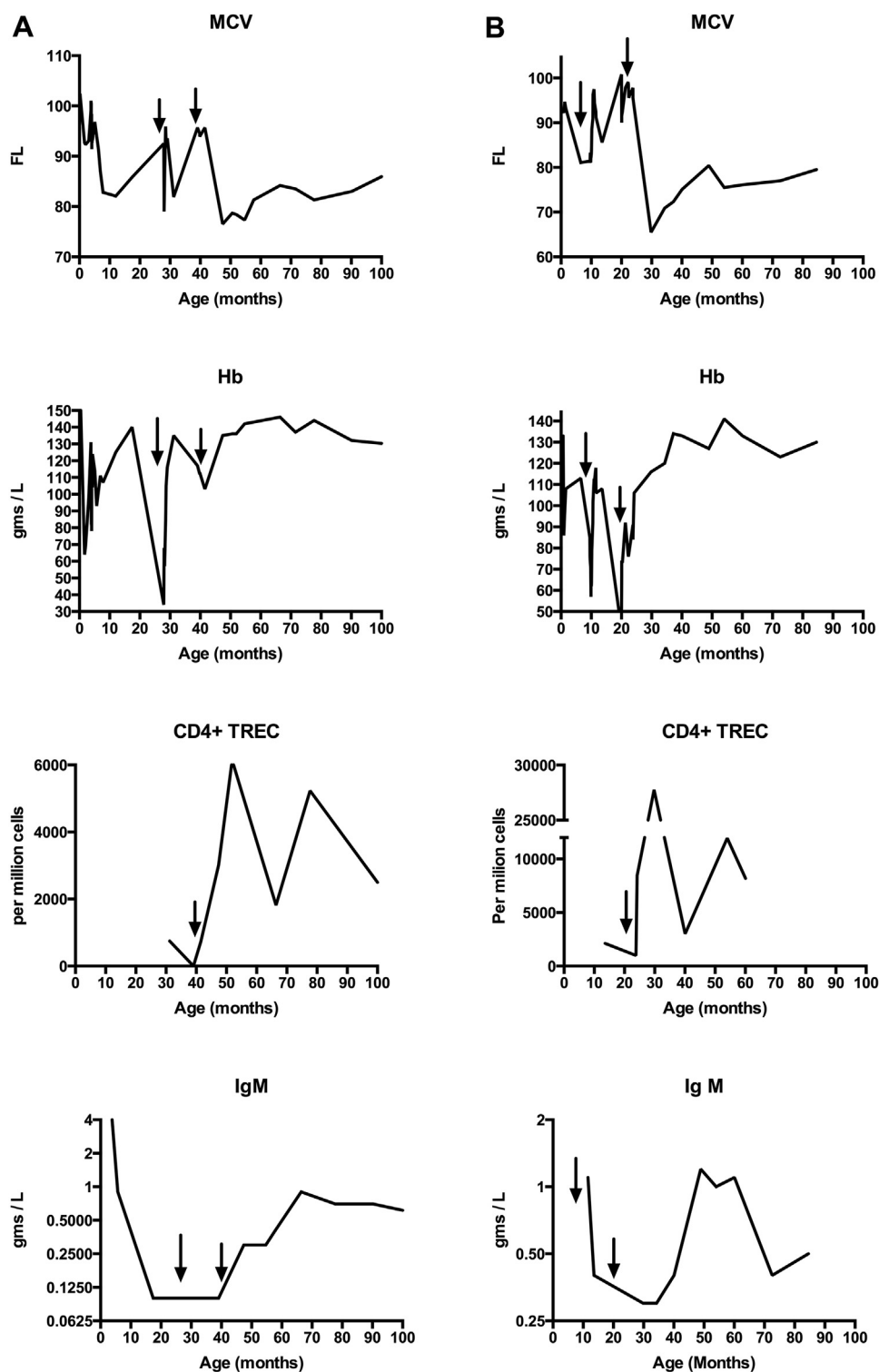


FIGURE 1. Response to folinic acid treatment in patients **A** and **B**. Both brothers received parenteral folinic acid treatment for 2 weeks at the time point indicated by the first arrow and regular enteral supplement from the time point indicated by the second arrow. *FL*, femtoliter; *Hb*, hemoglobin; *MCV*, mean corpuscular volume; *TREC*, T-cell receptor excision circle.

article's Online Repository at www.jaci-inpractice.org). Both have had persistently low thymic output of T cells as measured by T-cell receptor excision circles (Figure 1). T-lymphocyte subsets and T-cell receptor repertoire were normally distributed

(data not shown). Lymphocyte proliferation to polyclonal (phytohemagglutinin) and T-lymphocyte-specific (anti-CD3 and 28) stimuli have repeatedly been comparable to healthy controls (data not shown).

Both patients had low immunoglobulin levels involving all isotypes before immunoglobulin replacement therapy (Figure 1; also see Tables E4 and E5 in this article's Online Repository at www.jaci-inpractice.org). Patient A did not complete his primary course of vaccines in infancy before immunoglobulin replacement therapy. Patient B showed defective response to conjugated polysaccharide vaccines in infancy (see Table E5).

Subsequent to establishing the molecular diagnosis, we undertook further *in vitro* assays to assess T-lymphocyte function. These demonstrated normal T-lymphocyte activation, cytokine production, and terminal differentiation. Taken together, these data suggested that although T-lymphocyte numbers were low, T-cell function was normal (see Figure E2, A-C). In addition, assessments of antigen-presenting cells including monocytes and myeloid and plasmacytoid dendritic cells were comparable to healthy controls (see Figure E1, B).

Response to folinic acid treatment

A presumed diagnosis of a defect in the folate metabolic pathway was made and both siblings were empirically treated with regular folinic acid supplement. As shown in Figure 1, the mean corpuscular volume normalized within weeks of supplementation. There have been no further episodes of anemia. In the first months of therapy, there was a partial reconstitution of the lymphocyte compartment, with absolute lymphocyte and all subsets returning to lower limits of normal for age (see Tables E2 and E3). The CD4+ T-cell receptor excision circle count improved 10-fold in both patients (Figure 1). IgM (Figure 1) and IgA (see Tables E4 and E5) levels also improved to normal levels over months, suggesting a reconstitution of the humoral compartment. Immunoglobulin supplements were discontinued, and both patients have maintained normal levels of all isotypes during the subsequent 4 years of follow-up (see Tables E4 and E5). They have both been reimmunized and have responded well to both protein and conjugate vaccines (see Tables E4 and E5). Both patients have remained free of significant infections without prophylactic antibiotics or immunoglobulin replacement for 8 years since starting folinic acid therapy.

Exome analysis

Suspecting a defect in folate metabolism without a single candidate gene, we undertook whole-exome sequencing to analyze multiple gene variants.⁶ Interrogation of variants within genes involved in folic acid metabolism (GO:0046655⁷) for which the brothers were concordant identified a maternally inherited novel point mutation in exon 3, heterozygous T- to -C transition at c.152 position (c.152T>C; NM_005956.3), that would lead to a substitution of a conserved amino acid (p.Leu51Pro). This amino acid is situated within the methylenetetrahydrofolate dehydrogenase/cyclohydrogenase domain of C1-THF synthase and the amino acid substitution is predicted to adversely impact protein function. The base within the Leu-51 residue is well conserved evolutionarily, with a Genomic Evolutionary Rate Profiling (GERP++) score of 3.2.⁸ Leu-51 is located within an α -helix, with the adjacent Tyr-52 and the proximal Lys-56 involved in substrate binding within the dehydrogenase/cyclohydrogenase active site.⁹ Proline residues are well documented to distort α -helices. The lack of an amide proton, coupled with the conformational rigidity of the cyclic residue, canonically results in an approximately 30° bend in the helix¹⁰ and

the identified L51P mutation would be expected to distort this α -helix, reducing the affinity of the active site for the substrates.

In the previous report of C1-THF synthase deficiency, the heterozygous parents were reported to be asymptomatic.² Structural variations (SVs) that are also important for Mendelian disease are not easily detected using an exome approach.¹¹ Most exome studies conducted so far on specific diseases focus on single-nucleotide variants, rather than SVs such as insertion/deletion variants.¹² This is because insertion/deletion bioinformatics analysis methodologies are still being refined. Read depth and split reads provide a clue to structural variants within exome data.¹³ Therefore, we extended our bioinformatic interrogation of the *MTHFD1* gene by manually examining sequencing depth coverage of the raw alignments in affected brothers compared with an unrelated individual concurrently sequenced. For exons with apparent reduced coverage, read depth was enumerated, normalized according to total aligned reads for the sample, and assessed statistically (1-tailed *t* test) alongside 13 unrelated samples from the same sequencing batch processed identically (Figure 2, B). This process identified a paternally inherited deletion encompassing exon 13 in both brothers (Figure 2, C).

Confirmation of mutation by Sanger sequencing

PCR amplification and Sanger sequencing using primers (primers EX3F and EX3R; see Table E1) confirmed that the novel point mutation in exon 3 was present in both patients and the mother but not the father (Figure 2, D).

Identification by traditional sequencing of the breakpoints of exon deletions suspected on the basis of read depth coverage in the exome data can be arduous as the breakpoints are likely to be located in the intronic regions upstream and downstream of the identified exon, which may span many thousands of base pairs (bps). However, we noted that in the read pile up of our patients, there were anomalous read pairs spanning over 2000 bps (Figure 2, C). Primers were designed (Ex13LF and EX13LR; see Table E1) to encompass this region. The predicted PCR product size in the reference template is 2350 bps (see Figure E4, A, in this article's Online Repository at www.jaci-inpractice.org). PCR amplification of this region revealed an expected 2350-bps product in the mother but only a 600-bps product in the patients, confirming a deletion involving exon 13 (Figure 2, E). We were subsequently able to delineate the breakpoints accurately by Sanger sequencing of the above PCR products from the mother and patients (see Figure E3, A and B, in this article's Online Repository at www.jaci-inpractice.org). We were also able to confirm the heterozygous state for deletion of exon 13 in the patients by allele-specific PCR (see Figure E4, A and B). Further analysis revealed that the deletion boundaries were within homologous positions of flanking, parallel, AluS repeats (see Figure E5, A and B, in this article's Online Repository at www.jaci-inpractice.org), previously observed as a pathogenic mutational mechanism in other genes, such as in *BRCA1*.¹⁴ For interested readers, further discussion is provided in this article's Online Repository at www.jaci-inpractice.org.

Allelic expression at the mRNA level

The deletion of exon 13 is predicted to introduce a premature stop codon resulting in a truncated protein of 422 amino acids. The nonsense-mediated decay pathway exists in eukaryotic cells to ensure fidelity of transcription and prevent translation of truncated proteins with potentially deleterious gain-of-function

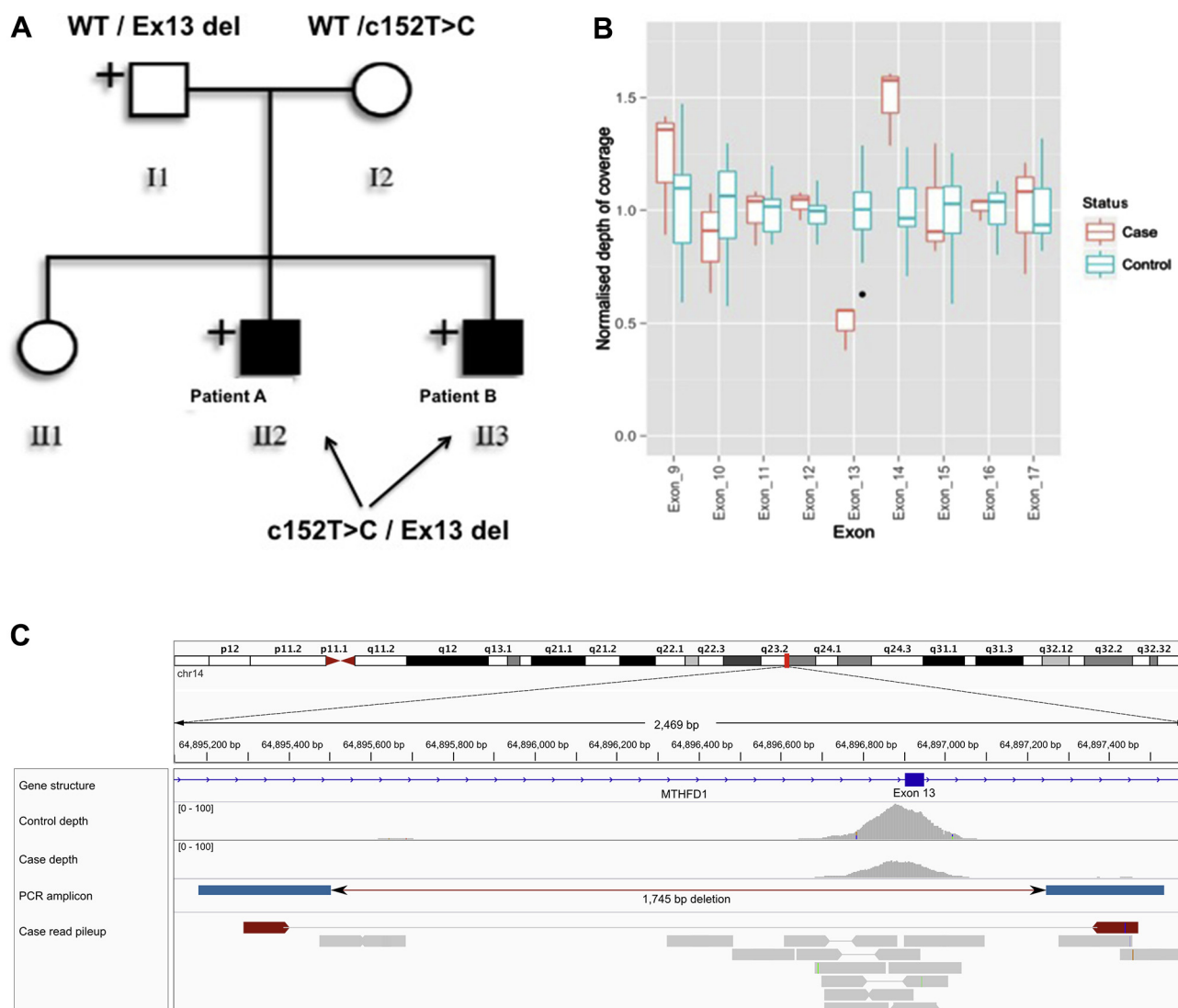


FIGURE 2. Molecular diagnosis. Where not shown, patient A's results were representative of patient B. **A**, Pedigree showing affection with disease. Black fill, affected; +, whole-exome sequenced. **B**, Depth of coverage for exons in *MTHFD1* in 13 control samples and the 3 cases, indicating an exon 13 deletion in the 3 whole-exome-sequenced family members ($P = .00019$ for depth between cases and controls). **C**, Summary of evidence for exon 13 deletion in NGS data. Chromosomal location is indicated on karyogram, expanded below into coordinates. Read depth in representative case (patient A) is shown, alongside a representative unrelated control sample from the same batch. It can be seen that read depth in our case is approximately half that of the control. Furthermore, in the case read pileup, an anomalous excessive read pair span can be seen (highlighted in red). Read pairs are derived from opposing ends of a single contiguous fragment of DNA. Presuming that the DNA fragment sequenced falls within the expected length range (representative reads pairs can be seen in pileup in gray), and that the reads are aligned to the reference genome correctly, the extended apparent read pair span would indicate a deletion within the encompassed genomic region. As such, PCR primers were designed flanking the anomalous read pair. Upon Sanger sequencing of the resultant amplicon, a 1745-bps deletion was observed. **D**, Sanger sequencing of *MTHFD1* exon 3 showing c.T152C:p.L51P, which is present in both brothers and the mother but not the father. **E**, Sequencing of *MTHFD1* gene yielded the expected PCR product of 2350 bps in the mother (lane 5) and a PCR product of around 600 bps in the patients and the father (lane 2-4). **F**, Sanger sequencing of gDNA and cDNA of patient A revealed the presence of the 2 alleles at the genomic level but the expression of only the mother's allele at the cDNA level. NGS, Next-generation sequencing; WT, wild-type.

or dominant-negative activity.¹⁵ We hypothesized that the deletion of EX13 would target the mRNA from paternal allele (with a premature stop codon), for nonsense-mediated decay.¹⁶ Allelic expression of the *MTHFD1* gene was undertaken using the point mutation in the exon 3 to analyze the relative

expression of the maternal and paternal allele.¹⁷ *MTHFD1* cDNA was amplified using gene-specific primers covering the point mutation in the EX3 (inherited from their mother). As shown in Figure 2, F, the mRNA from the paternal allele was almost extinct, confirming our hypothesis.

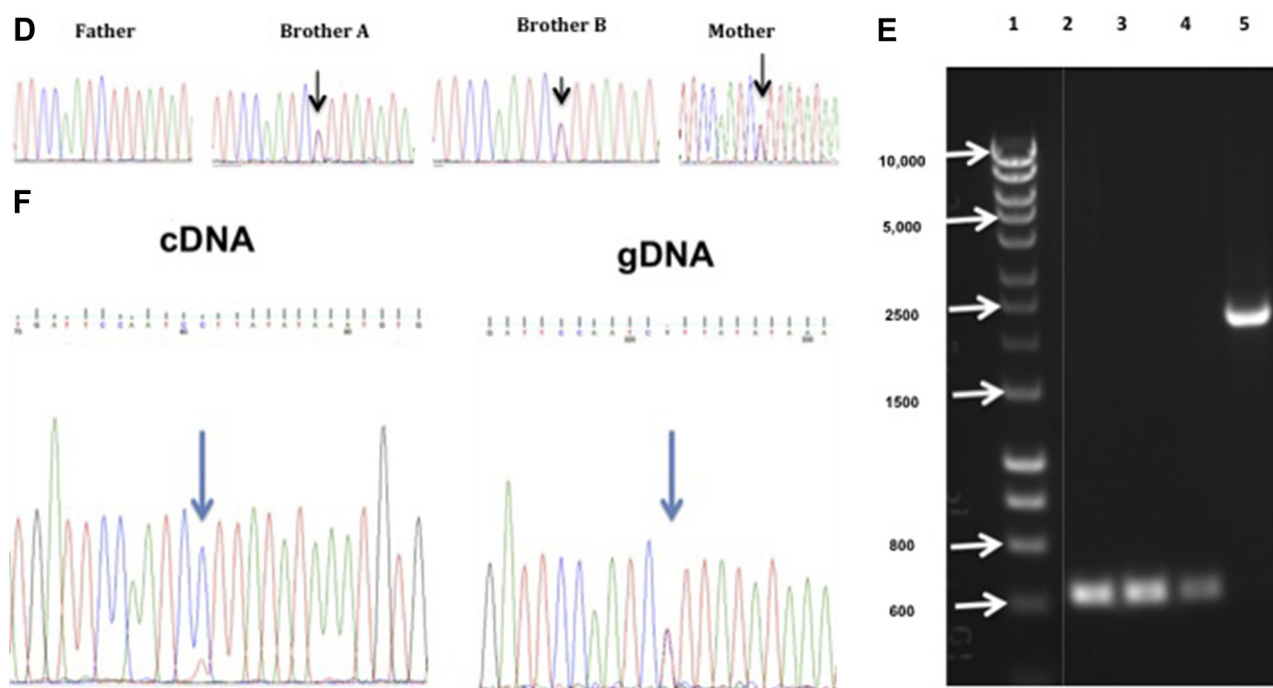


FIGURE 2. (Continued).

DISCUSSION

Patients A and B carry compound heterozygous mutations consistent with autosomal-recessive inheritance (Figure 2, A), L51P inherited from their mother and deletion of exon 13 inherited from their father. *In silico* analysis of mutant L51P predicts an adverse impact on the function of the protein. A mutation in the vicinity (S49F) has recently been shown to be significantly less functional in biochemical studies.⁴ Allelic expression data are consistent with the deletion of exon 13 producing haploinsufficiency. The partial response to folinic acid therapy is consistent with a residual functioning enzyme as previously described.⁴

MTHFD1 mutations cause highly variable clinical phenotypes, with megaloblastic anemia being the only consistent feature.^{3,4} This is likely the result of variable degree of residual enzyme function, but the limited number of patients currently reported makes it impossible to draw conclusions regarding genotype to phenotype correlation. Clinical manifestations described in a smaller proportion of patients include severe immunodeficiency, hemolytic uremic syndrome, mild mental retardation, epilepsy, and retinopathy. Severe immunodeficiency has been reported in 3 of 5 patients and was the most likely cause of mortality in 2 of the patients. Macrocytosis, lymphopenia involving all subsets, and low T-cell receptor excision circles (Figure 1) were present from infancy in the patients reported here and may be a clue to the need to investigate folate metabolic pathways. *MTHFD1* deficiency should be considered in the etiology of megaloblastic anemia, where there are normal levels of vitamin B₁₂ and folate, and in Combined Immunodeficiency (CID), where there is macrocytosis.

We have characterized the immune function in our kindred that delineate the immune function in *MTHFD1* deficiency. The antigen-presenting cells in the peripheral blood were present

in normal numbers and responded to Toll-like receptor ligands in a comparable fashion to controls (data not shown). Both cell-mediated immunity and humoral immunity were defective in our patients. The clinical improvement in susceptibility to infections, partial reconstitution of cell numbers, and improvement in both overall immunoglobulin levels and vaccine responses suggest that the immunodeficiency caused by this condition can be reversed to a large extent with folinic acid treatment.

The major limitation of exome sequencing is the inability to comprehensively represent genomic SVs such as deletions, inversions, or duplications.¹¹ Many groups have designed algorithms that use a read depth or read pair-based approach for predicting structural variation. However, these approaches are not very efficient at identifying SVs with exome data because of the discontinuous nature of the data, with only approximately 1% of the genome having significant read coverage.¹⁹ Our approach was successful in identifying the exon 13 deletion due to strong clinical suspicion. Although it may not be possible to detect all forms of SVs, we have demonstrated the possibility of analyzing specific genes using read depth, split reads, and read pairs to provide clues to structural variants. Where improved detection of structural variants is required, whole-genome sequencing should be used.

The importance of establishing a definitive molecular diagnosis in children with primary immunodeficiency cannot be overemphasized. Both patients were considered to have SCID and were on the bone marrow transplant list for the first few years of life. Knowledge of the specific molecular diagnosis has led to the identification of a precision treatment in the form of life-long folinic acid supplementation. Other prophylactic therapies have been able to be discontinued. The responsiveness of this disorder to folinic acid supplementation emphasizes the importance of precision diagnostics in the diagnosis of primary immunodeficiency before the consideration of stem cell

transplantation, and to stratify appropriate treatment modalities to reduce risk and improve outcomes. Clinical exome sequencing, even in single families, is a powerful adjunct to the investigation of metabolic pathways that may lead to distinctive immunodeficiency disorders.

Acknowledgments

Y.G. and A.P.W. were supported by Cancer Research UK and the Experimental Cancer Medicine Centre, Southampton. K.R. and S.N.F. received support from the Southampton National Institute for Health Research Wellcome Trust Clinical Research Facility.

REFERENCES

- Whitehead VM. Acquired and inherited disorders of cobalamin and folate in children. *Br J Haematol* 2006;134:125-36.
- Watkins D, Schwartzenruber JA, Ganesh J, Orange JS, Kaplan BS, Nunez LD, et al. Novel inborn error of folate metabolism: identification by exome capture and sequencing of mutations in the MTHFD1 gene in a single proband. *J Med Genet* 2011;48:590-2.
- Keller MD, Ganesh J, Heltzer M, Paessler M, Bergqvist AG, Baluarte HJ, et al. Severe combined immunodeficiency resulting from mutations in MTHFD1. *Pediatrics* 2013;131:e629-34.
- Burda P, Kuster A, Hjalmarson O, Suormala T, Burer C, Lutz S, et al. Characterization and review of MTHFD1 deficiency: four new patients, cellular delineation and response to folic and folinic acid treatment. *J Inher Metab Dis* 2015;38:863-72.
- Pengelly RJ, Upstill-Goddard R, Arias L, Martinez J, Gibson J, Knut M, et al. Resolving clinical diagnoses for syndromic cleft lip and/or palate phenotypes using whole-exome sequencing. *Clin Genet* 2015;88:441-9.
- Harding KE, Robertson NP. Applications of next-generation whole exome sequencing. *J Neurol* 2014;261:1244-6.
- The Gene Ontology. Available from: <http://www.geneontology.org/>. Accessed April 22, 2015.
- Davydov EV, Goode DL, Sirota M, Cooper GM, Sidow A, Batzoglou S. Identifying a high fraction of the human genome to be under selective constraint using GERP++. *PLoS Comput Biol* 2010;6:e1001025.
- Allaire M, Li Y, MacKenzie RE, Cygler M. The 3-D structure of a folate-dependent dehydrogenase/cyclohydrolase bifunctional enzyme at 1.5 Å resolution. *Structure* 1998;6:173-82.
- Rey J, Deville J, Chabbert M. Structural determinants stabilizing helical distortions related to proline. *J Struct Biol* 2010;171:266-76.
- Biesecker LG, Shianna KV, Mullikin JC. Exome sequencing: the expert view. *Genome Biol* 2011;12:128.
- Lescai F, Bonfiglio S, Bacchelli C, Chanudet E, Waters A, Sisodiya SM, et al. Characterisation and validation of insertions and deletions in 173 patient exomes. *PloS One* 2012;7:e51292.
- Zhao M, Wang Q, Wang Q, Jia P, Zhao Z. Computational tools for copy number variation (CNV) detection using next-generation sequencing data: features and perspectives. *BMC Bioinform* 2013;14:S1.
- Tancredi M, Sensi E, Cipollini G, Lombardi G, Di Cristofano C, et al. Haplotype analysis of BRCA1 gene reveals a new gene rearrangement: characterization of a 19.9 KBP deletion. *Eur J Hum Genet* 2004;12:775-7.
- Biesecker LG. Editorial comment on "Whole exome sequencing identifies compound heterozygous mutations in WDR62 in siblings with recurrent polymicrogyria". *Am J Med Genet Part A* 2011;155A:2069-70.
- Chang YF, Imam JS, Wilkinson MF. The nonsense-mediated decay RNA surveillance pathway. *Annu Rev Biochem* 2007;76:51-74.
- Hsu AP, Johnson KD, Falcone EL, Sanalkumar R, Sanchez L, Hickstein DD, et al. GATA2 haploinsufficiency caused by mutations in a conserved intronic element leads to MonoMAC syndrome. *Blood* 2013;121:3830-7. S1-7.

APPENDIX

Case Description

The first child (patient A) presented at age 4 months with a 2-week history of breathlessness, poor feeding, and weight loss. His chest X-ray was consistent with *Pneumocystis jirovecii* pneumonia (PCP), which was confirmed on bronchoalveolar lavage. He was suspected to have SCID on the basis of lymphopenia ($0.9 \times 10^9/L$), which involved all the lymphoid subsets. HIV tests were negative, and extensive investigations excluded all known SCID gene defects. Although he had T lymphopenia, he had normal distribution of subsets, T-cell-receptor repertoire, and his proliferation to PHA was comparable to control. A diagnosis of undefined SCID was made and he was listed for a hematopoietic stem cell transplant (HSCT). After his recovery from PCP, he was discharged home on cotrimoxazole, fluconazole, and subcutaneous immunoglobulin, awaiting a matched donor. Patient A was born to a South Asian mother and white father at 36+6 weeks' gestation. His elder sister was reported to be healthy and there was no significant family history of immunodeficiency or metabolic disorders. The mother was known to carry hepatitis B virus. At age 2 months, he was found to be anemic despite apparently thriving normally. Investigation for hemolytic anemia was negative and symptoms resolved with folate and iron supplements for 2 weeks. HSCT was postponed despite finding a well-matched unrelated donor, as he remained clinically well with an improving immunological picture. Although he did not suffer significant infections, he remained lymphopenic and the evolving hypogammaglobulinemia led to reconsideration of HSCT. In the interim, his mother became pregnant and HSCT was postponed in view of his progress, the lack of ongoing opportunistic infections, and a potential for getting a better donor match when the sibling was born.

His brother (patient B) was born when his elder sibling was 18 months old and displayed similar lymphopenia to his elder patient. He was started on fluconazole and cotrimoxazole prophylaxis soon after diagnosis, but intravenous immunoglobulin was withheld to try to establish better immunological assessment. He was immunized with inactivated vaccines between age 2 and 5 months according to the UK infant immunization program. His immunoglobulin levels dropped below normal limits by the age of 6 months and although he responded to tetanus he did not respond to conjugated HiB vaccine (Table E5) and he was therefore started on azithromycin prophylaxis at age 6 months. Subsequently, he presented at age 9 months with septic arthritis of his left hip. Blood culture and joint aspiration pus identified *Streptococcus pneumoniae*. While patient B was still hospitalized, patient A presented with a 1-week history of pallor and was found to be severely anemic (hemoglobin, 34 g/L). Bone marrow examination showed a marked megaloblastic picture. RBC folate and vitamin B₁₂ levels were within normal limits; therefore, cotrimoxazole was considered to be a potential cause. Patient B also became anemic during the course of his septic arthritis treatment (hemoglobin, 6.4 g/dL). Bone marrow examination performed at this time showed a megaloblastic picture and cotrimoxazole was discontinued. Both boys were treated with a 2-week course of parenteral vitamin B₁₂ and calcium folinate. Patient A was started on pentamidine nebulizers for PCP prophylaxis. Because of parental concern regarding medicalization and therapy safety, patient B was not given any PCP prophylaxis. Patient B was started on subcutaneous immunoglobulin therapy

at age 10 months. Despite this, patient B suffered further pyogenic infections that included pneumonia (organism not isolated) at age 19 months, periorbital cellulitis at age 22 months, and pneumococcal bacteremia at age 24 months. The infection at age 19 months was accompanied by a further episode of megaloblastic anemia.

Immunological evaluations revealed that both patients were lymphopenic for all subsets throughout their follow-up (see Tables E2 and E3). Both patients had low IgM levels while on immunoglobulin replacement therapy, suggestive of antibody deficiency. Patient A was not immunized in infancy, whereas patient B was vaccinated with inactive vaccines and showed adequate tetanus titers but did not develop protective HiB titers (Table E5).

T-cell subsets expressed memory markers in a pattern similar to healthy controls (data shown in main article). T-cell subsets expressed activation marker CD69 in response to T-cell-receptor-specific stimuli (data shown in main article). T-helper subset enumeration showed comparable numbers of T_H1 and T_H17 development (data shown in main article). Both siblings continued to have normal T-cell proliferation, but their T-cell receptor excision circle counts remained low, suggestive of low thymic output. Following the initiation of calcium folinate supplementation, both patients have remained free of infections and have mounted protective vaccine responses following the cessation of immunoglobulin replacement therapy (Tables E4 and E5). Both patients are in regular schools with no disabilities identified. Both had normal magnetic resonance imaging brain and plasma/cerebrospinal fluid folate studies before regular folinic acid supplements were started. Following their successful treatment and identification of the molecular cause, the family has been informed that HSCT is no longer a therapeutic consideration.

Immunological Experiments

Methods

Cell culture. Lithium heparin anticoagulated blood samples were obtained from the patients and experiments were set up within 4 hours of sampling. Whole blood was diluted 1:1 with RPMI + 2 mM glutamine and cultured with ligands at 37° and 5% CO₂ for 6-hour durations. Ligands used to stimulate specific population of cells at final concentrations were soluble anti-CD3 (clone: OKT3) + anti-CD28 (clone: CD28.6) (10 µg/mL each); PHA (10 µg/mL; Sigma-Aldrich, Poole, UK); phorbol myristate acetate (50 ng/mL; Sigma-Aldrich) + ionomycin (0.5 µg/mL; Sigma-Aldrich), and R848 (5 µg/mL; Invivogen, Toulouse, France). Brefeldin A (10 µg/mL) was added after the first hour of culture to inhibit protein secretion for assays measuring intracellular cytokines. For cytokine measurement, supernatants were harvested immediately at the end of cell culture and stored at -20°C until analysis by multiplexed particle-based flow cytometry assay on a Luminex analyzer (Bio-Plex, Bio-Rad, Hertfordshire, UK). Samples from healthy volunteers were set up in parallel as positive controls.

Flow cytometry. The following fluochrome-tagged antihuman mAbs were obtained from BD Biosciences (Oxford, UK): anti-CD69 (catalog no. 555531), anti-CD45RA (catalog no. 555488), anti-CD8 (catalog no. 555369), anti-CD62L (catalog no. 559772), anti-CD3 (catalog no. 3010928), anti-TNF-α (catalog no. 559321), anti-CD123 (catalog no.

560087), anti-CD19 (catalog no. 339190), anti-CD14 (catalog no. 560180), anti-CD11c (catalog no. 560370), anti-HLA-DR (catalog no. 347402), anti-IFN- γ (catalog no. 560741), and anti-CD4 (catalog no. 560251). Antihuman IFN- α (PBL interferon source; 21112-3) and IL-17 (Ebiosciences, Cheshire, UK; 11071-79-71) were obtained from other sources. A 2-step staining process for surface and intracellular antigens was performed according to manufacturer instructions using BD fix-perm kit. Flow cytometry data were acquired on a BD FACS canto II flow cytometer. At least 200,000 events were collected for lymphocyte assays and 500,000 for dendritic cell assays. Data were analyzed using FACS Diva software and gating strategy is illustrated in Figure E1, A and B.^{E1,E2}

Results. *In vitro* stimulation of PBMCs with T-lymphocyte-specific stimuli led to T-cell subset expression of activation marker CD69 (Figure E2, A) and IL-2 (Figure E2, B) comparable to control. We analyzed the terminal differentiation of CD4+ T lymphocytes by enumeration of the T_H1 (IFN- γ producing) and T_H17 (IL-17 producing) subsets in the patients. As shown in Figure E2, C, T_H1 and T_H17 development was similar to a control and within normal laboratory limits (data not shown). Similarly, CD8+ and CD4- CD8- (double-negative) T lymphocytes produced IFN- γ comparably to a healthy control on mitogen stimulation (Figure E2, C). T-cell subsets expressed memory markers in a similar pattern to healthy controls (Figure E2, D). Taken together, these data suggest that although T-lymphocyte numbers were low, T-cell function was normal.

Methods: Allele-specific PCR

In a heterozygous state with 1 allele carrying a deletion, PCR products of 2 sizes are anticipated. We were unable to demonstrate the undeleted allele in the patients with primers EX13LF and EX13LR despite varying PCR conditions. To demonstrate the heterozygous state in the patients, we used allele-specific primer (EX13SF) that is predicted to anneal to the deleted region. We set the PCR conditions to exclude the longer product (2350 bps) by limiting the extension time. As shown in Figure E4, A, the PCR product from the primers EX13SF and EX13LR is possible only from the undeleted allele and is predicted to be around 1500 bps. With extension time limited to exclude the 2350-bps product, PCR product (from EX13LF and EX13LR) is possible only from the allele carrying exon 13 deletion and is expected to be of 600-bps size. As shown in Figure E4, B, analysis by agarose gel electrophoresis of PCR products from patient A and the mother shows a heterozygous state for EX13 deletion in the patient.

TABLE E1. Primer sequences used in this study

Primers (5' to 3')	
EX3F	ATGGCAGCAGAAATCCCACAT
EX3R	AAAAACTCACGGGCAAACCAG
EX13LF	GAGTTAGCTTTCTCTGCTTGGT
EX13LR	GTGTGTTGGTGCTTCCCGAT
EX13SF	GCACTGATAATGTGGTAACCTGG
cDNAF	ATCTCCGCGCAAATAAGGGC
cDNAR	CACATCCTTCTCGGGTGCAA

AluS repeats as the mechanism for exon 13 deletion

Following breakpoint identification, further analysis revealed that the deletion boundaries around exon 13 were within homologous positions of flanking, parallel, AluS repeats (Figure E5, A and B). Alu elements are short interspersed elements of approximately 300 bp in length and derive their name from a single recognition site for the restriction enzyme AluI located near the middle of the Alu element. Alu elements have amplified in primate genomes through a RNA-dependent mechanism termed retroposition and human chromosomes contain about 1,000,000 Alu copies, which equal 10% of the total genome.^{E3,E4} Alu elements continue to amplify at a rate of 1 in 200 births and insertion of Alu elements contributes to approximately 0.1% of human genetic disorders. In addition to disease caused by insertion of Alu elements, their dispersion throughout the genome provides ample opportunity for unequal homologous recombination, which can lead to deletion or insertion mutations and most often occur intrachromosomally.^{E3} As illustrated (Figure E5, A and B), these nearby low-complexity sequence regions likely permitted replication slippage to occur, excising the 1745-bp region; this has been previously observed as a pathogenic mutational mechanism in other genes, such as in *BRCA1*.^{E5}

REFERENCES

- E1. Blimkie D, Fortuno ES III, Thommai F, Xu L, Fernandes E, Crabtree J, et al. Identification of B cells through negative gating—an example of the MIFlow-Cyt standard applied. *Cytometry Part A* 2010;77:546-51.
- E2. Jansen K, Blimkie D, Furlong J, Hajjar A, Rein-Weston A, Crabtree J, et al. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. *J Immunol Meth* 2008;336:183-92.
- E3. Deininger PL, Batzer MA. Alu repeats and human disease. *Mol Genet Metabolism* 1999;67:183-93.
- E4. Rowold DJ, Herrera RJ. Alu elements and the human genome. *Genetica* 2000;108:57-72.
- E5. Tancredi M, Sensi E, Cipollini G, Aretini P, Lombardi G, Di Cristofano C, et al. Haplotype analysis of *BRCA1* gene reveals a new gene rearrangement: characterization of a 19.9 KBP deletion. *Eur J Hum Genet* 2004;12:775-7.

TABLE E2. T-, B-, and NK-cell count (patient A)

Age (mo)	Lymphocytes (10 × 9/L)	CD3+ absolute nos. (10 × 9/L)	CD3+ CD4+ absolute nos. (10 × 9/L)	CD3+ CD8+ absolute nos. (10 × 9/L)	CD19+ absolute nos. (10 × 9/L)	NK-cell absolute nos. (10 × 9/L)
4	1	0.23	0.19	0.05	0.69	0.07
6	1.8	0.27	0.2	0.07	0.99	0.29
17	2.3	0.81	0.53	0.21	0.87	0.58
28	0.7	0.57	0.37	0.17	0.09	0.02
29	1.7	0.78	0.46	0.27	0.53	0.32
31	1.3	0.66	0.38	0.21	0.43	0.18
39	0.9	0.62	0.38	0.21	0.25	0.03
47	1.2	0.53	0.36	0.17	0.4	0.24
51	1.1	0.47	0.3	0.15	0.3	0.25
52	1.6	0.72	0.46	0.21	0.58	0.27
66	1.3	0.72	0.34	0.21	0.26	0.27
72	1.5	0.9	0.53	0.29	0.32	0.24
78	1.4	0.85	0.52	0.24	0.32	0.18
Reference range	1.5-4.0	0.7-4.2	0.3-2.0	0.3-1.8	0.2-1.6	0.09-0.9

NK, Natural killer.

TABLE E3. T-, B-, and NK-cell count (patient B)

Age (mo)	Lymphocytes (10 × 9/L)	CD3+ve absolute nos. (10 × 9/L)	CD3+ve CD4+ve absolute nos. (10 × 9/L)	CD3+ve CD8+ve absolute nos. (10 × 9/L)	CD19+ve absolute nos. (10 × 9/L)	NK-cell absolute nos. (10 × 9/L)
10	1.1	0.84	0.69	0.13	0.24	0.01
12	2.6	1.69	0.88	0.78	0.47	0.36
14	1.9	1.12	0.7	0.4	0.57	0.19
30	1.9	0.95	0.67	0.25	0.55	0.36
34	3.1	1.61	1.09	0.43	0.68	0.71
49	2.4	1.18	0.65	0.36	0.46	0.7
54	3.5	1.65	0.95	0.6	0.56	1.16
60	3	1.65	0.93	0.57	0.66	0.66
Reference range	1.5-4.0	0.7-4.2	0.3-2.0	0.3-1.8	0.2-1.6	0.09-0.9

TABLE E4. Immunoglobulin levels and vaccine responses in patient A

Immunoglobulin levels and vaccine responses	Age (mo)											
		4	5	21	42	47	58	63	68-70	72	90	102
IgG (g/L)	Two doses DTP, OPV, Men C	1.5*	IVIG replacement started	9.1	Folinic acid supplements started; IVIG continued	10.1	9	IVIG discontinued	Three doses DTP/IPV/HiB; 1 dose Prevanar 13/Men C	7.3	8.2	8.1
IgA (g/L)		0.1*		0.4*		1.2	1.2			1.5	1.2	1.4
IgM (g/L)		4†		0.1*		0.3*	0.4*			0.9	0.7	0.6
Tetanus IgG total (IU/mL)		0.04								>4		
Hemophilus b antibody (μg/mL)		0.1*								>10		
Pneumococcal antibody (μg/mL)												
Serotype 14										2.8		
Serotype 18C										1.96		
Serotype 19F										5.53		
Serotype 23F										2.94		
Serotype 4										0.89		
Serotype 9V										>3.45		
Serotype p6B										3.48		

DTP, Diphtheria, Tetanus, and Pertussis; OPV, oral polio vaccine; Men C, Meningitis C; IVIG, intravenous immunoglobulin therapy.

*Levels below the local laboratory reference range for age.

†Levels above local laboratory reference range for age.

TABLE E5. Immunoglobulin levels and vaccine responses in patient B

Immunoglobulin levels and vaccine responses	Age (mo)										
		6	10	14	27	37	45	51-53	54	67	85
IgG (g/L)	Three doses DTP, IPV, Men C	2.2*	IVIG replacement started	10.8	Folinic acid supplements started; IVIG continued	9.2	IVIG discontinued	Three doses DTP/IPV/HiB; 1 dose Prevanar 13/Men C	9.4	7.8	8
IgA (g/L)		0.37*		1.3		0.9			1.6	1.1	1.1
IgM (g/L)		0.4*		0.4*		0.2*			1.2	1	0.6
Tetanus IgG total (IU/mL)		0.08							>4		
Hemophilus b antibody (μg/mL)		<0.15*							>10		
Pneumococcal antibody (μg/mL)											
Serotype 14									1.76		
Serotype 18C									0.84		
Serotype 19F									0.78		
Serotype 23F									0.35		
Serotype 4									0.42		
Serotype 9V									0.67		
Serotype p6B									0.63		

DTP, Diphtheria, Tetanus, and Pertussis; OPV, oral polio vaccine; Men C, Meningitis C; IVIG, intravenous immunoglobulin therapy.

*Levels below the local laboratory reference range for age.

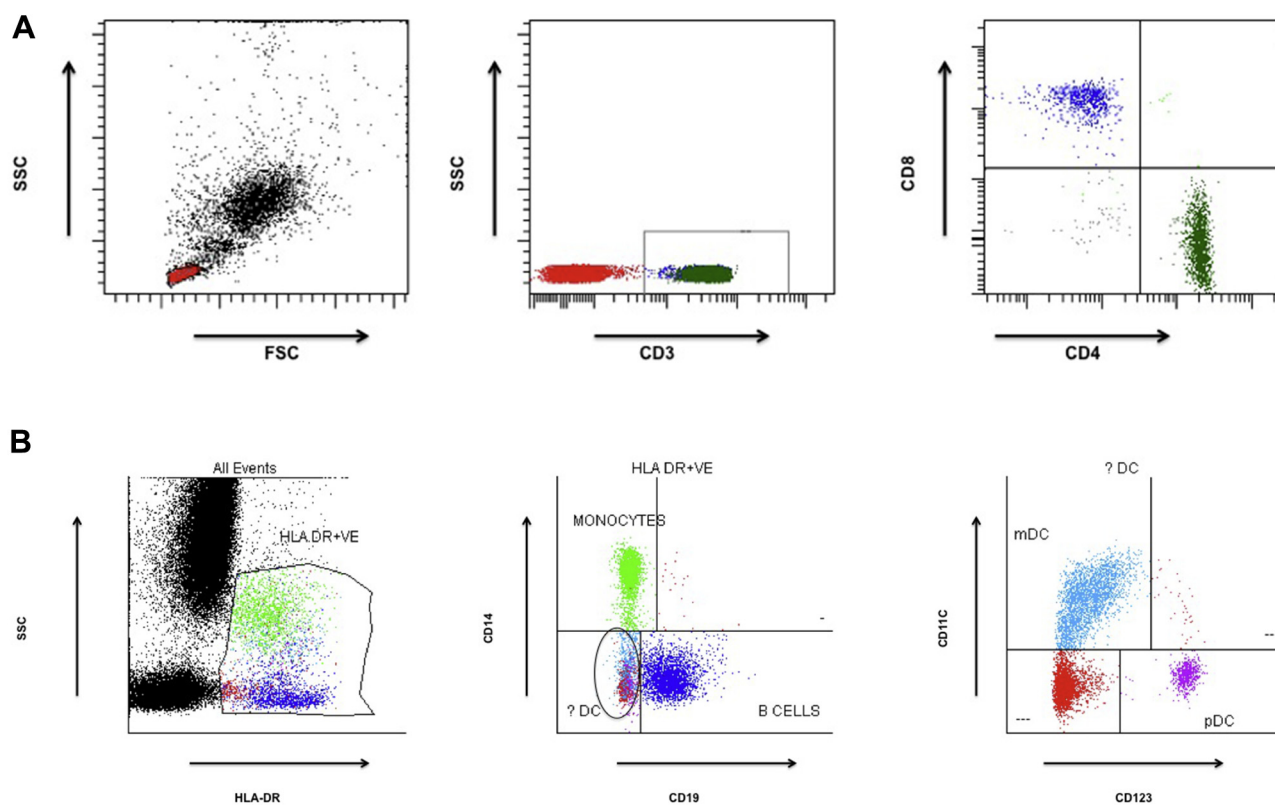


FIGURE E1. Gating strategy. **A**, Lymphocyte subsets. **B**, Antigen-presenting cells. Monocytes: HLA DR+ CD14+; Myeloid DC: HLA DR+ CD14– CD19– CD11c+ CD123–; Plasmacytoid DC: HLA DR+ CD14– CD19– CD11c– CD123+. DC, Dendritic cell; FSC, forward scatter; mDC, myeloid DC; pDC, plasmacytoid DC; SSC, side scatter.

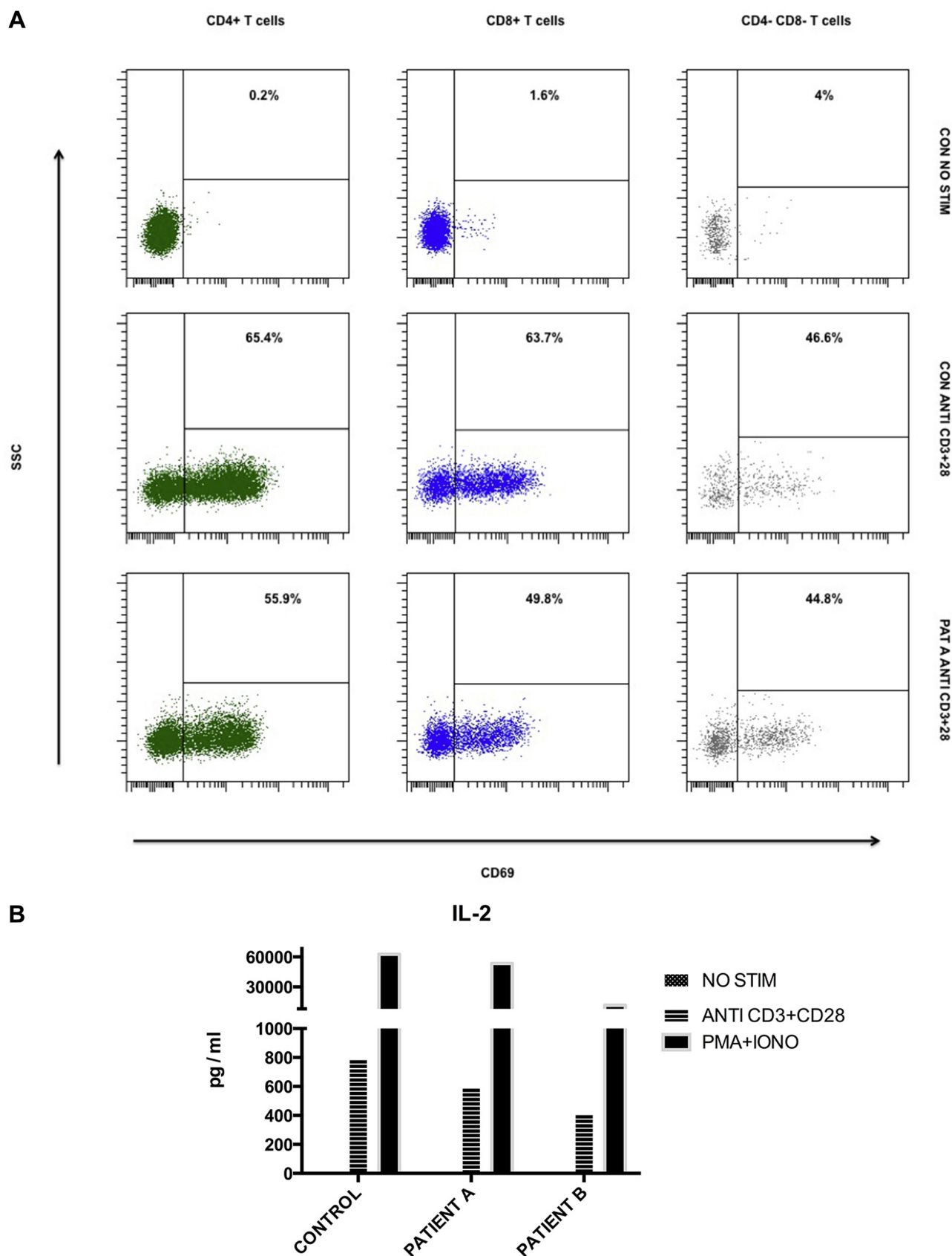


FIGURE E2. *In vitro* functional assessment of T lymphocytes and antigen-presenting cells. Patient A's responses were representative of patient B's responses where data are not shown. **A**, Patient A's T-lymphocyte subsets expressed activation marker CD69 comparably to a healthy control (CON). **B**, IL-2 production was comparable to a healthy control. **C**, Cytokine (IFN- γ and IL-17) production by T-lymphocyte subsets was comparable to a healthy control (CON). **D**, Memory phenotyping of T-lymphocyte subsets was comparable to a healthy control (CON). *IONO*, ionomycin; *PAT*, patient; *PMA*, phorbol 12-myristate 13-acetate; *SSC*, side scatter; *STIM*, stimulation.

C

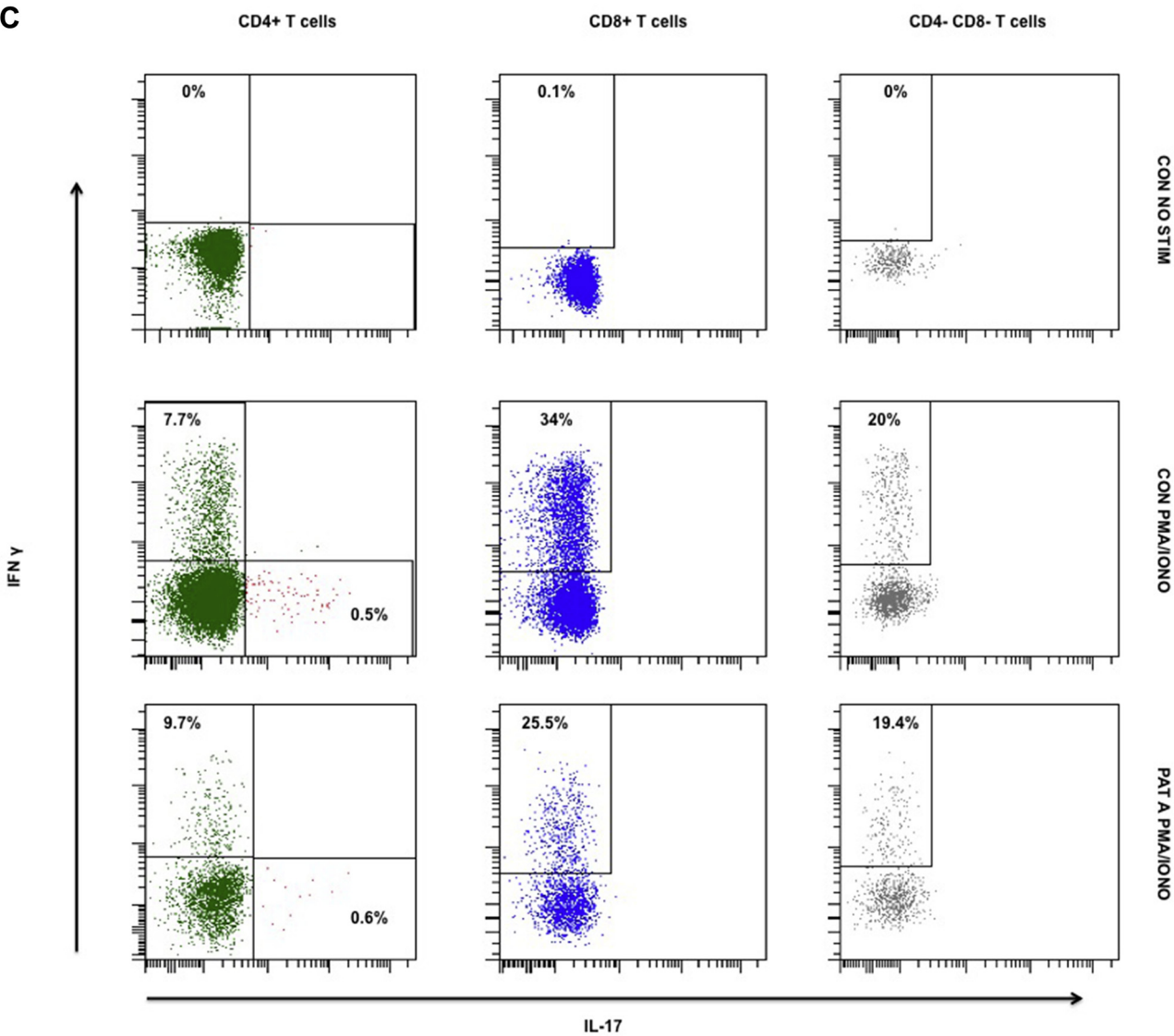


FIGURE E2. (Continued).

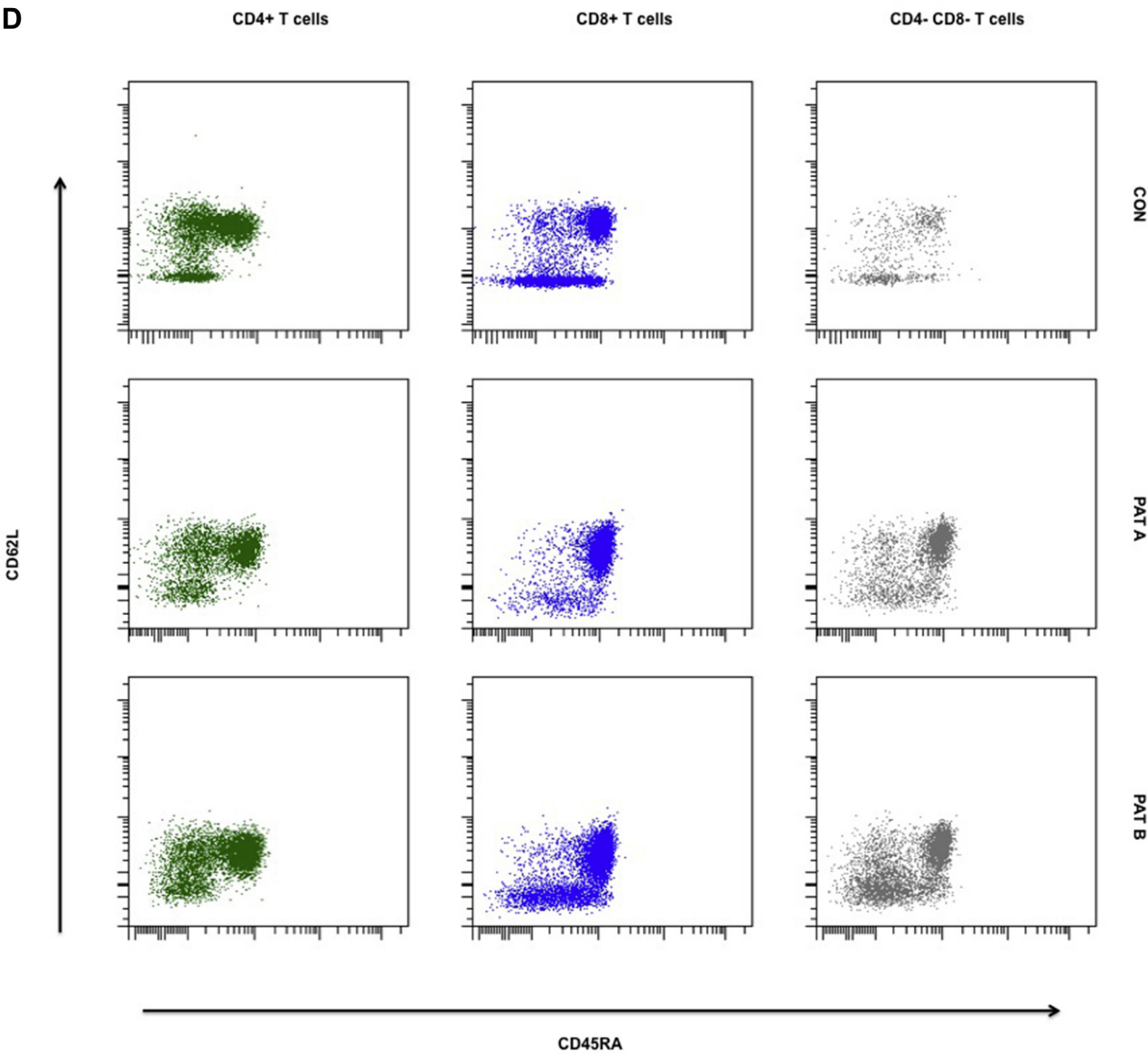


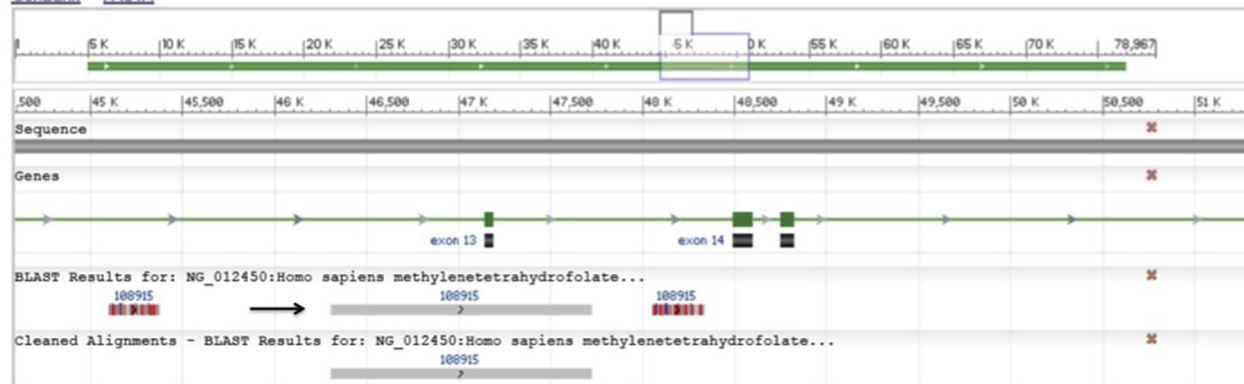
FIGURE E2. (Continued).

A

Homo sapiens methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase (MTHFD1), RefSeqGene on chromosome 14

NCBI Reference Sequence: NG_012450.1

[GenBank](#) [FASTA](#)



B

Homo sapiens methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase (MTHFD1), RefSeqGene on chromosome 14

NCBI Reference Sequence: NG_012450.1

[GenBank](#) [FASTA](#)

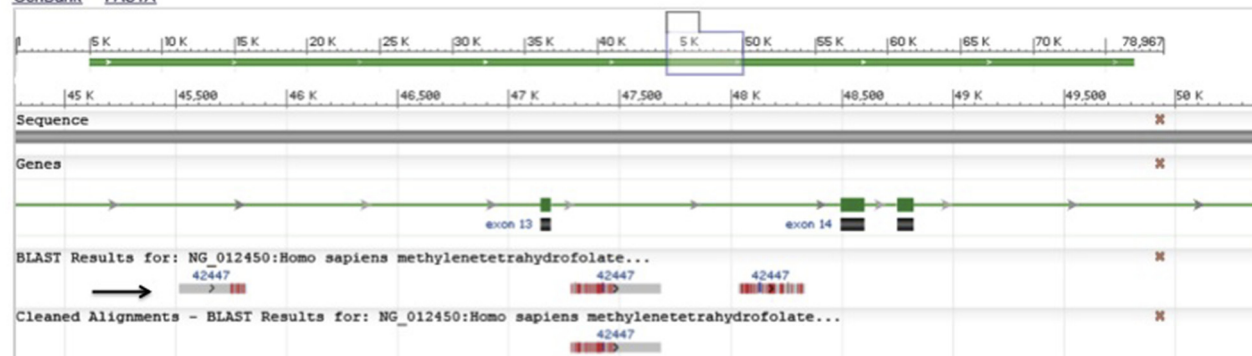


FIGURE E3. Delineation of break points of exon 13 deletion by Sanger sequencing of PCR products. Analysis was performed on the publicly available nucleotide blast program. (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP_9606_9558). **A**, The 2350-bps PCR product obtained (using primers EX13LF and EX13LR) from the mother aligned one fragment against the reference sequence of MTHFD1 (arrow). **B**, The 600-bps PCR product obtained (using primers EX13LF and EX13LR) from patient A aligned against the reference sequence of MTHFD1 (arrow) with a gap of 1745 bps confirming deletion that involves exon 13.

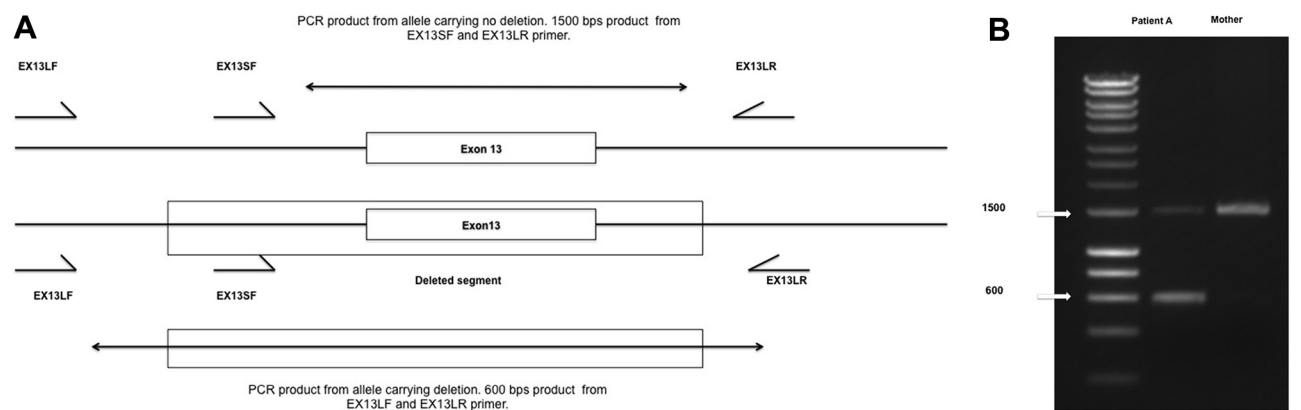


FIGURE E4. Allele-specific PCR. **A**, PCR conditions were set to exclude the 2350-bps product from the EX13LF and EX13LR primers. In this condition, PCR amplification of the patients showed 2 products of approximately 1500 and 600 bps. PCR amplification of the mother showed only 1 product of approximately 1500 bps as expected. **B**, Agarose gel electrophoresis of the allele-specific PCR product from patient A and the mother demonstrating the heterozygous state.

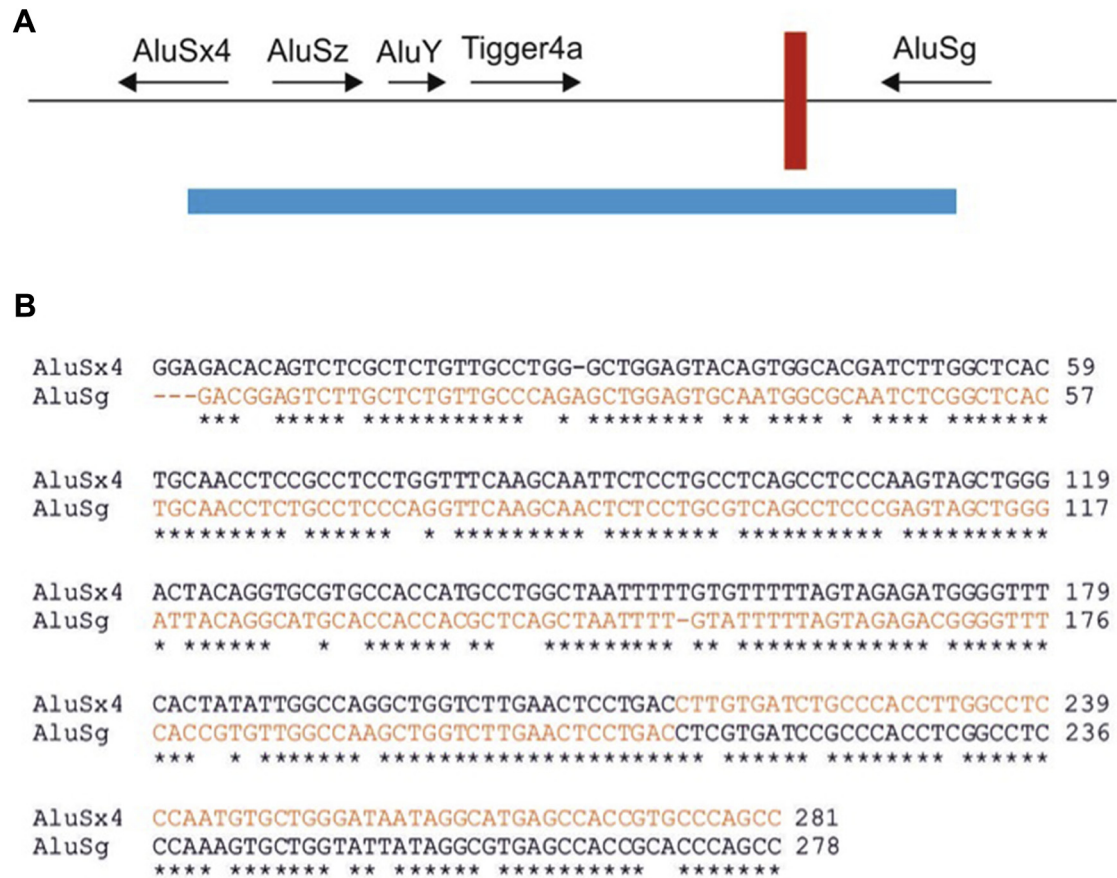


FIGURE E5. Identification of an Alu-mediated deletion in MTHFD1. **A**, Schematic representation of repetitive elements encompassing the deletion. Introns are shown as lines, exon 13 as a red rectangle, deletion as a blue rectangle. Annotation of transposed elements is based on a reference sequence (hg19). **B**, Sequence alignment of 2 AluS elements across the deletion breakpoint. Deleted nucleotides are in red.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.