

Immunologic heterogeneity in two Cartilage-Hair Hypoplasia (CHH) patients with distinct clinical course

Short Title: Immunological analysis of CHF patients.

Gamliel A^{1,2}, Nee Lee Y^{1,2}, Lev A^{1,2}, AbuZaitun O³, Rechavi E^{1,2}, Levy S¹, Simon AJ*^{1,4}, Somech R*^{1,2}

¹Pediatric Department A and the Immunology Service, Jeffrey Modell Foundation Center, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Hashomer, Israel

²Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

³Ambulatory Pediatrics, Nablus, Palestinian Authority

⁴Hematology Laboratory, Hemato-Immunology Unit and Sheba Cancer Research Center, Sheba Medical Center, Hashomer, Israel

*These authors jointly supervised this work

Corresponding authors:

Raz Somech

Pediatric Department and the Immunology Service, "Edmond and Lily Safra" Children's Hospital, Sheba Medical Center, Hashomer, Israel.

Email: raz.somech@sheba.health.gov.il

Amos J Simon

Hematology Laboratory, Hemato-Immunology Unit and Sheba Cancer Research Center, Sheba Medical Center, Hashomer, Israel.

Email: amos.simon@sheba.health.gov.il

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Abstract

Introduction: Cartilage-hair hypoplasia (CHH) syndrome is a rare autosomal recessive syndrome associated with skeletal dysplasia, varying degrees of combined immunodeficiency (CID), short stature, hair hypoplasia, macrocytic anemia, increased risk of malignancies and Hirschsprung disease.

Objective: To provide clinical and immunological insights obtained from two unrelated patients who displayed clinical characteristics of CHH.

Methods: Two patients with suspected CHH syndrome due to skeletal dysplasia and immunodeficiency, underwent immunological and genetic work-up using flow cytometry, immune repertoire next-generation sequencing (NGS), and Sanger sequencing to identify the underlying defects.

Results: P1 presented with low birth weight and skeletal dysplasia. Newborn screening (NBS) was suggestive of T cell immunodeficiency as T cell receptor excision circle (TREC) levels were undetectable. Both T cell receptor (TCR) - V β and T-cell receptor gamma (TRG) repertoire were restricted with evidence of clonal expansion. Genetic analysis identified compound heterozygous variants inherited from both parents in *RMRP*. P2 presented with recurrent lung and gastrointestinal infections, skeletal dysplasia, failure to thrive and hepatomegaly. TCR β repertoire revealed a normal polyclonal pattern with only slight overexpression of TCR- β V20 and several restricted expression of V β s. TRG expressed a normal diverse repertoire, similar to the healthy control sample. Genetic analysis identified bi-allelic novel regulatory variants in *RMRP*. Both parents are carriers of this mutation.

Conclusion: Our findings demonstrate how immunological work-up, supported by genetic findings can dramatically change the suggestive treatment and future outcome in patients with the same clinical syndrome.

Key words: *RMRP*. Severe combined immunodeficiency (SCID). Cartilage-Hair Hypoplasia (CHH). NGS. TCR repertoire.

Resumen

Introducción: El síndrome de hipoplasia de cartílago-cabello (CHH) es un síndrome autosómico recesivo poco frecuente, asociado con displasia esquelética, diversos grados de inmunodeficiencia combinada (CID), baja estatura, hipoplasia del cabello, anemia macrocítica junto a un mayor riesgo de desarrollar tumores malignos y enfermedad de Hirschsprung.

Objetivo: Proporcionar conocimientos clínicos e inmunológicos obtenidos de dos pacientes, sin relación familiar, que mostraron características clínicas de CHH.

Métodos: Dos pacientes con sospecha de síndrome CHH debido a la presencia de displasia esquelética e inmunodeficiencia, se sometieron a un estudio inmunológico y genético mediante citometría de flujo, secuenciación de próxima generación (NGS) del repertorio inmune y secuenciación de Sanger, para identificar los defectos subyacentes.

Resultados: Paciente 1: Presenta al nacimiento bajo peso y displasia esquelética. El cribado de recién nacidos (NBS, por sus siglas en inglés) sugirió una inmunodeficiencia de células T, ya que los niveles del receptor de células T (TREC, por sus siglas en inglés) eran indetectables. El estudio posterior reveló anemia macrocítica, linfopenia grave e hipogammaglobulinemia. Tanto el repertorio del receptor de células T (TCR) - V β como el repertorio del receptor de células T gamma (TRG) estaban restringidos y con evidencia de expansión clonal. El análisis genético identificó variantes heterocigóticas compuestas heredadas de ambos padres en RMRP. Paciente 2: presentaba una clínica de infecciones pulmonares y gastrointestinales recurrentes, displasia esquelética, retraso en el crecimiento y hepatomegalia. El estudio inmunológico, el hemograma completo (CSC) y los análisis de subpoblaciones de linfocitos fueron normales. El repertorio de TCR β reveló un patrón policlonal normal con solo una ligera sobreexpresión de TCR- β V20 y varias expresiones restringidas de V β s. TRG expresó un repertorio diverso normal, similar a la muestra de controles sanos. El análisis genético identificó nuevas variantes reguladoras bialélicas en RMRP. Ambos padres son portadores de esta mutación.

Conclusión: Nuestros hallazgos demuestran cómo el estudio inmunológico, respaldado por hallazgos genéticos, puede cambiar drásticamente el tratamiento adecuado y el pronóstico clínico, en pacientes con un mismo síndrome clínico.

Palabras clave: RMRP. Inmunodeficiencia combinada severa (SCID). Hipoplasia de cartilago-cabello (CHH). NGS. Repertorio TCR.

Introduction

Cartilage-hair hypoplasia (CHH) syndrome is a rare autosomal recessive syndrome characterized by chondrodysplasia, variable degree of combined immunodeficiency (CID), short stature, hair hypoplasia, anemia, increased risk of malignancies and Hirschsprung disease. The syndrome is caused by mutations in *RMRP*, a gene encoding for the untranslated RNA component of mitochondrial RNA processing endoribonuclease. This ribonucleoprotein cleaves mitochondrial RNA at a priming site of mitochondrial DNA replication. RMRP also interacts with the telomerase reverse transcriptase (TERT) catalytic subunit in order to form a distinct ribonucleoprotein complex that has RNA-dependent RNA polymerase activity, and produces double-stranded RNAs that can be processed into small interfering RNA [1]. CHH disease prevalence is exceptionally high among specific populations including the Finnish and Amish [2]. So far, more than 70 different *RMRP* mutations have been identified. Mutations are predominantly found in both the transcribed region and the promoter region (from the TATA box to the transcription initiation site) of the *RMRP* gene. The most frequently found mutation is the +70A>G point mutation [3-6]. CHH patients demonstrate high overall mortality, mostly from malignancies and lung disease that can be related to their immunodeficiency. Median age at death from immunodeficiency-related causes was found to be 40.9 years [7].

Clinical heterogeneity and variable degrees of immunodeficiency have been previously reported among patients harboring *RMRP* variants. Abnormalities of cellular immunity can range from severe impairment causing severe combined T and B cell deficiency (SCID) to near normal with clinical insignificance [6, 8-10]. In addition, immune dysregulation leading to a wide spectrum of autoimmune manifestations is also frequent in the CHH patient [11]. For some CHH patients who display severe T cell immunodeficiency, early diagnosis is possible through newborn screening panel and is of clinical importance. This may prevent future infections and clinical deterioration since adequate isolation and treatment are immediately commenced.

Newborn screening for severe T cell immunodeficiency is widely performed using detection of the T-cell receptor excision circles (TREC) in dried blood spots (DBS) collected on Guthrie cards. This assay is increasingly being used for newborn screening for SCID worldwide as well as in Israel [12]. Interestingly, the TREC assay can also identify newborns with syndromes (those associated with severe T cell immunodeficiency), newborns with secondary causes of T cell immunodeficiency and newborns with T cell lymphopenia. Thus, it is possible to identify some, but not all, CHH patients with profound T-cell lymphopenia [13]. Hematopoietic stem cell transplantation (HSCT) has profoundly changed the natural history of severe T-lymphocyte deficiencies by curing many patients with SCID and CID [14]. In contrast to SCID, not all CHH patients should be considered for HSCT. Only those with chronic or recurrent infections in association with autoimmunity or bone marrow hypoplasia are good candidates if a well-matched donor is available [15].

In this study, we describe the immune work up of two unrelated patients who presented with clinical findings suggestive of CHH. One was diagnosed through newborn screening for SCID and interestingly displayed severe immunodeficiency including abnormal T cell receptor repertoire, while the other was diagnosed at the age of 1 year mainly due to his skeletal dysplasia, recurrent lung and gastrointestinal infections. We used next generation analysis (NGS) to determine their T cell receptor (TCR) repertoire. In both patients, Sanger sequencing identified distinct mutations in the *RMRP* gene. This research extends current understanding of CHH in patients suspected to have immunodeficiency and the correlation between their TRG (T-cell receptor gamma) repertoire and their diseases.

Methods

Clinical Data

Clinical information was obtained from the patients' electronic medical records. All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards.

Immune Function

Cell surface markers of peripheral blood mononuclear cells (PBMCs) were determined by immunofluorescent staining using flow cytometry, as previously described [16]. Lymphocyte proliferation was assessed in response to the mitogens phytohemagglutinin (PHA) and anti-CD3 using tritiated thymidine incorporation, as previously described [16]. Serum concentration of immunoglobulins was measured by nephelometry (BN-II, Siemens).

TREC analysis was performed using DNA extracted from the study patients' PBMCs, as previously described [16].

TCR-V β Repertoire

The expression of the representatives of specific TCR variable β families were detected and quantified using patients' PBMCs with flow cytometry (NAVIOS, Beckman Coulter) according to the manufacturer's instructions (Beta Mark TCR V β repertoire kit, Beckman Coulter), as previously described [16].

TCR-gamma (TRG) repertoire determination using next generation sequencing

TCR libraries were generated from patient and control genomic DNA extracted from PBMCs using primers to the conserved regions of V and J genes in the TRG (TCR-gamma) locus according to the manufacturer's protocol (Lymphotrack; Invivoscribe Technologies®, Carlsbad, CA, USA). Quantified libraries were pooled and sequenced using Mi-Seq Illumina Technology®. FASTA files from the filtered sequences were submitted to ImMunoGeneTics (IMGT) HighV-QUEST webserver® (<http://www.imgt.org>), further filtered for productive sequences only (no stop codons or frameshifts), and analyzed [3]. Analyses were performed on CDR3 amino acid sequences. For TCR repertoires, V and J gene usage patterns were analyzed. Repertoire diversity was calculated using Shannon and Gini-Simpson's diversity indices [4].

Sanger sequencing

Sanger sequencing (NCBI Reference Sequence: NR_003051.3) was used for mutation identification and familial segregation of both nucleotide changes in the *RMRP* gene.

Bone marrow transplantation

Patients received the transplantation in house, at our hemato-oncology unit. Standard prophylactic medications consisted of trimethoprim/sulfametaxazole for *Pneumocystis jiroveci* (PCP), IVIG (Intra Venous Immunoglobulins) to maintain IgG level above 6 g/L, acyclovir in the occasion of donor–recipient serologic cytomegalovirus (CMV) disparity, and cyclosporine A (CSA) for GVHD. Granulocyte colony-stimulating factor (G-CSF) was given from the day of transplant until neutrophil counts were above $1.0 \times 10^9/L$ for three consecutive days. The HSCT infusion itself originated from bone marrow. Conditioning regimens were based on the European group for blood and marrow transplantation (EBMT) and European Society for Immunodeficiencies (ESID) guidelines for HSCT in PID.

Engraftment and immunologic assessment

Neutrophil engraftment was defined after three consecutive days with an absolute count of over $0.5 \times 10^9/L$. Lymphocyte engraftment and chimerism were evaluated mainly by analysis of microsatellite variable numbers of tandem repeats, and FISH studies using Y-specific probes in gender-mismatched couples. Absolute numbers and percentages of lymphocytes were quantified by assessment of cell surface markers using immuno-fluorescent staining and flow cytometry (Epics V; Beckman Coulter, Hialeah, FL, USA) with antibodies purchased from Beckman Coulter. Serum immunoglobulin concentrations (IgG, IgM, IgA) were measured using standard nephelometry.

Results

Clinical description

We present two unrelated patients that were evaluated for suspected CHH. Patient 1 (P1) was born to a healthy non-consanguineous Ashkenazi-Jewish couple after spontaneous pregnancy. In the 3rd trimester short long bones were demonstrated by fetal ultrasound. Premature labor of female fetus, at 34 weeks of gestation with birth weight of 1800 gr and length of 35 cm (-2 SD). Post-natal period was uneventful. Newborn screening was suggestive of T cell immune deficiency as TREC levels were undetectable. At the 4th week of life, blood workup showed severe lymphopenia and anemia (Table 1).

Patient 2 (P2) presented at the age of 1 year to our clinic, mainly to evaluate his recurrent infections. He was born to healthy non-consanguineous Palestinian parents. His birth weight was 3.3 Kg. After birth, P2 was noticed to have short limbs. At the 2nd month of life, examination revealed hepatosplenomegaly. Later on, he presented with poor weight gain. At the first year of life weight was only 7.6 Kg and 4 months later 7.8 kg (-3 SD). Since birth, P2 suffered from recurrent pneumonia once a month and multiple episodes of gastroenteritis, some of which required hospitalization. Initial findings in our hospital showed short stature (72cm, -3SD), short limbs, skeletal dysplasia, Failure to thrive (FTT) and hepatomegaly.

Immunological evaluation

Initial immunologic evaluation for Patient 1 (P1) at 4th week of life, revealed severe lymphopenia with depleted both T and B cells (Table 1). Lymphocyte proliferation was markedly reduced following stimulation with PHA. In order to evaluate thymus activity and naïve T cell production, TRECs in peripheral blood were quantified. TREC's level was remarkably low (Table 1). Hypogammaglobulinemia was noticed, as reflected by the absence of IgA, and low levels of IgM. IgG levels were borderline, as expected in the post-natal period. At the age of 3 months, IgG levels were 342 mg/dl (470-1230 mg/dl normal range), monthly replacement therapy was started of IVIG.

The TCR-V β repertoire was assessed with the traditional TCR-V β assay using flow cytometry. P1 had a monoclonal pattern with overexpression of TCR- V β 12, compared to healthy controls (Fig. 1). Taken together, his lab tests were suggestive of SCID. P1 was also assessed for cell chimerism upon diagnosis, due to suspected trans placental-acquired maternal T cells, which is a pathognomonic feature of SCID and showed no chimerism after birth. Patient 2's (P2) immunologic evaluation was performed at the age of 1 year. CBC and lymphocyte sub-population were normal (Table 1). TREC's level was low (Table 1). Immunoglobulins levels were normal for age (Table 1). The TCR-V β repertoire revealed a normal polyclonal pattern with only slight overexpression of TCR-V β 20 and several restricted TCR-V β expression, compared to healthy controls (Fig. 1).

TRG repertoire analyses

In order to further characterize the TCR repertoire, high-throughput immune sequencing of the TRG (T cell receptor γ chain) repertoire was performed on PBMCs from both patients and a healthy age-matched control. Of the four chains of TCR ($\alpha, \beta, \gamma, \delta$), the TRG repertoire was specifically selected for sequencing for the following reasons: it is rearranged earlier in the development of T cells; it is often used for sensitive and comprehensive detection of clonal expansions [4, 5, 17]. As shown in Figure 2A, the Treemaps graphically represent T cell receptor Gamma (TRG) repertoire determine that P1 expressed a restricted diverse repertoire with evidence of clonal expansion consistent with abnormal T cell selection within the thymus. Unlike P1, P2 expressed a normal diverse repertoire, similar to the healthy control. We used Shannon's H and Simpson's D indices [4] to measure the diversity of the repertoires, which are commonly used in ecology [18-20]. These diversity indices take into account the unique and total sequences and the evenness of the clonal size. Thus, the Shannon's H index reflects the overall diversity of the repertoire whereas the Simpson's D index focuses on how unevenly the clones are distributed in a given repertoire due to the presence of clonal expansions, as was previously shown [19, 20]. The Shannon's H diversity index for P1 repertoires was relatively low, compared with P2 and the controls (Fig. 2C). Calculation of the Simpson's D index showed unevenness in the TRG repertoire for P1 as compared to P2 and controls (Fig. 2B). Thus, in accordance with the Treemaps, the diversity indices showed that P1 but not P2 has restricted and clonally expanded TRG repertoire compared to the controls. Non-productive sequences of the TRG were also analyzed, there was no difference from the productive sequences results (Fig. S1).

Genetic analyses

Sanger sequencing revealed three distinct mutations in the *RMRP* gene of P1 and P2, two of them are known [1, 21] and one is novel. Familial segregations were elucidated (Figs. 3 and 4). In P1, bi-allelic compound heterozygous known mutations were found (Fig. 3). The first mutation, maternally inherited, is a small duplication of 17 nucleotides, 4 nucleotides upstream to the transcription initiation site (TIS) (1). The second mutation, paternally inherited, is a regulatory mutation of C to T at +238

relative to TIS (20). In P2, a homozygous novel regulatory mutation was found at +222 relative to TIS. Both parents are carriers of this mutation (Fig. 4).

HSCT for Patient 1

P1 was 10 months when transplantation was done. Underwent conditioning with Treosulfan/fludarabine. A brother was found to be matched related donor. As described, GVHD prophylaxis, when used, was based mainly on CSA. MMF and corticosteroids were added when GVHD was suspected or diagnosed. Acute and chronic GVHD appeared only in conditioned patients, all of which received adequate pro-phylaxis.

Immune reconstitution

Neutrophil engraftment exhibited on day +12 from HSCT, lymphopenia (defined as an ALC < 2500) recovered 5 months post HSCT and stayed stable until present. T cell deficiency (CD3 < 1000) has recovered 6 months post HSCT and immunoglobulins levels recovered on 3 months post HSCT.

Normal TREC levels (> 400 copies) were detected on 6 months post HSCT.

Chimerism based on FISH studies using Y-specific probes in gender-mismatched couples was 100% 3 months post HSCT and since 1 year post HSCT remained stable on 60%.

Discussion

CHH is a well-known syndromic primary immunodeficiency (PID) with a wide spectrum of clinical presentations specifically regarding the immune system. Patients can be diagnosed immediately after birth through newborn screening for SCID or later in life, due to a clinical suspicion.

In this report, we describe two patients with a clinical and molecular diagnosis of CHH. Patient 1 has been identified through the Israeli New born screening (NBS) program. Besides making the diagnosis process much simpler, CHH patients with absent TREC tend to have a greater degree of immunodeficiency. CHH patients positively screened by the NBS program are likely to go in a different route of

treatment. [13, 22]. Recent studies summarizing the latest results of the SCID-NBS programs revealed several outcomes. Besides the diagnosis of typical SCID, syndromes such as DiGeorge syndrome, Trisomy 21, Trisomy 18, and CHARGE accompanied by severe lymphopenia, were also identified using the TREC test [23-25]. These studies also detected two CHH patients. The first CHH patient, diagnosed with SCID and clinical presentation of skeletal dysplasia and Hirschsprung disease, unfortunately died at the age of 1 month. Her *RMRP* mutation was discovered postmortem. The second patient initially diagnosed as leaky-SCID, had IUGR and short limbs [23, 24]. Scott et al described 8 Amish CHH patients with abnormal TREC levels positively screened in the NBS. Four patients had absent TREC and tend to have a greater degree of immunodeficiency [22]. P2 had also presented with low TREC levels. Unfortunately, there is not NBS-TREC program in the Palestinian territories, and the evaluation was started, based on clinical suspicion.

As often seen in patients with CHH who present with variable clinical symptoms and therefore undergo a complete different treatment and outcome, we detected such differences in our reported cases, specifically when evaluating their immune function. P1 had lymphopenia from birth with severe T and B cell depletion, while P2 had lymphocytes within normal range. Interestingly, P1 had restricted TCR regardless of any underlying infection, which potentially could lead to an abnormal T cell selection or Omenn syndrome. In contrast, P2 had diverse TCR, which was normal compared to a healthy control. Respectively, The Shannon's H diversity index for P1 repertoires was relatively low, compared with P2 and the controls. Simpson's D index also showed similar results. Using advanced methods, like NGS, we were able to show the difference in the immunologic profile between the patients, leading eventually to different clinical outcomes.

Moreover, a genotype-phenotype correlation existed in our patients, which is also supported by the literature [10, 13, 26, 27]. In P1, the positions of the two known mutations: 4 base pairs before the TIS (maternal) and in +238 (paternal) can explain her severe clinical phenotype of immune deficiency and bone dysplasia. Thiel et al. [10] suggested that insertions, duplications, and triplications within the conserved 24–26 bp between the TATA box and TIS, as in the maternal mutation, cause reduced *RMRP* transcription, due to binding impairment of the RNA polymerase III

transcription factor complex (TFIIIB), leading to a cellular phenotype of defects in rRNA cleavage and ribosome assembly and clinical phenotype of bone dysplasia [10]. The other mutation lies within the P4 region of *RMRP* which is responsible for the regulation of cell cycle progression at G2 through mRNA cleavage, leading to immune deficiency in the case of a mutation there [10, 11]. P2 harbors a novel homozygous regulatory mutation in position +222 which lies in the P1, P2 region of *RMRP* which is responsible for its rRNA cleavage activity and ribosome assembly, but not immune deficiency. A mutation in +220, two nucleotides upstream to our mutation, is associated with milder phenotypes [10]. Indeed, the clinical manifestations of milder bone dysplasia and a very mild immune deficiency of P2, compared to P1, correlates well with the genetic finding. Although TCR for P2 was diverse we have seen low TREC level on age of 1 year. We cannot assume the TREC results on the newborn period, but we can learn about the high sensitivity of the test regarding T-cell immunodeficiency. We can try explaining it with the mild restrictions found in the TCR-V β examination.

The therapy in CHH is focused on immunodeficiency. Most of the other available therapies for CHH patients are mainly palliative. Hematopoietic stem cell transplantation (HSCT) has profoundly changed the natural history of severe T-lymphocyte deficiency patients including SCID and other syndromes associated with severe T cell lymphopenia. SCID and other moderate T-lymphocyte dysfunction were shown to have excellent outcome rates. After HSCT patients have been reported to reach the age of 30 [14, 28, 29]. The current understanding is that not all CHH patients should be considered for HSCT but only those with chronic or recurrent infections and availability of a matched donor. Profound immunodeficiency associated with CHH can be cured by stem cell transplantation. Unfortunately, replacing hematopoietic stem cells does not seem to improve the skeletal dysplasia [23, 25]. In our report, P1 went through successful HSCT from a matched related donor and currently she is 3 years after the procedure, alive and well, not on any therapy for her immune system and without significant complications. In contrast, P2 was advised to continue regular follow up and to be treated symptomatically.

In summary, we describe herein two unrelated CHH patients with distinct clinical presentations and immune workup findings and different modes of diagnosis. In order to delineate the difference between these patients, which eventually led to different

treatment approaches, we included analyses of their TCR using advanced methodologies.

We showed how this could help to choose the appropriate treatment for each patient. Detection of patients through NBS program for severe T cell immunodeficiency can help in the decision-making process of treating newborns with immunodeficiency, as in our CHH case, benefiting the patient with early HSCT.

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None

Conflict of interest

The authors have no conflict of interest to declare.

Ethics approval

Patient information was obtained from the electronic medical record of our hospital. The guardians were interviewed, and the authors examined the patients. Informed consent was obtained, and all procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards.

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TABLE

Table. Immunoprofile at presentation.

	P1 (4wk)	P2 (16mo)	Normal range
Hemoglobin (g/dl)	7.06		11.5-13.5
Platelet count (K/microL)	416		130-440
Lymph count (cells/mm³)	870	2002	2900-11400/1400-12000
Lymphocytes subsets			
CD3 (cells/mm³)	87	1211	1900-8400/700-8800
CD4 (cells/mm³)	35	480	1500-6000/400-7200
CD8 (cells/mm³)	139	360	300-2700/200-2800
B cells (cells/mm³)	165	320	180-3500/160-3700
NK cells % (cells/mm³)	55	29	6-30
TREC	12	64	>400
Immunoglobulins			
IgG (mg/dl)	496	1100	590-1430
IgA (mg/dl)	undetectable	59	38-222
IgM (mg/dl)	24	105	56-208
Proliferation capacity[†]			Healthy donor
No mitogen	1381		1097
PHA 6 mcg/ml	2327		103158
PHA 25 mcg/ml	3822		135014
Anti-CD3 antibody	2379		36154

[†] Counts per minute of H3-thymidine uptake in response to mitogens.

(NK-Natural Killers, TREC-T cell receptor excision circle, PHA- Phytohemagglutinin)

FIGURES

Figure 1. TCR-V β repertoire analysis. Twenty-four TCR-V β families were detected and quantified using flow cytometry, performed on total lymphocytes obtained from two *RMRP* deficient patients. Levels of expression of the patient (black bars) were compared to normal controls values (clear bars) which were obtained from the IOTest Beta Mark- Quick Reference Card. values that are below or above 2 standard errors (SE) are marked with asterisk (*).

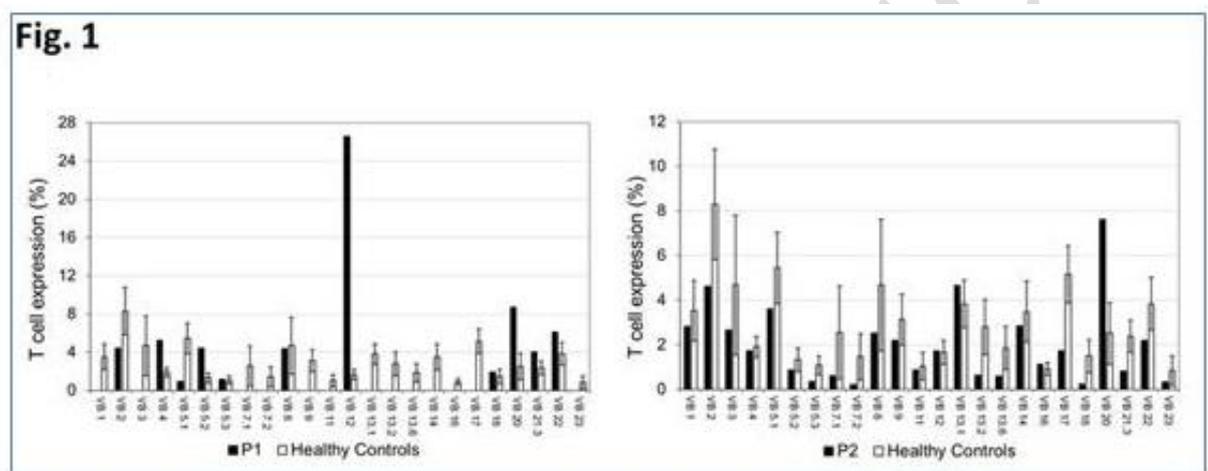


Figure 2. TRG repertoire determined by NGS. **A.** Treemaps representation of T cell receptor Gamma (*TRG*) repertoire in peripheral blood mononuclear cells (PBMCs) samples from patients with *RMRP* deficiency and one representative healthy control. Each square represents a unique V to J joining and the size of the square represents relative frequency within that sample. **B and C.** Quantification of the unevenness and the diversity the *TRG* repertoire using the Simpson-D index of unevenness (**B**) and the Shannon's H index of diversity (**C**) in four healthy controls and in patients with *RMRP* deficiency. Controls are the same that were used in previous studies [30].

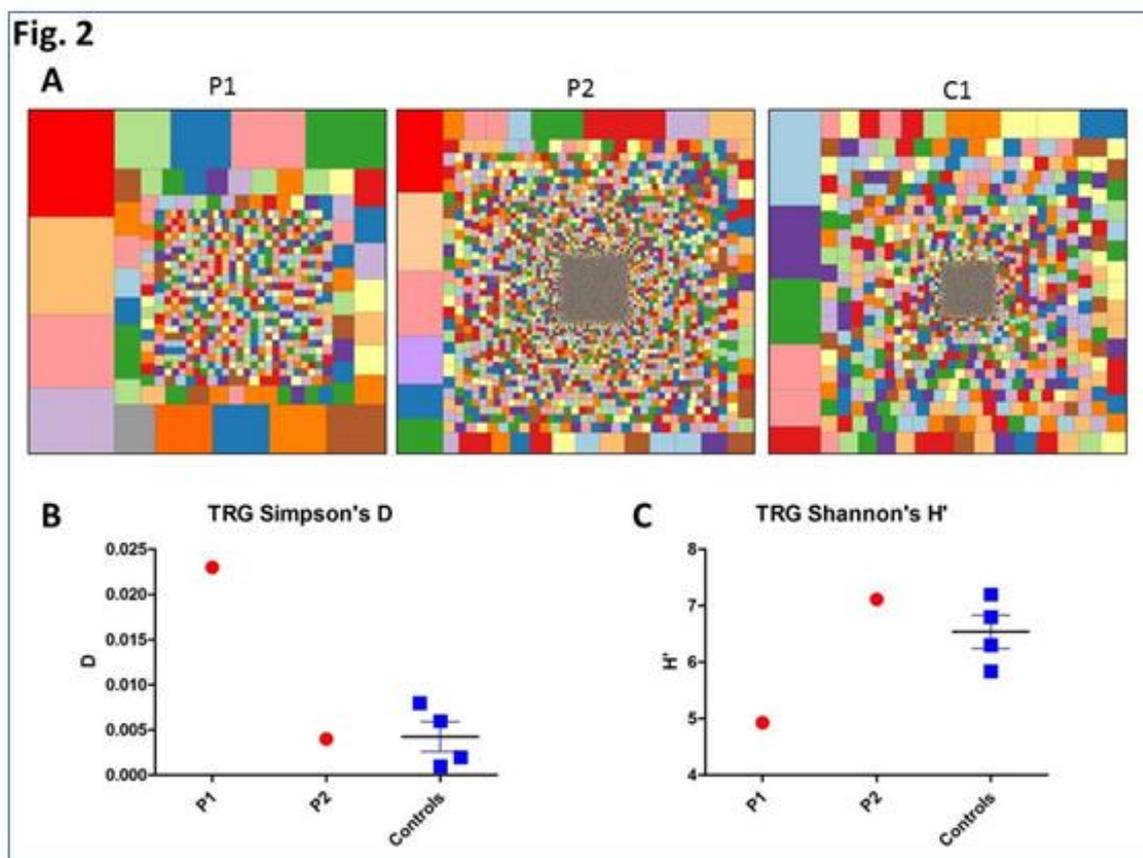


Figure 3. Sanger sequencing of the *RMRP* compound heterozygous variants in P1. A. The duplicated 17 nucleotides in the maternal allele are boxed. M=mother, F=father. **B.** The variant in the paternal allele is marked by an arrow.

Figure 3

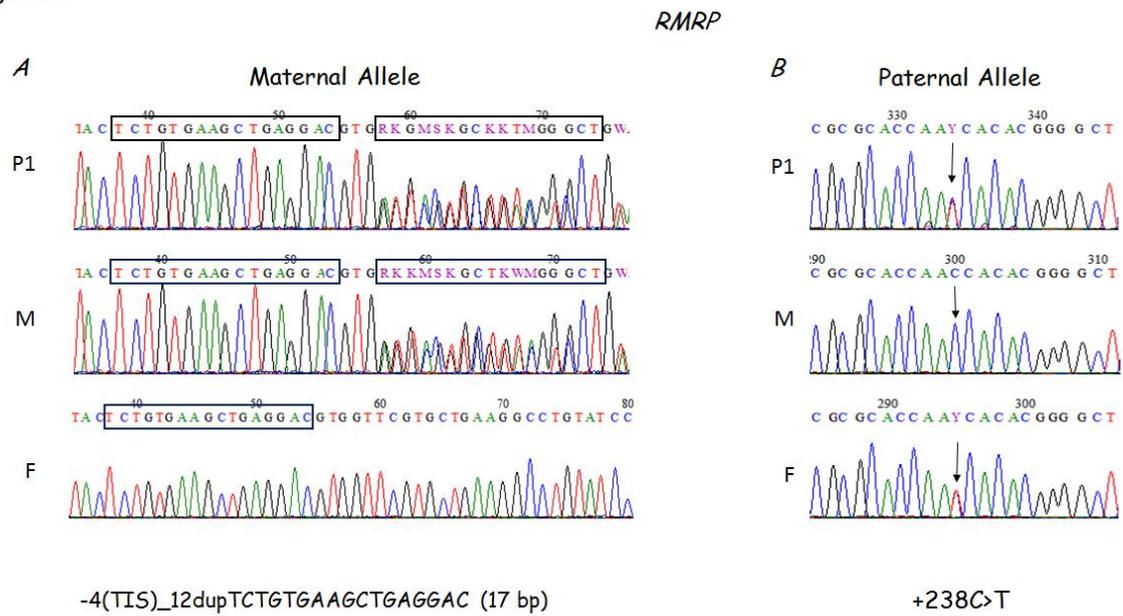


Figure 4. Sanger sequencing of the *RMRP* homozygous novel variant in P2. The variant is marked by an arrow.

