Application of aCGH Analysis in Patients with Primary Immunodeficiency of Unknown Genetic Origin – Identification of Atypical SAP Deficiency and Coronin-1a Deficiency

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Abstract

Objective: Patients with primary immunodeficiencies (PID) verified by clinical disease and standard immunological diagnostics but without a genetic diagnosis remain a challenge. Genetic diagnosis is important in order to plan follow-up and treatment possibilities including bone marrow transplantation and genetic counselling.

Methods: We used array comparative genomic hybridization (aCGH) analysis to investigate a cohort of pediatric patients with PID of unknown genetic origin in order to investigate the diagnostic yield of applying this technique to such patients and in an attempt to identify new possible genetic explanations for immunodeficiencies.

Results: Chromosomal imbalances were found in four of 14 investigated patients. In two cases a genetic diagnosis was established involving the SH2 domain-containing protein 1A gene (SH2D1A) and the Coronin-1a gene (CORO1A) causing atypical SLAM (signaling lymphocyte activation molecule)-associated protein (SAP) deficiency and Coronin-1a deficiency, respectively. Identification of the heterozygote deletion including the CORO1A gene by aCGH analysis led to sequencing of the other allele and identification of a novel point mutation. The clinical relevance of the deletions in the remaining two patients is not yet clarified but involves genes (the dedicator of cytokinesis 4 gene (DOCK4) and the protease, serine, 16 (thymus) gene (PRSS16) with suspected immunological functions.

Conclusion: Applying aCGH analysis in case of rare severe symptoms of immunodeficiency may be of great importance in order to establish a diagnosis and treat the patients appropriately. We identified a case of atypical SAP deficiency with no comparable cases in the literature, and a case of Coronin-1a deficiency of which only two families have previously been described.

Keywords: Array comparative genomic hybridization; Immunological deficiency syndromes; Duncan's disease SH2-protein, human; X-linked lymphoproliferative disorder; Coronin; CORO1A protein, human; Coronin, actin binding protein, 1A, human

Introduction

The use of novel genomic techniques, coupled with an increased knowledge of molecular mechanisms of the immune system, have resulted in the identification of several novel primary immunodeficiencies (PIDs), but still many clinical entities remain without a genetic diagnosis [1,2]. Patients with PID determined by molecular defects and clinical symptoms but without a genetic diagnosis remain a challenge in the clinical setting. A genetically based diagnosis is important in order to plan follow-up and treatment possibilities including possible bone marrow transplantation and genetic counseling [3-5].

We used array comparative genomic hybridization (aCGH) analysis to investigate a cohort of pediatric patients with PID of unknown genetic origin in order to ascertainment the diagnostic yield of applying the technique to this group of patients and to identify new possible genetic explanations for immunodeficiencies. Chromosomal imbalances were found in four of 14 investigated patients. In two cases a genetic diagnosis was established involving the SH2 domain-containing protein 1A (SH2D1A) and Coronin-1a (CORO1A) genes. The clinical relevance of the deletions in the remaining patients has yet to be fully elucidated but involves the dedicator of cytokinesis 4 gene (DOCK4) and the protease, serine, 16 (thymus) gene (PRSS16), both with suspected immunological functions.

Materials and Methods

Patients

From 1970 to 2011, 43 patients below 18 years of age with PID received allogeneic bone marrow transplants at the Department of
PEDIATRICS AND ADOLESCENT MEDICINE, RIGSHOSPITALET, COPENHAGEN, DENMARK. OF THESE, ELEVEN PATIENTS HAD PID OF UNKNOWN GENETIC ORIGIN. THERE WAS NO PRE-TRANSPLANTATION MATERIAL AVAILABLE FOR aCGH ANALYSIS IN THREE OF THESE PATIENTS. THE aCGH ANALYSIS WAS RETROSPECTIVELY APPLIED TO THE REMAINING EIGHT PATIENTS, ALONG WITH SIX NON-TRANSPLANTED PATIENTS WITH PID OF UNKNOWN ORIGIN FOLLOWED IN THE OUTPATIENT CLINIC AT THE END OF 2011. ALL PATIENTS UNDERWENT COMPREHENSIVE STANDARD QUANTITATIVE AND FUNCTIONAL IMMUNOLOGICAL TESTS AND WELL-KNOWN IMMUNODEFICIENCIES WERE EXCLUDED EITHER BY IMMUNOLOGICAL OR GENETIC TESTING, ACCORDING TO INTERNATIONAL PROTOCOLS [1,6].


Methods

Peripheral blood was obtained from the patients and DNA was isolated by NaCl extraction [7] or QIAGEN Genomic-tip 20 according to the manufacturer’s protocol (Qiagen, Hilden, Germany). The aCGH analysis was performed using the Agilent SurePrint G3 Human CGH Microarray kit 2 × 400K (Agilent Technologies, Santa Clara, CA, USA). Labeling and hybridization were performed according to the protocol provided by Agilent (Protocol v6.0, November 2008). Briefly, 1.5 μg of patient DNA and of a sex-matched control was double-digested with AluI and Rsal (Promega, Madison, WI, USA) for 2 hrs at 37°C. The digested DNA was labeled by random priming using the Agilent Genomic DNA Enzymatic Labeling Kit. Patient DNA and control DNA were labeled with Cy3-dUTP and Cy5-dUTP, respectively. Labeled products were purified by Amicon Ultra 30K filters (Millipore, Billerica, MA, USA). Patient and control DNAs were pooled and hybridized with 25 μg of Human Cot I DNA at 65°C with rotation for 40 hours. Washing was performed according to the Agilent protocol. Arrays were analyzed using an Agilent DNA Microarray scanner and the Agilent Feature Extraction software (v10.5). Results were presented on an Agilent Genomic Workbench (v. 5.0).

Real-time PCR was used for confirmation of aCGH findings and investigation of parental DNA. Primers were designed using Primer Express Version 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1). Amplification was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) was used for quantitative PCR according to the manufacturer’s instructions. Relative copy numbers were measured relative to GAPDH. DNA extracts from two healthy individuals were included as a negative control in each PCR run. Each assay was duplexed and evaluated by a comparative method validated by Applied Biosystems with the formula 2^ΔΔCt.

We further sequenced the coding regions (exon 2-11) and exon/intron boundaries of the CORO1A gene in all patients included and exon 37 of the DOCK4 gene in the family of case 4. Purified PCR products were sequenced using BigDye Terminator cycle sequencing kit v3.1 and the automated sequencer ABI PRISM 3130 DNA Analyzer, (Applied Biosystems, Foster City, CA, USA). The results were analyzed and interpreted using Mutation Surveyor (Softgenetics, LLC, PA, USA).

Results

The aCGH analysis led to an established genetic diagnosis in two patients

Case 1: Atypical SLAM (signaling lymphocyte activation molecule)-associated protein (SAP) deficiency: A 15 year old male patient presented with a 2-year history of weight loss, partial alopecia/grey hair (Figure 1a), hyperkeratotic exanthema (without eosinophilia in the skin biopsy) (Figure 1b), repeated cutaneous abscesses, nail abnormalities, hepatosplenomegaly, markedly reduced pulmonary function, and alveolitis with eosinophilia and lymphocytic infiltration on the lung biopsy. In the family history, there was a deceased maternal uncle at 1 and a half years of age of unknown cause.
Paraclinical peripheral blood samples showed elevated concentration of eosinophil leucocytes and lymphocytosis with increased concentrations of CD4 T-cells, CD8 T-cells, and B-cells, and normal concentration of NK cells (Table 2). Subpopulation analyses showed a decreased fraction of naive CD4 T-cells, increased fraction of memory CD4 T-cells, and slightly increased expression of HLA-DR on TCRαβ positive T-cells. The CD8 T-cells had increased expression of CD57 compared to CD8 T-cells from age matched controls. The polyclonal proliferative response of CD4 T-cells to pokeweed mitogen (PWM) and to CD3/CD28/CD2 stimulation was normal. There were decreased fractions of IgD-positive and isotype switched memory B-cells (Table 2). Tetanus vaccination titre was low, and the fraction of somatic hypermutation measured as Igk-REHMA was slightly decreased [8]. Immunoglobulin concentration, complement function, cystic fibrosis analysis, and reumatological markers were all normal, with a negative HIV test. The aCGH analysis showed a 5.3 Mb maternally derived deletion of chromosome Xq25 (arr [hg19] Xq25 (123,284,279-128,572,938) x 0 mat). This deletion encompasses 5 genes including the SH2D1A gene associated with X-linked lymphoproliferative disease type 1 (Duncan’s syndrome), Table 3. The SH2D1A gene encodes the protein SAP and flow cytometry of peripheral blood confirmed that the patient’s T-cells did not express SAP. The patient was Epstein-Barr virus (EBV) antibody and DNA negative. The patient never experienced lymphoproliferative disease, and hemophagocytosis was never proven. The patient was bone marrow transplanted one year after diagnosis with a matched unrelated donor. He was conditioned with treosulfan, fludarabine, and antithymocyte globulin. The patient was treated with methylprednisolone pulses pre-transplant, with improved forced expiratory volume in 1 second (FEV1) from 20% to 70%. He is almost two years from transplantation with improvement of skin, hair, and nail symptoms, normalization of lung function, no opportunistic infections, and no graft-versus-host disease.

Figure 1: Case 1. SAP deficiency.
Table 2: Immunologic features in patients. In brackets are age-matched normal values for lymphocyte counts according to Shearer et al. [27]. Normal values for recent thymic emigrants are from Mayo Medical Laboratories. Normal values for B-cell subpopulation are from Huck et al. [28]. Normal values for naive CD8 T cells are from Schatorje et al. [29]. Normal values for naive CD4 T cells T-proliferation and somatic hypermutation are established by the performing laboratory. Values in italic are adult reference values. * MACSi Beads from Miltenyi Biotec diluted 1:4.

Case 2: Coronin-1a deficiency: This male patient, now aged 10 years, had suffered from recurrent laryngitis, pneumonia and otitis media since 8 months of age and had been repeatedly treated for obstructive bronchitis over many years. His psycho-motor development and language skills were delayed.

The patient was lymphopenic with a markedly reduced concentration of CD4 T-cells, CD8 T-cells, and B-cells (his B-cell concentration normalized in later samples). The concentration of NK cells was normal. There was no maternal engraftment. Subpopulation analyses showed a severely reduced fraction of naive CD4 and CD8 T-cells, and reduced concentration of recent thymic emigrants, Table 2. The subpopulation profile was consistent from the first analysis at 2½ years of age to 8 years of age. The proliferative response of CD4 T-cells to PWM and to CD3/CD28/CD2 stimulation was markedly decreased. The immunoglobulins (IgM and IgG) were continuously decreasing.
with age, and were at last evaluation moderately decreased, but the frequency of somatic hypermutation was normal. Diphtheria and pneumococcus vaccination titers were comparatively low. EBV antibody and DNA was negative, as was HIV antibodies. No deficiencies in adenosine deaminase, purine nucleoside phosphorylase, common gamma chain, IL-7Ra, JAK3, CD3D or p56/Lck were identified.

The aCGH analysis revealed a 0.8 Mb duplication of chromosome 16p13.11 and a 0.6 Mb deletion of chromosome 16p11.2 (arr[hg19] 16p13.11(15,491,527-16,292,235) × 3, 16p11.2(29,652,999-30,199,351) × 1 mat). The 16p11.2 deletion includes the CORO1A gene (an actin regulatory gene expressed in hematopoietic cells). When sequencing the other allele, we detected a novel nucleotide substitution in exon 6, c.616C>T, p.R206C in the CORO1A gene. The amino acid changed is highly conserved and in silico evaluation show a large physicochemical difference between Arginine and Cysteine. The pedigree is shown in Figure 2. The 16p11.2 deletion was of maternal origin, and was shared by all children in the family. The novel mutation was only identified in the patient. Accordingly, no other family members suffered from clinically or para-clinically proven immunodeficiency. All evaluated family members had moderate intellectual disability. This is most likely associated with the 16p11.2 deletion and the 16p13.11 duplication as both are recurrent chromosomal imbalances reported in a number of patients with intellectual disability [9,10]. At the moment, the patient is well without recurrent infections but still with reduced relative wellness of the patient and the lack of a fully matched donor.

Chromosomal imbalances of uncertain clinical significance

**Case 3:** A seven year old boy was diagnosed with T-B-NK’ SCID at around 5 years of age following investigations for recurrent pulmonary infections (Table 2). His total IgG and pneumococcal vaccination titres were marginally reduced. The aCGH analysis detected a 0.2 Mb deletion of chromosome 6p22.1 and a 0.4 Mb duplication of chromosome 14q32.33 (arr[hg19] 6p22.1(27,172,130-27,367,948) × 1 pat, 14q32.33(105,603,450-106,009,354) × 3 dn). The 6p22.1 deletion includes a thymic-specific serine protease gene (PRSS16) which in mice is involved in the positive selection of some CD4 T-cells (Table 3) [11-13]. The deletion was paternally derived but the father displayed no clinical phenotype. None of the genes in the 14q32.33 duplication were suspected to be associated with PID.

![Pedigree of case 2 (Coronin-1a deficiency).](image)

**Table 3:** aCGH results.

<table>
<thead>
<tr>
<th>Patients</th>
<th>aCGH result</th>
<th>Genes included in deletion duplication *</th>
<th>Mutation in CORO1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Del Xq25</td>
<td>SH2D1A, LOC100129520, DCAF12L, DCAF12L1, ACTRT1</td>
<td>No</td>
</tr>
<tr>
<td>Case 2</td>
<td>Dup 16p13.11</td>
<td>MPV17L, C16orf45, KIAA0430, NDE1, MIR484, MYH11, FOPN1L, ABCC1, ABCB5</td>
<td>c.616C&gt;T, p.R206C</td>
</tr>
<tr>
<td>Case 3</td>
<td>Del 6p22.1</td>
<td>PRSS16, VNYR1R10P, ZNF391</td>
<td>No</td>
</tr>
<tr>
<td>Case 4</td>
<td>Del 7q31.1</td>
<td>DOCK4</td>
<td>No</td>
</tr>
</tbody>
</table>

* According to Refseq genes UCSC Genome Browser hg19.

**Case 4:** The aCGH analysis detected a 5 Kb intragenic deletion of the DOCK4 gene at chromosome 7q31.1 (arr[hg19] 7q31.1(111,423,909-111,428,559) × 1 pat), Table 3. This patient had recurrent skin infections (warts and abscesses) and severe exanthema since early childhood. He had normal concentrations of CD4 and CD8 T-cells. His B cell concentration was initially within the normal range but had decreased to subnormal levels over a period of two years. His NK cell concentration was persistently reduced. Subpopulation analysis showed a normal naive/memory ratio of CD4 and CD8 T-cells. The proliferation response of CD4 T-cells to PWM and to CD3/CD28 stimulation was normal (Table 2). He had eosinophilia and markedly increased immunoglobulin E (around 20,000 × 103 U/L). His other immunoglobulins were normal to slightly increased. He had no DOCK8 or STAT3 mutations, despite the apparent resemblance between this patient and patients suffering from hyper IgE syndromes.
14 pediatric patients investigated the genetic diagnosis was established by aCGH analysis. Identification of a heterozygote deletion including the CORO1A gene in one allele was found in combination with a 2 bp deletion in CORO1A exon 3, c.248-249delCT, resulting in a frame shift and premature stop codon (p.Pro83ArgfsX10) in the opposite allele. In this patient psycho-motor development was also impaired and she was successfully treated with HCT for her immunodeficiency [23,24]. In another family, three siblings with hypomorph Coronin-1a deficiency were found. They were homozygous for a missense mutation (c.717G>A, p.V134M) that caused severely reduced expression of Coronin-1a, and presented with EBV-associated B-cell lymphoproliferation at an early age. Two of the patients died (one during the induction therapy, the other of graft versus host disease after stem cell transplantation). The third patient recovered and is in complete remission with few infections at the age of 11½ years [22]. Of note, our patient is still EBV negative and is at the moment well, without infections on supportive treatment, but he may be susceptible for EBV-induced lymphoproliferation when he encounters EBV. However, bone marrow transplantation is at the moment being postponed because of his general wellbeing and lack of a fully matched donor. Time will show the nature of this relative newly discovered PID under these awaiting circumstances.

Coronin-1A is one of seven mammalian coronin family members regulating the act cytoskeletal network involved in cell motility. CORO1A is preferentially expressed in cells of hematopoietic origin, for example neutrophils, macrophages and activated T-cells. In murine Coro1A knockout models, T-cell migration from the thymus is impaired leading to a deficient peripheral T-cell compartment. Furthermore, T-cell homing to secondary lymphocyte organs and T-cell survival also seems to be decreased in Coro1A knockouts. T-cell receptor function, though, appears not to be affected by mutation of the Coro1A gene [23,25]. To conclude, aCGH analysis is a valuable tool in the diagnosis of patients with PID of unknown genetic origin. The aCGH analysis may provide insight into new possible genetic mechanisms involved in the pathogenesis of immunodeficiencies. Further investigation of patients with PID of unknown genetic origin by whole genome sequencing modalities may be the next step towards improved understanding of the etiology of these diseases and once identified, may aid in developing effective therapies [26].

Conflict of Interest

The authors have no financial or commercial conflicts of interest to be disclosed.

References

4. Gennery AR, Slatter MA, Grandin L, Taupin P, Cant AJ, et al. (2010) Transplantation of hematopoietic stem cells and long-term survival for [14,15]. The DOCK4 deletion was paternally derived, but the father displayed no clinical phenotype; however the same deletion was found in a sister of the patient who suffered from the same symptoms as the patient and died of rapidly progressing T-cell lymphoma as a young teenager. One may speculate that the patient and his sister have a mutation on the other allele derived from the mother. Whole exome sequencing (Medical Genetics Laboratory, Baylor College of Medicine, Houston, US) were performed on DNA from the patient, and a novel heterozygote nucleotide substitution, c.3830G>A, p.R1277Q in exon 3 of the DOCK4 gene was identified. The variant change a highly conserved nucleotide and a moderately conserved amino acid. In silico evaluation show a small physiochemical difference between Arginine and Glutamine and results from different prediction tools were from tolerated to disease causing. We investigated parents and siblings in the family using Sanger sequencing. The mutation was inherited from the mother and was additionally identified in a healthy sister but not in the deceased sister also harboring the DOCK4 deletion. Further, microsatellite analyses of D7S523at 7q31.1 in the DOCK4 locus indicate that the proband and the deceased sister had not inherited the same maternal allele at that position. Thus, lack of segregation of the disease and the combination of the deletion and the mutation makes an association unlikely. Mutations in the DOCK4 gene in humans have been associated with myeloid leukaemias and dyslexia [16-19], while in vitro DOCK4 knockout model in bone marrow CD34+ stem cells led to decreased hematopoiesis [17].

Discussion

We applied aCGH analysis in a cohort of pediatric patients with PID of unknown molecular-genetic origin. Application of aCGH analysis in this clinical setting was of a valuable clinical utility. Out of 14 pediatric patients investigated the genetic diagnosis was established in two patients and in another two patients possible candidate genes were identified.

The patient (case 1) with SAP deficiency did not present with symptoms of Duncan’s syndrome. He was EBV naive and he mainly presented with a variety of severe clinical symptoms from the ectodermal derived organs and lungs. The immunological investigations in this patient indicated underlying dysgammaglobulinemia i.e. decreased fractions of isotype switched memory B-cells, low vaccination titres to T-cell dependent antigens (e.g. Tetanus), and a slightly decreased fraction of somatic hypermutations. Dysgammaglobulinemia is a common characteristic in X-linked lymphoproliferative disease occurring in EBV-positive as well as in EBV-negative patients [20], where it is believed that SAP is required in CD4 T-cells to promote the full differentiation of B-cells [21]. All symptoms resolved after bone marrow transplantation. According to the UCSC Genome Browser (hg19) six genes, in addition to SH2D1A, were included in the deletion. So far none of these genes have been associated with disease or with function in the immune system, yet it remains a possibility that the patient’s ectodermal and pulmonary symptoms are related to the deletion. There are no comparable cases in the literature. Applying aCGH analysis in cases of rare severe symptoms may be of great exploratory value in the diagnosis and appropriate treatment of these patients.

In case 2, Coronin-1A deficiency was also suspected by findings of the aCGH analysis. Identification of a heterozygote deletion including the CORO1A gene by aCGH analysis led to sequencing of the other allele and identification of a novel point mutation. Two families with Coronin-1a deficiency have previously been described [22-24]. These patients had a severe deficiency of naive CD4 T-cells and normal concentrations of B and NK cells. In one patient deletion of 16p11.2 involving the CORO1A gene in one allele was found in combination with a 2 bp deletion in CORO1A exon 3, c.248-249delCT, resulting in a frame shift and premature stop codon (p.Pro83ArgfsX10) in the opposite allele. In this patient psycho-motor development was also impaired and she was successfully treated with HCT for her immunodeficiency [23,24]. In another family, three siblings with hypomorph Coronin-1a deficiency were found. They were homozygous for a missense mutation (c.717G>A, p.V134M) that caused severely reduced expression of Coronin-1a, and presented with EBV-associated B-cell lymphoproliferation at an early age. Two of the patients died (one during the induction therapy, the other of graft versus host disease after stem cell transplantation). The third patient recovered and is in complete remission with few infections at the age of 11½ years [22]. Of note, our patient is still EBV negative and is at the moment well, without infections on supportive treatment, but he may be susceptible for EBV-induced lymphoproliferation when he encounters EBV. However, bone marrow transplantation is at the moment being postponed because of his general wellbeing and lack of a fully matched donor. Time will show the nature of this relative newly discovered PID under these awaiting circumstances.

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To conclude, aCGH analysis is a valuable tool in the diagnosis of patients with PID of unknown genetic origin. The aCGH analysis may provide insight into new possible genetic mechanisms involved in the pathogenesis of immunodeficiencies. Further investigation of patients with PID of unknown genetic origin by whole genome sequencing modalities may be the next step towards improved understanding of the etiology of these diseases and once identified, may aid in developing effective therapies [26].
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