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Original article

Pair analysis and custom array CGH can detect a small copy number variation in *COQ6* gene

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Abstract

Background Recently, comprehensive genetic approaches for steroid-resistant nephrotic syndrome (SRNS) using next-generation sequencing (NGS) have been established, but causative gene mutations could not be detected in almost 70% of SRNS patients. Main reason for the low variant detection rate is that most of them are SRNS caused not by genetic but by immunological factors. But some of them are probably because of the difficulty of detecting copy number variations (CNVs) in causative genes by NGS.

Methods In this study, we performed two novel analytical methods of NGS data-dependent pair analysis and custom array comparative genomic hybridization (aCGH) in addition to NGS analysis in an infantile nephrotic syndrome case.

Results We detected only one known pathogenic heterozygous missense mutation in exon 7 of *COQ6* (c.782C>T, p.(Pro261Leu) by NGS. With pair analysis, heterozygous exon 1-2 deletion was suspected and was confirmed by custom aCGH. As a result, a small CNV was successfully detected in the *COQ6* gene. Because we could detect variants in *COQ6* and could start treatment by Coenzyme Q10 (CoQ10) in his very early stage of SRNS, the patient achieved complete remission.

Conclusions These novel methods should be adopted in cases with negative results in gene tests by NGS analysis. Especially, in cases with CoQ10 deficiency, it is possible to delay initiating dialysis by starting treatment at their early stages.

Keywords

COQ6, CoQ10 glomerulopathy, copy number variation, pair analysis, custom array CGH

Introduction

Comprehensive genetic analysis using next-generation sequencing (NGS) of causative genes for steroid-resistant nephrotic syndrome (SRNS) has been widely established globally [1-3]. It enables us to treat certain monogenic SRNS patients with specific therapies, such as coenzyme Q10 (CoQ10) supplementation for patients with CoQ10 deficiency-related gene variants, including *PDSS2*, *COQ2*, *COQ6*, and *ADCK4*, exhibiting so-called CoQ10 glomerulopathies [4].

CoQ10 is a ubiquitous lipophilic substance that is biosynthesized in mitochondria and plays essential roles in adenosine triphosphate production through the mitochondrial respiratory chain, beta-oxidation of fatty acids, and pyrimidine biosynthesis [5]. Another important role of CoQ10 is modulating cellular oxidant stress. At least 15 genes have been reported to be causative of CoQ10-related diseases [6]. Among these, some genes have also recently been reported to be causative of SRNS [7]. These include *COQ2* variants, which were detected for the first time in CoQ10 glomerulopathies [8]. The main extra-renal symptoms were nervous system involvement such as encephalopathy and myopathy. Nephropathy was also reported, usually occurs at an age of under one year old, and progresses to end-stage renal disease [9]. *ADCK4* variants were also reported as being causative of CoQ10 glomerulopathies, but extra-renal symptoms are seldom reported in such cases [10]. The onset of nephrotic syndrome

is usually in adolescence [11]. Moreover, in the Asian population with SRNS, it was reported that the rate of patients having *ADCK4* gene variants seemed to be higher than that found elsewhere [2].

COQ6 mutations have also been reported to be causative of diseases in some patients with inherited nephrotic syndrome; these patients usually exhibit hearing loss [7]. In these patients, proteinuria occurs before the age of 6 years old and rapidly develops to end-stage renal disease before 10 years old [12].

CoQ10 glomerulopathies show massive proteinuria, for which immunosuppressant drugs including steroids are not usually effective; most of these patients progress to end-stage renal disease. Recently, CoQ10 has been reported to be an effective therapy for patients with CoQ10 glomerulopathies [12, 10]. Thus, there is currently a growing need for a comprehensive gene screening system for screening genes associated with CoQ10 synthesis.

Comprehensive NGS analysis has revealed that about 30% of SRNS patients possess a monogenic gene variant in genes causative of SRNS; however, comprehensive NGS analysis has the disadvantage of it having difficulty detecting copy number variations (CNVs).

CNVs are present throughout the human genome and are considered to be a significant source of human genetic variation. They have also been reported to cause diseases [13]. To detect CNVs, karyotype analysis for chromosome size variations or in situ hybridization for specific

gene variations have conventionally been performed. Recently, array comparative genomic hybridization (aCGH) for detecting genome-wide CNVs or multiplex ligation and probe amplification (MLPA) for detecting specific genes has been conducted [14]; however, commercial aCGH kits that can detect CNVs expanded over more than 2–5 kbp and commercial MLPA kits are only available for a limited number of genes.

NGS-data-based CNV analysis has been reported to be an effective method for detecting CNVs; its sensitivity was 92% and specificity was 100% to detect deletions as small as 180 bp and duplications of more than 300 bp in specific targeted gene panels [15]. We also recently published data about the effectiveness of NGS-data-based CNV analysis for inherited kidney diseases [16]. Moreover, custom aCGH has been reported to be effective for detecting CNVs [17]. The main differences between custom aCGH and original whole-genome aCGH are as follows. First, with custom aCGH, we can analyze target regions including the candidate genes. Second, we can set probes more densely, every 100 bp, in targeted regions, which would be expected to result in higher sensitivity and specificity to detect CNVs by custom aCGH than by original aCGH.

In this study, we conducted NGS analysis on an infantile SRNS patient; moreover, to detect any CNV in this subject, we performed NGS-data-based pair analysis and custom aCGH. Based

on the results obtained by these analyses, we also initiated CoQ10 treatment in this case and assessed its efficacy.

Materials and methods

Genetic analysis

A list of all of the genes screened for causative of SRNS in this study is shown in Supplementary Table S1.

Genomic DNA was extracted from peripheral blood leukocytes of the patient and his parents with the QuickGene Mini 80 system (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), in accordance with the kit's protocol. The patient sample was prepared for targeted sequencing with a HaloPlex target enrichment system kit (Agilent Technologies, Santa Clara, CA), in accordance with the kit's protocol. Approximately 225 ng of genomic DNA was extracted, subjected to a restricted reaction for fragmentation, and hybridized with NGS probes at 54 °C for 16 h.

Subsequently, the patient's genomic DNA was amplified by polymerase chain reaction and sequenced using the MiSeq platform (Illumina, San Diego, CA). After completing the NGS analysis, we analyzed the data with SureCall 4.0, by which we can combine the data with the algorithms of alignment NGS data analysis (Agilent Technologies).

Pair analysis

We conducted the NGS-data-based CNV analysis, in other words, the pair analysis, using SureCall. This pair analysis involves five steps as follows. First, the read depth is calculated for each base pair of targeted regions using the NGS analysis data. Second, the targeted region is divided into 125-base-pair fragments. Third, the average read depth of each fragment is calculated. Fourth, the read depths are normalized with the total read depths and the sample specimen is compared with the reference one. As references, we selected control samples that had been analyzed for gene mutations using the same protocol of NGS, in which disease-causing variants other than those in the targeted regions were detected, and for which there was a small possibility of CNVs occurring at the targeted regions. Fifth, $\log_2[\text{sample read depth (normalized)} / \text{reference read depth (normalized)}]$ was calculated. Deletion sites have scores of about -1.0 for this, while duplication sites have scores of about 1.58 , in autosomal genes.

Custom aCGH

Before we conducted custom aCGH, we selected genes for analysis. Here, we selected the *COQ6* gene and constructed probes for it and the regions surrounding it. We used a custom HD-CGH Microarray, 8X15K (Agilent Technologies), in accordance with the kit's protocol.

Using 1 µg of genomic DNA from a reference human control (Promega) and from the patient, we obtained fragments by heating at 95°C for 10 min.

Next, we used the SureTag Complete Labeling Kit (Agilent Technologies) to label fragmented DNA of the reference sample with Cy3 and of the patient sample with Cy5 fluorescent dUTP.

Next, we conducted purification of the samples and removed the unincorporated nucleotides and dyes using purification columns (Agilent). After purification, we obtained the labeled fragmented samples along with human Cot-1 DNA and hybridized them on array slides.

Labeled samples were hybridized to custom CGH arrays with heating at 65°C and 20 rpm for 24 h in a hybridization oven. The slides were scanned by Agilent Microarray Scanner System (Agilent Technologies) at 3-µm resolution. We used Agilent CytoGenomics software (Agilent Technologies) to analyze chromosomal patterns within the microarray profiles.

Semi-quantitative polymerase chain reaction (PCR)

We conducted semi-quantitative PCR for detecting a large deletion of exon 1 in *COQ6* on the paternal allele using capillary electrophoresis (2100 Bioanalyzer; Agilent Technologies). The concentration of genomic DNA was approximately 50 ng/µL, and PCR was performed with primers covering the regions of exon 1 and exons 6–7 in *COQ6* and exon 12 in NUP107 as an

internal control. The quantity of PCR products was calculated using the Agilent DNA1000 kit (Agilent Technologies).

Results

Patient

The patient was a one-year-old male. After birth, he was diagnosed with tetralogy of Fallot. When he was admitted for intracardiac repair at 10 months old, severe proteinuria and hypoalbuminemia (urine protein creatinine ratio of 50 g/gCr and serum albumin of 1.2 g/dL) were detected. He was diagnosed with infantile nephrotic syndrome, for which treatment with steroid was initiated. However, the therapy was not effective and he was diagnosed with SRNS, after which the steroid was tapered and angiotensin-converting enzyme inhibitor (ACE-I) and angiotensin 2 receptor blocker (ARB) were started. At the same time, we decided to conduct a genetic test on the patient. He also underwent auditory brain-stem response (ABR) screening and was shown to have no hearing loss at one year old.

Pathological findings

The patient underwent a kidney biopsy; images of the obtained pathological findings are shown in Figure 1. On light microscopy, the glomeruli showed no remarkable changes, while the

results of immunofluorescence staining were all negative. However, electron microscopic examination revealed abnormal proliferation of dysmorphic mitochondria in podocytes, diffuse foot process effacement, and microvillous transformation of podocytes (Fig. 1).

Genetic analysis

Targeted sequencing with NGS showed one known pathogenic heterozygous missense mutation in exon 7 of *COQ6* [c.782C>T, p.(Pro261Leu)] [18]. We also conducted Sanger sequencing and detected the same mutation in both the patient and his mother (Supplemental Fig. 1).

Pair analysis

Next, we conducted pair analysis using the SureCall application. The log ratio of exon 1 in *COQ6* was -1.465 and that of exon 2 in *COQ6* was -1.679 (Supplemental Fig. 2). We suspected that the patient had a large heterozygous deletion including exons 1 and 2 of *COQ6*.

Custom aCGH

By employing custom aCGH, a large heterozygous deletion including the first half of the *COQ6* gene was successfully detected (Figure 2).

Semi-quantitative PCR

The results of semi-quantitative PCR showed that, in their genomic DNA, the patient and his father had about half the level of exon 1 of *COQ6* compared with the levels in the patient's mother and the control. However, the levels of exons 6 and 7 in *COQ6* were the same (Supplemental Fig. 3). These results indicated that the patient had a compound heterozygous mutation involving deletion of the paternal allele and a known pathogenic missense mutation in the maternal allele.

Effect of CoQ10 supplementation

After detecting the *COQ6* gene defect, the patient was started on CoQ10 supplementation in addition to ACE-I/ARB. As a result, his proteinuria was gradually alleviated and finally achieved a negative state. His serum albumin level was also increased (Fig. 3).

Discussion

Recently, more than 40 disease-causing genes of SRNS have been reported. NGS analysis has also recently become a standard approach for gene screening. However, NGS cannot detect CNVs, so another approach is needed for their detection. In this study, we conducted NGS, pair

analysis, and custom aCGH, enabling the detection of two disease-causing variants, including a large heterozygous deletion in the *COQ6* gene, in an infantile SRNS patient.

The use of the comprehensive screening system including pair analysis for SRNS patients enabled us to detect CoQ10 glomerulopathy-related genes at early disease stages [12]. In a previous report, it was suggested that early initiation of CoQ10 supplementation is important to prevent progression to end-stage renal disease and worsening neurological involvements in diseases involving CoQ10 deficiency [19]. In line with this, in our patient, CoQ10 was extremely effective because it was started at quite an early stage in the clinical course, at which point there was no hearing loss or glomerular sclerosis.

In a previous report, it was described that all cases with *COQ6* defects showed hearing loss and some showed epilepsy of mental retardation [18]. These extra-renal symptoms provide a clue for the diagnosis of CoQ10 glomerulonephropathies. However, it should be noted that cases with *ADCK4* variants do not show extra-renal symptoms.

In pathological examinations, the proliferation of abnormally shaped mitochondria in the cytoplasm of podocytes was consistent with the findings in a previous report on CoQ10 glomerulopathies related to *COQ6* mutation [18], providing a pathological clue for diagnosing this disease.

To improve our comprehensive screening system by adding effective assays such as pair analysis or custom aCGH, we can achieve a high rate of detection of causative genes in SRNS cases. Furthermore, the identification of CoQ10 glomerulopathy at an early stage can improve the prognosis of these serious disorders.

Conclusions

The use of a comprehensive gene screening system with NGS was effective to detect causative gene variants in SRNS. In addition, pair analysis and custom aCGH were remarkably useful to detect CNVs. Specific therapy of CoQ10 for the patient carrying *COQ6* mutations was very effective when started at an early stage, so it is important not to miss the opportunity to initiate supplementation at an early stage in cases with CoQ10 glomerulopathies.

Compliance with ethical standards

Conflict of interest statement

The authors have nothing to disclose.

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Ethical approval

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine.

Informed consent

Informed consent was obtained from the parents.

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Figure Legends

Figure 1 Electron microscopy images of the patient

- a. In the podocytes, the abnormal proliferation of swollen mitochondria was observed.
- b. Foot process of podocytes showed diffuse effacement. Microvillous transformation was prominent.

Figure 2 Custom aCGH results of the patient

We conducted custom aCGH in the patient using primers for a region including the *COQ6* gene.

We detected a large deletion, including part of the *COQ6* gene. The deleted area extended from exon 1 to the middle of the *COQ6* gene.

Figure 3 Clinical course of the patient after treatment with coenzyme Q10 (CoQ10)

After starting CoQ10 supplementation, the proteinuria remarkably decreased and finally disappeared. The patient's serum albumin level gradually increased to the normal range.

UPCR: urine protein creatinine ratio

ACE-I: angiotensin-converting enzyme inhibitor

ARB: angiotensin receptor blocker

Figure 1

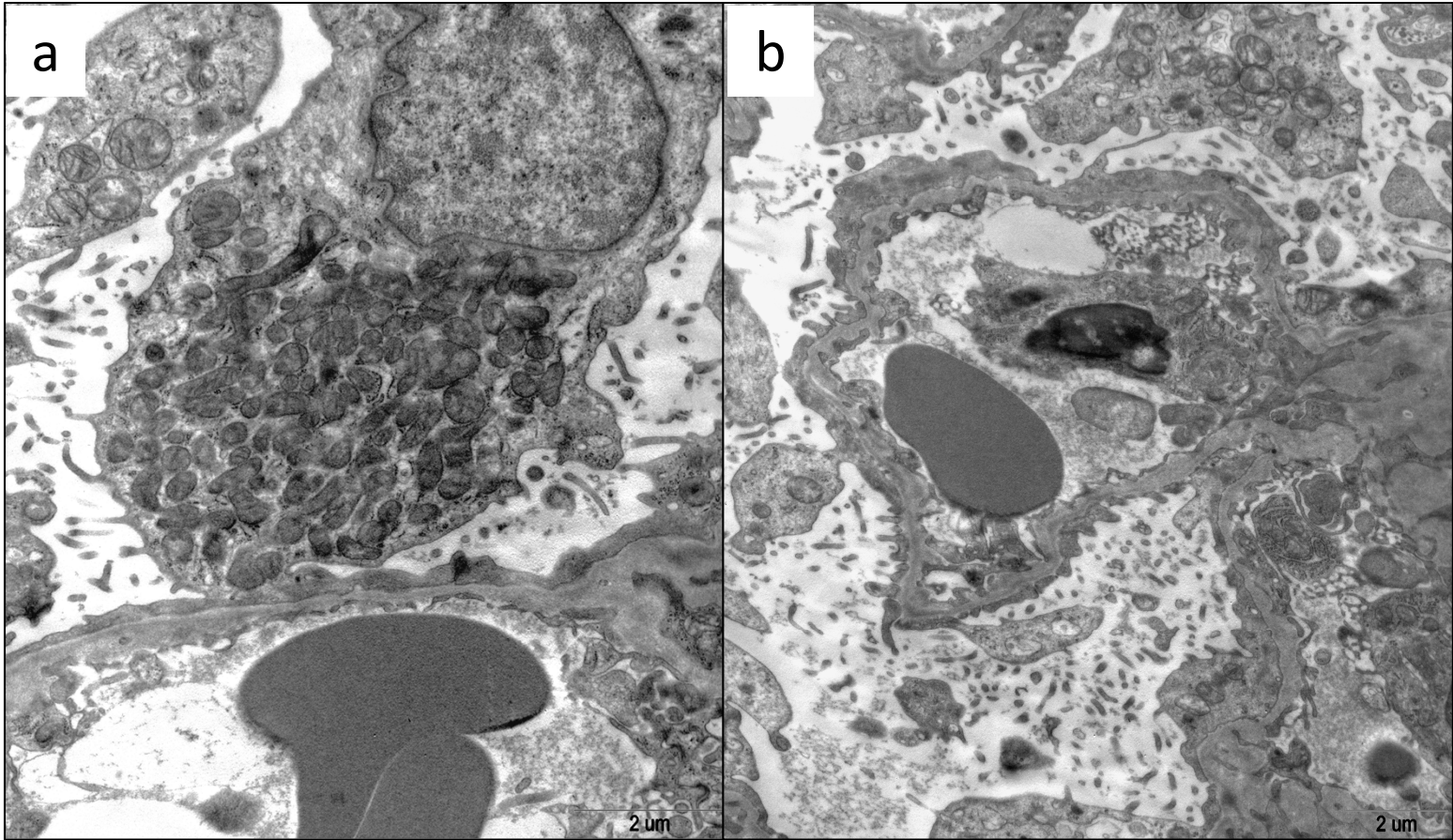


Figure 2

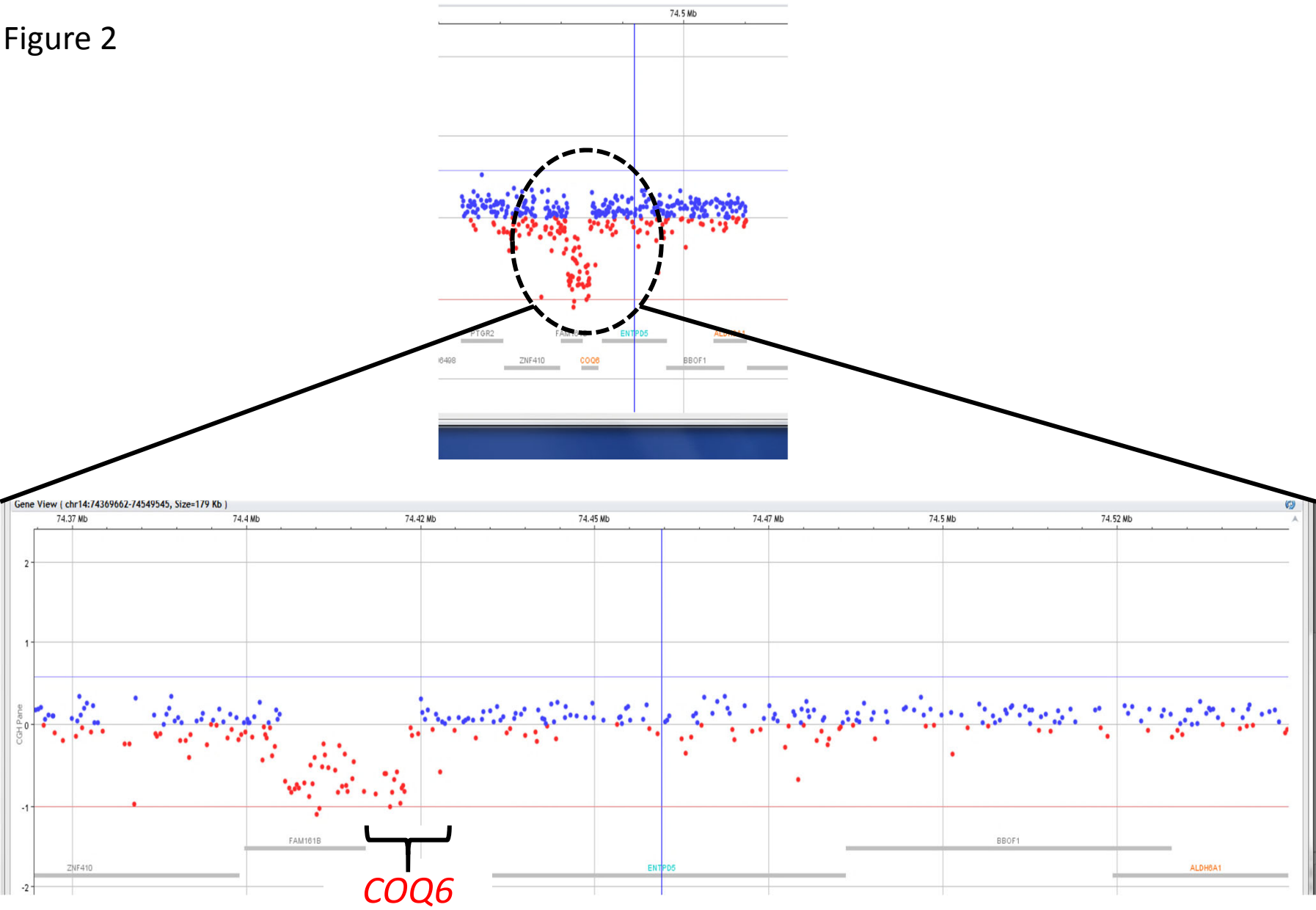
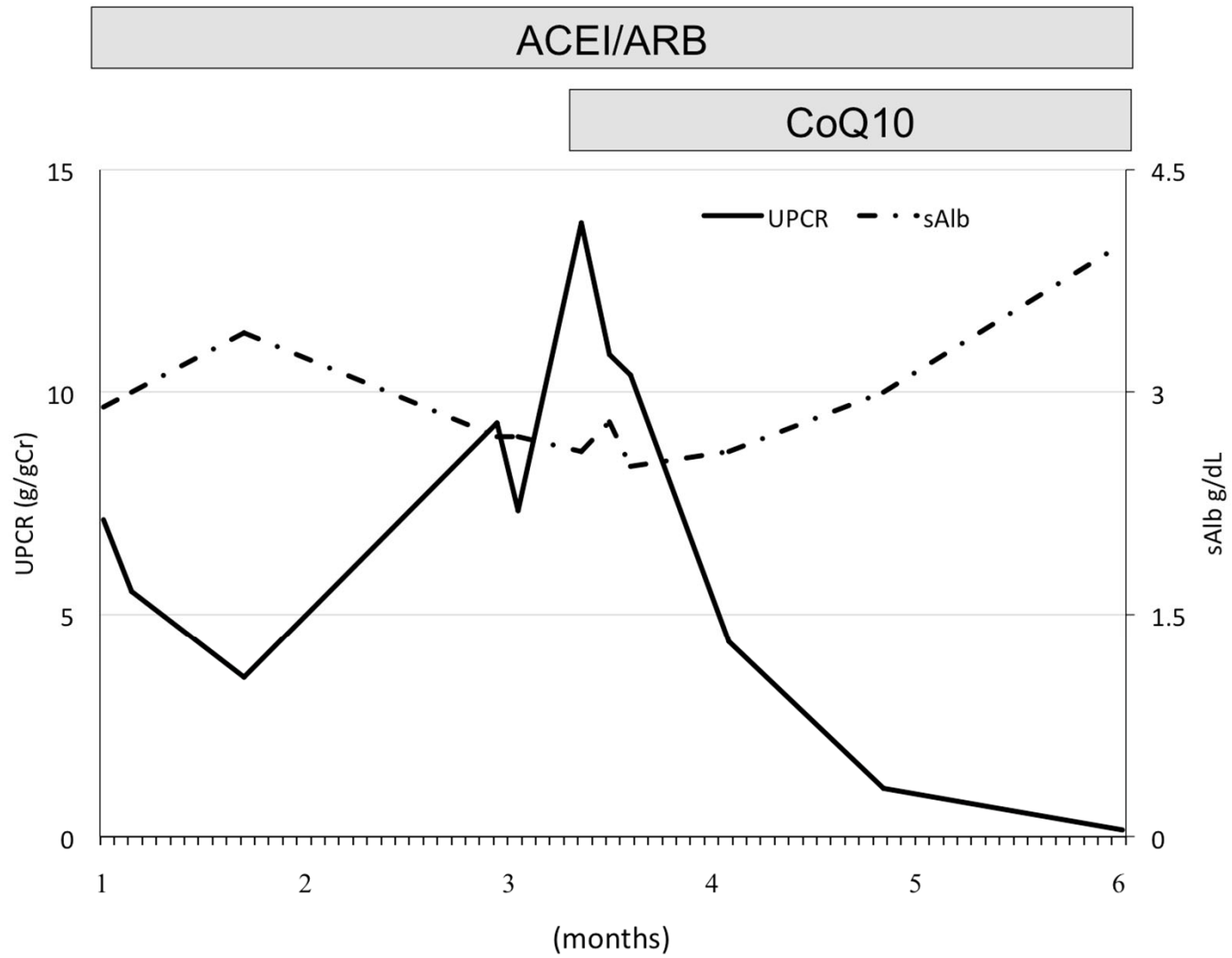


Figure 3



Supplementary Data



Supplementary Figure 1. Sequence results

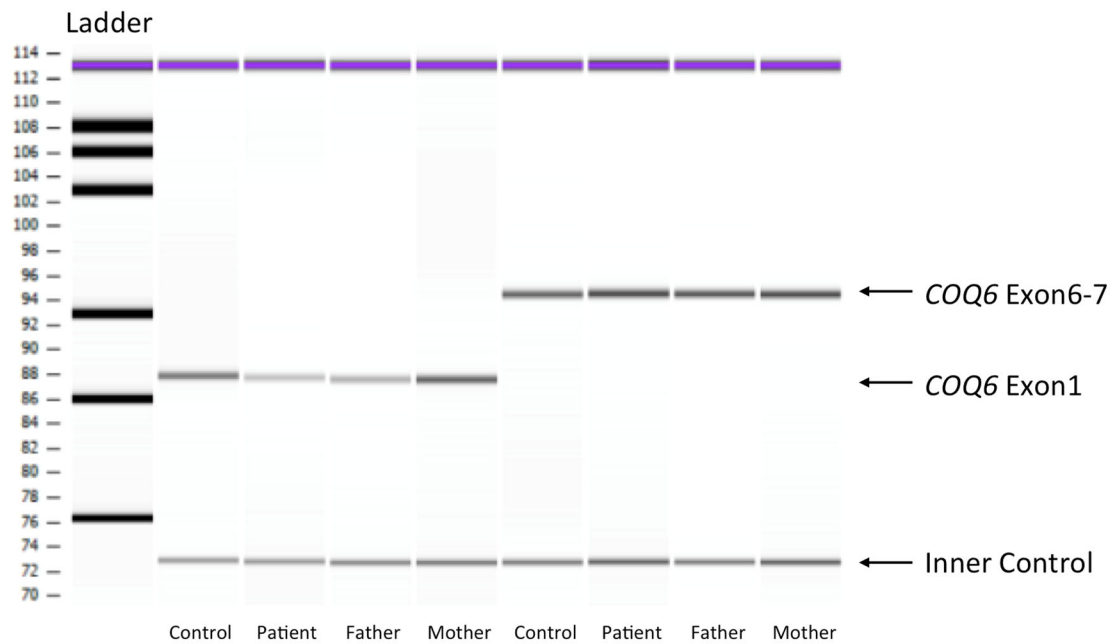
In the sequences of both the patient and his mother, a *COQ6* gene (NM_0182476.2) (c.782 C>T) [p.(Pro261Leu)] mutation was detected. This mutation has previously been reported.

Aberration	Read Depth	Score	Log Ratio	Gene
Deletion	49	1.0	-1.052	FAM161B, <i>COQ6</i>
Deletion	17	1.0	-1.465	<i>COQ6</i>
Deletion	35	1.0	-1.679	<i>COQ6</i>

} *COQ6* exon1-2 deletion

Supplementary Figure 2. Pair analysis results of the patient using targeted sequence data

Upon calculation with the SureCal application, calling of exons 1 and 2 in the *COQ6* gene was performed. The log ratio of the region was under -1 . We thus suspected that, in this region, the patient had one allele.



Supplementary Figure 3. Semi-qPCR results of the patient

Semi-qPCR showed that the bands of the patient and his father representing *COQ6* exon 1 were weaker than the other bands. The quantitative ratios (relative to the internal control) were 0.49 (the patient) and 0.49 (his father), while for almost all of the other bands, the quantitative ratio was almost 1.0. Thus, both the patient and his father had a large heterozygous deletion in exon 1 of *COQ6*.

Supplemental Table S1 Gene list for the targeted sequencing

<i>ACTN4</i>	<i>COQ2</i>	<i>INF2</i>	<i>MYO1E</i>	<i>SCARB2</i>
<i>ADCK4</i>	<i>COQ6</i>	<i>ITGA3</i>	<i>NPHS1</i>	<i>SMARCAL1</i>
<i>ANLN</i>	<i>CRB2</i>	<i>ITGB4</i>	<i>NPHS2</i>	<i>TRPC6</i>
<i>ARHGAP24</i>	<i>CUBN</i>	<i>KANK1</i>	<i>NUP107</i>	<i>TTC21B</i>
<i>ARHGDIA</i>	<i>DGKE</i>	<i>KANK2</i>	<i>NUP205</i>	<i>UMOD</i>
<i>CD2AP</i>	<i>EMP2</i>	<i>KANK4</i>	<i>NUP93</i>	<i>WDR73</i>
<i>COL4A3</i>	<i>FAT1</i>	<i>LAMB2</i>	<i>PAX2</i>	<i>WT1</i>
<i>COL4A4</i>	<i>GLEPP1</i>	<i>LMX1B</i>	<i>PDSS2</i>	<i>XPO5</i>
<i>COL4A5</i>	<i>HNF1B</i>	<i>MYH9</i>	<i>PLCE1</i>	
