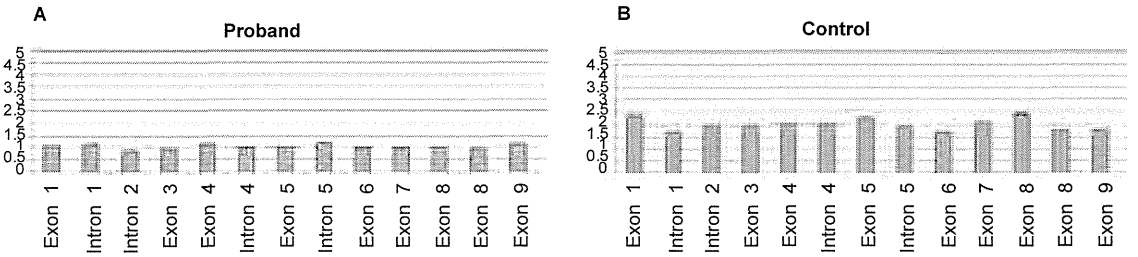


Figure 5. Results of MLPA analysis of *PAX3* in family 2. (A, B) Relative ratios of DNA quantity in each exon compared with that in the control region are shown for the proband (A) and control (B).

missense mutation may be a rare normal variant. Thus, the pathogenicity of such mutations needs to be verified by detection of the same mutation in multiple families with the same phenotype or by functional analysis. The functional consequences of a few *PAX3* mutations have been tested and reduced DNA-binding properties have been reported [13–15]. The p.I59F mutation was reported in a Japanese family [8], but functional analysis has not been conducted. We analyzed the predicted 3D structures of the paired domain of the PAX3-DNA complex and showed that this mutation was likely to distort the structure of the DNA-binding site of PAX3 and lead to functional impairment. This result substantially supports the hypothesis that the p.I59F mutation is pathogenic, although it is based on a theoretical prediction rather than functional experiments.

In family 2, the distinct phenotypes of the proband, the proband's mother, and the proband's grandmother were congenital unilateral hearing loss, heterochromia iridis, and early graying, respectively. Because of these differences, they were not aware of the hereditary nature of the symptoms. Identification of the *PAX3* mutation in the proband and the proband's grandmother led to an accurate diagnosis of WS1 and facilitated understanding of the symptoms. In this family, direct sequencing of *PAX3* did not detect any mutations, but MLPA analysis detected a large heterozygous deletion. Furthermore, quantitative PCR analysis revealed that the deleted region spanned 1759–2554 kb and included 12–18 genes. Large deletions of *PAX3* in patients with WS1 have been reported in several families [6,16–18]. To our knowledge, however, this is the largest deletion identified in patients with WS1 and has, therefore, expanded the spectrum of *PAX3* mutations. There is no reported correlation between the nature of the mutation (deleted vs truncated or missense) or

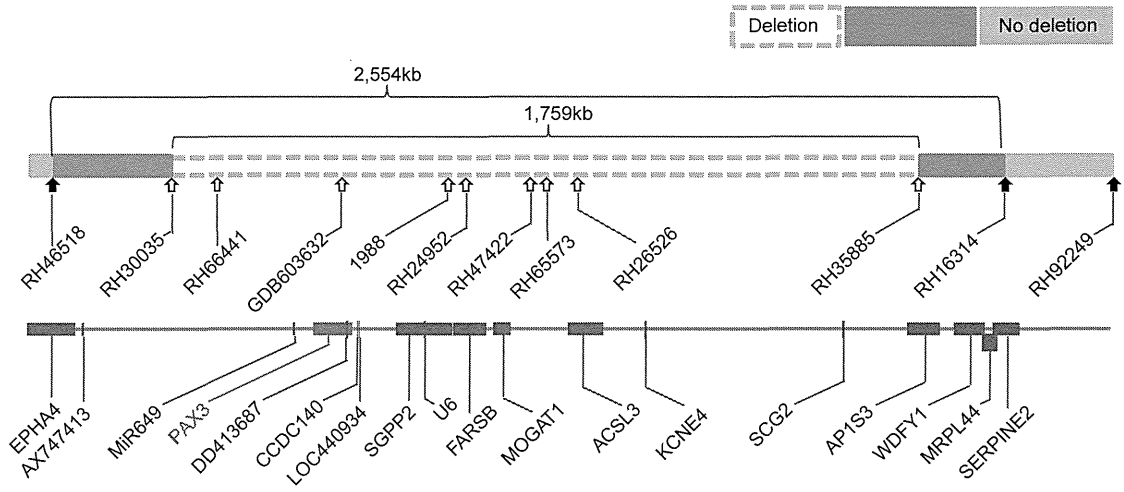


Figure 6. Genetic map showing the estimated location of the *PAX3* deletion together with the regions surrounding *PAX3*. Sites examined by quantitative PCR are indicated by arrows. Blank and white arrows indicate that the quantities of DNA at these sites are half or identical to the quantities of DNA at the corresponding sites in the control, respectively. The 5′ and 3′ ends of the deletion are located within the blue regions flanking the white region, designated as 'deletion,' and flanked by the green regions, designated as 'no deletion.' All genes mapped within this region, including *PAX3*, are shown in the lower map.

its location in *PAX3*, and the severity of the WS1 phenotype [19,20]. Similarly, no evidence of such a correlation was found in the data presented in this study.

In the present study, *PAX3* genetic diagnosis contributed to the accurate diagnosis of WS1. Such diagnosis could help provide genetic counseling to patients with isolated or few phenotypic symptoms, those with mild phenotypes or few first-degree relatives, or those who have yet to develop any symptoms. In addition, analysis of the predicted 3D structure of PAX3 facilitated the verification of pathogenicity of a missense mutation, and MLPA analysis increased the sensitivity of genetic diagnosis of WS1.

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***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

# Heterozygous Tandem Duplication Within the *PTCH1* Gene Results in Nevoid Basal Cell Carcinoma Syndrome

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Nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant disorder characterized by developmental defects and tumorigenesis. The gene responsible for NBCCS is *PTCH1*. Using multiplex ligation-dependent probe amplification, we identified a heterozygous tandem duplication within the *PTCH1* gene in a 14-year-old girl with typical NBCCS. We have sequenced the chromosomal breakpoint and determined the duplication as tandem in orientation and 18,814 bp in size. The fusion occurred between non-repetitive elements with an overlap of three nucleotides. The duplicated segment began at exon 10 and ended at intron 17. Subsequent analysis of cDNA from the patient showed the expression of mutant mRNA species containing a duplicated segment spanning exons 11–17, resulting in a frameshift and premature stop codon. This is the first reported case of NBCCS due to a tandem multiexon duplication of *PTCH1* representing a novel mechanism leading to the NBCCS phenotype, and highlights the importance of copy number analysis as an adjunct to exon sequencing in identifying infrequent mutational events in *PTCH1*. 

**Key words:** nevoid basal cell carcinoma syndrome; *PTCH1*; tandem duplication

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## INTRODUCTION

Nevoid basal cell carcinoma syndrome (NBCCS) (OMIM 109400), also known as Gorlin syndrome, is an autosomal dominant disorder characterized by developmental defects including bifid ribs, palmar or plantar pits and tumorigenesis such as the development of basal cell carcinoma, medulloblastoma, or keratocystic odontogenic tumor (KCOT) (formerly known as odontogenic keratocysts) [Gorlin, 1987]. The gene responsible for NBCCS is the human homologue of the *Drosophila patched* gene, *PTCH1* [Hahn et al., 1996; Johnson et al., 1996]. The human *PTCH1* gene contains 23 coding exons spanning approximately 70 kb and encodes a protein of 1447 amino-acid residues containing 12 transmembrane-spanning domains and two large extracellular loops [Johnson et al., 1996]. To date, we have analyzed 42 NBCCS families. Nine of the families (21%) did not harbor *PTCH1* mutations detectable by PCR-based direct sequencing of the exons. Five of these mutation-negative families could be explained by large deletions involving complete loss of one copy of *PTCH1* [Fujii et al., 2007; Nagao et al., 2011].

Here we report on a novel mutation involving duplication of eight exons detected in one of the four remaining families. This is the first report of a multiexon duplication event in NBCCS.

## CLINICAL REPORT

The propositus was a Japanese girl who was referred to our genetic clinic at the age of 14 years for evaluation. The parents were non-consanguineous and phenotypically normal. The propositus was a second child and had been delivered vaginally at 38 weeks of gestation. Her birth weight was 3,676 g (+1.8 SD), her crown-to-heel

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length was 54 cm (+2.7 SD), and her head circumference was 36 cm (+1.0 SD).

Her development was normal. She gained head control at the age of 3 months, sat at the age of 6 months, and walked alone at the age of 14 months. At the age of 10 years, she developed bilateral maxillary and mandibular cysts, which were surgically enucleated. A histological examination of the cystic linings showed the presence of KCOT, prompting a clinical diagnosis of NBCCS. The presence of numerous pits over her palms and soles and lamellar calcification on the falx detected using cranial computed tomography was compatible with the diagnosis. Two additional surgeries were subsequently performed to remove KCOTs. Vertebral defects, including fusion of the vertebral bodies or hemivertebrae, were not noted on a skeletal survey.

At the age of 15 years and 6 months, she weighed 54.8 kg (+0.3 SD) and was 168.5 cm tall (+2.1 SD). Physical examination identified macrocephaly, ocular hypertelorism and several pits over her palms and soles. Ten patches of basal cell nevi were noted on her back. Radiological examination did not show rib or vertebrate anomalies. Her parents did not have NBCCS manifestations.

## METHODS

### DNA and RNA Extraction

All experiments described below were approved by the ethics committee at Kitasato University. DNA was extracted from peripheral blood lymphocytes using a QIAamp DNA blood midi kit (QIAGEN, Germantown, MD) and RNA from established lymphoblastoid cell lines derived from the patient and a healthy control using a QIAamp RNA Blood Mini Kit (QIAGEN) and cDNA prepared using standard laboratory protocols. Lymphoblastoid cell lines were grown in the presence or absence of puromycin (100 μg/ml) for 6 hr prior to RNA extraction.

### PCR and Sequence Analysis

The complete coding region of the *PTCH1* gene, including all splice junctions, was amplified from constitutional DNA as described previously [Fujii et al., 2003]. Amplified products were gel-purified using a QIAEX II gel extraction kit (QIAGEN) and cycle sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) in both directions. The sequence was analyzed on a 3130 Genetic Analyzer (Applied Biosystems). The duplication junction was determined by direct sequencing for the PCR product obtained with a forward primer P1 (5′-TGCGAAGCTCAGCTTCTGTGC-3′) hybridizing to intron 16 and a reverse primer P2 (5′-TACTGGGTCAGACTGAGGAA-3′) hybridizing to intron 11. The mutant mRNA was sequenced for the RT-PCR product obtained with a forward primer P3 (5′-TTACGACCTACACAGGAGTT-3′) hybridizing to exon 15 and a reverse primer P4 (5′-ATCCAGTCTCCTGTCCTCGC-3′) hybridizing to exon13.

### MLPA Analysis

The multiplex ligation-dependent probe amplification (MLPA)-Gorlin kit was obtained from MRC-Holland (Amsterdam, The Netherlands). MLPA reactions were performed according to the manufacturer's instructions. Products were analyzed using a 3130 Genetic Analyzer (Applied Biosystems) and GeneMapper software (Applied Biosystems).

### Southern Blotting

Southern blotting was performed using standard laboratory protocols. The probe DNA hybridizing with the intron 16 sequence was amplified by PCR with a forward primer P5 (5′-CTTGGAATCAGAGTGGCTGC-3′) and a reverse primer P6 (5′-TCTGGCCCAATCCCATTTGT-3′) and radiolabeled with α-$^{32}$P-dATP during the PCR procedure.

## RESULTS

In this case, direct sequencing of all *PTCH1* exons amplified from peripheral blood DNA failed to identify a pathogenic germline mutation. However, MLPA analysis showed an increase in the copy number of exons 11–17, suggestive of a duplication event involving one copy of the *PTCH1* gene (Fig. 1A). This observation warranted further investigation to establish the effect of exon duplication on gene expression and the phenotype of this patient.

MLPA alone cannot provide an insight as to the precise location and potential functional outcomes of the duplication. Therefore, we next determined the breakpoint of the duplication. An aberrant PCR product was obtained from the genomic DNA of this patient with the P1 and P2 primers (Fig. 1B, lane 3), and sequencing of the PCR product suggested a tandem duplication involving 8 of the 23 coding exons of *PTCH1* (g.39362_58175dup18814 based on NG_007664.1 sequence) (Fig. 1C). The duplicated region was predicted to be 18,814-bp long and the fusion occurred between non-repetitive elements in exon 10 and intron 17 with an overlap of three nucleotides, TGC (Fig. 1D). No microhomology was observed around the duplication junction except for the 3-bp overlap. According to Repeatmasker (http://www.repeatmasker.org), the proximal breakpoint was located 314 bp upstream of an MER5A element and 2,128 bp upstream of an LTR element, LTR85b. The distal breakpoint was 2,773 bp downstream of an MIR3 element. Her parents did not carry the breakpoint sequence, thus this duplication was a de novo event (Fig. 1B, lanes 4 and 5).

To confirm the tandem duplication, we performed Southern blotting using genomic DNA digested with each of the three restriction enzymes and a probe hybridizing to intron 16 (Fig. 2A). As shown in Figure 2B, rearranged bands of expected sizes were detected in the patient but not in a healthy control, verifying the predicted gene structure of the mutant allele.

To investigate the effect of the duplication on the transcription and translation, we next performed an RT-PCR analysis using RNA extracted from lymphoblastoid cell lines derived from the patient and a healthy control. mRNA species harboring a premature termination codon (PTC) usually undergo nonsense-mediated mRNA decay (NMD) [Holbrook et al., 2004]. NMD is a highly conserved surveillance process leading to the detection and selective reduction of PTC-harboring mRNAs to prevent the synthesis of abnormal proteins. Since NMD is a translation-dependent process, the degradation is suppressed by translation inhibitors such as puromycin [Carter et al., 1995]. An aberrant

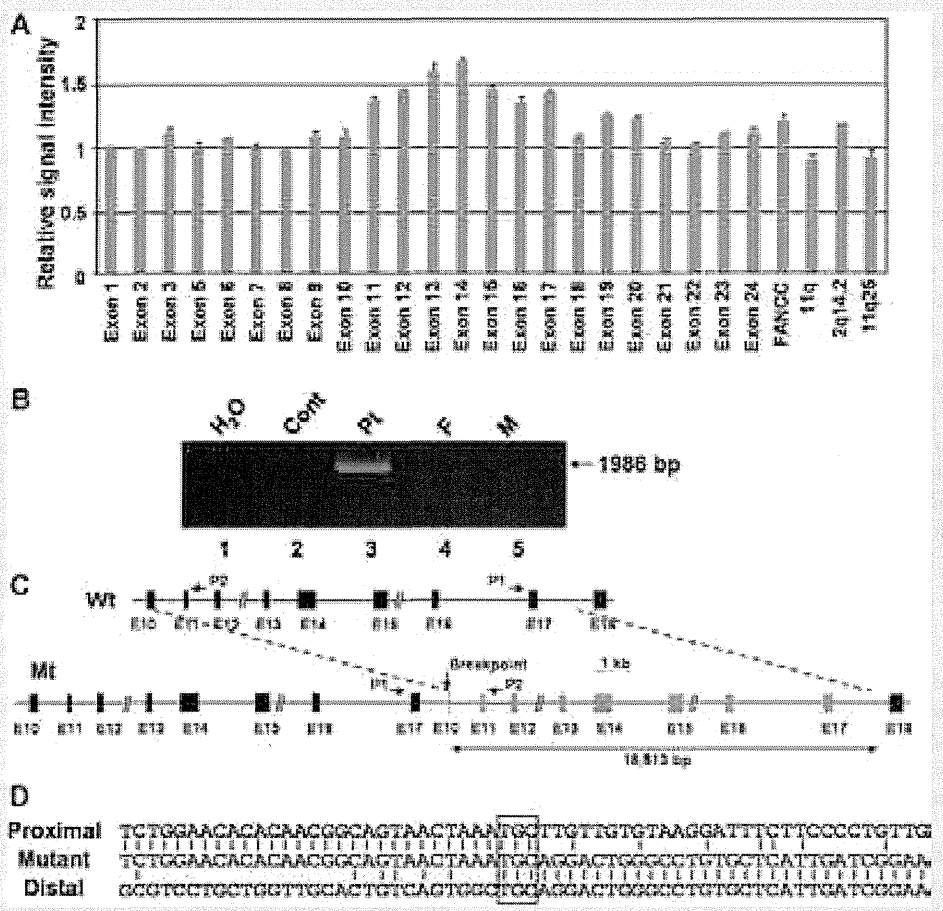


**FIG. 1. Assessment of copy number changes. A: MLPA profile. The value of the control average was set to one for all exons. FANCC is an intrachromosomal control while 11q, 2q14.2 and 11q25 are interchromosomal controls. B: Amplified genomic DNA fragment containing a breakpoint. Genomic PCR was performed using the P1 and P2 primers (see panel C) and genomic DNA obtained from the patient (Pt), her father and mother (F and M), and a healthy control (Cont). No template control ($H_2O$) was also included in the analysis. C: Predicted gene structure. The gene structure of the mutated allele (Mt) was predicted from the PCR result. Exons are depicted as boxes and introns are lines. Duplicated exons are depicted as gray boxes. D: Breakpoint sequence of the junction fragment. The DNA sequence for the duplication-specific junction fragment determined from the PCR product was aligned to the wild-type flanking genome sequence for both proximal and distal breakpoints. The three-nucleotide overlap is depicted as a box.**

product was amplified with the P3 and P4 primers specifically from the patient's RNA in the presence of, but not in the absence of puromycin, suggesting the presence of a PTC in the mutant sequence (Fig. 3A). Sequencing of the aberrant product identified a junction between exon 17 and exon 11, generating the duplicated segment composed of exons 11–17 (Fig. 3A,B). According to the junctional sequence, the duplication event was predicted to introduce 12 novel amino acids and then a PTC (Fig. 3C). This provided an explanation for the pathogenesis in this individual.

## DISCUSSION

Using MLPA analysis, we identified an approximate 19-kb duplication of the *PTCH1* gene in an individual with NBCCS. The duplicated segment harbored a part of exon 10 and exons 11–17. To date, more than 10 NBCCS patients with a constitutional interstitial deletion involving *PTCH1* have been reported [Shimkets et al., 1996; Olivieri et al., 2003; Haniffa et al., 2004; Midro et al., 2004; Boonen et al., 2005; Fujii et al., 2007; Takahashi et al., 2009; Nagao et al., 2011]. However, this is the first report of a multiexon

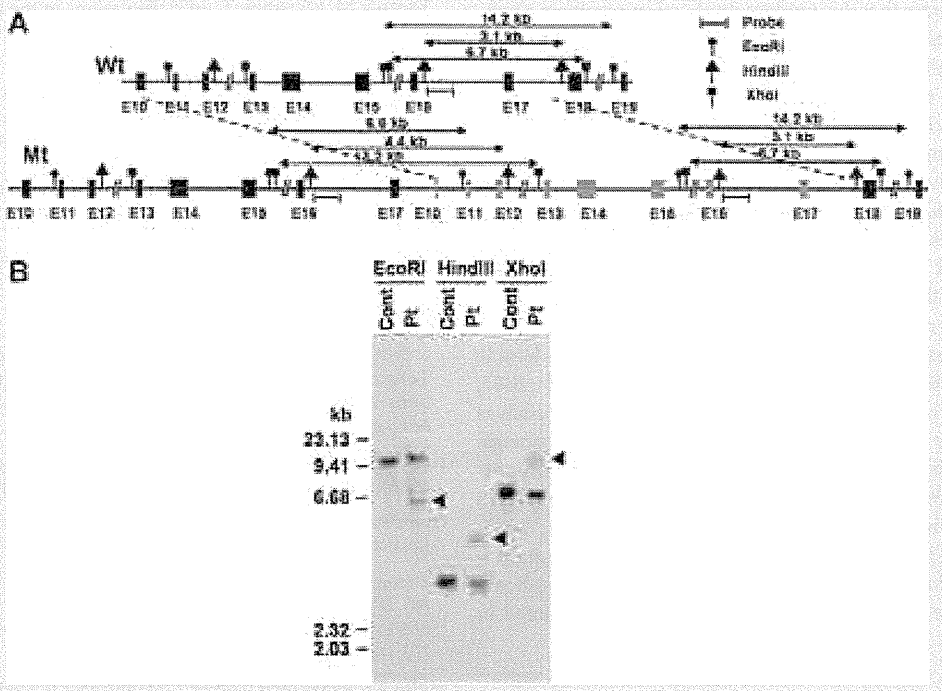


FIG. 2. Southern blot analysis of the patient's genomic DNA. A: Gene structures of both wild-type and mutant (Wt and Mt) alleles are depicted with restriction enzyme sites. The location of the probe and size of the DNA fragment predicted to be detected by Southern blotting are also indicated. B: Autoradiogram of Southern blotting. Genomic DNA of the patient as well as a healthy control was digested with the restriction enzymes indicated at the top and subjected to Southern blotting. Patient-specific rearranged bands are indicated by arrowheads.

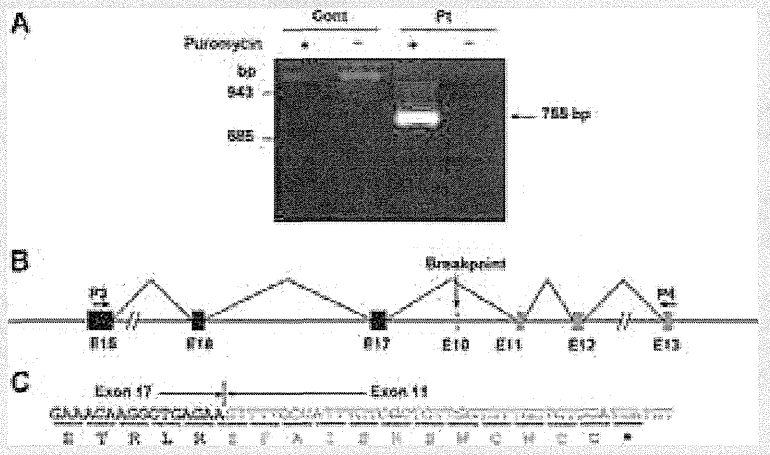


FIG. 3. Analysis of the mutant RNA transcribed from the mutant allele. A: Duplication-specific RT-PCR product. cDNA synthesized from lymphoblastoid cell lines established from the patient and a healthy control cultured in the presence or absence of an NMD inhibitor, puromycin (100 μg/ml), was subjected to RT-PCR using the P3 and P4 primers. B: Genomic structure surrounding the breakpoint. Duplicated exons are depicted by gray boxes. The location of the P3 and P4 primers used for RT-PCR is indicated. The splicing pattern deduced by sequencing the RT-PCR product is also depicted. C: Sequence of the mutant cDNA carrying the breakpoint. Direct sequencing of the duplication-specific RT-PCR product showed that the splicing took place between exon 17 and exon 11, skipping the partial sequence of exon 10 as well as intron 10. Consequently, a frameshift is generated leading to a PTC (*). The duplicated sequence is presented in gray letters.

duplication event that occurred in one of the *PTCH1* alleles. Our findings demonstrate that this tandem duplication results in a loss-of-function mutation and caused NBCCS due to haploinsufficiency of *PTCH1*. Accordingly, this patient did not exhibit manifestations inconsistent with the NBCCS phenotype.

As to the mechanism of the duplication, Alu-Alu-mediated homologous recombination is reported to be responsible for 27% of all segmental duplications in humans [Bailey et al., 2003]. In our study, the comparison of the proximal and distal normal sequences that span the duplication junctions did not identify apparent sequence motifs common to both parental strands at or near the junctions, suggesting that a non-homologous recombination is responsible.

Since large deletions or duplications are found in a considerable fraction of mutation-negative NBCCS patients [Fujii et al., 2007; Nagao et al., 2011], copy number analysis using techniques such as MLPA is strongly recommended to complement DNA sequence analysis. Although cost- and labor-intensive, copy number microarray is another powerful method to detect copy number alterations as reported previously in NBCCS patients [Fujii et al., 2007; Nagao et al., 2011]. When a duplication is detected, RNA analysis is necessary to elucidate the effect on coding since the splicing event taking place in the affected allele is not always predicted in silico.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

# A Transient Myelodysplastic/Myeloproliferative Neoplasm in a Patient With Cardio-Facio-Cutaneous Syndrome and a Germline *BRAF* Mutation

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A male infant, born at 32 weeks gestation by cesarean because of hydrops fetalis, presented with multiple anomalies, such as sparse and curly scalp hair, absent eyebrows, frontal bossing, an atrial septal defect, pulmonary artery stenosis, and whole myocardial thickening. He was clinically diagnosed with cardio-facio-cutaneous (CFC) syndrome, and was confirmed to have a germline V-raf murine sarcoma viral oncogene homologue B1 (*BRAF*) c.721 A>C mutation. At 1 month of age, he presented with a transient myelodysplastic/myeloproliferative neoplasm (MDS/MPN), which improved within a month without the administration of antineoplastic agents. This is the first report of CFC syndrome with MDS/MPN. The coexistence of MDS/MPN may be related to this *BRAF* c.721 A>C mutation. © 2013 Wiley Periodicals, Inc.

**Key words:** cardio-facio-cutaneous syndrome; myelodysplastic/myeloproliferative neoplasm; BRAF; RAS/MAPK syndromes; juvenile myelomonocytic leukemia

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## INTRODUCTION

Cardio-facio-cutaneous (CFC) syndrome is genetic disorder characterized by clinical features such as congenital heart defects, a characteristic facial appearance, ectodermal abnormalities and growth failure [Reynolds et al., 1986]. V-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) is one of rat sarcoma viral oncogene homolog/mitogen activated protein kinase (RAS/MAPK) signaling pathway genes, and has been identified as a causative gene of CFC syndrome [reviewed in Aoki et al., 2008 and Denayer and Legius, 2007]. We report on a male infant with CFC syndrome, who was confirmed to have a germline *BRAF* mutation, and then presented with a myelodysplastic/myeloproliferative neoplasm (MDS/MPN) at 1 month of age.

## CLINICAL REPORT

A male was born through cesarean at 32 weeks gestation as the first product of healthy nonconsanguineous Japanese parents. His birth weight, length and head circumference were 2,370 g (−0.8 SD), 40.0 cm (+2.3 SD), 34.2 cm (+3.2 SD), respectively. Due to hydrops fetalis and neonatal asphyxia, he required immediate resuscitation. Mechanical ventilation was needed until age 3 months. He presented with multiple anomalies, such as sparse and curly scalp hair, absent eyebrows, frontal bossing with temporal narrowing, ocular hypertelorism, low set ears, a short and webbed neck, and cryptorchidism (Fig. 1). His complete blood counts at age 1 day revealed the following: WBC 12,770/μl (neutrophils 80%,

Conflict of interest: none.

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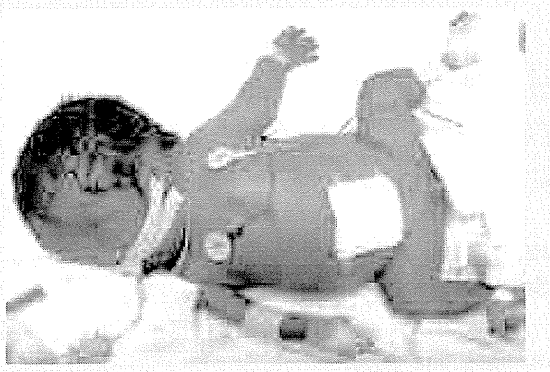

**FIG. 1. Full-body image of the patient at birth and his facial features at 3 hours of age. The patient showed severe generalized edema at birth. He presented with sparse and curly hair, frontal bossing, hypertelorism, low-set ears, a short and webbed neck, and cryptorchidism.**

lymphocytes 12%, monocytes 6%, myelocytes 2%), RBC 343 × $10^4$/μl, erythroblasts 2,430/μl, hemoglobin 14.2g/dl, and platelets 3.2 × $10^4$/μl. A chromosome analysis of his peripheral blood lymphocytes showed a 46, XY karyotype. He had an atrial septal defect (ASD), pulmonary artery stenosis (PS), whole myocardial thickening, a pulmonary arteriovenous fistula, an intrahepatic portal systemic shunt, hepatosplenomegaly, right cryptorchidism, a right double renal pelvis, and ureter and agenesis of the corpus callosum. These clinical features were all compatible with CFC syndrome.

At age 1 month, a peripheral blood examination indicated monocytosis of 17% (2,370/μl), with WBC 13,930/μl, RBC 295 × $10^4$/μl, and platelets 11.2 × $10^4$/μl, with giant platelets. Bone marrow aspiration revealed a nucleated cell count of 9.4 × $10^4$/μl, megakaryocyte count 56.2/μl, and did not contain pathologic blasts. The karyotype of the bone marrow cells was 46, XY. The granulocyte-macrophage colony-forming unit (CFU-GM) assay using a semi-solid methylcellulose method showed spontaneous CFU-GM formation of bone marrow (5/5 × $10^4$ mononuclear cells) and peripheral blood (35/5 × $10^4$ mononuclear cell), without growth factors. Based on these laboratory findings, this patient was diagnosed with MDS/MPN. However, the peripheral blood monocytosis improved without the administration of antineoplastic agents after 1 month with 13% monocytes (1,140/μl), WBC of 8,780/μl, RBC 314 × $10^4$/μl, and platelets 26.3 × $10^4$/μl. At age 3 years, his complete blood counts revealed 11% monocytes (903/μl), WBC 8,210/μl, RBC 428 × $10^4$/μl, and platelets 31.1 × $10^4$/μl (Table I). He smiled normally. He demonstrated generalized hypotonia without normal head control and was unable to produce meaningful speech.

## CYTOGENETIC AND GENOMIC ANALYSIS

The *BRAF* sequencing analysis showed a heterozygous A>C change at nucleotide 721, resulting in a p.T241P amino acid change in exon 6, which was a previously known mutation in CFC syndrome [Schulz et al., 2008]. No mutations were noted in the Kirsten rat sarcoma viral oncogene homologue (*KRAS*) or protein-tyrosine phosphatase, nonreceptor-type11 (*PTPN11*).

## DISCUSSION

A male infant, born via cesarean section because of hydrops fetalis, presented with multiple anomalies suggestive of CFC syndrome. A pulmonary arteriovenous fistula, an intrahepatic portal systemic shunt, hepatosplenomegaly, cryptorchidism, a double renal pelvis, and ureter have been reported as rare complications in CFC syndrome [Narumi et al., 2007]. At 1 month of age, he presented with MDS/MPN, which improved within a month. He showed a germline mutation of *BRAF* c.721 A>C, resulting in a p.T241P amino acid change in exon 6, within a cysteine-rich domain. This mutation was previously described in CFC syndrome [Schulz et al., 2008].

The clinical findings of CFC syndrome are similar to those of other RAS/MAPK or neuro-cardio-facial-cutaneous syndromes, such as Noonan and Costello syndrome [reviewed in Aoki et al., 2008; Denayer and Legius, 2007]. The RAS/MAPK signaling pathway genes, not only *BRAF*, but also *KRAS*, MAPK kinase/ERK kinase 1 (*MEK1*), and MAPK kinase/ERK kinase2 (*MEK2*) have been reported as causative genes for CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. CFC syndrome had been considered to have a low risk of malignancy among the various RAS/MAPK syndromes, but a few patients with CFC syndrome due to *BRAF* mutation have presented with malignancies, such as acute lymphoblastic leukemia [van Den Berg and Hennekam, 1999; Makita et al., 2007], and precursor T-lymphoblastic lymphoma [Ohtake et al., 2011].

MDS/MPNs include clonal myeloid neoplasms that at the time of initial presentation have clinical, laboratory or morphologic findings supporting a diagnosis of MDS, and other findings more consistent with MPN. They are usually characterized by hypercellularity of the

TABLE I. Peripheral Blood Examinations of This Patient

| Age (months) | 0 | 1 | 2 | 12 | 21 | 38 |
|---|---|---|---|---|---|---|
| WBC (/μl) | 12,770 | 13,930 | 8,730 | 8,660 | 9,040 | 8,210 |
| Monocytes (/μl) | 766 | 2,370 | 1,140 | 866 | 633 | 903 |
| RBC (×$10^4$/μl) | 343 | 295 | 314 | 396 | 404 | 428 |
| Platelets (×$10^4$/μl) | 3.2 | 11.2 | 26.3 | 24.2 | 38.6 | 31.1 |

bone marrow due to proliferation in one or more of the myeloid lineages [Swerdlow et al., 2008]. Juvenile myelomonocytic leukemia (JMML) is one type of MDS/MPN. Peripheral blood and bone marrow from JMML patients demonstrate spontaneous proliferation according to a CFU-GM assay [Estrov et al., 1986]. Transient monocytosis is not rare in preterm infants [Rajadurai et al., 1992]. Monocytosis in preterm infants is not usually considered a sign of MPD/MPN. In this case, the monocytes proliferation independent of growth factors was noticed according to a CFU-GM assay. The spontaneous proliferation was in favor of MPN. In RAS/MAPK syndromes, occasionally young infants with Noonan syndrome develop a JMML-like disorder which spontaneously resolves without treatment in some, and behaves more aggressively in others [Bader-Meunier et al., 1997; reviewed in Choong et al., 1999]. These children carried germline mutations in *PTPN11* [Tartaglia et al., 2003] or in *KRAS* [Kratz et al., 2005]. *BRAF* mutations had not previously been detected in patients with JMML [de Vries et al., 2007]. This is the first report of a germline *BRAF* mutation and MDS/MPN in a patient with CFC syndrome. The MDS/MPN improved without the administration of antineoplastic agents. This clinical course is similar to the JMML-like disorder observed in Noonan syndrome. This suggests a common mechanism for the development and progression of MDS/MPN in patients with RAS/MAPK syndromes. The MDS/MPN in RAS/MAPK syndrome patients has parallels with the transient leukemia of newborns with Down syndrome. However, the transient leukemia associated with Down syndrome has a high concentration of blasts in the peripheral blood and a GATA binding protein 1 (*GATA1*) mutation as somatic molecular marker [Xu et al., 2003].

The germline *BRAF* mutation site of this patient, c.721 A>C in exon 6, had been reported in two previous patients. One had CFC syndrome [Schulz et al., 2008], and the other had Noonan syndrome with multiple lentigines, previously referred to as LEOPARD syndrome [Sarkozy et al., 2009]. These two patients did not present with malignancies. Garnett and Marais [2004] reviewed the *BRAF* mutations in various adult cancers, and showed that up to 90% of mutations occurred in exon 12. The *BRAF* mutation site of this patient, exon 6, may be related to the spontaneous improvement of his MDS/MPN. A long-term follow-up and additional bone marrow assays might be needed if the patient demonstrates suspicious symptoms with or without peripheral blood monocytosis, because of the risk that MDS/MPN may recur. Further accumulated data about CFC syndrome with a *BRAF* mutation may help to elucidate the basic mechanisms of malignancy, and may suggest a therapeutic strategy.

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Case report

# Cardio-facio-cutaneous syndrome with infantile spasms and delayed myelination

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**Abstract**

A girl with cardio-facio-cutaneous (CFC) syndrome due to a *BRAF* gene mutation (c.1454T→C, p.L485S) experienced repetitive epileptic spasms at the corrected age of 4 months. Electroencephalograms revealed hypsarrhythmia, and magnetic resonance imaging identified delayed myelination and a hypoplastic corpus callosum. Various antiepileptic treatments, including adrenocorticotropic hormone therapy, were ineffective, although transient seizure control was achieved by a ketogenic diet and clorazepate dipotassium. However, seizures with epileptic foci at the bilateral posterior temporal areas re-aggravated and remained intractable; severe psychomotor delay persisted. This case indicated that infantile spasms in CFC syndrome can be difficult to control and may be accompanied by severe psychomotor retardation and abnormal myelination.


*Keywords:* Cardio-facio-cutaneous syndrome; Infantile spasms; *BRAF*; Abnormal myelination

## 1. Introduction

Cardio-facio-cutaneous (CFC) syndrome is characterized by a distinctive facial appearance, congenital heart defects, ectodermal abnormalities, and psychomotor retardation [1]. *BRAF*, *MEK1*, *MEK2*, and *KRAS*, which are involved in the RAS/mitogen-activated protein kinase (MAPK) pathway, have been identified as the causative genes for CFC syndrome. The RAS-MAPK pathway is essential in regulation of the cell cycle, differentiation, growth, and cell senescence [2]. In particular, *BRAF* mutants are the most common in CFC syndrome; their gene product is highly expressed in the brain and plays important roles in myelination and hippocampus-dependent learning [3,4].

A few recent reports have focused on the neurological symptoms of CFC syndrome, describing the complication of epilepsy and neuroradiological findings in this entity [5,6]. However, detailed information on individual patients, including the treatment course of epilepsy, has not been provided. We here describe a CFC syndrome patient with a *BRAF* gene mutation, who experienced infantile spasms and showed delayed myelination on magnetic resonance imaging (MRI).

## 2. Case report

A girl was born to nonconsanguineous Japanese parents at 33 weeks of gestation after a pregnancy complicated by polyhydramnios. There was no family history of epilepsy or neuromuscular disease and no previous

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pregnancy with spontaneous abortion. Birth weight was 2360 g (+2.1 SD), height 42 cm (−0.1 SD), and head circumference 32 cm (+1.7 SD). The Apgar score was 2 at 1 min and 6 at 5 min, and the patient needed artificial ventilation for several days. Mild laryngomalacia was noted, and cardiac ultrasonography revealed pulmonary valve stenosis, hypertrophic cardiomyopathy, and atrial septal defect. G-band analysis showed a karyotype of 46,XX. Metabolic screening tests of blood amino acids and urine organic acids showed negative results.

The patient was fed by a nasogastric tube because of poor milk intake. Repetitive brief tonic seizures emerged at the corrected age of 2 months and consisted of extension of the trunk, accompanied by staring and a few seconds of ocular bobbing. Asynchronous, high-voltage slow waves with multifocal sharp waves appeared with bilateral parieto-occipital predominance on interictal electroencephalogram (EEG) (Fig. 1A). The epileptic seizures remained intractable despite treatment with valproic acid and vitamin B6. Subsequently, the seizure type changed to tonic spasms in clusters when hypsarrhythmia was noted on EEG. Zonisamide (ZNS), clobazam (CLB), phenobarbital, and adrenocorticotropic hormone (ACTH) therapy (0.01 mg/kg/day for 2 weeks) were also ineffective.

On physical examination at the corrected age of 4 months, her weight was 3950 g (−3.5 SD), height 59 cm (−1.2SD), and head circumference 36 cm (−3.4 SD). She presented with a prominent forehead; a coarse face; a wide, depressed nasal bridge with broad anteverted nares; thick lips; and low-set ears. Her hair was sparse and curly, and cutaneous findings included eczema of the face, skin redundancy in the proximal extremities, and deep palmar and plantar creases. Based on her characteristic facial appearance and cardiac anomalies, CFC syndrome was suspected. The patient manifested with generalized hypotonia and showed scarce responses to auditory or visual stimuli and no purposeful movements of the extremities. Deep tendon reflexes were normal. She did not smile or vocalize during this period and showed continuous laryngeal stridor. Laboratory findings revealed hypogammaglobulinemia but were otherwise unremarkable. MRI of the brain revealed a hypoplastic corpus callosum with moderate brain atrophy, delayed myelination, and an ambiguous corticomedullary boundary in the right posterior temporal lobe (Fig. 2A–C). Magneto-

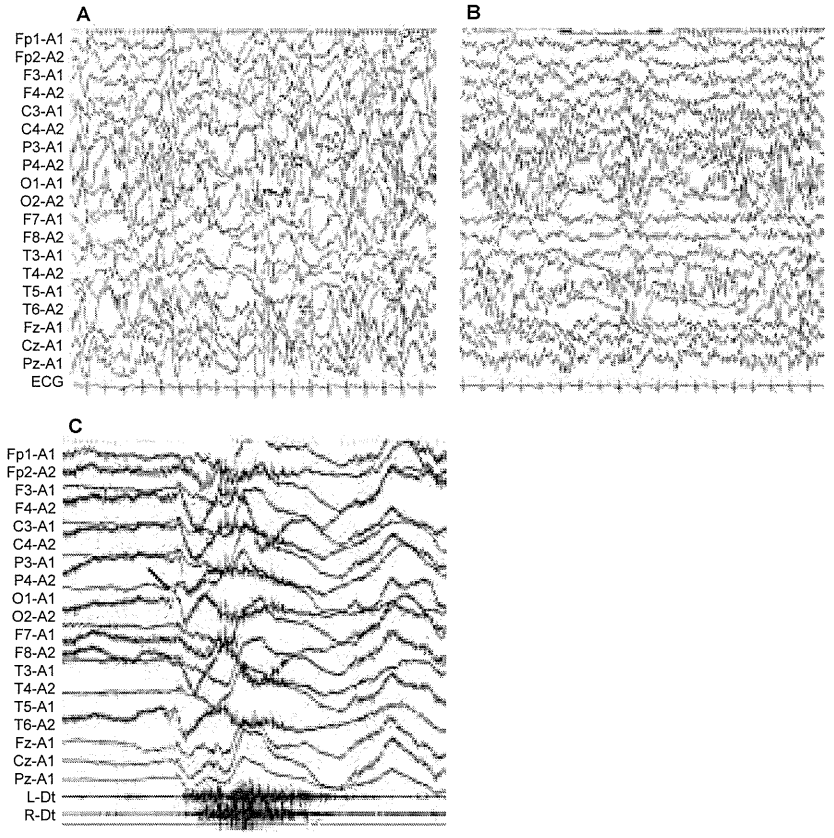


Fig. 1. Electroencephalograms (EEG) of the patient. (A and B) Interictal sleep EEG at the corrected ages of 5 (A) and 9 (B) months. (C) Ictal EEG of epileptic spasms at the corrected age of 5 months. L-Dt, left deltoid muscle; R-Dt, right deltoid muscle.

encephalography identified dipole sources at the bilateral temporo-parietal areas (Fig. 2D). Ictal EEG identified isolated spikes over the right parieto-occipital areas, which heralded the onset of repetitive epileptic spasms (Fig. 1C). Relative hypoperfusion was noted in the left temporo-parietal cortex on interictal single-photon emission computed tomography (SPECT) (Fig. 2E), and subtraction ictal SPECT coregistered to MRI during an episode of brief tonic seizure suggested an epileptic focus in the left temporal cortex (Fig. 2F).

Replacement of ZNS and CLB with potassium bromide (50 mg/kg/day) and topiramate (20 mg/kg/day) and re-administration of ACTH (up to 0.025 mg/kg/day) at the corrected age of 7 months did not improve seizure control. Tonic spasms with oculocephalic deviation in series gradually increased to 10 times per day, and continuous oxygen supply was needed because of recurrent airway infection and increased salivation with frequent desaturation. After initiation of a ketogenic diet with a ketogenic ratio of 3:1 in combination with 0.3 mg/kg/day clorazepate dipotassium, epileptic seizures decreased in frequency by the fifth day and temporarily disappeared at the corrected age of 9 months. Partial improvement in the EEG findings (Fig. 1B) was noted during this period. Her respiratory condition also improved, and thus, oxygen therapy could be terminated. Body weight gradually increased to 6 kg in parallel with improvement in the general condition. However, her development was still stagnated and MRI (Fig. 2A–C) did not show any progress in myelination. Seizures were exacerbated 1 month later and remained intractable.

Based on molecular studies, the patient was finally diagnosed with CFC syndrome. Genetic testing for mutations in *BRAF*, *MEK1*, *MEK2*, *KRAS*, and *HRAS* was performed, and a heterozygous *BRAF* gene mutation (c.1454T→C, p.L485S) was identified.

## 3. Discussion

Intellectual disabilities in CFC syndrome are more severe than those in Noonan and Costello syndromes, which are also caused by mutations in the constituents of the RAS/MAPK pathway [5,6]. However, the marked developmental delay in the present patient was more serious than that in usual cases of this entity [6]. This may be attributed to the complication of infantile spasms in this patient; the prevalence of this complication in CFC syndrome has been reported to be approximately 10% in large series [5,6]. Clinical information on these previous cases is very limited, and this is the first report on the detailed course of treatment for epileptic encephalopathy. This needs to be recognized to better understand the pathomechanism and management of this syndrome. In the central nervous system, *BRAF* is most densely expressed in hippocampal neurons [4], where the product of this gene is critically involved in the process of long-term potentiation and learning [7]. This preferential distribution may account for the severe psychomotor

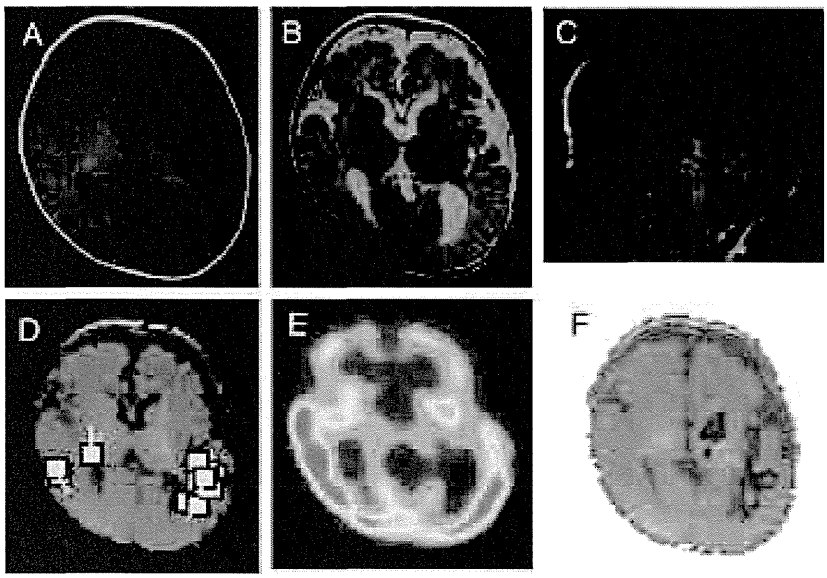


Fig. 2. Neuroimaging of the patient. (A–C) Magnetic resonance images at the corrected age of 9 months. Myelination is visible in the posterior limb of the internal capsule, optic radiation, and deep white matter of the frontal lobe (A and C, T1-weighted images; B, T2-weighted image), which corresponds to the pattern of early infancy. (D–F: examined at the corrected age of 5 months) Magnetic electroencephalogram (D), interictal single-photon emission computed tomography (SPECT) (E), and subtraction of ictal SPECT coregistered to MRI (F) based on a brief tonic seizure.

retardation in CFC syndrome, but the role of this genetic defect in epileptogenesis remains unclear. The presence of brain anomalies, including pachygyria and heterotopia, in CFC syndrome supports the contribution of *BRAF* to the morphogenesis of the brain and may be partly responsible for epilepsy in CFC syndrome. The ambiguous corticomedullary boundary in the right temporal lobe in the present patient may represent cortical dysgenesis in this area, which may have played a role in the evolution of infantile spasms in this patient. We observed another patient with a *BRAF* mutation (c.1495A→G, p.G499E) and lobar dysplasia in the left temporal cortex [8] (initially reported as Noonan syndrome). This suggests a propensity toward involvement of this region in CFC syndrome, possibly through the specific spatial distribution of the *BRAF* gene in corticogenesis and epileptogenesis. Although abundant *BRAF* expression in the fetal cerebrum has been confirmed, further studies on the detailed distribution of this gene in cell populations and cortical regions are required [9].

Another characteristic of the present patient was markedly delayed myelination throughout the course of the disease. Nonspecific delayed myelination and thinning of the corpus callosum are not uncommon findings in infantile spasms, which may be related to the persistence of hypsarrhythmic discharges [10]. However, these findings have also been recognized in some CFC syndrome patients without infantile spasms [5]. Conditional ablation of the *BRAF* gene in neuroglial precursors in mice resulted in severe dysmyelination and defective oligodendrocyte differentiation [3], thus supporting the idea that CFC syndrome can be regarded as a type of myelination disorder. Given that the presence of delayed myelination does not always correlate with seizure control and developmental outcomes in infantile spasms [11], the significance of this MRI finding in epileptogenesis and prognosis of CFC syndrome should be specifically explored.

Another patient with CFC syndrome due to an L485S mutation in the *BRAF* gene, identical to the defect in the present case, also showed infantile spasms [5]. This mutated residue is located in the protein kinase domain, a defect of which would critically affect the activity of BRAF protein. Although some mutations in this domain could result in a less severe phenotype [5], the neurological complications observed in the present patient, i.e., epileptic encephalopathy and abnormal myelination, may be specifically linked to certain genetic defects in CFC syndrome and may be characteristic in patients with a severe phenotype. Further analysis of genotype–phenotype correlations is required in this regard.

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RESEARCH ARTICLE

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# Prevalence and Clinical Features of Costello Syndrome and Cardio-Facio-Cutaneous Syndrome in Japan: Findings From a Nationwide Epidemiological Survey

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Costello syndrome and cardio-facio-cutaneous (CFC) syndrome are congenital anomaly syndromes characterized by a distinctive facial appearance, heart defects, and intellectual disability. Germline mutations in *HRAS* cause Costello syndrome, and mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) cause CFC syndrome. Since the discovery of the causative genes, approximately 150 new patients with each syndrome have been reported. However, the clinico-epidemiological features of these disorders remain to be identified. In order to assess the prevalence, natural history, prognosis, and tumor incidence associated with these diseases, we conducted a nationwide prevalence study of patients with Costello and CFC syndromes in Japan. Based on the result of our survey, we estimated a total number of patients with either Costello syndrome or CFC syndrome in Japan of 99 (95% confidence interval, 77–120) and 157 (95% confidence interval, 86–229), respectively. The prevalences of Costello and CFC syndromes are estimated to be 1 in 1,290,000 and 1 in 810,000 individuals, respectively. An evaluation of 15 adult patients 18–32 years of age revealed that 12 had moderate to severe intellectual disability and most live at home without constant medical care. These results suggested that the number of adult patients is likely underestimated and our results represent a minimum prevalence. This is the first epidemiological study of Costello syndrome and CFC syndrome. Identifying patients older than 32 years of age and following up on the patients reported here is important to estimate the precise prevalence and the natural history of these disorders.



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## INTRODUCTION

Costello syndrome (OMIM 218040), a rare, multiple congenital anomaly syndrome, was first described by Costello in 1971 [Costello, 1971]. Costello syndrome is characterized by intellectual disability, a high birth weight, neonatal feeding problems, short stature, congenital heart defects, curly hair, distinctive facial features, nasal papillomata, and loose integuments of the back of the hands [Hennekam, 2003]. Cardio-facio-cutaneous (CFC) syndrome (OMIM 115150) was first described in 1986 [Reynolds et al., 1986]. Affected individuals present with heart defects, short stature, frequent intellectual disability, and ectodermal abnormalities such as sparse, fragile hair, hyperkeratotic skin lesions, and a generalized ichthyosis-like condition. These syndromes overlap phenotypically with Noonan syndrome (OMIM 163950). We discovered that *HRAS* mutations of are causative of Costello syndrome [Aoki et al., 2005], and we and other group subsequently identified mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) in patients with CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Missense mutations in *PTPN11*, *SOS1*, *KRAS*, *RAF1*, and *NRAS* have been identified in individuals affected by Noonan syndrome or Noonan syndrome with multiple lentigines, previously known as LEOPARD syndrome (OMIM 151100, 611554) [Tartaglia et al., 2001; Schubbert et al., 2006; Pandit et al., 2007; Razzaque et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007; Cirstea et al., 2010]. Mutations in *SHOC2* have been identified in patients with Noonan-like disorder with loose anagen hair (OMIM 613563) [Cordeddu et al., 2009]. Because the clinical manifestations of these diseases are similar, a novel disease entity was proposed that consists of a syndrome characterized by a dysregulation of the RAS/MAPK signaling pathway [Aoki et al., 2008; Tidyman and Rauen, 2009].

Evaluation of the clinical manifestations of Costello and CFC syndromes revealed the similarities and differences between individuals with the diseases. Individuals with either syndrome have distinctive facial features; full cheeks and a large nose and mouth are characteristic of individuals with Costello syndrome, and a high cranial vault, bitemporal narrowing and a hypoplastic supraorbital ridge are characteristic of individuals with CFC syndrome. Wrinkled palms and soles have been thought to be characteristic features of individuals with Costello syndrome. A recent evaluation showed that 30% of individuals with CFC syndrome also have wrinkled palms and soles [Narumi et al., 2007]. Heart defects have been frequently reported in individuals with Costello and CFC syndromes; 61% of patients with Costello syndrome have hypertrophic cardiomyopathy, while 44 and 56% of Costello syndrome patients have congenital heart defects and arrhythmia, respectively. In contrast, hypertrophic cardiomyopathy, congenital heart defects, and arrhythmia have been observed in 36, 45, and 9%, respectively, of patients with CFC syndrome [Lin et al., 2011].

Approximately 10–15% of individuals with Costello syndrome develop malignant tumors, including transitional carcinomas in the bladder, rhabdomyosarcomas, and neuroblastomas [Aoki et al., 2008; Kratz et al., 2011]. Although association of malignant tumors has been rarely reported in individuals with CFC syndrome, we observed patients with *BRAF* mutations who developed acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma [Niihori et al., 2006; Makita et al., 2007; Ohtake et al., 2011].

The number of patients known to have these diseases is growing due to the identification of the causative genes. At least 150 genotyped patients with Costello syndrome have been reported [Lin et al., 2011]. In addition, more than 100 individuals with CFC syndrome have been reported in the literature [Rauen, 2007]. Till date, however, an epidemiological study has not been conducted. In order to identify the precise number of patients with these diseases, the natural history of the diseases, the prognosis and the rate of tumor development, we performed a nationwide investigation of both Costello syndrome and CFC syndrome.

## MATERIALS AND METHODS

### First-Stage Survey

The protocol we followed was established by the Research Committee on the Epidemiology of Intractable Diseases funded by the Ministry of Health, Labour and Welfare of Japan [Kawamura et al., 2006]. The prevalence of intractable diseases, including moyamoya disease, pancreatitis and sudden deafness, were all reported using this protocol [Teranishi et al., 2007; Kuriyama et al., 2008; Satoh et al., 2011]. The protocol consists of a two-stage postal survey. The first-stage survey aimed to estimate the number of individuals with Costello syndrome or CFC syndrome, and the second-stage survey aimed to identify the clinico-epidemiological features of the two syndromes.

The pediatric departments of all hospitals were identified based on a listing of hospitals as of 2008 supplied by the R & D Co.LTD (Nagoya, Japan). These hospitals were classified into seven categories according to the type of institution (i.e., university hospital or general hospital) and the number of hospital beds. Hospitals were then randomly selected from each of these categories for sampling. The sampling rate was approximately 5, 10, 20, 40, 80, and 100% of general hospitals with less than 100 beds, 100–199 beds, 200–299 beds, 300–399 beds, 400–499 beds, and 500 or more beds, respectively, and 100% of university hospitals [Kuriyama et al., 2008]. To increase the efficiency of the study, we sent a survey form to 205 pediatricians and 44 clinical geneticists working in the departments of gynecology, genetics, or ophthalmology in university hospitals (See Supplemental eTable I in supporting information online). We also selected 29 physicians who previously sent patient samples to our facility for molecular analysis. These hospitals were separately classified into a "selected hospitals" category, and all hospitals in this category were surveyed. Another 205 institutions that treat the disabled were included in order to identify adult patients.

The survey was mailed out to the targeted departments of health institutes in October 2009 along with cover letters. A simple questionnaire was used to ask about the number of patients with Costello syndrome known to have an *HRAS* mutation, CFC syndrome patients with mutations in *KRAS*, *BRAF*, or *MAP2K1/2*

(*MEK1/2*) and clinically suspected patients. Photographs of patients, obtained with their specific consent, were printed on the brochure describing the disease overview. In December 2009, a second request was sent to departments that had not responded by the earlier deadline (the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

## Genetic Testing of Clinically Suspected Patients

Blood samples from 42 individuals clinically suspected to have Costello or CFC syndrome were sent to our facility. After DNA was extracted by a standard protocol, we performed genetic screening for all four exons of *HRAS* and 14 exons of *BRAF*, *MAP2K1*, *MAP2K2*, and *KRAS* in which mutations have been previously identified (*BRAF* exons 6 and 11–16, *MAP2K1* exons 2 and 3, *MAP2K2* exons 2 and 3 and *KRAS* exons 1, 2, and 5) (Fig. 1). In samples negative for the first screening, we further analyzed all of the known causative genes for Noonan syndrome and related disorders (including the remaining exons in *BRAF*, *KRAS*, *MAP2K1*, and *MAP2K2*, all 17 exons in *RAF1*, all 23 exons in *SOS1*, all 4 exons in *NRAS*, and exon 1 of *SHOC2*). The clinical manifestations of the patients were evaluated by clinical dysmorphologists (K.K., H.O., H.K., N.O., S.M.).

## Second-Stage Survey

The second questionnaires were forwarded to the departments that reported patients with Costello or CFC syndrome on the first questionnaires. Detailed clinical information was collected, including the age, gender, growth and development pattern, cardiac defects, central nervous system defects, craniofacial characteristics, musculoskeletal characteristics, skin characteristics, tumors, identified mutations, and the facility where the genetic analysis had been performed. Duplicate results were excluded using the information regarding the patient's age, gender, and the type of mutations, if available. The Ethics Committee of Tohoku University School of Medicine approved this study. We obtained informed consent from all subjects involved in the genetic testing and specific consent for the photographs from three patients shown in Figure 1.

## Estimation of Prevalence

We first estimated the number of patients in departments who responded the first survey, using the number of mutation-positive patients from the first-stage postal survey and the number of newly identified patients by mutational analysis in the current study. $PR_k$ denotes the number of mutation-positive patients reported in the first-stage survey. The estimate was made based on the assumption that mutation-positive patients equally existed in the clinically

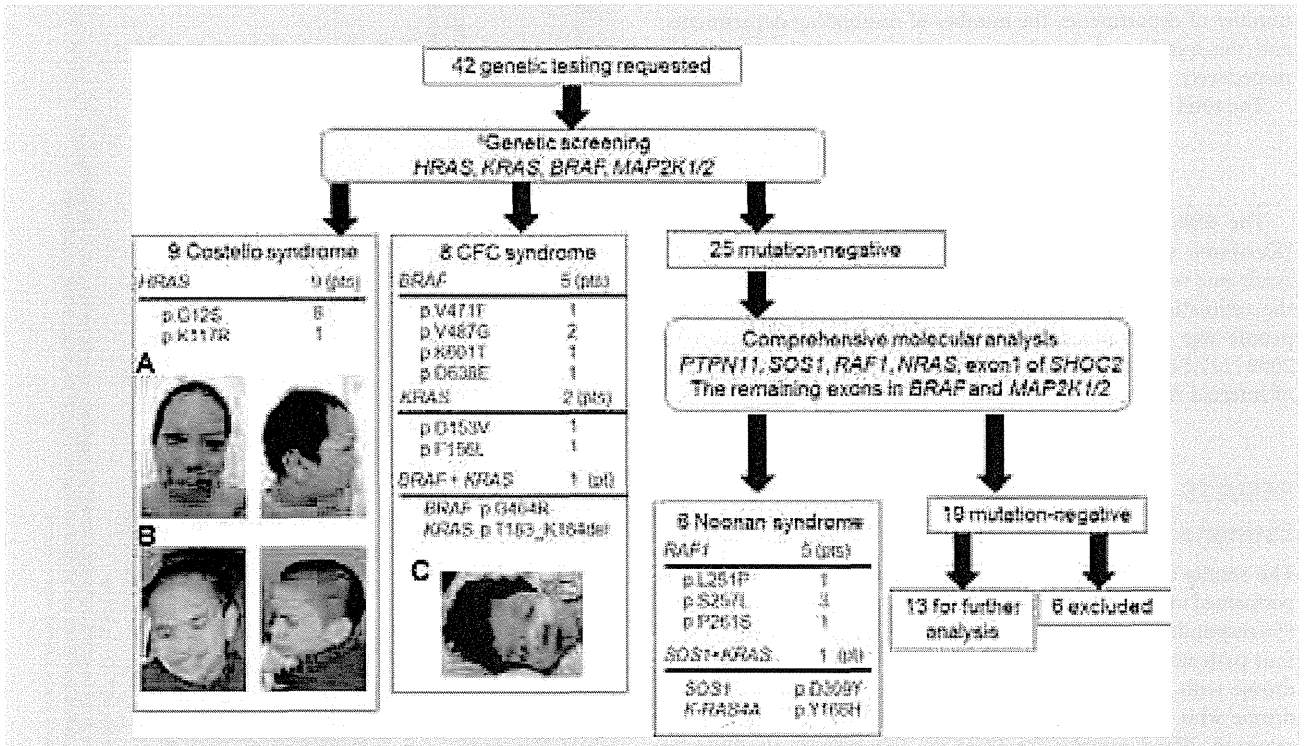


**FIG. 1. Flow chart of the genetic testing results for 42 patients whose blood samples were submitted for this study. A, B: Patients harboring *HRAS* p.G12S, (C) patient with *BRAF* p.K601T.[a] For the first screening, all exons in *HRAS* and *KRAS*, exons 6 and 11–16 in *BRAF*, and exons 2 and 3 in *MAP2K1/2* were sequenced.**

suspected patients who did not receive the genetic testing. The number of mutation-positive patients estimated by the mutation analysis was calculated using the number of the clinically suspected patients reported in the first-stage survey ($PS_k$), the ratio of the number of newly identified mutation-positive patients ($PD_k$), and the total number of patients examined ($PA_k$). Therefore, the total estimated number of patients in hospitals in stratum k $\sum_i iN_{ki}$, which responded to the first survey, was calculated as follows:

$$\sum_i iN_{ki} = PR_k + PS_k \frac{PD_k}{PA_k}$$

To calculate the total number of patients in all hospitals listed, we estimated that the mean number of patients among the departments that responded to the survey was equal to that of those departments that did not respond.

The number of patients in stratum k was therefore estimated as

$$\begin{aligned}\hat{\alpha}_k &= \frac{1}{SRT_k RRT_k} \sum_i iN_{ki} \\ &= \frac{1}{\frac{NS_k}{n_k}\frac{N_k}{NS_k}} \sum_i iN_{ki} \\ &= \frac{n_k}{N_k} \sum_i iN_{ki}\end{aligned}$$

where $SRT_k$, $RRT_k$, $NS_k$, $n_k$, $N_k$, and $N_{ki}$ denote the sampling rate, the response rate, the number of sampled departments, the total number of departments, the number of responding departments, and the number of departments with i patients in stratum k, respectively.

The total number of patients, $\hat{\alpha}$, was computed as follows:

$$\hat{\alpha} = \sum_k \hat{\alpha}_k$$

The 95% CI of $\hat{\alpha}_k$ was calculated as previously described [Kuriyama et al., 2008]. Five deceased patients with Costello syndrome reported in the first survey (Table I) were excluded in the estimation of prevalence. The prevalence rate per 100,000 people was determined based on the population of Japan in 2009 (127,510,000) with data from the Statistics Bureau, Ministry of Internal Affairs and Communications.

## RESULTS

### Estimated Number of Patients

The results of the first postal survey and the molecular analysis performed in this study are shown in Table I. Of 1,127 departments, 856 responded to the first-stage survey questionnaire (76%). Fifty-four patients, including five deceased patients, with Costello syndrome with mutations in *HRAS* and 54 patients with CFC syndrome who had mutations in *KRAS*, *BRAF*, or *MAP2K1/2* were reported. Blood samples for 42 of the 114 individuals clinically suspected to have Costello syndrome or CFC syndrome were sent to our laboratory. Molecular screening identified nine patients with Costello syndrome and eight with CFC syndrome (described below, Fig. 1 and Table I). Results from the second-stage survey followed by

TABLE I. Results of the First Postal Survey and the Number of Newly Identified Patients

| | Total departments | Surveyed departments | Sampling rate (%) | Departments that responded | Response rate (%) | Reported in the first-stage postal survey | | | Genetic testing performed | Newly identified | Newly identified |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CS[c] (deceased) | CFCS[c] | CS/CFCS suspected | | CS | CFCS |
| University hospitals | 166[b] | 163 | 98.2 | 158 | 96.9 | 11(2) | 13 | 44 | 15 | 5 | 1 |
| Selected hospitals[a] | 29 | 29 | 100 | 18 | 62.1 | 28(2) | 33 | 16 | 1 | 0 | 1 |
| Institutions for the mentally and physically disabled | 208 | 205 | 98.6 | 142 | 69.3 | 10(1) | 5 | 16 | 5 | 2 | 1 |
| General hospitals with ≥500 beds | 261 | 254 | 97.3 | 205 | 80.7 | 5 | 1 | 25 | 12 | 0 | 5 |
| General hospitals with 400–499 beds | 212 | 151 | 71.2 | 124 | 82.1 | 0 | 0 | 5 | 6 | 2 | 0 |
| General hospitals with 300–399 beds | 402 | 150 | 37.3 | 106 | 70.7 | 0 | 0 | 5 | 1 | 0 | 0 |
| General hospitals with 200–299 beds | 362 | 70 | 19.3 | 43 | 61.4 | 0 | 0 | 1 | 1 | 0 | 0 |
| General hospitals with 100–199 beds | 740 | 67 | 9.1 | 42 | 62.7 | 0 | 2 | 2 | 1 | 0 | 0 |
| General hospitals with ≤99 beds | 830 | 38 | 4.6 | 18 | 47.4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 3210 | 1127 | 35.1 | 856 | 76 | 54(5) | 54 | 114 | 42 | 9 | 8 |

CS, Costello syndrome; CFCS, CFC syndrome.
[a]Hospitals that had asked for genetic testing of Costello/CFC syndrome to our laboratory prior to the survey.
[b]131 university hospitals were listed, and we sent survey forms to 249 physicians in 166 departments.
[c]Possible duplications among patients were excluded.

exclusion of duplicates showed that in total, 63 patients with Costello syndrome and 62 patients with CFC syndrome were identified. Taking into consideration the sampling rates in each stratum of the general hospitals and the number of undiagnosed patients in the clinically suspected patients, we estimated the total numbers of patients in Japan with Costello syndrome and CFC syndrome to be 99 (95% confidence interval, 77 to 120) and 157 (95% confidence interval, 86 to 229), respectively. Therefore, the prevalence of Costello syndrome and CFC syndrome was estimated to be 1 in 1,290,000 (95% confidence interval, 1 in 1,061,000 to 1 in 1,660,000), and 1 in 810,000 (95% confidence interval, 1 in 556,000 to 1 in 1,490,000) individuals, respectively.

## Results of the Molecular Analysis

Screening of 42 clinically diagnosed patients identified nine patients with Costello syndrome and eight patients with CFC syndrome (Fig. 1). Eight of the nine patients with *HRAS* mutations had a p.G12S mutation, and the remaining one had a p.K117R mutation. Six of the eight patients with CFC syndrome had *BRAF* mutations (p.G464R, p.V471F, p.K601T, and p.D638E in a single patient, and p.V487G in two patients), and two patients had *KRAS* mutations (p.D153V and p.F156L). One patient had *BRAF* p.G464R, which has previously been reported in a patient with CFC syndrome [Nava et al., 2007], and a novel *KRAS* variation, c.547_552delACCAAG (p.T183_K184del). Parental samples were not available for this patient, and it is unknown if this variation was pathogenic or not. A subsequent, comprehensive mutation analysis showed that *RAF1* mutations, including p.L251P, p.S257L, and p.P261S, were identified in five patients. Four of the five patients had severe perinatal problems, including polyhydramnios, fetal distress, pleural effusion, and hypertrophic cardiomyopathy. An *SOS1* p.D309Y mutation was identified in a single patient diagnosed with Noonan syndrome. The patient also had another novel variation (p.Y166H) in *K-RAS4A*. Her asymptomatic father had the same variation, suggesting that this variation is a benign polymorphism. The five patients with *RAF* mutations and one patient with the *SOS1* mutation were diagnosed as having Noonan syndrome. In the remaining 19 patients who had no mutations, six patients were excluded based on the review of dysmorphologists because of non-matching facial features and clinical manifestations. The remaining 13 patients will be further analyzed.

## Clinical-Epidemiological Features of the Patients

We collected detailed clinical-epidemiological information on 43 of 63 Costello syndrome patients and 54 of 62 CFC syndrome patients who were reported in the first postal survey and newly diagnosed by the current study (Table II). Seventeen male and 25 female patients with Costello syndrome and 28 male and 24 female patients with CFC syndrome were reported. Twenty-six of the patients with Costello syndrome [Aoki et al., 2005; Niihori et al., 2011] and 10 of the patients with CFC syndrome [Niihori et al., 2006; Narumi et al., 2008] had been previously studied. Of the Costello syndrome patients, 27 of the 43 patients had *HRAS* p.G12S, five had p.G12A and two had p.G13D, p.G12C, p.G12V, p.G12D, and p.K117R were identified in a single patient. In the patients with CFC syndrome, 38 (70%), eight (15%) and eight (15%) of the 54 patients had *BRAF*, *MAP2K1/2*, and *KRAS* mutations, respectively.

Evaluation of clinical manifestations showed that postnatal failure to thrive and intellectual disability were reported at a rate of more than 95% in both disorders (Table II). Short stature was reported in 72 and 82% of patients with Costello syndrome and CFC syndrome, respectively. The frequency of hypertrophic cardiomyopathy and arrhythmia was significantly higher in patients with Costello syndrome compared to CFC syndrome. In contrast, the frequency of pulmonic stenosis was significantly higher in patients with CFC syndrome compared to Costello syndrome. Abnormal brain structure as detected by CT and/or MRI was reported in eight Costello syndrome patients. Of these eight patients, two were reported as having Arnold–Chiari type I, two had hydrocephalus, one had cortical atrophy, one had hydrocephalus and cortical atrophy, one had tonsillar descent, and one had ventricular dilation and a thinning of the corpus callosum. Abnormal brain structure was also observed in seven CFC patients; two had thinning of the corpus callosum, one had cortical atrophy, one had cortical atrophy, thinning of the corpus callosum and a reduction in white matter volume, one had ventricular dilatation, and one had ventricular dilatation and vermis hypoplasia. Regarding the skin characteristics, the frequency of soft, loose skin and deep palmer/plantar creases was significantly higher in patients with Costello syndrome than in CFC syndrome. Four patients with Costello syndrome developed malignant tumors, including bladder carcinomas, ganglioneuroblastomas and rhabdomyosarcomas. Two patients with CFC syndrome were previously reported as developing ALL and non-Hodgkin lymphoma [Makita et al., 2007; Ohtake et al., 2011]. Five patients with Costello syndrome were deceased. Two patients died from ganglioneuroblastoma and rhabdomyosarcoma. One patient died from tachycardia-induced cardiomyopathy at age 18 months.

The age distribution of the 38 patients with Costello syndrome and the 53 CFC syndrome patients whose ages were reported in the second-stage survey is shown in Figure 2. There were major peaks at 5 years of age in both diseases. The oldest patient diagnosed with Costello syndrome was 22 years of age, while the oldest patient with CFC syndrome was 32 years. Six patients with Costello syndrome and nine patients with CFC syndrome age 18–32 years were identified (Table III). Analysis of their daily living activities showed that 10 individuals could walk independently, one had an abnormal gait, one had a cane-assisted gait, and one used a wheelchair. Two patients with *BRAF* mutations were bedridden. All patients showed intellectual disability, and eight (severe in three patients with Costello syndrome and three patients with CFC syndrome, very severe in two patients with CFC syndrome) were severely disabled. Daily conversation was possible for three individuals. Simple conversations and two-word sentences were possible for four and three patients, respectively. Eleven patients lived at home. Three individuals had graduated from a school or public school for disabled children. Eight adults worked in vocational training facilities. Thirteen patients were able to feed themselves, but two of them sometimes needed assistance with feeding. Two patients with CFC syndrome were bedridden and needed full assistance with feeding and toileting.