ORIGINAL ARTICLE

A family of oculofaciocardiodental syndrome (OFCD) with a novel *BCOR* mutation and genomic rearrangements involving *NHS*

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Oculofaciocardiodental syndrome (OFCD) is an X-linked dominant disorder associated with male lethality, presenting with congenital cataract, dysmorphic face, dental abnormalities and septal heart defects. Mutations in *BCOR* (encoding BCL-6-interacting corepressor) cause OFCD. Here, we report on a Korean family with common features of OFCD including bilateral 2nd–3rd toe syndactyly and septal heart defects in three affected females (mother and two daughters). Through the mutation screening and copy number analysis using genomic microarray, we identified a novel heterozygous mutation, c.888delG, in the *BCOR* gene and two interstitial microduplications at Xp22.2–22.13 and Xp21.3 in all the three affected females. The *BCOR* mutation may lead to a premature stop codon (p.N297IfsX80). The duplication at Xp22.2–22.13 involved the *NHS* gene causative for Nance–Horan syndrome, which is an X-linked disorder showing similar clinical features with OFCD in affected males, and in carrier females with milder presentation. Considering the presence of bilateral 2nd–3rd toe syndactyly and septal heart defects, which is unique to OFCD, the mutation in *BCOR* is likely to be the major determinant for the phenotypes in this family.

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INTRODUCTION

Oculofaciocardiodental syndrome (OFCD, Mendelian Inheritance in Man (MIM) #300166), an X-linked dominant disorder, is characterized by ocular, facial, cardiac and dental abnormalities associated with male lethality.^{1,2} Mutations in the BCL-6 corepressor gene (*BCOR*, MIM *300485) at Xp11.4 cause OFCD.³ *BCOR/Bcor* is ubiquitously expressed in human tissues and is strongly and specifically expressed in the eye, brain, neural tube and branchial arches during mouse embryonic development, which are affected in OFCD.^{4,5} In 2009, Hilton *et al.*⁶ clinically reviewed 35 cases with *BCOR* mutations and summarized the frequency of phenotypes: congenital cataract (100%), microphthalmia and/or microcornea (82%), facial dysmorphism (96%) including long narrow face and high nasal bridge, cardiac anomalies (74%, commonly septal defects), dental abnormalities (100%) such as delayed and/or primary dentition, root radiculomegaly, and absent/duplicated/fused teeth and mental retardation (18%).⁶ Additionally, skeletal abnormalities such as 2nd–3rd toe syndactyly, hammer toes, and radioulnar synostosis are also observed in 97% patients. Various types of mutations in *BCOR* have been described including nonsense, small insertions or deletions and splice

site mutations, suggesting that the loss of functions (null allele) might result in OFCD. In addition, microdeletions involving *BCOR* have been also reported in individuals with OFCD. Most mutations predicted to generate premature stop codons, likely suffering from nonsense-mediated mRNA decay, although nonsense-mediated mRNA decay was unable to be confirmed because of the severe skewed X-inactivation in blood leukocytes.^{3,6}

Nance–Horan syndrome (NHS) is an X-linked cataract-dental syndrome (MIM #302350) characterized by congenital cataract, dental abnormalities, facial dysmorphism and mental retardation.⁷ Congenital cataract in affected male usually requires early surgery.⁸ Dental abnormalities include maxillary and mandibular diastema of both central and lateral incisors, and screwdriver-shaped teeth because of narrow gingival and incisal margins.⁹ Carrier females typically display posterior Y-sutural lens opacities, and the dental and facial anomalies of the syndrome may be observed, but with a milder presentation.⁸ Mutations in *NHS* (MIM *300457) at Xp21.1–p22.3 cause NHS.^{9–11} The most pathogenic mutations are truncating mutations. Coccia *et al.*⁸ reported complex duplication-triplication rearrangements of the *NHS* gene in a family with congenital cataract and congenital

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heart defects in affected males, suggesting that genomic rearrangements of *NHS* are able to cause the X-linked cataract.

In this report, mutation screening and genomic microarray revealed a heterozygous mutation in *BCOR* and genomic rearrangements involving *NHS* in the three affected females of a Korean family with congenital cataract, dental abnormalities and 2nd–3rd toe syndactyly. Detailed molecular analysis will be presented.

MATERIALS AND METHODS

Clinical report

The Korean family with congenital cataract was previously described (as family 4) (Figure 1a).¹² Clinical features are summarized in Table 1. In the elder sister (MC17, the proband), bilateral congenital cataracts were noted 100 days after birth. Bilateral lensectomy and secondary intraocular lens insertion were performed. Ventricular septal defect, atrial septal defect, patent ductus arteriosus, delayed dentition, bilateral broad halluces, bilateral 2nd–3rd toe syndactyly, bilateral hammer toes and right brachyphalangia of fourth toe were also recognized. Mental development was normal. In the younger sister (MC18), bilateral congenital cataracts were also recognized. Bilateral lensectomy and secondary intraocular lens insertion were performed at ages of 2 months and 3 years, respectively. Right inguinal hernia, delayed dentition, and bilateral broad halluces, bilateral 2nd–3rd toe syndactyly, and bilateral hammer toes were noted (Figures 1b and c). She had learning difficulties at school, but IQ was not measured. In the mother (MC17b), bilateral congenital cataracts were noted and left lensectomy was performed at age of 10 years. Because of her dental anomalies and hypodontia, all her teeth were surgically removed. Bilateral 2nd–3rd toe syndactyly and bilateral hammer toes were noted. Her intelligence was normal. All the three affected members shared bilateral congenital cataracts, delayed dentition, bilateral 2nd–3rd toe syndactyly and bilateral hammer toes. Dysmorphic facial features were unseen.

DNA sequencing

Experimental protocols were approved by Institutional Review Boards for Ethical Issues at Yokohama City University School of Medicine and the Committee for the Ethical Issues on Human Genome and Gene Analysis, Seoul National University. Informed consent was obtained from all individuals. Genomic DNA was obtained from peripheral leukocytes using QIAGEN Blood and Cell Culture DNA Midi Kit (QIAGEN, Hilden, Germany). DNA was amplified using GenomiPhi V2 kit (GE healthcare, Buckinghamshire, UK). In *BCOR*, there are three isoforms: isoform a (GenBank accession number NM_017745.5), isoform b (GenBank accession number NM_001123384.1) and isoform c (GenBank accession number NM_001123385.1). In *NHS*, there are two isoforms: isoform 1 (GenBank accession number NM_198270.2) and isoform 2 (GenBank accession number NM_001136024.2). Nucleotide sequences of 1st to 15th exons of *BCOR* and 1st to 8th exons of *NHS* covering all the protein coding region as well as exon–intron borders were analyzed. Polymerase chain reaction (PCR) conditions and primer information are shown in Supplementary Table 1. PCR products were purified with ExoSAP (USB, Cleveland, OH, USA) and sequenced with BigDye terminator 3.1 (Applied Biosystems, Foster City, CA, USA) on 3100 and 3500×1 Genetic Analyzer (Applied Biosystems). Sequences of patients were compared with the reference human genome sequences (based on the UCSC Genome Browser coordinate, February 2009) with Sequencher 4.10.1. (Gene Codes, Ann Arbor, MI, USA).

Copy number analysis

Copy number variations (CNVs) were investigated by Cytogenetics Whole-Genome 2.7M. Array (Affymetrix, Santa Clara, CA, USA) based on the manufacturer's instruction using 100 ng genomic DNA from three affected females (MC17b, MC17 and MC18). Copy number alterations were analyzed by Chromosome Analysis Suite (Affymetrix) with NetAffx 30.1 annotations (hg18 assembly). Any filters such as minimum size and probe numbers of CNVs were not applied. The selection criteria for putative pathogenic CNVs were as follows: (1) CNVs were shared with three affected females, (2) CNVs

Figure 1 Pedigree, foot malformation and a *BCOR* mutation found in the family. (a) Familial pedigree. Black and open symbols denote affected and unaffected individuals, respectively. (b, c) Bilateral broad halluces (the big toe, arrows in b), bilateral 2nd–3rd cutaneous syndactyly (arrowheads in c) and bilateral hammer toes in MC18. (d) Schematic representation of the *BCOR* gene (top). UTR and coding region are open and filled rectangles, respectively. Alternative splicing by three different isoforms is shown. The isoform b is absence of exon 5 and the isoform c is 102bp and 156bp longer than the isoform a and b, respectively. The location of the c.888delG mutation is indicated by an arrow. The protein structure of BCOR (isoform a, bottom). Three consecutive ankyrin motifs are indicated as a dark-gray box. The three binding sites for BCL-6, AF9 and NSPC1 are indicated with horizontal bars. (e) Electropherograms showing the mutation in the affected patient (MC17b) (top) and a control (bottom). A single nucleotide deletion in exon 4 results in a frameshift. mut, a mutant allele; wt, a wild type allele.

encompassed exons and (3) CNVs were not present in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>).

Cloning of duplication breakpoints

DNA of the affected mother (MC17b) was digested with restriction enzymes: *EcoRI*, *NsiI*, *XbaI*, *BamHI* and *BglII* (New England Biolabs, Beverly, MA, USA). Digested DNA was self-ligated by Ligation High ver. 2 (Toyobo, Osaka, Japan), precipitated with ethanol, and dissolved in 20 µl EB buffer (QIAGEN, Tokyo, Japan). Inverse PCR was performed in 25 µl volume containing 2 µl ligated DNA, 1× PCR buffer for KOD FX, 0.4 mM each dNTPs, 0.5 µM each primer and

Table 1 Clinical features of patients with a *BCOR* mutation

	MC17b	MC17	MC18
Age	41	11	8
Sex	Female	Female	Female
<i>Ocular features</i>			
Congenital cataract	+	+	+
Microphthalmia/microcornea	—	—	—
Coloboma	—	—	—
Ptosis	—	—	—
Secondary glaucoma	—	+	+
Nystagmus	—	—	—
<i>Cardiac features</i>			
Septal defects	—	+	—
Patent ductus arteriosus	—	+	—
<i>Dental features</i>			
Delayed/persistent/unerupted dentition	+	+	+
Root radiculomegaly (secondary teeth)	ND	ND	ND
Hypodontia (secondary teeth)	+	ND	ND
Duplication/fusion (secondary teeth)	ND	ND	ND
<i>Skeletal features</i>			
Hammer toes (camptodactyly)	+	+	+
Second-third toe syndactyly	+	+	+
Broad halluces (big toe)	—	+	+
Brachyphalangia of the fourth right toe	—	+	—
Radioulnar synostosis	ND	ND	ND
Lodosis/scoliosis/vertebral fusion	ND	ND	ND
<i>Other features</i>			
Mental retardation	—	—	+
Hearing impairment	—	ND	ND
Inguinal hernia	—	—	+

Abbreviation: ND, Not determined.

A plus (+) or minus (—) sign denotes the presence or absence of a particular physical feature.

0.5 U KOD FX polymerase (Toyobo). PCR were cycled 35 times at 98 °C for 10 s, 68 °C for 10 min. PCR products electrophoresed in 1% agarose gel were stained with ethidium bromide and the aberrant band was extracted using QIAEXII Gel Extraction Kit (QIAGEN, Tokyo, Japan) and sequenced. Primer information is available on request.

X inactivation study and haplotype analysis

X inactivation pattern was studied using the human androgen receptor assay and fragile X mental retardation locus methylation assay as previously described.^{13–15} Briefly, genomic DNA of a patient (MC17), the parents (MC17a and MC17b), a control male and a control female was digested with two methylation-sensitive enzymes, *HpaII* and *HhaI*. PCR was performed with human androgen receptor assay primers (FAM-labeled ARf: 5'-CCAGAATCTGTCCAGAGCGTGC-3'; ARr: 5'-CTCTACGATGGGCTTGGGAGAA C-3')¹⁶ and fragile X mental retardation assay primers (FAM-labeled FMR1f: 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'; FMR1r: 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3'). Fluorescent-labeled products were analyzed on an ABI PRISM 3100 or 3130×1 Genetic analyzer and GeneMapper Software version 4.0 (Applied Biosystems). One of affected females (MC18) was not analyzed because of insufficient amount of genomic DNA. According to published criteria, X inactivation ratios of ≤80:20 were considered random and ratios >80:20 were considered skewed and ratios >90:10 were considered highly skewed.^{16,17}

X chromosome haplotype was analyzed using 12 microsatellite markers (*DXS1060*, *DXS8051*, *DXS987*, *DXS1226*, *DXS1214*, *DXS1068*, *DXS993*, *DXS991*, *DXS986*, *DXS8055*, *DXS1047* and *DXS1073*). Fluorescent-labeled (either FAM, VIC or NED) forward primers and tailed reverse primers were purchased from Applied Biosystems. These markers were based on the Marshfield genetic map (<http://research.marshfieldclinic.org/genetics>). PCR was cycled 40 times at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s in 10 µl volume containing 50 ng DNA, 1× ExTaq buffer, 0.2 mM each dNTP, 0.4 µM each primer and 0.1 U ExTaq HS polymerase. Haplotypes were manually constructed.

RESULTS

We detected a *BCOR* mutation, c. 888delG in MC17, MC17b and MC18 (Figures 1d and e). The mutation may result in insertion of 80 new amino acids after the mutation site with a premature stop codon at position 377 (p.N297IfsX80). The mutation was completely co-segregated with OFCD phenotypes in this family (Figure 1a). Sequencing of the entire *NHS* coding region detected no pathological mutations in this family.

The Cytogenetics Whole-Genome 2.7 M array detected a total of 48 CNVs (12 duplications and 36 deletions) in any of the affected females. The CNVs, which encompassed exons, were 8 duplications and 13 deletions. Among them, two duplications and one deletion were shared with three affected females. The one deletion was present in the Database of Genomic Variants. Thus, the CNVs fulfilling the criteria for pathogenic CNVs were two interstitial duplications. The 740-kb duplication at Xp22.2–22.13 encompassed exons 2–18 of *REPS2* (MIM *300317) and exons 1–3 of *NHS*. The other 110-kb duplication at Xp21.3 contained exon 2 of interleukin-1 receptor accessory protein-like 1 (*ILIRAPL1*) (MIM *300206) (Figure 2a). We were unable to examine the duplications by fluorescent *in situ* hybridization because only DNA samples were available. Instead, inverse PCR of self-ligated DNA with different sets of primers was able to amplify an expected fragment from normal *NHS* allele in all sets of primers, suggesting that the presence of one or more normal *NHS* alleles (Figures 2b and c). In addition, several attempts successfully cloned one of rearrangement junctions in relation to *NHS* (Figure 2c). This aberrant band showed that the sequences of intron 1 of *ILIRAPL1* followed the sequences of intron 3 of *NHS*, suggesting that two duplications were tandemly connected (Figure 2d, upper cases). More complicatedly, 62-bp sequences of intron 3 of *ILIRAPL1* with inverted orientation were inserted between intron 3 of *NHS* and intron 1 of *ILIRAPL1* (Figure 2d, lower cases). The other possible breakpoints, which may result in disruption of *NHS* locus could not be determined regardless of rigorous attempts. Thus there seems to be two normal *NHS* alleles and an extra *NHS* allele, in which exons 1–3 of *NHS* was connected to exon 2 of *ILIRAPL1*. Copy number of the *BCOR* gene was normal. In human androgen receptor assays, the mother (MC17b) was skewed pattern (8%), whereas the elder sister (MC17) showed a random pattern (26%). In fragile X mental retardation assays, the mother (MC17b) and elder sister (MC17) showed a highly skewed pattern (<1%) and a random pattern (48%), respectively. X-chromosome haplotype analysis was able to separate all the alleles in this family (Supplementary Figure 1). The inactivated allele in the mother harbored the *BCOR* mutation and the *NHS* rearrangement.

DISCUSSION

BCOR functions as a corepressor of BCL-6, which is a POZ/zinc finger transcription repressor.⁴ *BCOR* have three consecutive ankyrin motifs, an AF9 (ALL1 fused gene from chromosome 9) binding site and an NSPC1 (nervous system polycomb-1) binding site.^{4,18,19} Recently, the

Figure 2 Two microduplications at Xp22.2–22.13 and Xp21.3. (a) Array profile of a part of chromosome X in the elder sister (MC17). *x* and *y* axis show the genomic location from the p telomere of chromosome X (UCSC coordinates, May, 2006) (upper) and $\log_2$ (Signal ratio) values (lower), respectively. The 740-kb duplication at Xp22.2–22.13 and the 110-kb duplication at Xp21.3 are indicated by arrows. (b) Upper shows schematic representation of two duplicated regions (highlighted in gray). Three genes (*REPS2*, *NHS* and *IL1RAPL1*) are oriented in the same direction (from centromere to telomere). Lower indicates scheme of inverse PCR using self-ligated DNA after digestion with *EcoRI*. Primers are shown by arrows (F, forward; R, reverse). (c) Gel image of inverse PCR products performed as shown in (b). Upper and lower bands represent wild-type allele (WT allele) and rearranged allele (Rea allele), respectively. The DNA of both bands was extracted from agarose gel and sequenced. (d) Sequence of a Rea allele is shown. The top, middle and bottom indicate sequences of the intron 3 of *NHS*, rearranged junction and intron 1 of *IL1RAPL1*, respectively. The 62 nucleotides of intron 3 of *IL1RAPL1* (lower cases) were inserted in an inverted orientation between intron 3 of *NHS* and intron 1 of *IL1RAPL1*. Matched sequences are highlighted with gray shadows.

minimal BCL-6 binding site was identified within residues 498–514, which is located in exon 4.²⁰ In this study, a novel *BCOR* mutation, c.888delG (p.N297IfsX80) in exon 4, was found in this family. We assumed the mutant transcript with this mutation may undergo nonsense-mediated mRNA decay, but we could not confirm it as no living cells were available from the patients. A total of 31 mutations in *BCOR* were registered in Human Gene Mutation Database (<http://www.biobase-international.com/>). All the mutations result in premature stop codons. Exon 4 harbors 40% of mutations (12/30), leading to truncated proteins lacking BCL-6, AF9 and NSPC1 binding sites, and ankyrin motifs if translated. Lack of BCL-6 binding site could not

explain the OFCD phenotypes because BCL-6-deficient mice did not show ocular, dental and skeletal phenotypes.^{21,22} Moreover, an OFCD mutant protein, that lacked ankyrin motifs and NSPC1 binding site, showed transcriptional repression activity similar to that of wild type.³ Thus, the c.888delG mutation in *BCOR* may be associated with loss of *BCOR* transcripts through nonsense-mediated mRNA decay.

By genomic microarray, two microduplications were detected in MC17, MC18 and MC17b: one at Xp22.2–22.13 involving a part of *REPS2* and *NHS*, and the other at Xp21.3 containing exon 2 of *IL1RAPL1*. Previously, complex duplication-triplication rearrangements of the *NHS* gene in a family with congenital cataract were reported, suggesting that genomic rearrangements of *NHS* are able to cause the X-linked cataract.⁸ This family did not show the typical features of *NHS* such as dental anomalies, dysmorphism and developmental delay, and congenital heart defects were diagnosed in four out of six affected males. The complex rearrangement consists of triplication embedded within a duplication region. The triplicated region includes the *NHS* genes except exon 1, and the entire *SCML1* and *RAI2* genes. Coccia *et al.*⁸ described that the additional phenotype of congenital heart defects observed in some affected males could be because of perturbed *NHS* gene transcription or increased dosage of the *NHS*, *SCML1* or *RAI2* genes. In our cases, two normal *NHS* alleles may exist in addition to an extra *NHS* allele, in which exons 1–3 of *NHS* was connected to individual exon 2 of *IL1RAPL1*, keeping the protein coding frame if properly spliced. Because the transcript from the extra *NHS* allele did not have polyA signal sequences, the allele is likely to produce no functional protein. Thus, the situation was totally different between our cases and Coccia's *et al.*'s⁸ cases.

Recently, Honda *et al.*²³ reported two unrelated X-linked mental retardation Japanese families, which possessed the similar duplication found in our Korean family: the 737-kb duplication at Xp22.2, which contains a part of *REPS2* and *NHS*, and the 100-kb duplication at Xp21.3, which contains a part of *IL1RAPL1*. In their report, fluorescent *in situ* hybridization analysis revealed that the clone RP11-438J7, which entirely covered the duplication at Xp21.3, demonstrated two distinct signals at Xp in metaphases, suggesting that the duplication at Xp21.3 was inserted separately from the original site. The clone RP11-2K15 at Xp22.2, spanning the breakpoint of *REPS2*, showed one bright strong signal, suggesting that the duplication at Xp22.2 occurred in the proximity. Interestingly, the one of two signals of the RP11-438J7 was close to that of RP11-2K15 at Xp22.2, suggesting that the duplication involving *IL1RAPL1* was inserted at Xp22.2. Their result is consistent with our data of the duplication breakpoint, in which the breakpoint in *NHS* was connected with the breakpoint of *IL1RAPL1*. Based on our experiences, high-density array experiments of >500 Japanese cases never showed such the genomic rearrangement involving *NHS*, implying that the rearrangement is very rare in Japanese population. It is noteworthy that congenital cataract and dental abnormalities were not pointed out in all the members (males and carrier females).²³ Thus, it is unlikely that the genomic rearrangement involving *NHS* causes congenital cataract and dental abnormalities as found in our family.

IL1RAPL1 is a causative gene for X-linked mental retardation,²⁴ and the microduplication at Xp21.3 containing exon 2 of *IL1RAPL1* was suggested to cause MR in affected males.²³ Most carrier females of *IL1RAPL1* mutations and the carrier mother of the microduplication involving *IL1RAPL1* showed normal intelligence.^{23–25} In this study, one of the three affected females (MC18) had learning difficulties at school, which could be mild presentation of MR. As 18% of patients with *BCOR* mutations showed MR,⁶ it is reasonable that the *BCOR* mutation, rather than *IL1RAPL1* rearrangement, causes mild MR in MC18.

Skewed and random X-inactivation in the mother (MC17b) and the elder daughter (MC17) was confirmed, respectively, in this family. In OFCD, skewed X-inactivation with the preferential inactivation of the mutated allele were recognized in eight affected females (like the mother, MC17b), suggesting that the *BCOR* mutations may lead to a selective disadvantage in blood leukocytes.^{3,26} However, it has been reported that the X-inactivation pattern in blood leukocytes are unable to determine the severity of disease phenotypes as X-inactivation pattern may vary among tissues.²⁷ We suspect that X-inactivation pattern is different between peripheral blood leukocytes and respective tissues associated with OFCD phenotype.

In conclusion, a new OFCD family is described with a novel *BCOR* mutation. Clinical features overlap between OFCD and NHS, both of which belong to a spectrum of X-linked microphthalmia disorders.⁶ Our cases have a *BCOR* mutation and genomic rearrangements involving *NHS*, confusing us to address the genetic etiology in the family. However, the presence of bilateral 2nd–3rd toe syndactyly and septal heart defects, which is unique to OFCD, can lead us to the conclusion that the *BCOR* mutation is the major determinant for the phenotypes in this family. Careful examination of associated anomalies will be useful for genetic testing of X-linked microphthalmia disorders.

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Web Resources

The URLs for data presented herein are as follows: UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>

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