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# 〔VI〕

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INVITED REVIEW ARTICLE

# Role of rare cases in deciphering the mechanisms of congenital anomalies: CHARGE syndrome research

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**ABSTRACT** **In this review, our work on CHARGE syndrome will be used to exemplify the role of rare cases in birth defects research. The analysis of 29 cases with mutations of *CHD7*, the causative gene for CHARGE syndrome, clarified the relative importance of the cardinal features, including facial nerve palsy and facial asymmetry. Concurrently, *in situ* hybridization using chick embryos studies were performed to delineate the expression pattern of *Chd7*. The *Chd7*-positive regions in the chick embryos and the anatomical defects commonly seen in patients with CHARGE syndrome were well correlated: expression in the optic placode corresponded with defects such as coloboma, neural tube with mental retardation, and otic placode with ear abnormalities. The correlation between expression in the branchial arches and nasal placode with the clinical symptoms of CHARGE syndrome, however, became apparent when we encountered two unique CHARGE syndrome patients: one with a DiGeorge syndrome phenotype and the other with a Kallman syndrome phenotype. A unifying hypothesis that could explain both the DiGeorge syndrome phenotype and the Kallman syndrome phenotype in patients with CHARGE syndrome may be that the mutation in *CHD7* is likely to exert its effect in the common branch of the two pathways of neural crest cells. As exemplified in CHARGE syndrome research, rare cases play a critical role in deciphering the mechanisms of human development. Close collaboration among animal researchers, epidemiologists and clinicians hopefully will enhance and maximize the scientific value of rare cases.**

**Key Words:** CHARGE syndrome, *CHD7*, dysmorphology, London Dysmorphology Database, methimazole embryopathy

The key components of birth defects research include animal experiments, epidemiological studies, and detailed case studies. Animal studies involve experimental procedures, including prenatal exposure to potential teratogens or gene targeting; in human studies, on the other hand, experimental approaches are not feasible and observational studies must instead be undertaken. Collectively, birth defects are relatively common in humans. Nevertheless, individual disorders are relatively uncommon, and information obtained through detailed analyses of individual cases, including genetic analyses, are thus invaluable. This notion constitutes the basis for dysmorphology. In this review, our work on CHARGE syndrome will be used to exemplify the role of rare cases in birth defects research.

CHARGE syndrome is one of the most common multiple malformation syndromes. Its characteristic features include C – coloboma, H – heart defects, A – choanal atresia/stenosis, R – retardation of growth, G – genital hypoplasia, and E – external ear abnormalities (Pagon *et al.* 1981). Vissers *et al.* identified *CHD7* at chromosome 8q12.1 as the causative gene for CHARGE syndrome in 2004 (Vissers *et al.* 2004). The causative gene was identified through physical mapping; thus, the biological function of *CHD7* was unknown at the time of its discovery.

## EPIDEMIOLOGICAL STUDY

In 2006, 24 cases were identified in Japan (Aramaki *et al.* 2006). Seventeen of these 24 cases had mutations in the *CHD7* gene. The frequency of the cardinal features of CHARGE syndrome among 17 mutation-positive cases is shown in Table 1.

In a nationwide study of CHARGE syndrome that was performed recently, we sent a questionnaire regarding CHARGE syndrome to 179 hospitals in which members of the Japan Society of Pediatric Genetics belonged at the time of study. Eighteen hospitals responded that at least one patient with CHARGE syndrome had been managed at the hospital; among these 18 hospitals, 132 patients with CHARGE syndrome were being followed. Among these 132 patients, at least 29 patients had tested positive for the *CHD7* mutation. The questionnaire contained items regarding the presence or absence of 50 characteristic features of CHARGE syndrome, including those used in the original criteria defined by Blake *et al.* (Blake and Prasad 2006).

The results of the questionnaire are summarized in Table 2. The first column contains the names of the features that were relatively common among the 29 mutation-positive cases, the second column (parameter a) contains the number of patients with that particular feature, and the third column (parameter b) contains the frequency

**Table 1** Frequency of cardinal features of CHARGE syndrome (Aramaki *et al.* 2006)

| |
|---|
| C – coloboma: 15/17 |
| H – heart defects: 13/17 |
| A – choanal atresia/stenosis: 5/17 |
| R – retardation of growth: 14/17 and development: 14/14 |
| G – genital hypoplasia: 8/8 (male), 5/9 (female) |
| E – external ear abnormalities and hearing loss: 17/17 |
| Cleft lip and palate: 8/17 |
| Tracheoesophageal fistula: 3/17 |

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**Table 2** Delineation of specific features of CHARGE syndrome

| Features | **a**: Number of patients among 29 mutation-positive cases | **b**: Frequency a/29 | **c**: Number of syndromes in London Dysmorphology Database | **d**: b/c × 100 |
|---|---|---|---|---|
| Choanal atresia or stenosis | 8 | 0.28 | 74 | 0.37 |
| Coloboma (iris, optic nerve, retina/choroid) | 24 | 0.83 | 178 | 0.46 |
| Characteristic external ears | 29 | 1.00 | 202 | 0.50 |
| Cleft palate | 15 | 0.52 | 466 | 0.11 |
| Congenital heart defects | 20 | 0.69 | 817 | 0.08 |
| Undescended testes or micropenis | 13 | 0.45 | 427 | 0.10 |
| Esophago-tracheal anomalies | 7 | 0.24 | 104 | 0.23 |
| Facial nerve palsy or asymmetric face | 26 | 0.90 | 103 | 0.87 |
| Developmental delay | 29 | 1.0 | 1137 | 0.088 |
| Short stature | 29 | 1.0 | 1392 | 0.072 |

of the feature. To evaluate the specificity of each cardinal feature, we searched the London Dysmorphology Database (Winter and Baraitser 1987), which contains more than 3000 syndromes with 700 query features. The fourth column (parameter c) contains the number of syndromes with that particular feature as registered in the London Dysmorphology Database. Thus, smaller numbers indicate more specific features. Finally, to define the relative importance of the features in supporting the diagnosis of CHARGE syndrome, we divided the number in the third column (parameter b) by the number in the fourth column (parameter c). The resulting parameter is shown in the fifth column (parameter d).

An analysis of these parameters revealed the following observations: first, developmental delay and short stature had high values (i.e. 100%) for parameter b. Nevertheless, the values of parameter c were also high, resulting in a low parameter d-values for developmental delay and short stature. Second, the value of parameter d for facial nerve palsy and/or an asymmetric face was very high and thus may be considered as a useful feature. In the Blake criteria (Blake and Prasad 2006), both facial nerve palsy and swallowing function were included in the cranial nerve palsy. However, swallowing dysfunction had a very high value for parameter c and thus could be excluded from the criteria. Based on the relative importance of these cardinal features, as outlined above, the author suggests that the existing clinical criteria for CHARGE syndrome could be revised (Table 3). The validity of this proposed revision of the diagnostic criteria needs to be evaluated in a separate group of *CHD7* mutation-positive CHARGE syndrome patients.

**Table 3** Proposed revision of the clinical criteria for CHARGE syndrome

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| **Essential features** |
| Bilateral hearing loss with external ear anomalies |
| Short stature |
| Developmental delay of variable degree |
| **Major criteria** |
| Ocular coloboma of any kind |
| Choanal atresia or cleft palate |
| Facial nerve palsy or facial asymmetry |
| **Minor criteria** |
| Congenital heart defects |
| Tracheoesophageal anomalies |
| Micropenis or undescended testes (male) |

Clinical diagnosis of CHARGE syndrome can be made when the patient fulfils the essential features and has two or more major features or has one major feature with two or more minor features.

## CHICK *IN SITU* HYBRIDIZATION STUDY

As the clinical spectrum of CHARGE syndrome has now been clarified, we wished to know whether the anatomical distribution of defects was correlated with the expression pattern of CHD7 in early embryos. We performed *in situ* hybridization using chick embryos to delineate the expression pattern of *Chd7* (Aramaki *et al.* 2007). First, we identified partial fragments of chicken *Chd7* sequences using a bioinformatics analysis and determined the missing portion of the transcript using reverse transcriptase polymerase chain reaction (RT-PCR). The presumable chicken *Chd7* mapped to chicken chromosome 2. The order of genes surrounding the *Chd7* gene was conserved between humans and chickens. Based on this finding, we concluded that a true homolog or ortholog of human *Chd7* was identified in the chicken genome. Using a probe that is complementary to the putative chicken *Chd7* cDNA sequence, the expression pattern of the *Chd7* gene was delineated.

At Hamburger and Hamilton stage 8, *Chd7* expression was detected along the entire rostrocaudal axis of the neuroectoderm. At stages 12 and 13, *Chd7* expression was seen in the neural ectoderm and was uniformly expressed at high levels. Two paraxial crescent signals representing the dorsal halves of the otic placodes were identified at the hindbrain level. At stage 14, *Chd7* was expressed at the optic vesicles. At stage 20, *Chd7* was expressed in the brain and the optic placode, including the lens vesicle. *Chd7* expression was also observed in the branchial arches and olfactory placodes. The *Chd7*-positive regions in the chick embryos and the anatomical defects commonly seen in patients with CHARGE syndrome were well correlated: expression in the optic placode corresponded with defects such as coloboma, neural tube with mental retardation, and otic placode with ear abnormalities. The correlation between

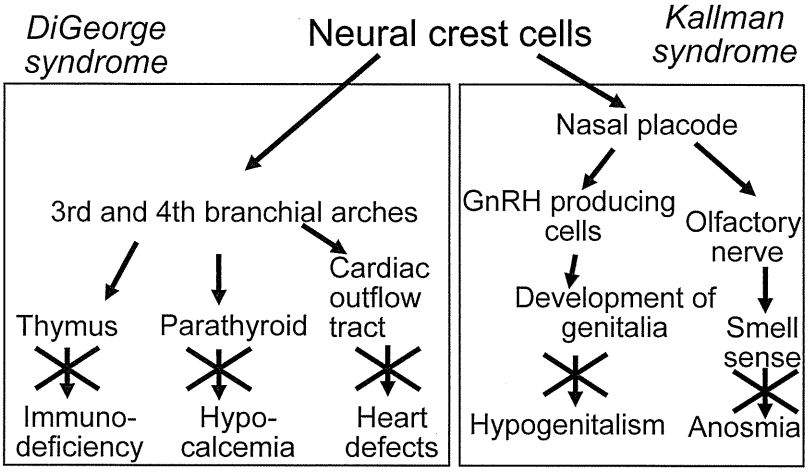


**Fig. 1** Two developmental pathways affected in DiGeorge syndrome phenotype and Kallman syndrome phenotype are depicted. The two pathways share a common feature: the involvement of the neural crest.

expression in the branchial arches and nasal placode with the clinical symptoms of CHARGE syndrome, however, was not obvious until we encountered two unique cases (Ogata *et al.* 2006; Inoue *et al.* 2010).

## SIGNIFICANT CASES

Interestingly, we had the opportunity to analyze a patient with CHARGE syndrome and a *CHD7* mutation who exhibited a DiGeorge syndrome phenotype (Inoue *et al.* 2010). DiGeorge syndrome is characterized by cellular immunodeficiency as a result of thymus hypoplasia, hypocalcemia arising from parathyroid hypoplasia, and heart defects. Similar cases have been reported from other groups recently (Hoover-Fong *et al.* 2009). Hence, the association between the *CHD7* mutation and DiGeorge syndrome is unlikely to have occurred by chance. The developmental abnormality leading to DiGeorge syndrome is accounted for by defects in the formation of the neural crest that contributes to the third and fourth branchial arch derivatives including the thymus, parathyroid, and thyroid glands. The observation that patients with CHARGE syndrome phenotype and *CHD7* mutation exhibited a DiGeorge syndrome phenotype does not prove but strongly suggests that *CHD7* contributes to either the formation or the maintenance of neural crest cells of the third and fourth branchial arches.

We also encountered a patient with CHARGE syndrome who also exhibited a Kallman syndrome phenotype, a combination of central hypogonadism accompanied by anosmia, or a lack of the sense of smell (Ogata *et al.* 2006). Furthermore, our collaborators have shown that a defect in the olfactory bulb is a common finding among patients with CHARGE syndrome (Asakura *et al.* 2008). This finding was subsequently confirmed by other groups as well. The formation of these two apparently different defects in a patient (CHARGE syndrome and Kallman syndrome) is accounted for by a defect in a common developmental pathway: the nasal placode contributes to both gonadotropin releasing hormone-producing cells in the hypothalamus and olfactory nerve cells. Defects in the origin of both cell lineages, the nasal placode or its upstream structures neural crest cells, may lead to the Kallman syndrome phenotype. The observation that patients with the CHARGE syndrome phenotype and *CHD7* mutation exhibited Kallman syndrome phenotype suggests that *CHD7* contributes to either the formation or maintenance of the neural nasal placode. So, based on observations of rare cases, we suggested that expression in the branchial arches (Aramaki *et al.* 2007) may be correlated with the DiGeorge syndrome phenotype (Inoue *et al.* 2010) and that expression in the nasal placode (Aramaki *et al.* 2007) may be correlated with the Kallman syndrome phenotype (Ogata *et al.* 2006).

A unifying hypothesis that could explain both the DiGeorge syndrome phenotype and the Kallman syndrome phenotype in patients with CHARGE syndrome may be that the mutation in *CHD7* is likely to exert its effect in the common branch of the two pathways of neural crest cells (Fig. 1). Indeed, the notion that *CHD7* plays a critical role in neural crest formation was recently demonstrated by Dr Wysocka's group (Bajpai *et al.* 2010). They induced neural crest cells from human embryonic stem cells (ES) cells and abolished the function of the *CHD7* gene using small interfering RNA (SiRNA), documenting the subsequent defects in the migration of multipotent neural crest cells. In other words, *CHD7* plays a critical role in the formation of multipotent migratory neural crest cells. Hence, what was strongly suggested by clinical observation was documented using *in vitro* studies.

Overall, animal (i.e. chicken) experiments have provided insight that was later proven to be relevant in humans. More specifically, expression in the branchial arch or expressions in the nasal placode (Aramaki *et al.* 2007) may account for the DiGeorge syndrome phenotype (Inoue *et al.* 2010) or the Kallman syndrome phenotype (Ogata *et al.* 2006) that can appear in patients with CHARGE syndrome who have a *CHD7* mutation.

## METHIMAZOLE EMBRYOPATHY AS PHENOCOPY OF CHARGE SYNDROME

Here, the author wishes to illustrate how detailed case studies can contribute to epidemiological studies, using methimazole embryopathy as an example (Aramaki *et al.* 2005). Whether methimazole, an antithyroid drug, represents a teratogen has been the subject of debate. The vast majority of infants prenatally exposed to methimazole are normal. Nevertheless, several reports have suggested a possible causal relationship between methimazole exposure and birth defects, including aplasia cutis, esophageal malformations, and persistent vitelline duct (Johnsson *et al.* 1997; Clementi *et al.* 1999). Interestingly, choanal atresia, one of the cardinal features of

CHARGE syndrome, has been reported several times. Greenberg reported a case of prenatal exposure to methimazole resulting in choanal atresia and hypoplastic nipples (Greenberg 1987). Subsequently, Wilson *et al.* reported another patient prenatally exposed to methimazole who exhibited choanal atresia (Wilson *et al.* 1998). Barbero *et al.* recently reported three cases of prenatal exposure to methimazole resulting in choanal atresia (Barbero *et al.* 2004).

Choanal atresia is a congenital failure of the communication of the nasal cavity and nasopharynx and is a highly specific feature for CHARGE syndrome. So, the natural question to ask would be whether methimazole may be associated with another very specific feature of CHARGE syndrome, coloboma of the eyes. Indeed, the author recently evaluated a newborn female who had been prenatally exposed to methimazole (Aramaki *et al.* 2005). The patient exhibited multiple anomalies, including vitelline duct anomalies and nipple hypoplasia. In place of choanal atresia, however, the baby exhibited ocular coloboma. Because choanal atresia and coloboma occur together more frequently than otherwise expected and are features of CHARGE syndrome, we suspected that this case may expand the phenotypic spectrum of prenatal methimazole exposure. Furthermore, we suggested that the pathogenesis of methimazole embryopathy and the CHARGE syndrome phenotype may be causally associated. The molecular mechanism leading to methimazole embryopathy is completely unknown at present, and our case may provide a new clue. It would be important to test whether *CHD7* expression is affected after prenatal methimazole exposure using animal models.

## FUTURE DIRECTIONS

What can we do to better exploit the scientific value of rare cases among specialists in various fields of teratology? First of all, descriptive terms for congenital malformations should be standardized to enable better interdisciplinary communication. Fortunately, an international working group has proposed a standard terminology for human teratology, and a consensus has been published together with hundreds of pictures in the *American Journal of Medical Genetics* (Allanson *et al.* 2009). The Japanese Teratology Society finalized similar standard terminology for mice, and hopefully comparisons between humans and mice will be easier to perform with the help of such standard terminology (Makris *et al.* 2009). Second, I would propose that a detailed postnatal physical examination be performed when epidemiological studies on prenatal exposure to teratogens are performed. The use of standard terminology will be extremely helpful for precise communication and documentation. Again, collaboration between epidemiologists and dysmorphologists would be invaluable and essential. The standardization of phenotypic information should also help to establish national or international registries for rare conditions. Such registries would be even more valuable if biological samples were available for *in vitro* research.

In summary, rare cases play a critical role in deciphering the mechanisms of human development. Close collaboration among animal researchers, epidemiologists and clinicians hopefully will enhance and maximize the scientific value of rare cases.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

# Ophthalmic Features of CHARGE Syndrome With CHD7 Mutations

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Coloboma and various ocular abnormalities have been described in CHARGE syndrome, although the severity of visual impairment varies from case to case. We conducted a multicenter study to clarify the ophthalmic features of patients with molecularly confirmed CHARGE syndrome. Thirty-eight eyes in 19 patients with CHARGE syndrome and confirmed CHD7 mutations treated at four centers were retrospectively studied. Colobomata affected the posterior segment of 35 eyes in 18 patients. Both retinochoroidal and optic disk colobomata were bilaterally observed in 15 patients and unilaterally observed in 3 patients. The coloboma involved the macula totally or partially in 21 eyes of 13 patients. We confirmed that bilateral large retinochoroidal colobomata represents a typical ophthalmic feature of CHARGE syndrome in patients with confirmed CHD7 mutations; however, even eyes with large colobomata can form maculas. The anatomical severity of the eye defect was graded according to the presence of colobomata, macula defect, and microphthalmos. A comparison of the severity in one eye with that in the other eye revealed a low-to-moderate degree of agreement between the two eyes, reflecting the general facial asymmetry of patients with CHARGE syndrome. The location of protein truncation and the anatomical severity of the eyes were significantly correlated. We suggested that the early diagnosis of retinal morphology and function may be beneficial to patients, since such attention may determine whether treatment for amblyopia, such as optical correction and patching, will be effective in facilitating the visual potential or whether care for poor vision will be needed.



**Key words:** CHARGE syndrome; *CHD7*; coloboma; ophthalmic features

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## INTRODUCTION

CHARGE syndrome is a multiple malformation syndrome named from the acronym of its major features: coloboma, heart defects, atresia of the choanae, retarded growth and/or development, genital anomalies, and ear abnormalities [Pagon et al., 1981; Zentner et al., 2010]. The major ocular feature of CHARGE syndrome is coloboma, and a previous investigation by ophthalmologists revealed an incidence of up to 86%, although the severity

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of coloboma and visual impairment varied from case to case [Russell-Eggitt et al., 1990].

Recently, the gene *Chromodomain helicase DNA-binding protein-7* (*CHD7*) at chromosome 8q12.1 was identified as a causative gene of CHARGE syndrome [Vissers et al., 2004]. Up to 70% of patients clinically diagnosed as having CHARGE syndrome exhibit mutations in the *CHD7* gene [Aramaki et al., 2006a; Jongmans et al., 2006; Lalani et al., 2006]. Although the exact function of this gene product remains unknown, it may have an important effect on an early stage of ocular morphogenesis.

We conducted the present multicenter study to clarify the ophthalmic features of patients with molecularly confirmed CHARGE syndrome and to explore the role of *CHD7* in ocular development.

## PATIENTS AND METHODS

Thirty-eight eyes in 19 patients clinically diagnosed as having CHARGE syndrome at the National Center for Child Health and Development, the Osaka Medical Center and Research Institute for Maternal and Child Health, the Kanagawa Children's Medical Center, or the Institute for Developmental Research, Aichi Human Service Center were retrospectively studied. All the patients had been molecularly confirmed to carry *CHD7* mutations at the Keio University School of Medicine [Aramaki et al., 2006a]. The clinical diagnosis of CHARGE syndrome was made based on the Blake criteria [Blake et al., 1998]. Molecular screening for mutations in the *CHD7* gene was conducted as reported previously [Aramaki et al., 2006b]. Ophthalmic features were examined using slit-lamp biomicroscopy and binocular indirect ophthalmoscopy. Two patients were also examined using a spectral domain optical coherence tomography (SD-OCT). The SD-OCT images were obtained with RS-3000 (NIDEK Co., Ltd., Gamagori, Japan). The best-corrected visual acuity (BCVA) was measured with a standard Japanese VA chart using Landolt rings or pictures at 5 m, then converted to Snellen VA.

The anatomical severity of the eye defect was classified as follows: Grade 1, Normal; Grade 2, colobomata with macular formation; Grade 3, colobomata including the macula; and Grade 4, colobomata, macular defect, and microphthalmos. Then, Cohen's kappa coefficient [Cohen, 1960] was used to measure the agreement of the severity in the two eyes among 19 CHD7-mutation positive patients. The potential correlation between the anatomical severity of the eyes in an individual and the amino acid position where the truncation of the *CHD7* protein occurred in the same individual was evaluated among 14 patients with protein-truncating mutations.

This study was approved by the institutional ethics committee; the patients or the parents of the patients provided informed consent prior to enrollment in the study.

## RESULTS

The characteristics of the 38 eyes of the 19 patients with CHARGE syndrome carrying *CHD7* mutations are summarized in Table I. Ten patients (53%) were male and 9 (47%) were female. The age of the patients at the time of the examination ranged from 1 to 21 years (mean 7.9 ± 5.0 years). Ocular abnormalities were found in 18 patients (94.7%), bilateral abnormalities were observed in 17 patients (89.4%), and unilateral abnormalities were observed in 1 patient (5.3%). Among these 18 patients, all 35 abnormal eyes had varying severities of colobomata.

TABLE I. Characteristics of Patients of CHARGE Syndrome With CHD7 Mutations (n = 9)

| Variable | Number |
|---|---|
| Gender | |
| Male | 10 (53%) |
| Female | 9 (47%) |
| Age at examination | 1–21 years |
| Mean | 7.9 ± 5.0 years |
| Ocular abnormalities (colobomata) | |
| Bilateral | 17 (89.4%) |
| Unilateral | 1 (5.3%) |
| None | 1 (5.3%) |
| BCVA | |
| <20/400 | 4 (21.1%) |
| 20/400 to <20/60 | 7 (36.8%) |
| 20/60 to 20/20 | 6 (31.6%) |
| Not measured | 2 (10.5%) |

BCVA, best-corrected visual acuity.

The ocular features of the individual patients are summarized in Table II. Colobomata affected the posterior segment in 35/38 eyes (92.1%), retinochoroidal coloboma was present in 33 eyes (86.8%), and optic disk coloboma was present in 33 eyes (86.8%). Both retinochoroidal coloboma and optic disk coloboma were bilaterally present in 15 patients (78.9%) and unilaterally present in 3 patients (15.8%). The coloboma involved the macula totally or partially in 21 eyes (55.3%) of the 13 patients (68.4%): bilaterally in 8 patients

TABLE II. Ocular Features of the Patients (n = 19 patients, 38 eyes)

| | Number of patients (%) | | | Number of |
|---|---|---|---|---|
| Findings | Bilateral | Unilateral | Total | eyes (%) |
| Colobomata | 17 (89.5) | 1 (5.3) | 18 (94.7) | 35 (92.1) |
| Retinochoroidal | 15 (78.9) | 3 (15.8) | 18 (94.7) | 33 (86.8) |
| Optic disk | 15 (78.9) | 3 (15.8) | 18 (94.7) | 33 (86.8) |
| Macula | 8 (42.1) | 5 (26.3) | 13 (68.4) | 21 (55.3) |
| Iris | 1 (5.3) | 0 (0.0) | 1 (5.3) | 2 (5.3) |
| Lens | 0 (0.0) | 1 (5.3) | 1 (5.3) | 1 (2.6) |
| Microphthalmos | 3 (15.8) | 2 (10.5) | 5 (26.3) | 8 (21.1) |
| Microcornea | 3 (15.8) | 1 (5.3) | 4 (21.1) | 7 (18.4) |
| Ptosis | 1 (5.3) | 1 (5.3) | 2 (10.5) | 3 (7.9) |
| PFV | 0 (0.0) | 1 (5.3) | 1 (5.3) | 1 (2.6) |
| Cataract | 0 (0.0) | 1 (5.3) | 1 (5.3) | 1 (2.6) |
| High myopia (>6.0 D) | 2 (10.5) | 1 (5.3) | 3 (15.8) | 5 (13.2) |

PFV, persistent fetal vasculature.

(42.1%) and unilaterally in 5 patients (26.3%). The SD-OCT demonstrated a partially formed macula and cystic changes in the colobomatous area in 1 case (Fig. 1).

Only 2 eyes of 1 patient (5.3%) were identified as having iris colobomata, and 1 eye (2.6%) of another patient was revealed by examination under general anesthesia to have a dislocated and colobomatous lens. No cases of eyelid colobomata were seen, but congenital ptosis was present in 3 eyes (7.9%) of 2 patients who had undergone surgical treatment. All the cases of ptosis were not pseudoptosis associated with microphthalmos and/or cranial nerve palsy, but were true congenital ptosis associated with poor levator function. We evaluated the levator muscle function in each case. None of the patients had a history of acquired causes or signs of oculomotor palsy, such as paralytic strabismus and limited ocular movement.

Microphthalmos was found in 8 eyes (21.1%) of 5 patients (26.3%): bilaterally in 3 patients (15.8%) and unilaterally in 2 patients (10.5%). Microcornea was also present in 7 eyes (18.4%) of 4 patients (21.1%): bilaterally in 3 patients (15.8%) and unilaterally in 1 patient (5.3%). Persistent fetal vasculature was identified in 1 eye (2.6%). Cataracts had developed in 1 eye (2.6%), but neither glaucoma nor retinal detachment was observed in this series.

The refraction could be estimated in 23 eyes of 12 patients (63.2%). Of these eyes, 10 were myopic, 7 were emmetropic, and 6 were hypermetropic. High myopia (−6.00 diopters or more) was found in 5 eyes (13.2%) of 3 patients (15.8%).

The BCVA are shown in Table I. The measurement of VA was possible in 17 patients (89.5%) older than 3 years of age. The remaining 2 patients were infants or mentally retarded. The binocular BCVA or BCVA in the better eye was less than 20/400 in 4 patients (21.1%), less than 20/60 but no less than 20/400 in 7 patients (36.8%), and 20/60 to 20/20 in 6 patients (31.6%) with macular formation (Fig. 1). The overall prevalence of blindness and visual impairment (less than 20/60) [World Health Organization, 1992] among the 17 patients was 65%.

The agreement of anatomical severity between the 2 eyes in each of the 19 patients was evaluated using Cohen's Kappa statistics. The $\kappa$ statistic of 0.41 suggested a moderate degree of agreement, per the guidelines by Landis and Koch [1977]. Because there was a moderate, if not a substantial, agreement between the severity of the 2 eyes, the severity grading of the more severely affected eye was used as the representative grade for the severity of the eyes in an individual. The correlation between the anatomical severity of the eyes in an individual and the amino acid position where the truncation of the CHD7 protein occurred in the same individual is illustrated in Figure 2. Patients with truncated protein devoid of the SANT domain tended to have severer anatomical defects of the eyes. Subcategorization of the patients according to the presence or absence of the SANT domain (4 cases with intact SANT domain and 10 other cases), and the subcategorization of the anatomical severity of the eyes in an individual (7 cases classified as Grade 1 or 2 vs. 7 cases classified as Grade 3 or 4) revealed a statistically

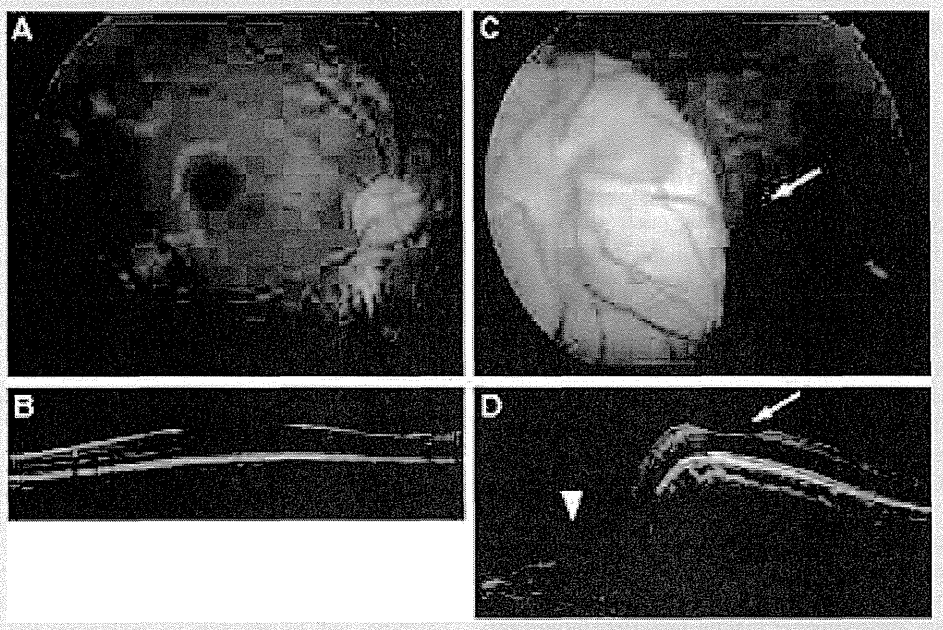


FIG. 1. Fundus photographs and spectral domain optical coherence tomography (SD-OCT) scan of the retina in the right eye (A,B) and the left eye (C,D) in a 6-year-old girl. A: Retinochoroidal colobomata inferior to the optic disk is visible in the right eye. B: The SD-OCT shows a good macular formation in the right eye, resulting in a BCVA of 20/20. C: Retinochoroidal and optic disk coloboma are seen in the left eye. The colobomata partially involved the macula (arrow). D: The SD-OCT shows a partially formed macula (arrow) and cystic changes in the colobomatous area (arrow head) in the left eye, resulting in a BCVA of 20/50 after amblyopia treatment.

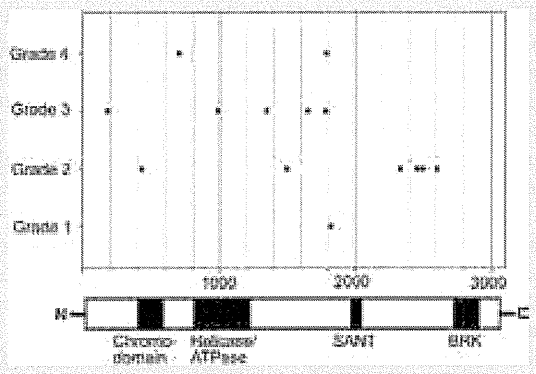


**FIG. 2. The correlation between the anatomical severity of the eyes in an individual and the amino acid position where the truncation of the CHD7 protein occurred in the same individual. Horizontal axis indicates amino acid position of the CHD7 protein together with the domains of the protein. Vertical axis indicates the anatomical severity of the eye defect classified as follows: Grade 1, Normal; Grade 2, colobomata with macular formation; Grade 3, colobomata including the macula; and Grade 4, colobomata, macular defect, and microphthalmos.**

significant correlation between the location of protein truncation and the anatomical severity of the eyes ($P = 0.02$, chi-squared test).

## DISCUSSION

In the current series, the incidence of coloboma, the major ocular feature of CHARGE syndrome, was 94.7% (18/19), which was much higher than the previously reported incidence. Since most of the authors were ophthalmologists, the number of cases without eye defects might have been underrepresented. Hence, this high incidence should be viewed with caution. Nevertheless, attending clinical geneticists were on duty at all the participating children's hospitals, and thus the bias from such underrepresentation may be relatively small. The finding that there was one mutation-positive patient who did not have abnormal eye findings confirms that no finding in CHARGE syndrome has a 100% penetrance as is sometimes surmised.

Both retinochoroidal and optic disk coloboma occurred in 94.7% of the cases, mostly bilaterally, while the incidence of iris coloboma was only 5.3% (1/19). Coloboma also affected the macula in 68.4% of the cases. We confirmed that bilateral large retinochoroidal colobomata represent a typical ophthalmic feature of CHARGE syndrome with *CHD7* mutations.

The incidence of anomalies in the anterior segment was lower than that in the posterior segment, although microphthalmos, microcornea, PFV, and cataracts were present in some cases bilaterally or unilaterally. The presence of characteristic large retinochoroidal coloboma indicates the essential role of *CHD7* in the closure of the fetal fissure posteriorly between 5 and 6 weeks of gestation, and the malfunction of *CHD7* may have an effect so severe as to influence the entire ocular morphogenesis to some degree. Although most cases had bilateral colobomata in the posterior segment, the severity and associated features often differed between the two eyes. Other associated features in this series were ptosis in 10.5% and high myopia in 15.8%. Subtle-associated anomalies and refractive errors may have been underestimated in examinations that were not performed under general anesthesia.

The anatomical severity grading of the eye defect was evaluated in two ways: a comparison between the severity in one eye in comparison with that in the other eye and the correlation between the severity and the genotype. The low-to-moderate degree of agreement between the two eyes (i.e., left and right) reflects the general facial asymmetry in patients with CHARGE syndrome [Zentner et al., 2010]. In other words, the lack of substantial or perfect agreement between the anatomical severity of the right and the left eyes indicates a variable phenotypic effect of the same mutation. Yet, the location of protein truncation and the anatomical severity of the eyes were significantly correlated: if the chromodomain, helicase/ATP domain, and SANT domains are intact, the severity of the eyes tends to be milder. Interestingly, all four cases in which those domains were intact had less severe eye defects with intact macula. Further studies are warranted to verify this potential genotype–phenotype correlation.

The visual acuities of the eyes ranged between no light perception and 20/20, and the prevalence of blindness and visual impairment (less than 20/60) was 65% among 17 patients. A poor visual prognosis depended on the presence of a large coloboma involving the macula in the posterior segment and associated microphthalmos or microcornea, as reported previously [Russell-Eggitt et al., 1990; Hornby et al., 2000]. On the other hand, even eyes with large colobomata as a result of *CHD7* mutations were capable of forming maculas, resulting in good central visual acuity with superior visual field defects. As shown in the case illustrated in Figure 1, even a partially formed macula will enable useful vision following the adequate treatment of amblyopia as optical correction and patching during the earlier age of visual development. A recent report of a case examined using OCT revealed additional morphologic characteristics of eyes in patients with CHARGE syndrome carrying *CHD7* mutations [Holak et al., 2008]. Further investigation of retinal morphology and function using OCT and electroretinograms (ERG) may help to clarify the function of *CHD7* in ocular morphogenesis, including macular formation.

We suggested that the early diagnosis of retinal morphology and function, especially of macular lesions by way of OCT and ERG, may be beneficial to patients, since such attention may determine whether treatment for amblyopia, such as optical correction and patching, will be effective in facilitating the visual potential or whether care for poor vision will be needed. An infant's visual acuity rapidly develops during its first 2–3 years and continues up until 7–8 years of age, but plasticity decreases progressively thereafter. Thus, a better visual prognosis can be obtained with the earlier treatment of amblyopia during the critical period of visual development.

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## Original Article

# Exome sequencing in a family with an X-linked lethal malformation syndrome: clinical consequences of hemizygous truncating *OFD1* mutations in male patients

Tsurusaki Y, Kosho T, Hatasaki K, Narumi Y, Wakui K, Fukushima Y, Doi H, Saitsu H, Miyake N, Matsumoto N. Exome sequencing in a family with an X-linked lethal malformation syndrome: clinical consequences of hemizygous truncating *OFD1* mutations in male patients.
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Oral-facial-digital syndrome type 1 (OFD1; OMIM #311200) is an X-linked dominant disorder, caused by heterozygous mutations in the *OFD1* gene and characterized by facial anomalies, abnormalities in oral tissues, digits, brain, and kidney; and male lethality in the first or second trimester pregnancy. We encountered a family with three affected male neonates having an 'unclassified' X-linked lethal congenital malformation syndrome. Exome sequencing of entire transcripts of the whole X chromosome has identified a novel splicing mutation (c.2388+1G > C) in intron 17 of *OFD1*, resulting in a premature stop codon at amino acid position 796. The affected males manifested severe multisystem complications in addition to the cardinal features of OFD1 and the carrier female showed only subtle features of OFD1. The present patients and the previously reported male patients from four families (clinical OFD1; Simpson-Golabi-Behmel syndrome, type 2 with an *OFD1* mutation; Joubert syndrome-10 with *OFD1* mutations) would belong to a single syndrome spectrum caused by truncating OFD1 mutations, presenting with craniofacial features (macrocephaly, depressed or broad nasal bridge, and lip abnormalities), postaxial polydactyly, respiratory insufficiency with recurrent respiratory tract infections in survivors, severe mental or developmental retardation, and brain malformations (hypoplasia or agenesis of corpus callosum and/or cerebellar vermis and posterior fossa abnormalities).

### Conflict of interest

The authors have no conflict of interest to declare.

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Key words: exome sequencing – OFD1 – *OFD1* gene – splicing mutation – X-linked congenital malformation syndrome

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Oral-facial-digital syndrome type 1 (OFD1; OMIM #311200), originally described by Papillon-Leage and Psaume (1) and further delineated by Gorlin and Psaume (2), is an X-linked dominant developmental disorder with an estimated prevalence of 1:50,000, caused by mutations in the *OFD1* gene (OMIM #300170) (3–5). The disorder is characterized by facial anomalies and abnormalities in oral tissues, digits, brain and kidney (5). Almost all affected individuals with OFD1 are female, with highly variable expression, possibly resulting from random X inactivation (6). Affected males are generally lost in the first or second trimester of pregnancy (4). To date, only one liveborn male case with clinically definite OFD1 and a normal karyotype has been reported; the patient was born at 34 weeks of gestation and died 21 h after birth due to heart failure (7). In this report, we describe a family with three affected male neonates having an 'unclassified' X-linked lethal congenital malformation syndrome. Exome sequencing of entire transcripts of the whole X chromosome has successfully identified a causative splicing mutation in *OFD1*.

## Subjects and methods

### Clinical report

II-2, a 22-year-old woman, was referred to our clinic for genetic counseling (Fig. 1). Her deceased brother (II-4) had severe multiple congenital abnormalities. She had two sons (III-1 and III-5) with similar congenital abnormalities and a healthy boy (III-3) as well as two miscarriages (III-2, artificial; III-4, spontaneous). During genetic counseling and molecular investigations, she had another healthy boy (III-5). After identification of a heterozygous *OFD1* mutation, she was examined for features of OFD1. Only a few accessory frenulae and irregular teeth with no facial anomalies or tongue abnormalities were observed (Fig. 2a–e). A radiograph of her hands showed no abnormalities (Fig. 2f) and an abdominal ultrasonography detected no cysts in the kidneys, liver, or pancreas (data not shown). I-2, allegedly, had no apparent malformations or complications including renal diseases.

II-4 was born by caesarean section because of placental abruption at 33 weeks of gestation. Pregnancy was complicated by polyhydramnios. Apgar score was 3 at 1 min. His birth weight was 2056 g (+0 SD), length was 45.0 cm (+0.5 SD), and occipitofrontal circumference (OFC) was 34.0 cm (+2.0 SD). He manifested severe respiratory insufficiency and was transferred to a neonatal intensive care unit (NICU). His craniofacial features included a prominent forehead, a large fontanelle (5 × 5 cm), a low posterior hairline, microphthalmia, hypertelorism, short palpebral fissures, depressed nasal bridge, low-set ears, a small cleft lip and a soft cleft palate, narrowing of the tip of the tongue, and a hypoplastic gum (Fig. 2g). Additional physical features included redundant neck skin, postaxial polydactyly of the left hand (Fig. 2h), wide halluces (Fig. 2i), micropenis, and left cryptorchidism.

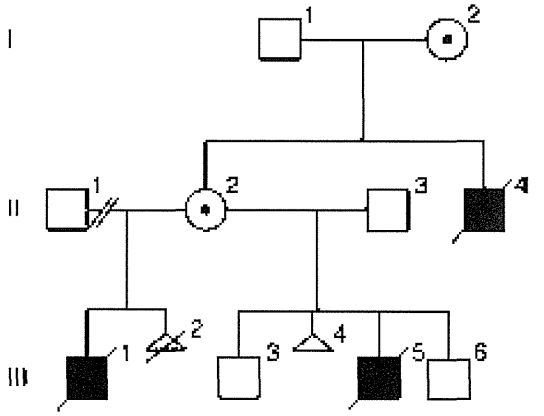


*Fig. 1.* Familial pedigree.

Ultrasonography revealed hypoplastic gyri, an atrial septal defect, and patent ductus arteriosus. Ophthalmological examination detected microcornea and retinal detachment. Intubation was impossible because of laryngeal anomalies and the patient died 11 h after birth. Additional autopsy findings included partial atelectasis and bilateral hydroureters.

III-1 was delivered by emergency caesarean section at 39 weeks of gestation. Pregnancy was complicated by polyhydramnios and intrauterine growth retardation, with moderate macrocephaly. His birth weight was 3064 g (+0.1 SD). He was admitted to a NICU because of respiratory insufficiency, and received mechanical ventilation. His craniofacial features included microphthalmia, hypertelorism, short palpebral fissures, epicanthus, low-set ears, and a cleft lip and palate. Additional physical features included bilateral polydactyly of hands (postaxial) and feet (preaxial), and an ectopic urethral opening. Ultrasonography revealed hydrocephalus, agenesis of the corpus callosum and cerebellar vermis, and a complete atrioventricular septal defect. Ophthalmological examination detected persistent pupillary membrane and optic disc coloboma. G-banded chromosomes were normal (46,XY). The patient died at age 14 days due to heart failure.

III-5 was delivered by caesarean section at 32 weeks of gestation. Pregnancy was complicated by polyhydramnios, intrauterine growth retardation, and congenital heart defects. His birth weight was 1704 g (−0.2 SD), length was 40.0 cm (−0.8 SD), and OFC was 33.3 cm (+2.0 SD). He was admitted to a NICU because of respiratory insufficiency, and received mechanical ventilation. His craniofacial features included a prominent forehead, hypertelorism, dysplastic ears, a small cleft lip, and a soft cleft palate (Fig. 2j,k). Ultrasonography revealed hydrocephalus with Dandy-Walker malformation and hypoplastic left heart syndrome. G-banded chromosomes were normal (46,XY). The patient died 1 day after birth. Additional autopsy findings included agenesis of the cerebellar vermis (Fig. 2l), enlargement of the fourth ventricle and aqueduct, anomalous positioning of the esophagus, mild

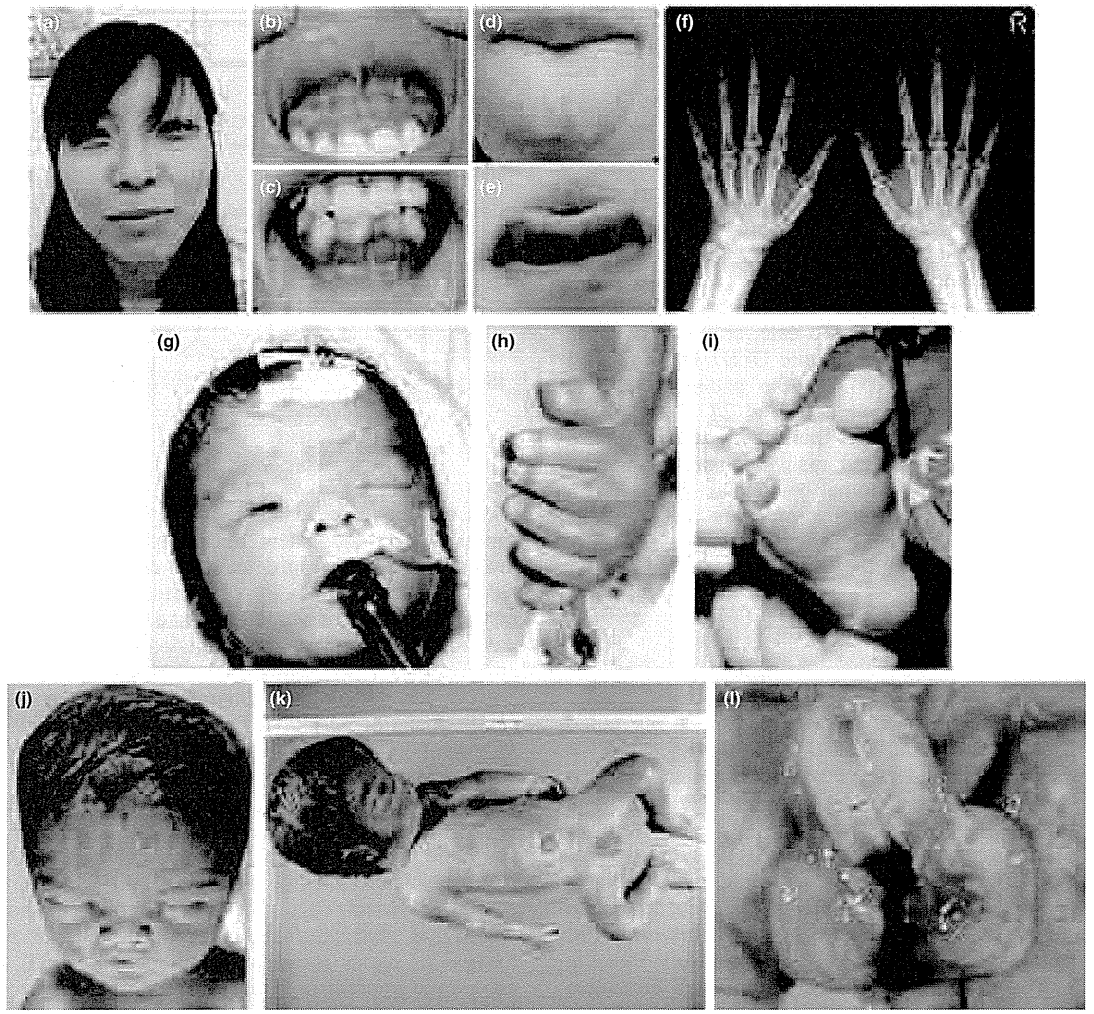


*Fig. 2.* Clinical photographs of II-2 (**a–f**), II-4 (**g–i**), and III-5 (**j–l**).

pulmonary congestion, and insufficient lobulation of the right lung.

The three affected male neonates, having strikingly similar clinical manifestations (Table 1), are considered to have had a congenital malformation syndrome with X-linked inheritance. Array-CGH analysis using 4200 BAC clones identified no pathologic genomic copy number abnormalities. Direct sequencing of *MID*, performed because of a partial similarity to these neonates' syndrome to X-linked Opitz-G/BBB syndrome (OMIM #300000) (8), revealed no mutation.

### Library preparation

Genomic DNA for II-2, II-3, III-3, and III-6 was extracted from peripheral blood using the Gentra PureGene Blood Kit (QIAGEN, Hilden, Germany), and genomic DNA for III-5 was extracted from the preserved dried umbilical cord using the DNeasy Blood & Tissue Kit (QIAGEN). Three micrograms of high-quality (absorbance at 260 nm/absorbance at 280 nm: 1.8–2.0) genomic DNA from II-2 was fragmented using the Covaris model S2 system (Covaris, Woburn, MA). The target peak size was 150 bp. After the size of sheared DNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), adapter sequences were ligated to the ends of DNA fragments, and amplified according to the manufacturer's protocol (Agilent Technologies).

### Exome capture and next-generation sequencing

Library DNA was hybridized for 24 h at 65°C using the SureSelect Human X Chromosome Demo Kit (Agilent Technologies). Captured DNA was diluted to a concentration of 8 pM and sequenced on a Genome Analyzer IIx (Illumina, San Diego, CA) with 76-bp paired-end reads. We used only one of the eight lanes in the flow