

Table 1 Definitions of categories

Category	Examples
Chromosomal syndromes	Down syndrome, trisomies 13 and 18, cri du chat syndrome, and Wolf–Hirschhorn syndrome
Recognizable malformation/dysplasia	22q11.2 deletion syndrome, CHARGE syndrome, and VATER association, Lowe syndrome, achondroplasia, Crouzon syndrome, Noonan syndrome, and Treacher–Collins syndrome
Multiple malformations	
Isolated malformations	
Congenital heart diseases	VSD ASD, AVSD, TGA, and DORV
Central nervous system malformations	Schistorrhachis, hydrocephalus, and meningoencephalocele
Gastrointestinal malformations	Diaphragmatic hernia, biliary atresia, and congenital intestinal obstruction
Respiratory system malformations	CCAM and tracheal stenosis
Other isolated malformations	Cleft palate and cleft lip
Single-gene defect	Metabolic diseases, spinal muscular atrophy, and spinocerebellar degeneration
Mitochondrion	

The classification of the underlying conditions of the 687 patients is shown in Table 3. In 13 cases, the data for identifying the underlying disease were insufficient (e.g., charts were missing). These cases were categorized as “unknown condition.” Of the total 687 patients, 372 (54.1%) had genetic disorders and the remaining 302 (44.0%) had acquired conditions unrelated to genetic disorders, including perinatal complications, neoplasm, and trauma. Among the 372 patients with genetic disorders, 72 had chromosomal abnormalities, with Down syndrome (29 cases) being the most common underlying disorder. Seventy patients had recognizable malformations and dysplasia, with conditions such as osteogenesis imperfecta, 22q11.2 deletion syndromes, CHARGE syndrome, and VATER association. Multiple malformations with unrecognizable patterns were present in 38 cases while isolated malformations, including CNS malformation, congenital heart disease, and gastrointestinal malformation were present in 160 cases.

We also summarized the reasons for the total of 884 admissions, according to the underlying condition (genetic

Table 2 Medical problems for admission (N=884)

Causes for admission	Number	Percent
Respiratory problems	298	33.7
Post-operative management	271	30.7
CNS problems	144	16.3
Gastrointestinal problems	35	4.0
Cardiac diseases	23	2.6
Other infectious state	23	2.6
Examination	21	2.4
Kidney and urinary tract problems	14	1.6
Other	55	6.2
Total	884	100.0

or acquired). Of these admissions, 200 were readmissions. Patients with genetic disorders and malformations had a greater tendency to be hospitalized repeatedly as compared with those with acquired conditions (Fig. 1). In both genetic and acquired condition categories, respiratory disease, post-operative management, and CNS problems were the major medical problems leading to admission.

We further compared age distribution and the lengths of hospitalization between the groups with genetic and acquired disorders (Table 4). The patients with genetic

Table 3 Classification of underlying diseases in 678 patients

Underlying diseases	Number	Percent
Genetic disorders and malformations (subtotal)	372	54.1
Chromosomal abnormalities	(72)	10.5
Recognizable malformation/dysplasia	(70)	10.2
Multiple malformations	(38)	5.5
Isolated malformations (subtotal:160)		23.3
Central nervous system malformation	(71)	10.3
Congenital heart disease	(35)	5.1
Gastrointestinal malformation	(32)	4.7
Respiratory system malformation	(9)	1.3
Other isolated malformations	(13)	1.9
Single-gene defect	(26)	3.8
Mitochondrion	(6)	0.9
Acquired non-genetic conditions (subtotal)	302	44.0
Perinatal complications	(66)	9.6
Neoplasm	(38)	5.5
Trauma(non-accidental and accidental)	(27)	3.9
Infection	(16)	2.3
Other	(155)	22.6
Unknown	13	1.9
Total	687	100.0

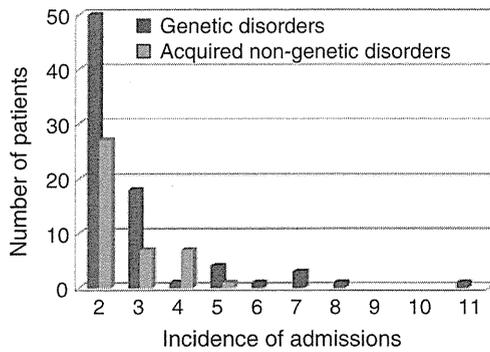


Fig. 1 Comparison of the incidence of admission between the groups with genetic disorders and acquired disorders. In both groups, a total of 200 patients were readmitted. The group with genetic disorders generally required frequent readmission

disorders were significantly younger than those with acquired conditions (median age, 2.0 vs. 4.9 years; $P < 0.0001$). There is no significant difference in the length of hospitalization between the patients with genetic disorders and those with acquired conditions (median, 4 vs. 4 days; $P = 0.26$), but some patients with genetic disorders had much longer hospitalization (mean, 13.0 vs. 7.0 days; $P = 0.007$; range, 1–979 days). Among the reasons for admission, respiratory problems tended to have a longer duration of hospitalization for patients with genetic disorders than for those with acquired conditions (median, 7 vs. 5 days; $P = 0.17$).

Discussion

Our study shows that genetic disorders and malformations account for a significant proportion of cases requiring admission to the HCU. Additionally, the rate of recurrent admission was higher among patients with genetic

disorders and malformations than among those with acquired non-genetic conditions. This finding is in agreement with those of previous reports for other countries [4, 13].

Several studies from different countries have previously suggested that genetic conditions and malformations and the associated mortality and morbidity have a significant impact on the cost burden for society and the patients' families. Cunniff et al. reported that 19% of deaths in a PICU were in cases of heritable disorders [1]. Stevenson and Carey reported that the 34.4% of deaths in a children's hospital were due to malformations and genetic disorders [15]. On the basis of a population-based study, Yoon et al. reported that the overall rate of hospitalization was related to birth defects and genetic diseases, and varied with age and race/ethnicity [16]. McCandless et al. reported the enormous impact of genetic disease on inpatient pediatrics and the health care system in both admission rates and the total hospital charges [11]. These studies emphasize the importance of understanding the impact that genetic diseases have on mortality and healthcare strategies [15]. Furthermore, it is also clear that early recognition of the underlying disorders is necessary for optimal management of patients with genetic disorders.

Our study highlights another aspect related to the impact of genetic disorders and malformations. In 1981, Matsui et al. analyzed the cases of 18,736 children of total admission during 1975–1979 to KCMC and found that 44% had genetic disorders and malformations [10]. Although our study period and ward are limited to those in the HCU, the patients with genetic disorders and malformations had consistently significant impact in KCMC during the ensuing three decades. Further, it emphasizes that medical care for acute conditions and surgical procedures frequently requires highly specialized knowledge of unusual disease conditions and should be provided in consultation with specialists such as clinical geneticists.

Table 4 Comparison of patients with genetic disorder vs. acquired condition on ages at admission and lengths of stay

	Genetic disorders		Acquired conditions		<i>P</i>
	Median (range)	<i>n</i>	Median (range)	<i>n</i>	
Ages	2.0 years (1 day–27.0 years)	372*	4.9 years (9 days–32.5 years)	302*	<0.0001
Length of hospitalization (days)					
Respiratory problem	7 (1–979)	182	5 (1–97)	109	0.17
CNS	4 (1–54)	73	4 (1–207)	68	0.61
Cardiovascular	4 (2–11)	13	4 (2–24)	8	0.94
Gastrointestinal	5.5 (1–37)	22	5 (2–15)	12	0.60
Kidney and urinary tract	3 (2–12)	5	8 (2–12)	9	0.32
Sepsis	3.5 (2–9)	14	7 (2–20)	9	0.19
Post-operative care	2 (1–49)	174	2 (1–62)	93	0.18
Total	4 (1–979)	518	4 (1–207)	366	0.26

*For the patients who have recurrent admissions, the only first admission was calculated

Although the strategies for management of respiratory infection, by means of newly developed antibiotics and mechanical ventilators, and surgical intervention for infants with malformations, have improved, the general strategies for the medical treatment of genetic disorders and malformations remain to be clarified. Hall commented on the report by Yoon et al. [16] and emphasized the significance of basic research on the human genome and developmental genetics [3]. As shown in Table 2, genetic disorders and malformations include rare diseases, which, although uncommon, remain an important public-health issue and a challenge for the medical community [12].

Our study had the limitations of genetic studies and evaluation in cases with multiple malformations and other isolated malformations. The underlying conditions of most patients in this study were ascertained by clinical geneticists, but high-resolution genome analysis with arrays using comparative genomic hybridization was applied in only limited cases. Recently, research attention has focused to a large extent on rare genetic disorders and Mendelian diseases, because of their significant effect on human health, with the aim of identifying disease-related genetic variations. Re-evaluation and classification of underlying disorders, especially in the case of multiple congenital anomalies in undiagnosed patients, are required for further analysis.

Another limitation of our study is estimation of the financial burden of the group of patients with a genetic background. McCandless et al. showed that the disorders with genetic determinant account for 81% of the total hospital charges [11]. Their results are consistent with those of Hall et al. in 1978 [4]. Further analysis of financial burden in our study may provide useful information for improvement of health care systems.

In conclusion, we report here the proportion of genetic disorders and malformations among cases encountered at the HCU of a tertiary children's medical center in Japan. Over 30 years, the proportion of admissions attributed to genetic disorders and malformations has impact and currently accounts for more than half of admissions to this facility. These results firstly indicate improvements in medical care for patients with genetic disorders and malformations and further highlight the large proportion of cases with genetic disorders. As these cases require highly specialized management, the involvement of clinical geneticists in HCUs at children's hospitals is crucial. Eventually, a better fundamental understanding of genetic disorders and malformations may lead to further improve-

ments in medical care and may reduce the impact of these conditions on the patients and their families.

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Conflict of interest The authors declare no conflict of interest.

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Trends in Occurrence of Twin Births in Japan

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The rise in the rate of multiple births since the 1980s is due to the effect of advanced maternal age and increased use of assisted reproductive technology (ART). To determine the trends of prevalence in twin births, we studied the data of a population-based birth defects monitoring system during 26 years in Kanagawa Prefecture, Japan. A total of 15,380 twins from 7,690 deliveries were ascertained from 990,978 births in the Kanagawa Birth Defects Monitoring Program (KAMP) during 1981–2008. From the start of KAMP in 1981, the incidence of twin births had been consistently increasing from 57.0 to 98.6 per 10,000 deliveries until 2003, but after this time, the incidence declined to 78.5 in 2007. While the rate of monozygotic twins has been stable (~40 per 10,000 deliveries) after 1990, that of dizygotic twins increased from 25.3 to 57.3 per 10,000 deliveries until 2002, and recovered to 40.1 in 2007. These results showed the most recent tendency of twin births and indicated that the single embryo transfer method can provide protection and reduction of perinatal risk caused by multiple births.

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Key words: assisted reproductive technology (ART); twin; Kanagawa Birth Defects Monitoring Program (KAMP); zygosity

INTRODUCTION

Multiple births including twin births have several implications for maternal and child health care. Twin pregnancy is associated with an increased incidence of anomalies [Bahtiyar et al., 2007; Glinianaia et al., 2008; Hardin et al., 2009a], a higher risk of perinatal mortality, and preterm births with low birth weight [Helmerhorst et al., 2004; McDonald et al., 2005] compared with singleton pregnancy. A tendency for an increasing rate of twin delivery has been observed in 14 out of 16 countries in Europe, Canada, Australia, Singapore, and Hong Kong [Imaizumi 1998]. This tendency has also been observed in Japan [Imaizumi 2000]. The rise in the rate of multiple births is due to the effect of advanced maternal age and increased use of assisted reproductive technology (ART) [Bondel and Kaminski, 2002]. In the USA and Europe, between 20 and 30% of deliveries following ART are twin births compared with 1% following spontaneous conception [Andersen et al., 2008; Wright et al., 2008]. However, the rate of twin pregnancies in the USA has stabilized at 32 per 1,000 births in 2006 [Chauhan et al., 2010]. In Australia, recent data

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indicated that the proportion of twin deliveries decreased in 2006 [Wang et al., 2008].

To determine the trends of prevalence in twin births, we studied the data of a population-based birth defects monitoring system during 26 years between 1981 and 2008 in Kanagawa Prefecture, Japan. Kanagawa Prefecture, which is adjacent to Tokyo, includes Yokohama City with a total population 3,687,000. To investigate the effects of ART, we analyzed the data of twins according to the zygosity during the study period.

MATERIALS AND METHODS

A total of 15,380 twins from 7,690 deliveries were ascertained from 990,978 births in the Kanagawa Birth Defects Monitoring Program (KAMP). This program has been in operation since October 1981 as the first population-based monitoring system in Japan. Details of KAMP are described elsewhere [Kuroki et al., 1982; Kuroki and Konishi, 1984, 1992; Kuroki, 1988; Kurosawa et al., 1994; Yuan et al., 1995]. KAMP covers one-half of the total births (40,000 births annually) in Kanagawa Prefecture. All live births and stillbirths are screened for 44–48 marker malformations (only surface anomalies), arranged in 10–11 groups, and they are examined by general obstetricians or occasionally by general pediatricians within 7 days after birth. During the study period between 1981 and 2008, the KAMP was divided into four stages according to a minor modification in marker anomalies and registration systems. The first two stages, for 1981–1983 and 1984–1988, had total birth registration systems including all the malformed infants, normal

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infants, and all multiple births. However, in the last two stages, 1989–2000 and 2001–2008, all malformed infants as well as all multiple births were registered with two consecutive normal infants. Information on zygosity is not available in the KAMP, and therefore, we used Weinberg's differential rule for zygosity estimation [Fellman and Eriksson, 2006; Hardin et al., 2009b]. The incidence of twin births was defined as the number of twin pairs per total deliveries.

RESULTS

During the period of analysis, the incidence of malformed infants was 0.88% in live births and 17.24% in stillbirths. The sex ratio was 1.05. From the start of KAMP at 1981, the incidence of twin births had been consistently increasing from 57.0 to 98.6 per 10,000 deliveries until 2003 (Fig. 1). This tendency is consistent with the results of previous studies [Imaizumi, 1998, 2000]. The incidence of twin births peaked at 98.6 per 10,000 deliveries in 2003, but after this time, the incidence declined to 78.5 per 10,000 deliveries in 2007. The incidence of monozygotic twins fluctuated during the first 10 years, but after 1990 the incidence was stable at 40 per 10,000 deliveries. The incidence of dizygotic twins increased from 25.3 to 57.3 per 10,000 deliveries in 2002, but rapidly decreased to 40.1 in 2007, while the incidence of monozygotic twins was stable (Fig. 2). These results indicated that the incidence of twins is directly affected by the rate of dizygotic twins, and that the incidence of dizygotic twin births has already reached its peak, at least in the urban area of Japan.

DISCUSSION

Our study found that during the last 20 years, the incidence of twin births increased from 57 to 98 per 10,000 deliveries, but after it reached a peak in 2003, it recovered to 78.5 per 10,000 deliveries in 2007. Our study demonstrated that the trend in twin births was affected by the incidence of dizygotic twins. The incidence of monozygotic twins was stable at 40 per 10,000 deliveries, while that of dizygotic twin births attained a peak in 2002 with 57.3 per 10,000 deliveries, and it declined to 40.1 after this time. To the best

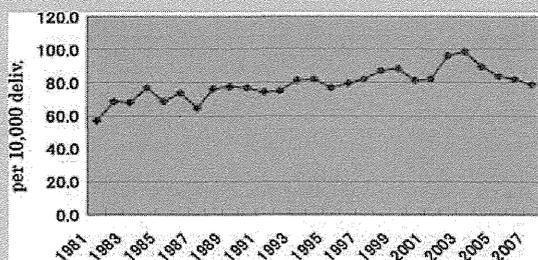


FIG. 1. Trends of the incidence of total births of twin pairs per total deliveries in Japan. The rate of twin births consistently increased from 57.0 per 10,000 deliveries in 1981 to 98.6 in 2003, but after this time, the rate recovered to that of the 1990s.

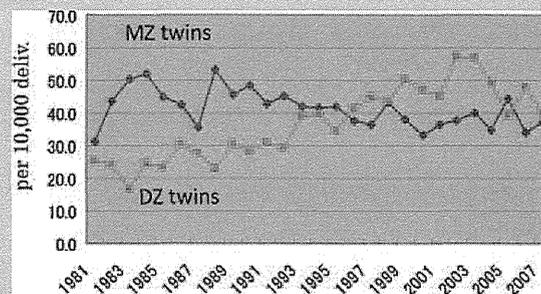


FIG. 2. Comparison of the rate of twin births between monozygotes and dizygotes. In the first 10 years, the fluctuation was remarkable in the rate of births in monozygotic twins for unknown reasons, but during the last 20 years, the rate was stable at approximately 40 per 10,000 deliveries. However, the rate of dizygotic twins consistently increased to reach a peak in 2003, and it then rapidly decreased.

of our knowledge, this is the first report describing the trends of a decrease in the rate of twin births in Japan. Because the rates of monozygotic twins are thought to be constant throughout the world, our results on the tendency of the rates of monozygotic twins have implication of the accuracy of the study. In the USA, between 1980 and 2006, the rate of twin pregnancies consistently increased from 18.9 to 32.1 per 1,000 births [Chauhan et al., 2010]. However, the rapid rise appeared to end in 2004 and the rate stabilized in 2006. A rise in the prevalence of twin births has also been observed in Austria, Finland, Norway, Sweden, Canada, Australia, Hong Kong, Israel, and Singapore [Imaizumi, 1997]. The rate of twin births in these countries stabilized between 2004 and 2006, and recent trends of a decreasing rate has been reported in some countries [Wang et al., 2008].

Clearly, the use of ART has contributed to the changes in the rate of twin pregnancies [Wright et al., 2008; Hansen et al., 2009]. ART twins have a greater risk of adverse perinatal outcome including preterm birth, low birth weight, and cerebral palsy compared with spontaneously conceived twins and singletons [Hansen et al., 2009]. The use of single embryo transfers reduces multiple birth rates and the risks of these adverse outcomes following ART. According to a report from the European Society of Human Reproduction and Embryology, compared with the number of cycles in 2003, fewer embryos were transferred in Germany in 2004, but there were still huge differences between countries [Andersen et al., 2008]. This transfer policy had a considerable impact in Belgium, Finland, Sweden, and several other countries [Andersen et al., 2008], and therefore, a reduced rate of twin births may be observed within a few years in these countries. In the case of Japan, the reduction of the rate was rapid, but a stable rate was not observed at the end of the study period. The rate of twin births may be stabilized when there is a balance between maternal age distribution in reproductive generation and establishment of technical standardization of single embryo transfer. Further analysis on the rates of multiple births based on the population-based monitoring system is required to determine the impact of ART.

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MBTPS2 Mutation Causes BRESEK/BRESHECK Syndrome

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BRESEK/BRESHECK syndrome is a multiple congenital malformation characterized by brain anomalies, intellectual disability, ectodermal dysplasia, skeletal deformities, ear or eye anomalies, and renal anomalies or small kidneys, with or without Hirschsprung disease and cleft palate or cryptorchidism. This syndrome has only been reported in three male patients. Here, we report on the fourth male patient presenting with brain anomaly, intellectual disability, growth retardation, ectodermal dysplasia, vertebral (skeletal) anomaly, Hirschsprung disease, low-set and large ears, cryptorchidism, and small kidneys. These manifestations fulfill the clinical diagnostic criteria of BRESHECK syndrome. Since all patients with BRESEK/BRESHECK syndrome are male, and X-linked syndrome of ichthyosis follicularis with atrichia and photophobia is sometimes associated with several features of BRESEK/BRESHECK syndrome such as intellectual disability, vertebral and renal anomalies, and Hirschsprung disease, we analyzed the causal gene of ichthyosis follicularis with atrichia and photophobia syndrome, *MBTPS2*, in the present patient and identified a p.Arg429His mutation. This mutation has been reported to cause the most severe type of ichthyosis follicularis with atrichia and photophobia syndrome, including neonatal and infantile death. These results demonstrate that the p.Arg429His mutation in *MBTPS2* causes BRESEK/BRESHECK syndrome. © 2011 Wiley Periodicals, Inc.

Key words: BRESEK/BRESHECK syndrome; IFAP syndrome; *MBTPS2*; mutation; S2P

INTRODUCTION

BRESEK/BRESHECK syndrome (OMIM# 300404), a multiple congenital malformation disorder characterized by brain anomalies, intellectual disability, ectodermal dysplasia, skeletal deformities, Hirschsprung disease, ear or eye anomalies, cleft palate or

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cryptorchidism, and kidney dysplasia/hypoplasia [Reish et al., 1997]. The acronym BRESEK refers to the common findings, whereas BRESHECK refers to all manifestations. Because the first two patients were maternally related half brothers, an X-linked disorder was proposed. Although each symptom of these patients is often observed in other congenital diseases, the combination of all symptoms is rare, and only one additional patient with BRESEK has been reported to date [Tumialán and Mapstone, 2006]. Here, we present the fourth male patient with multiple anomalies. The patient presented with a variety of clinical features that were consistent with those of the previously reported BRESHECK syndrome.

The syndrome of ichthyosis follicularis with atrichia and photophobia (IFAP, OMIM# 308205), an X-linked recessive oculocutaneous disorder, is characterized by a peculiar triad of ichthyosis follicularis, total or subtotal atrichia, and varying degrees

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of photophobia [MacLeod, 1909]. Martino et al. [1992] reported a male patient with IFAP syndrome presented with short stature, intellectual disability, seizures, hypohidrosis, enamel dysplasia, congenital aganglionic megacolon, inguinal hernia, vertebral and renal anomalies, and the classic symptom triad of IFAP syndrome. This report broadened the clinical features of IFAP syndrome. It should be noted that the clinical symptoms of this patient are quite similar to those of BRESHECK syndrome, with the exception of cleft palate, cryptorchidism, and photophobia (Patient 5; Table I). The gene mutated in patients with IFAP syndrome, *MBTPS2* (GenBank reference sequence NM_015884), was identified from a variety of clinical features of IFAP syndrome, including the triad and neonatal death [Oeffner et al., 2009]. Thus, the mode of inheritance and several clinical features are common to both BRESEK/BRESHECK and IFAP syndromes. These findings prompted us to perform mutation analysis of *MBTPS2* in the present patient, resulting in the identification of a missense mutation.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from the parents of the patient. Experiments were conducted after approval of the institutional review board of the Institute for Developmental Research, Aichi Human Service Center. The patient (II-1; Fig. 3) was born to a 31-year-old mother (I-2) and a 31-year-old father (I-1), both healthy Japanese individuals without consanguinity. His mother miscarried her first child at 5 weeks. The pregnancy of the patient reported here was complicated with mild oligohydramnios, and he was delivered by caesarean because of a breech position at 38 weeks of gestation. His birth weight was 1,996 g (-2.6 SD), and he measured 44 cm (-2.6 SD) in length with an occipitofrontal circumference of 32.5 cm (-0.5 SD). Apgar scores at 1 and 5 min were four and eight, respectively. The patient exhibited generalized alopecia and lacked eyelashes, scalp hair, and eyebrows (Fig. 1A). The skin on the entire body was erythematous with

TABLE I. Clinical Features of BRESEK/BRESHECK and IFAP Syndromes and *MBTPS2* Mutation

Patient	BRESEK/BRESHECK syndrome				IFAP syndrome		
	1	2	3	4	5	6	7
Clinical features							
Gender	M	M	M	M	M	M	M
Gestational age (weeks)	32	40	ND	38	30	ND	ND
Birth weight (g)	990	2,230	ND	1,996	2,040	ND	ND
Intrauterine growth retardation	+	+	ND	+	-	ND	ND
Major features							
Follicular ichthyosis	-	-	ND	-	+	+	+
Atrichia	+	+	+	+	+	+	+
Photophobia	-	-	-	+	+	+	+
Brain malformation	+	+	+	+	+	-	+
Mental and growth retardation	+	+	+	+	+	+	+
Skeletal (Vertebrate) anomalies	+	+	+	+	+	+	+
Hirschsprung disease	-	+	+	+	+	+	+
Eye malformation or	+	+	+	-	+	-	-
Large ears	+	+	+	+	+	-	-
Cleft lip/palate or	-	+	-	-	-	+	-
Cryptorchidism	+	+	-	+	-	-	-
Kidney malformation	+	+	-	+	+	+	+
Other features							
Microcephaly	+	+	+	+	+	-	+
Seizures	-	+	+	+	+	-	+
Deafness	-	+	-	+	-	-	-
Hand anomalies	+	+	+	-	+	+	+
Cardiac anomalies	-	-	+	-	-	-	+
Inguinal hernia	-	-	-	-	+	+	+
Trachea anomalies	-	-	-	+	-	-	-
Regression	-	-	-	+	-	-	-
Age	6 h d	7 y	1.5 y	8 y	3 y	9 m d	14 m d
<i>MBTPS2</i> mutation	NP	NP	NP	R429H	NP	R429H	R429H

+, present; -, not present; M, male; ND, not described; NP, not performed; h, hour; d, day; m, month; y, year; R429H, Arg429His; BRESEK/BRESHECK syndrome, [Patients 1-4]; IFAP syndrome, [Patients 5-7]; Patients: 1, Reish et al. [1997] patient 1; 2, Reish et al. [1997] patient 2; 3, Tumialán and Mapstone [2006]; 4, present case; 5, Martino et al. [1992]; 6, Oeffner et al. [2009] 3-III:3; 7, Oeffner et al. [2009] 3-III:4.

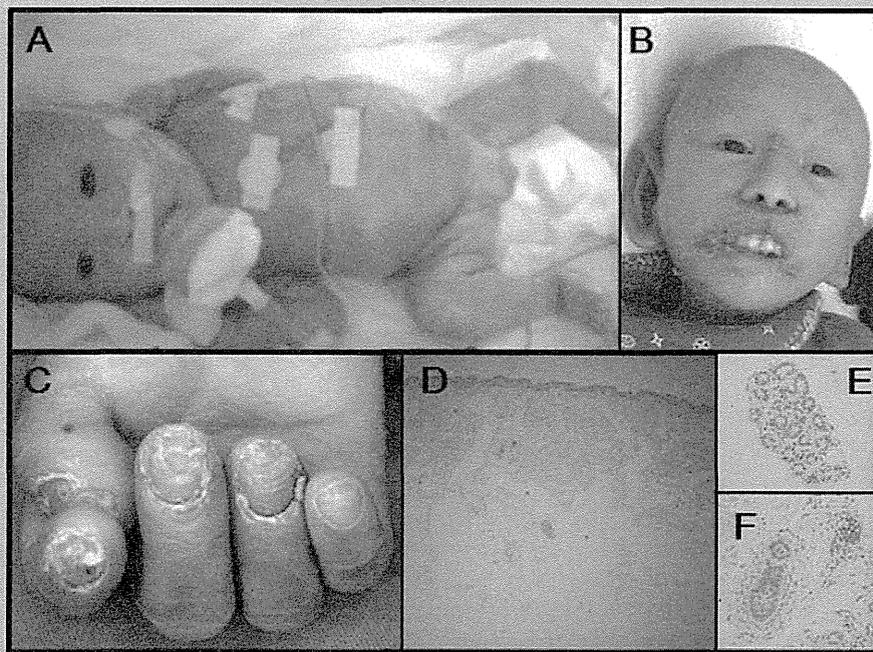


FIG. 1. Clinical appearance and dermatological findings of the patient. **A:** Lateral view of the patient at birth. Note the generalized alopecia with an absence of scalp hair, eyebrows, and eyelashes. The skin was dry and scaly, and an itchy erythema was observed over the entire body. **B:** Frontal view of the patient at 4 years of age. Note the characteristic facial appearance with long, malformed ears, a relatively high nasal bridge, and a wide nasal base. **C:** The patient had normal-sized but deformed and thickened nails. **D–F:** Histologic examination of the abdominal skin at the age of 15 months showed a reduced number of hair follicles (**D**), normal eccrine glands (**E**), and hypoplastic hair follicles (**F**).

continuous desquamation (Fig. 1A). He had malformed large ears, an inferiorly curved penis, and a bifid scrotum. The testicles were not palpable. He experienced persistent constipation, and total colonic Hirschsprung disease was confirmed through barium enema (Fig. 2E) and rectal biopsy at 2 months. A bone survey performed using three-dimensional (3D) computed tomography (CT) showed abnormal imbalanced hemivertebrae in the two lowest thoracic vertebral bodies (Fig. 2C). The patient's right kidney was smaller than normal. Brain magnetic resonance imaging (MRI) at 3 years of age demonstrated decreased volumes of the frontal and parietal lobes and thinning of the corpus callosum with dilatation of the ventricles (Fig. 2A,B). There were no abnormalities of the eyes or optic nerves. We concluded that the patient had BRESHECK syndrome. The patient had seizures at 5 months of age with an apneic episode and cyanosis. Electroencephalographic (EEG) analysis showed abnormal patterns of sharp waves in the posterior lobe. The seizures were almost completely controlled with phenobarbital. The patient was allergic to milk. At 7 months, tracheal endoscopy revealed subglottic tracheal stenosis and abnormal segmentation of the left lung. A chest CT performed at 3 years of age showed a congenital cystic adenomatoid malformation (CCAM) in the right upper lobe (Fig. 2D). Auditory brain stem responses showed bilateral 80 dB hearing loss at 8 months of age.

The patient exhibited delayed psychomotor development during his infancy. He could drink from a bottle at the age of 3 months and could sit up unsupported at 15 months. Abdominal skin biopsy at 15 months revealed reduced number of hair follicles (Fig. 1D). The eccrine glands were normal (Fig. 1E), and most of his hair follicles appeared to be hypoplastic (Fig. 1F). These findings were similar to ichthyosiform erythroderma. Photophobia was noted when the patient left the hospital and first went outside at 18 months of age. At 2 years and 6 months of age, he had a series of epileptic episodes. He experienced a maximum of 100 seizures per day, and EEG analysis showed continual abnormal spikes in the posterior lobe. The seizures were controlled with clonazepam therapy. At 2 years and 9 months of age, he could stand with support and displayed social smiles when interacting with other people. However, the patient developed psychomotor regression at the age of 3 years. He exhibited a progressive loss of emotional response to others, developed hypotonia, and could not stand or sit alone. At 4 years of age, he became bedridden and showed almost no response to people. He had highly desquamated skin, similar to that seen in ichthyosis (Fig. 1B), and easily developed erythema on the skin of the entire body. The patient had deformed and thickened nails (Fig. 1C). He had persistent corneal erosions, but ophthalmoscopy could not be performed at the age of 4 years because of corneal opacification.

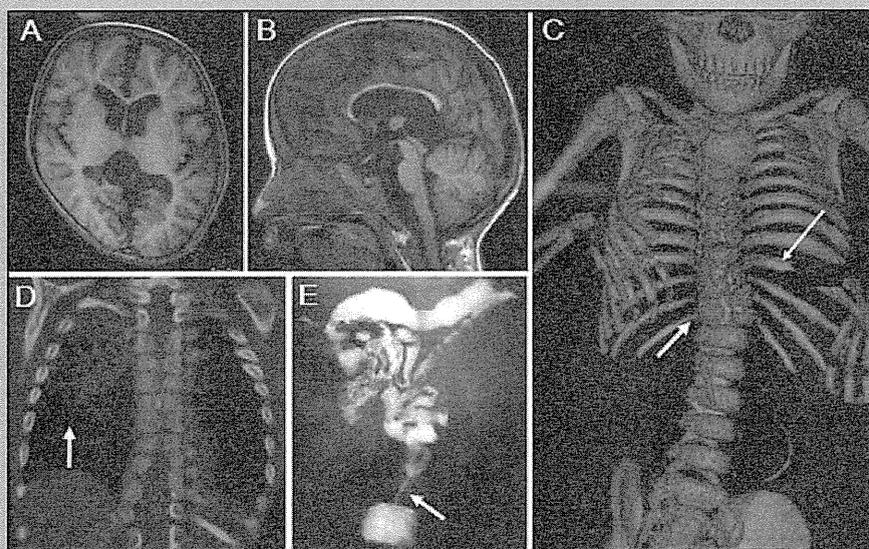


FIG. 2. CT and MRI findings of the patient. A,B: Brain MRI [T1-weighted image] at 3 years of age showed decreased volume of the cortex in the frontal and parietal lobes, the presence of a subdural cyst in the corpora quadrigemina, and dilatation of the lateral and fourth ventricle. C: A bone survey performed using 3D CT showed abnormal segmentation of the ninth rib and an imbalanced hemivertebrae in the two lowest thoracic vertebral bodies (shown with arrows). D: CT of the chest showed CCAM (indicated by the arrow) in the right upper lobe. E: Barium enema showed a reduced caliber rectum (indicated by the arrow), suggesting that the patient had Hirschsprung disease.

Chromosomal and Molecular Genetic Studies

Genomic DNA isolated from the patient's peripheral white cells by phenol/chloroform extraction was used for *MBTPS2* mutation analysis. PCR-amplified DNA fragments were isolated using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and purified using polyethylene glycol 6000 precipitation. PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Kit V1.1 and analyzed with the ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA). We also performed G-banded chromosome analysis at a resolution of 400–550 bands, genome-wide subtelomere fluorescence in situ hybridization (FISH) analysis, and array comparative genomic hybridization (array CGH) using Whole Human Genome Oligo Microarray Kits 244K (Agilent Technologies Inc., Palo Alto, CA) to identify genomic abnormalities.

RESULTS

G-banded chromosome analysis and genome-wide subtelomere FISH analyses did not show chromosomal rearrangements in the patient. Array CGH analysis did not show copy number changes in the patient's genome with the exception of known copy-number variations (CNVs). Since some patients with IFAP syndrome have been reported to present with several clinical features of BRESEK/BRESHECK syndrome, including severe intellectual disability, vertebral and renal anomalies, and Hirschsprung disease, we conducted a comprehensive sequencing analysis of all exons and intron–exon boundaries of *MBTPS2*. This analysis identified a

missense mutation (c.1286G>A, [p.Arg429His]) in exon 10, which was previously reported for IFAP syndrome (Fig. 3). The mutation was also found in one allele of the mother (I-2), indicating that the mutation was of maternal origin and that the mother was a heterozygous carrier (Fig. 3).

DISCUSSION

In this report, we describe the fourth male patient with BRESHECK syndrome in whom we identified a missense mutation (c.1286G>A, [p.Arg429His]) in *MBTPS2*, which is the causal gene for IFAP syndrome. *MBTPS2* encodes a membrane-embedded zinc metalloprotease, termed site-2 protease (S2P). S2P cleaves and activates cytosolic fragments of sterol regulatory element binding proteins (SREBP1 and SREBP2) and a family of bZIP membrane-bound transcription factors of endoplasmic reticulum (ER) stress sensors (ATF6, OASIS), after a first luminal proteolytic cut by site-1 protease (S1P) within Golgi membranes [Sakai et al., 1996; Ye et al., 2000; Kondo et al., 2005; Asada et al., 2011]. The SREBPs control the expression of many genes involved in the biosynthesis and uptake of cholesterol, whereas ATF6 and OASIS induce many genes that clean up accumulated unfolded proteins in the ER. Dysregulated SREBP activation, impaired lipid metabolism, and accumulation of unfolded proteins in the ER caused by *MBTPS2* mutations could lead to disturbed differentiation of epidermal structures, resulting in the symptom triad of IFAP syndrome [Cursiefen et al., 1999; Traboulsi et al., 2004; Elias et al., 2008]. Oeffner et al. [2009] first identified five missense mutations in *MBTPS2* in patients with IFAP

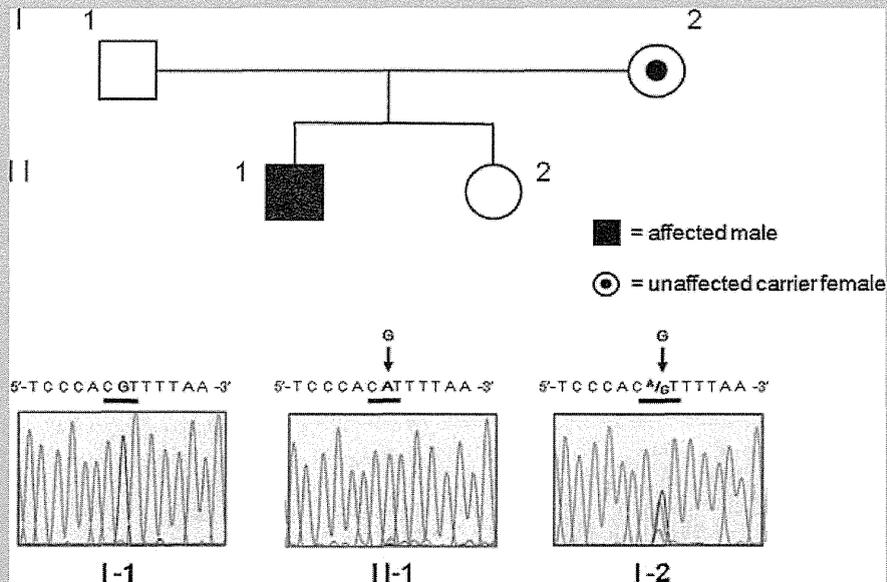


FIG. 3. Identification of a disease mutation. The sequence analyses of the patient (II-1) showed a c.1286G>A variant in exon 10 of *MBTPS2*, which predicts p.Arg429His, as indicated by the arrow (middle panel). The mother [I-2] was heterozygous for the mutation [C^A/G] (right panel).

syndrome. Transfection studies using wild type and mutant *MBTPS2* expression constructs demonstrated that the five *MBTPS2* mutations did not affect S2P protein amount and localization in the ER. However, enzyme activities, as measured by sterol responsiveness, were decreased in S2P-deficient M19 cells when the mutant *MBTPS2* was transiently expressed. Interfamilial phenotypic differences between male IFAP patients and the properties of mutants in functional assays predict a genotype–phenotype correlation, ranging from mild forms of the triad with relatively high enzyme activity (~80%) to severe manifestations of intellectual disability, various developmental defects, and early death with low enzyme activity (~15%). The identified p.Arg429His mutation in the patient reported here is one of the five missense mutations with the lowest enzyme activity. It was previously reported that all four patients harboring the p.Arg429His mutation died within 14 months of birth. The five mutations were not located in the HEIGH motif (amino acids [aa] 171–175) or in the LD₄₆₇G sequence, both of which are regions important for coordinating the zinc atom at the enzymatic active site for protease activity in the Golgi membrane [Zelenski et al., 1999]. However, among the five mutations, the p.Arg429His mutation is located closest to the intramembranous domain, and it strongly reduced the enzymatic activity and caused a severe phenotype. This finding suggests that mutations in the HEIGH motif or in the LD₄₆₇G sequence are fatal because they lead to a null function of the S2P. Although the detailed skin findings of the four patients with the p.Arg429His mutation have not been reported, it should be noted that one of the four patients (3-III:4) with the p.Arg429His mutation had brain anomaly, seizures, psychomotor retardation, vertebrae anomaly, Hirschsprung disease, absence of a kidney, atrial septum defect, and inguinal

hernia, in addition to the symptom triad of IFAP syndrome [Oeffner et al., 2009]. These symptoms overlap with the majority of symptoms observed in BRESHECK syndrome (BRESHK; six of eight symptoms observed in BRESHECK) (Table I), and the present patient has BRESHECK syndrome. Collectively, these observations suggest that the most severe form of the syndrome caused by the p.Arg429His mutation in *MBTPS2* shows features quite similar or identical to those of BRESEK/BRESHECK syndrome.

There are two major differences in the definitions of IFAP syndrome and BRESEK/BRESHECK syndrome. Ichthyosis follicularis, one of the triad symptoms of IFAP syndrome, is a clinical condition of the skin. However, several studies on IFAP syndrome have reported various skin eruptions such as psoriasis-like and ichthyosis-like eruptions [Martino et al., 1992; Sato-Matsumura et al., 2000]. In contrast, patients with BRESEK/BRESHECK syndrome showed severe lamellar desquamation with diffuse scaling [Reish et al., 1997], similar to that observed in the present patient. This could be because of the difference in features of the skin, namely, ichthyosiform erythroderma-like appearance versus ichthyosis follicularis, in patients with the most severe forms of *MBTPS2* mutation and patients with IFAP syndrome who were described earlier, respectively.

The second difference is that photophobia was not described in the reported three male patients with BRESEK/BRESHECK syndrome [Reish et al., 1997; Tumialán and Mapstone, 2006]. In the present patient, photophobia became evident after he was diagnosed with BRESHECK syndrome. Photophobia is a symptom of epithelial disturbances of the cornea, such as ulceration and vascularization, which result in corneal scarring [Traboulsi et al., 2004]. In the most severe cases of *MBTPS2* mutation, such as

patients with severe intellectual disability who are bedridden and die early, it is likely that the patients were treated in the hospital without being exposed to sunlight. Therefore, it would be difficult to observe photophobia as a main symptom in those cases. Moreover, two previously described patients with BRESEK/BRESHECK syndrome had initial maldevelopment of one eye or small optic nerves. In these patients, photophobia may not have been obvious because of malformations of the eyes and optic nerves [Reish et al., 1997]. In our study, the patient showed clinical features of BRESHECK syndrome and photophobia with *MBTPS2* mutation, indicating that the clinical features of the present patient are extremely broad compared to the features of IFAP syndrome caused by *MBTPS2* mutation that have been previously reported [MacLeod, 1909].

Recently, a missense mutation (c.1523A>G, [p.Asn508Ser]) in *MBTPS2* was identified from 26 cases of three independent families with keratosis follicularis spinulosa decalvans (KFS; OMIM# 308800), which is characterized by the development of hyperkeratotic follicular papules on the scalp followed by progressive alopecia of the scalp, eyelashes, and eyebrows in addition to childhood photophobia and corneal dystrophy [Aten et al., 2010]. A significant association was found between KFS and the p.Asn508Ser mutation. The specific localization of alopecia to the scalp, eyelashes, and eyebrows and the limited childhood photophobia of KFS indicate that KFS has a relatively mild phenotype. The authors postulate that IFAP syndrome and KFS are within the spectrum of one genetic disorder with a partially overlapping phenotype and propose that a new name should be chosen for KFS/IFAP syndrome with an *MBTPS2* mutation. In contrast, the BRESHECK syndrome observed in the present patient has a severe phenotype caused by the p.Arg429His mutation. The present patient and the two patients (3-III:3 and 3-III:4) with the p.Arg429His mutation displayed broader clinical features, including eight features (BRESHECK) and six features (RESCHK and BRESHK) of BRESEK/BRESHECK syndrome, respectively (patients 4, 6, and 7; Table I) [Oeffner et al., 2009]. There is a debate regarding whether the two patients harboring six features were correctly diagnosed with BRESEK/BRESHECK syndrome since the patients did not have "BRESEK" but rather a combination of six other clinical features. To better understand and clearly distinguish the clinical features of the present patient from those of the reported patients with *MBTPS2* mutations, we propose the nomenclature of "BRESHECK/IFAP syndrome" for the present patient because he has clinical features of BRESHECK syndrome. We also suggest that the BRESHECK/IFAP syndrome be used for a broader definition that would include patients harboring most features of BRESHECK syndrome, including the previously reported two patients (3-III:3 and 3-III:4) with p.Arg429His mutation in *MBTPS2* [Oeffner et al., 2009]. Data from further genetic and clinical studies on more patients are required to determine which genes or *MBTPS2* mutations are associated with BRESEK/BRESHECK or BRESHECK/IFAP syndrome, respectively.

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Original Article

Establishment and characterization of Roberts syndrome and SC phocomelia model medaka (*Oryzias latipes*)Akihiro Morita,^{1,*} Kumiko Nakahira,¹ Taeko Hasegawa,¹ Kaoru Uchida,¹ Yoshihito Taniguchi,^{2,†} Shunichi Takeda,² Atsushi Toyoda,^{3,§} Yoshiyuki Sakaki,^{3,¶} Atsuko Shimada,⁴ Hiroyuki Takeda⁴ and Itaru Yanagihara^{1,*}

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Roberts syndrome and SC phocomelia (RBS/SC) are genetic autosomal recessive syndromes caused by *establishment of cohesion 1 homolog 2 (ESCO2)* mutation. RBS/SC appear to have a variety of clinical features, even with the same mutation of the *ESCO2* gene. Here, we established and genetically characterized a medaka model of RBS/SC by reverse genetics. The RBS/SC model was screened from a mutant medaka library produced by the Targeting Induced Local Lesions in Genomes method. The medaka mutant carrying the homozygous mutation at R80S in the conserved region of *ESCO2* exhibited clinical variety (i.e. developmental arrest with craniofacial and chromosomal abnormalities and embryonic lethality) as characterized in RBS/SC. Moreover, widespread apoptosis and downregulation of some gene expression, including *notch1a*, were detected in the R80S mutant. The R80S mutant is the animal model for RBS/SC and a valuable resource that provides the opportunity to extend knowledge of *ESCO2*. Downregulation of some gene expression in the R80S mutant is an important clue explaining non-correlation between genotype and phenotype in RBS/SC.

Key words: *ESCO2*, *Oryzias latipes*, premature chromosomal separation, Roberts syndrome and SC phocomelia.

Introduction

Roberts syndrome (RBS) (Online Mendelian Inheritance in Man [OMIM] 268300) is a genetic autosomal recessive syndrome. RBS is characterized by intrauterine

fetal death, failure to thrive, mental and growth retardation, microcephaly, cleft lip and palate, and symmetrical limb defects (Vega *et al.* 2010). Chromosomal abnormality, for example, premature centromere separation (PCS), in the metaphase RBS cell is also observed. Recently, we identified *establishment of cohesion 1 homolog 2 (ESCO2)* as the gene responsible for RBS (Vega *et al.* 2005). Similar pathological features, although milder than RBS, are observed in SC phocomelia (SC) (OMIM 269000). SC is also an autosomal recessive disorder caused by *ESCO2* mutation (Schüle *et al.* 2005). In spite of the mutation being in the same gene, different diagnoses were previously made. Both RBS and SC were caused by truncating mutations (either nonsense mutation or frame-shift), eliminating the functionally important acetyltransferase domain, except for one family in which RBS was caused by the missense mutation in the acetyltransferase domain (Schüle *et al.* 2005; Vega *et al.* 2005, 2010; Gordillo *et al.* 2008). Gordillo *et al.* (2008) considered that RBS and SC were the same syndrome with varying phenotypic expression. Thus, clinical

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features are considerably different in severity, suggesting there is no phenotype–genotype correlation.

The expression of *ESCO2* is dependent on the cell cycle, namely, it is expressed in the S-phase (Whitfield *et al.* 2000; Nishihara *et al.* 2010). *ESCO2* is a homologue of *Ctf7/Eco1* in the yeast *Saccharomyces cerevisiae* (Hou & Zou 2005). *ESCO2* and *Eco1* encode a member of the acetyltransferase family (Skibbens *et al.* 1999; Vega *et al.* 2010). The *ECO1* protein lacks the N-terminal half of *ESCO2*. *ECO1* and the C-terminal half of *ESCO2* contain a PIP box, a C2H2 zinc finger and an acetyltransferase domain. PIP box and C2H2 zinc finger domains are involved in the interaction with proliferating cell nuclear antigen (Moldovan *et al.* 2006) and acetylation of cohesin subunits (Ivanov *et al.* 2001; Hou & Zou 2005; Rolef Ben-Shahar *et al.* 2008; Unal *et al.* 2008; Zhang *et al.* 2008; Onn *et al.* 2009), respectively. Cohesin acetylation by *ESCO2* and *ECO1* is a central determinant of replication fork processivity (Terret *et al.* 2009) and is essential for the establishment of cohesion from yeast to humans (Skibbens *et al.* 1999; Hou & Zou 2005).

Medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) are attractive vertebrate animal models because of their easy handling and large numbers of progeny per generation. To analyze gene function, an easy knock-down method using a morpholino injection has been established. Furthermore, medaka is suitable for genetic analysis because it has a lower ploidy level than zebrafish (Ishikawa 2000), and a high quality draft genome sequence of medaka (Kasahara *et al.* 2007) and large numbers of mutants (Furutani-Seiki *et al.* 2004) are available. In this study, we employed a reverse genetic approach in mutant medaka (*O. latipes*) produced using the Targeting Induced Local Lesions in Genomes (TILLING) method (Taniguchi *et al.* 2006) for translational research to explain RBS/SC pathobiology through the *ESCO2* mutation. The expression pattern of *ESCO2* in medaka is similar to that in humans. *ESCO2* mutant medaka showed RBS/SC-like divergence in their phenotype. This *ESCO2* mutant medaka is the animal model for RBS/SC. Moreover, downregulation of *notch1a* and its downstream gene expression was observed in *ESCO2* mutants. This is an important clue explaining the clinical variety of RBS/SC.

Materials and methods

Fish strain and embryos

This study was performed using K-Cab, a substrain of Cab, and K-Kaga, a northern strain of medaka.

K-Kaga was a kind gift from the National BioResource Project Medaka. The mutant strains were derived from K-Cab (Taniguchi *et al.* 2006). Eggs were collected from a laboratory colony. Embryos were incubated at 28.5°C after collection, and were staged according to Iwamatsu (1994).

Cloning of medaka ESCO2

A partial cDNA sequence of medaka *ESCO2* was obtained from a genome database (University of Tokyo Genome Browser Medaka [http://utgenome.org/medaka/]), and was amplified using the primers (*ESCO2* cloning primers, see Table S1). Because the 5' part was missing from the database, rapid amplification of 5' cDNA ends (5'-RACE) was performed using the SMART RACE cDNA Amplification Kit (Clontech). Two rounds of 5'-RACE were performed with cDNA prepared from embryos using the gene-specific primers (*ESCO2* RACE primers, see Table S1). The cDNA fragment of *ESCO2* (5'-untranslated region and open reading frame [ORF]) was amplified by polymerase chain reaction (PCR) from embryonic cDNA library using *ESCO2* full forward and reverse as primers, and was cloned into pSTBlue-1 vector (Novagen).

In situ hybridization

cDNA sequences of medaka *β -actin*, *lfn3* and *myf5* were obtained from a genome database (Ensembl genome browser in medaka [http://uswest.ensembl.org/Oryzias_latipes/Info/Index]; accession no., *β -actin*, ENSORLT00000017152; *lfn3*, ENSORLT00000005779; *myf5*, ENSORLT00000020353). cDNA fragments were cloned into pSTBlue-1 (Novagen) vector by reverse transcription (RT)–PCR using each primer set (see Table S1) and sequenced for confirmation. The other genes were previously cloned into vectors (Kage *et al.* 2004). The RNA probes were produced by *in vitro* transcription by using SP6, T7 or T3 RNA polymerase (Roche Applied Science) and DIG RNA Labeling Mix (Roche Applied Science) according to the manufacturer's instruction. Whole-mount *in situ* hybridization was performed as previously described (Terasaki *et al.* 2006). The fixative solution was modified as follows: 4% paraformaldehyde (PFA) in 0.85× phosphate-buffered saline containing Tween-20 (0.1%, 0.85× PBST) and 1.5× PBST, which were used for the embryos at the gastrula stage (stage 16) and at other stages, respectively. The length of proteinase K treatment was modified as follows: 0, 1, 7 and 12 min for the embryos at stage 11 or earlier, 12–18, 19–24 and 25 or later, respectively. The samples were observed using a microscope (Olympus BX51).

Cartilage staining

Cartilage staining was performed according to Yasutake *et al.* (2004). The whole-mount and pectoral fin samples were observed by using a stereoscopic microscope (Olympus SZX10) and a microscope (Olympus BX51), respectively.

Immunohistochemistry

Immunohistochemical analysis was performed according to Iijima and Yokoyama (2007) by using anti-phosphorylated histone H3 antibody (Upstate, at 1/100 dilution) and Alexa Fluor 488 anti-rabbit immunoglobulin (IgG) antibody (Invitrogen, at 1/1000 dilution). The samples were observed by using a fluorescence microscope (Olympus IX71).

5-Bromodeoxyuridine (BrdU) incorporation

Because medaka chorion is almost impermeable to BrdU (Nguyen *et al.* 1999), we injected 0.1% BrdU (in the kit FITC BrdU Flow Kit, BD Biosciences)/Yamamoto solution into the perivitelline space at 2.0 days postfertilization (dpf). Two hours after injection, the embryos were fixed by 4% PFA/PBST. After fixation, the experimental procedure was the same as in immunohistochemistry, but using anti-BrdU antibody (Thermo Scientific, at 1/500 dilution) and Alexa Fluor 488 anti-mouse IgG antibody (Invitrogen, at 1/1000 dilution).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

A whole-mount TUNEL assay was performed by using an *in situ* Apoptosis Detection Kit (Takara) according to the method of Iijima and Yokoyama (2007) and the manufacturer's instructions. The method was slightly modified as follows: The anti-fluorescein isothiocyanate (anti-FITC) antibody conjugated with horseradish peroxidase in the kit was replaced by anti-FITC antibody conjugated with alkaline phosphatase (at 1/500 dilution), 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP) (Roche Applied Science) were used for visualization of apoptotic cells. The samples were observed by using a microscope (Olympus BX51).

Genotyping

Genomic DNA was extracted from whole embryos and caudal fin of fry by treatment of proteinase K (100 µg/mL proteinase K, 10 mmol/L Tris-HCl (pH 8.0),

5 mmol/L ethylenediaminetetraacetic acid, 1% sodium dodecylsulfate), phenol extraction, phenol-chloroform isoamyl alcohol extraction and isopropyl alcohol precipitation. The genomic DNA was subjected to PCR by using a primer set (*ESCO2* Seq1 and *ESCO2* Seq2, see Table S1). After confirmation of amplicon length by separation electrophoresis in agarose gel, PCR products were subjected to direct sequence by using Big-Dye Terminator (Applied Biosystems), and primers *ESCO2* Seq3 or *ESCO2* Seq2 (see Table S1).

Investigation of the expression allele of *ESCO2*

First we crossed *ESCO2*^{240T, 242A, 581C/240G, 242T, 581C} females and *ESCO2*^{240G, 242A, 581A/240G, 242A, 581A} males. At several developmental stages, total RNA extraction by ISOGEN (Nippon Gene) and DNase (in the kit of SV Total RNA Isolation System, Promega) treatment and first strand cDNA production (Prime-Script 1st strand cDNA Synthesis Kit, Takara) was carried out according to the manufacturer's instructions. The first strand cDNA was subjected to PCR using TaKaRa Ex Taq (Takara) and the primer set (*ESCO2* full, see Table S1). The PCR products were separated by agarose electrophoresis. Direct sequencing was performed using the primer *ESCO2* Seq4 or *ESCO2* Seq3 (see Table S1). From the sequence, we assessed the expression alleles as follows: when the nucleotide sequence is 240T/G, 242A/T and 581C, the RNA is derived from two maternal alleles; when it is 240T/G, 242A/T and 581C/A, the RNA is from two maternal and paternal alleles; and when it is 240T/G, 242A and 581C/A or 240G, 242T/A and 581A/C, the RNA is from one maternal and paternal (i.e. embryonic alleles).

Morpholino injection

We constructed three morpholinos as follows: ATG mo, CGGATCATCTTCAGAGTTCACCGTC (start codon underlined); 5mis mo, CGCATGATCTTCACAGTTGACCCTC (mispaird bases underlined); and E212 mo, GCTTCCAGGAACCTCCGTACCTGTGTC (targets 3'-donor of exon 2). Morpholinos (50–300 nmol/L) diluted with Yamamoto solution were injected into one blastomere of one-cell stage embryos (stage 2) using FemtoJet (Eppendorff) and Injectman M2 (Eppendorff) according to the method of Rembold *et al.* (2006) under a stereoscopic microscope (Olympus SZX7).

Chromosomal analysis

Chromosomal analysis was performed according to the method of Westerfield (2000). Briefly, colchicine-treated (400 µg/mL at 28.5°C for 180 min) embryos

were treated with 1.1% trisodium citrate for 16 min. During trisodium citrate treatment, embryos were dechorionated and the yolk was dissected away. After hypotonization (50 mmol/L KCl at 30°C for 15 min), the dissociated cells were fixed using cold Carnoy's fixative for 20 min and washed. The fixed cell solution was divided into two aliquots. One of them was subjected to DNA extraction and genotyping (see genotyping). The other was spotted onto a cold slide glass and re-fixed by flame. The samples were stained using Giemsa solution (4% Giemsa Stock in 10 mmol/L phosphate buffer for 60 min, pH 7) and observed using a microscope (Olympus BX51).

Flow cytometry

Sample preparation was as previously described (Candal *et al.* 2007), but slightly modified. Briefly, after filtration of nuclei from frozen and dechorionized embryos, filtrates were treated with RNase A (100 µg/mL; Wako) and stained with 1 mg/mL propidium iodide (Dojindo). Nuclei (10 000) were analyzed using FACScan (BD Biosciences).

Quantitative RT-PCR

cDNA sequences of *notch1a*, *notch1b*, *notch3*, *ascl1a*, *ascl1b*, *GATA-1* and *EF-1α* were obtained from a genome database (Ensembl genome browser in medaka [http://uswest.ensembl.org/Oryzias_latipes/Info/Index]; accession no.: *notch1a*, ENSORLT00000006535; *notch1b*, ENSORLT00000000793; *notch3*, ENSORLT00000007526; *ascl1a*, ENSORLT000000020662; *ascl1b*, ENSORLT00000015163; *GATA-1*, ENSORLT00000017528; and *EF-1α*, ENSORLT00000009544). cDNA sequences of *sox9b*, *col2a1a* and *col2a1b* were obtained from GenBank (accession no.: *sox9b*, AY870393; *col2a1a*, AB617801; *col2a1b*, AB617803). Total RNA extraction and first strand cDNA production were carried out as described above. Before RT-PCR analysis, we performed sequence analysis of *ESCO2* similar to that performed during the investigation of expression alleles. For quantification of mRNA of *notch1a*, *notch1b*, *notch3*, *ascl1a*, *ascl1b*, *GATA-1* and *EF-1α*, real-time PCR analysis was applied to the first strand cDNA and the plasmid vector containing the target gene (as standard) using Applied Biosystems 7500 Fast RT-PCR System (Applied Biosystems), QuantiTect SYBR Green PCR Kit (Qiagen) and primer sets (see Table S1). For quantification of mRNA of the *sox9b*, *col2a1a* and *col2a1b*, Custom TaqMan Gene Expression Assays (assay name and ID: MF5OX9B, AIGJP3B; MFCOL2A1A, AIHSN9J; MFCOL2A1B, AII1MFR; Applied

Biosystems) was used. After estimating the amount of mRNA from the cycle threshold (Ct) value of samples and the standard curve, the amount of mRNA was normalized by the amount of *EF-1α* mRNA and represented by relative abundance against wild-type (WT) embryos. Some amplified fragments were cloned into pT7Blue vector (Novagen) and were sequenced for confirmation.

Statistical analysis

Several statistical analyses were performed according to Zar (2010).

Results

Cloning of medaka *ESCO2* cDNA

We cloned medaka *ESCO2* cDNA (DNA Data Bank of Japan accession no. AB601774) from embryos. It had a 1590-bp ORF encoding a 530-residue polypeptide, and a 5'-non-coding region of 177 nucleotides. The predicted *ESCO2* had a calculated molecular weight of 58.7 kDa. The similarity searches showed that the medaka *ESCO2* protein was similar to *ESCO2* from several species including humans (Fig. S1). The identities of the medaka *ESCO2* protein to that of humans, mice and zebrafish were 52.6%, 55.8% and 47.8%, respectively. Medaka *ESCO2* had several conserved regions that consisted of a nuclear localization signal, PIP domain, C2H2-zinc finger domain, acetyltransferase domain and regions with unknown functions.

Expression pattern of *ESCO2* in medaka

We investigated the *ESCO2* expression pattern during embryogenesis using whole-mount *in situ* hybridization. *ESCO2* mRNA was detected in all blastomeres at stage 5 (Fig. S2) and in the embryonic shield during gastrulation (stage 15–16, Fig. 1A). During early somitogenesis stages, *ESCO2* was expressed throughout the embryonic body, particularly in the optic vesicles, neural tube and somites (Fig. 1B). *ESCO2* expression was limited to the posterior region of optic tectum, prospective corpus cerebelli and pectoral fin buds in late somitogenesis stages at and after stage 28 (Fig. 1C), and to the genital ridge at stage 31 (Fig. 1D). These results are similar to the *ESCO2* expression pattern in human embryos (Vega *et al.* 2010). Because these *ESCO2*-expressing regions are thought to be proliferative zones (Nguyen *et al.* 1999; Candal *et al.* 2007), we

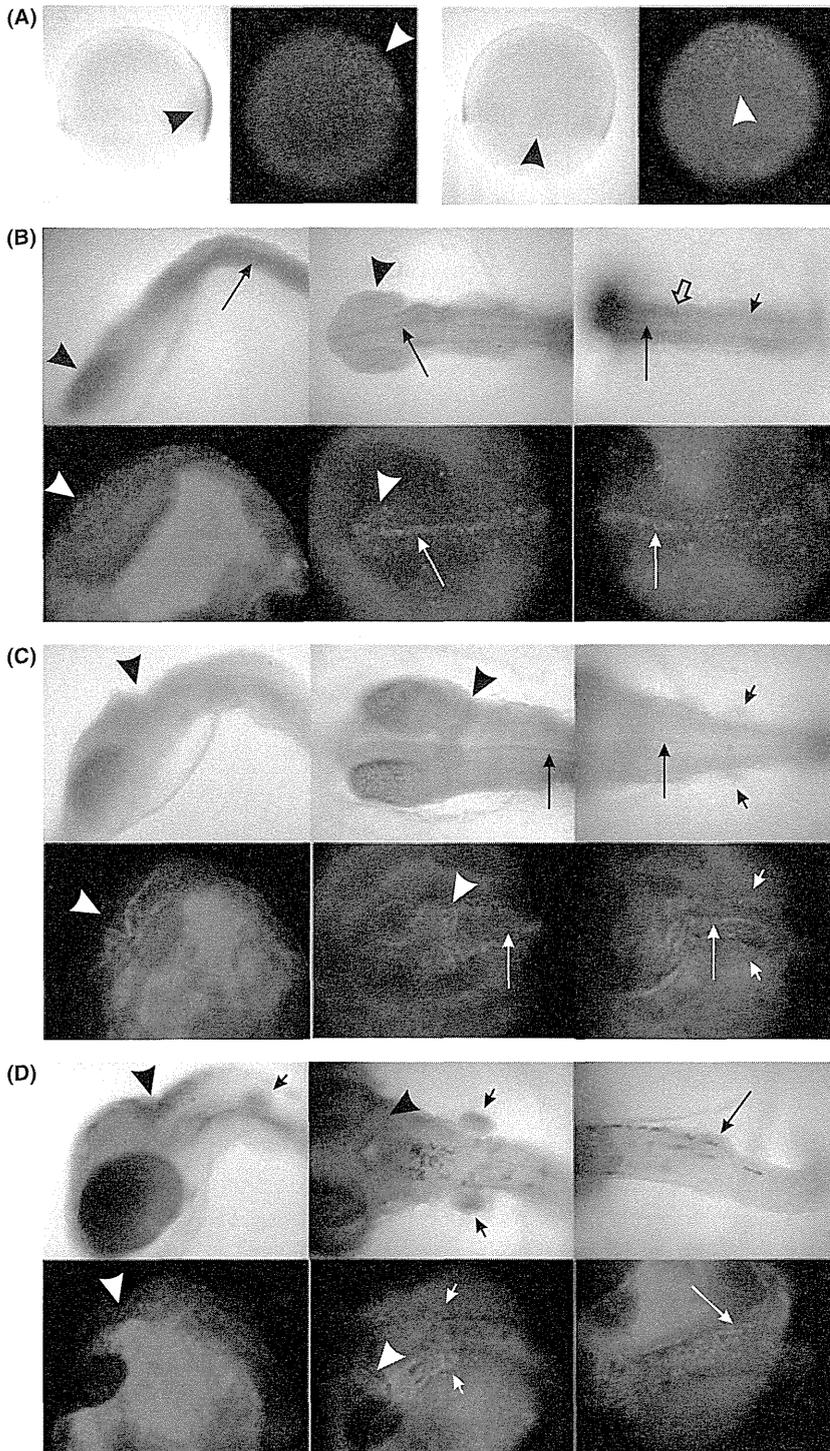


Fig. 1. *ESCO2* expression in medaka embryo. Whole-mount *in situ* hybridization analysis of *ESCO2* (micrograph) and immunohistochemical analysis of anti-phosphorylated histone H3 (M phase marker, fluorescence micrograph). (A) Gastrula stage (0.75 days postfertilization [dpf], stage 15). Left, lateral view; right, dorsal view; animal pole to top; arrowheads, embryonic shield. (B) Somitogenesis stage (1.0 dpf, stage 20). Left, lateral view; middle and right, dorsal view of head and tail, respectively; arrowheads, optic vesicles; long arrows, neural tube; open arrow, somite; short arrow, bilateral region in presomitic region. (C) Late somitogenesis stage (2.0 dpf, stage 28). Left, lateral view; middle and right, dorsal view of the head and pectoral regions, respectively; arrowheads, proliferative zone of optic tectum and prospective corpus cerebelli; long arrows, neural tube; short arrows, pectoral fin buds. (D) Gill blood vessel formation stage (3.0 dpf, stage 31). Left, lateral view; middle, dorsal view of the pectoral region; right, right dorsal view of abdomen; arrowheads, proliferative zone of optic tectum and prospective corpus cerebelli; short arrows, pectoral fin buds; long arrows, genital ridge.

performed immunohistochemical analysis of phosphorylated-histone H3, an M-phase marker. The immunopositive region was similar to the *ESCO2*-expressing region. These observations are in agreement with the fact that *ESCO2* is involved in cell division in other species (Skibbens *et al.* 1999; Hou & Zou 2005; Vega *et al.* 2005).

Developmental abnormalities in ESCO2 mutant medaka

In order to establish the RBS/SC model using medaka, we isolated mutant medaka from the TILLING library (Taniguchi *et al.* 2006). On sequence analysis, 18 mutations, including six missense, seven silent and five

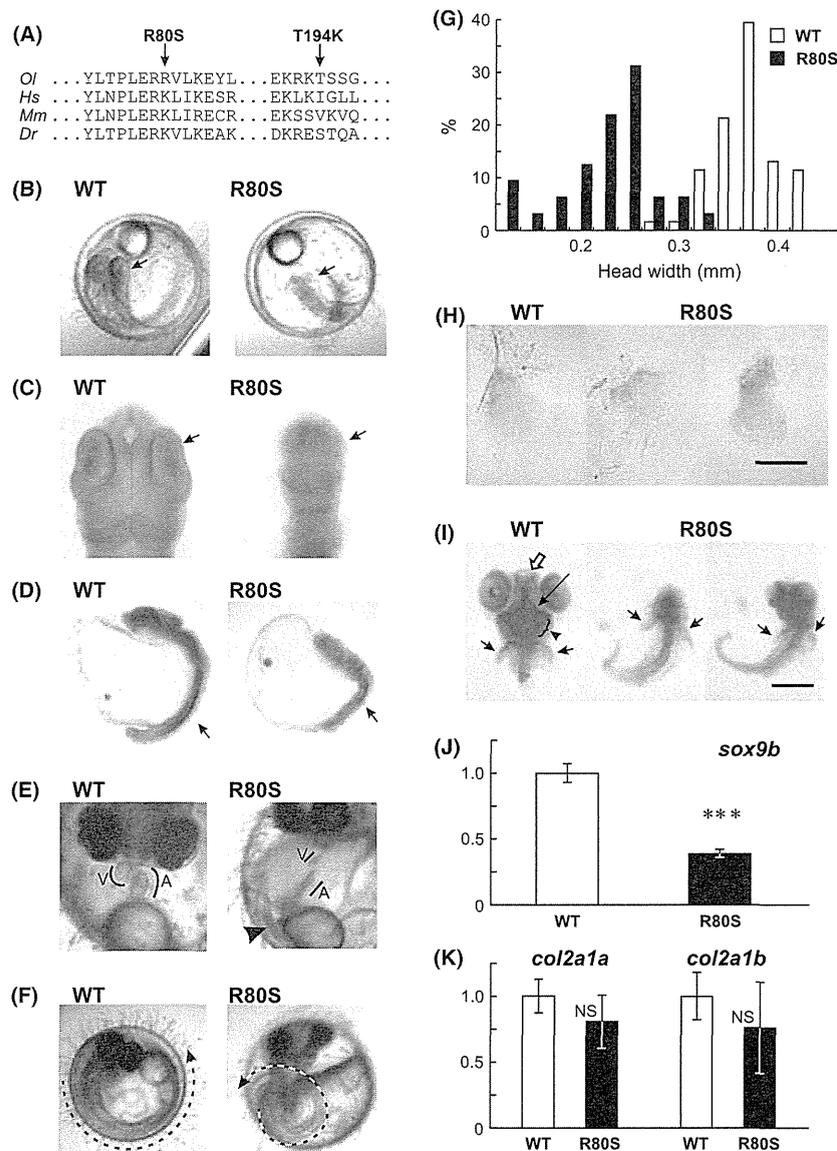


Fig. 2. Developmental abnormality of *ESCO2* mutant medaka. (A) Alignment of amino acid sequence of *ESCO2* (R80 and T194 adjacent sequences). Note that R80 and adjacent region is conserved. *Oi*, *Oryzias latipes*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Dr*, *Danio rerio*. (B–C) Head deformity of R80S homozygous mutant (B, 2.5 days postfertilization [dpf] optical observation; C, 2.0 dpf whole-mount *in situ* hybridization [WISH] with β -actin probe). Note: small head and lack of eyes (arrows). (D) Somite malformation of R80S mutant (2.0 dpf WISH with β -actin probe). The number of somites was decreased and disarranged (arrows). (E) Heart malformation of R80S mutant (5.0 dpf). In mutant, defects in looping of heart and stagnation in the tail (an arrowhead) were observed. V, ventricle; A, atrium. (F) Body axis abnormality in R80S mutant (5.0 dpf). Dashed lines indicate body axis. (G) The head width of wild-type (WT) and R80S mutant (2.0 dpf). R80S mutants were significantly smaller than WT embryos ($P < 0.001$, $n = 32$ –61). (H) Alcian blue staining of pectoral fin of WT and R80S mutant (6.0 dpf). Scale bar, 0.25 mm. In R80S mutants, the development of pectoral fins was normal (middle) or delayed (right). (I) Alcian blue staining of WT (head and pectoral region) and R80S mutant (whole body) (6.0 dpf, ventral view). In R80S mutant, defect of cartilage formation was observed. Open arrow, Meckel's cartilage; long arrow, ceratohyal; short arrow, pectoral fin; arrowheads, ceratobranchial arch. Scale bar, 0.5 mm. (J–K) *sox9b*, *col2a1a* and *col2a1b* expression in WT and R80S mutant (2.0 dpf) ($n = 13$ –14). *** $P < 0.01$; NS, not significant; Student's *t*-test.

intronic *ESCO2* mutations were identified from 5760 clones. Among the six missense mutations, we selected R80S (240G→T, nucleotide) and T194K (581C→A, nucleotide) mutations for artificial insemina-

tion because R80 was highly conserved among species and T194K substitution confers the charged amino acid (Fig. 2A). Heterozygous (*ESCO2*^{R80S/WT} and *ESCO2*^{T194K/WT}) embryos developed normally and did

Table 1. Genotyping and phenotype of *ESCO2* mutation[†]

Generation	<i>n</i>	WT/WT (%)	R80S/WT (%)	R80S/R80S (%)	Phenotype in R80S homozygous mutant (%)			
					Apparently normal	Developmental arrest [‡]	Cardiac abnormality	Body axis abnormality
F3 [§]	73	30.1	53.4	16.4	100.0	0.0	0.0	0.0
F4 [¶]	206	28.2	47.6	24.3	4.0	60.0	0.0	0.0
F5 ^{††}	198	19.7	56.4	24.2	0.8	60.4	16.7	20.8

[†]Embryos and adults were progenies of heterozygous parents in all generations examined. [‡]Developmental arrest with craniofacial abnormalities and somite malformation by 2.0 days post fertilization (dpf). [§]Data of adults only. Embryonic development was not examined. [¶]F4 embryos (at stage 28, 2.0 dpf). ^{††}F5 embryos (by hatch). Sampling was carried out at appearance of abnormality. Cumulative value is shown.

not show pathological phenotypes. However, when the heterozygotes were intercrossed, the resulting *ESCO2*^{R80S/R80S} homozygous mutants exhibited developmental delay and died before hatching. Homozygous *ESCO2*^{T194K/T194K} embryos developed normally. Therefore, we further analyzed the R80S homozygous mutants in detail. Genotyping F3–F5 generation of backcrossed mutants (*n* = 73–206) showed that this mutation was transmitted in a Mendelian fashion (Table 1). The mutants developed normally during gastrulation and neurulation. From 1.5 dpf (stage 19), 60% of the mutants began to exhibit developmental delay. By 2.0 dpf (stage 28), their development was arrested (Table 1) and they were smaller than WT (Figs. 2G, S5A). The mutants showed head deformities (particularly brain and eye malformation, Fig. 2B,C) and somite malformation (Fig. 2D), both of which expressed *ESCO2* during the somitogenesis stages (Fig. 1). The rest of homozygous mutants developed normally until 2 dpf (hereafter called “escapers”). However they exhibited the heart abnormality (looping defect), stagnation (16.7% of mutants, Fig. 2E) and body axis malformation (20.8%, Fig. 2F) subsequently, and 91.7% of F5 mutants died by hatching period (Table 1), indicating that the homozygous mutation causes embryonic lethality. Because phocomelia and craniofacial abnormality are characterized in RBS/SC, we observed cartilage of mutants. The mutants were deficient in clear structure of cartilage. However, the pectoral fins were normally developed by 6.0 dpf in most mutants (Fig. 2H, middle). Some mutants showed developmental delay (Fig. 2H, right). The ventral cartilage elements of heads were almost defective in mutants by 6.0 dpf (Fig. 2I). Furthermore, the gene expression of *sox9b*, a transcriptional factor for cartilage formation, and *col2a1a* and *col2a1b*, components of cartilage, was examined at 2.0 dpf embryos. In mutants, *sox9b* was suppressed (Fig. 2J) but both *col2a1a* and *b* were not (Fig. 2K). At 2.0 dpf, the cartilage was not observed even in WT embryos (data not

shown). Because the expression levels of *col2a1a* and *b* were very low, no significant difference between mutants and WT embryos may be observed.

It is known that ECO1 is essential for cellular viability in yeast (Skibbens *et al.* 1999). Although there are two related molecules in vertebrates, *ESCO1* and *ESCO2*, each plays a distinct role and both are required for normal cell cycle progression (Hou & Zou 2005). To investigate the reason the homozygous mutants developed normally up to 1.5 dpf, we examined the effect of maternal mRNA accumulated in oocytes. To address this, we examined expression alleles using point mutation and polymorphism of *ESCO2* (Fig. S3). Until stage 14, *ESCO2* mRNA was derived from two maternal alleles. The paternal or embryonic *ESCO2* mRNA began to be expressed from stage 12, in agreement with the previous finding that midblastula transition of medaka begins at stage 11 (Aizawa *et al.* 2003). These data suggest that maternal mRNA of *ESCO2* caused normal development in R80S homozygous mutants during the early stages of development.

We attempted the rescue experiment by injection of WT *ESCO2* mRNA into mutant embryos. Unfortunately, we produced no detectable effects on phenotypes of mutants.

Almost all the mutations reported so far in RBS/SC patients are protein-truncating mutations (Schüle *et al.* 2005; Vega *et al.* 2005, 2010; Gordillo *et al.* 2008). Because the mutant we recovered from the TILLING library was the missense mutation, we constructed three morpholinos (Fig. S4), ATG mo (translation inhibitor), E2I2 mo (splicing inhibitor) and 5mis mo (control), and injected them into eggs immediately after fertilization to further investigate the role of *ESCO2* in the embryogenesis. Because the maternal mRNA of *ESCO2* was accumulated until stage 14 as mentioned above, ATG mo was aimed at inhibiting both maternal and embryonic *ESCO2*, and E2I2 mo at inhibiting only embryonic *ESCO2*. The morpholinos had weak effect. Head deformities and somite malformation were not

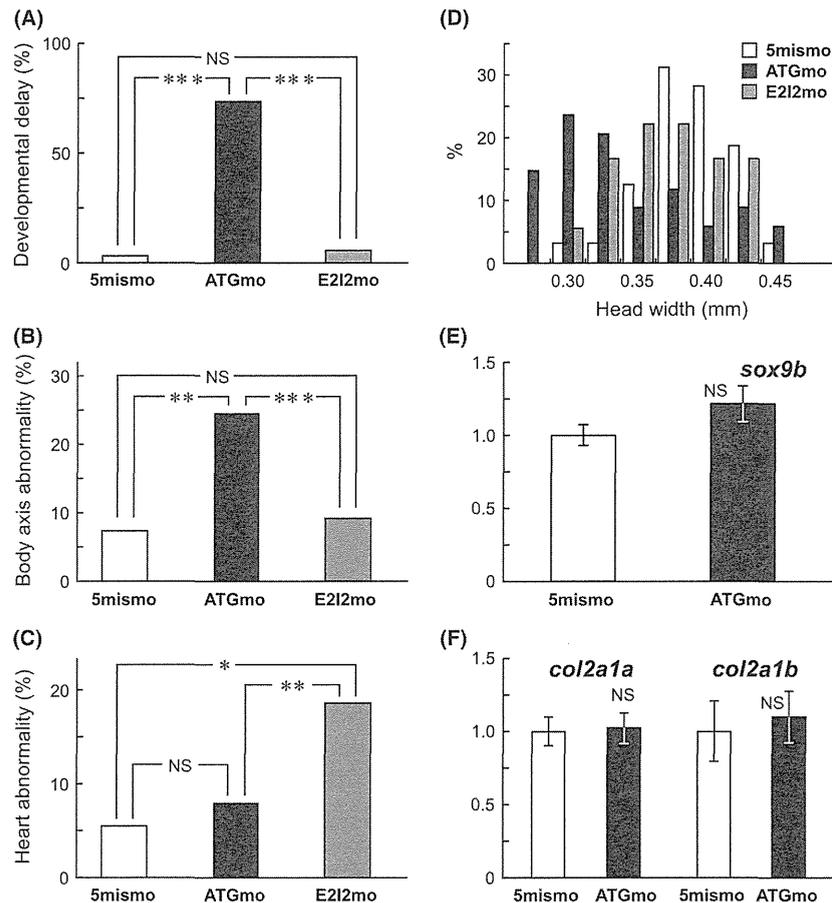


Fig. 3. Developmental abnormality of *ESCO2*-knockdown morphant. (A–D) The incidence of developmental delay (A), body axis abnormality (B, as in Fig. 2F) and heart malformation (C, as in Fig. 2E), and the head width (D) of morphants with 300 nmol/L of ATG mo (translation inhibitor, solid bars), E2I2 mo (splicing inhibitor, shaded bars) and 5mis mo (control, empty bars) injected just after fertilization. The 2.0-days postfertilization (dpf) embryos without heart beats were regarded as developmentally delayed. ATG mo morphants exhibited developmental delay. Body axis abnormality and heart malformation were observed by 5 dpf ($n = 54$ –101). ATG mo morphants showed body axis abnormality. E2I2 mo induced heart malformation, but ATGmo did not. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS, not significant; Tukey-type multiple comparison test (A–C); Tukey–Kramer test (D). ATG mo-injected morphants (2.0 dpf) were significantly smaller than control and E2I2 mo morphants ($P < 0.001$, $n = 18$ –34). (E–F) *sox9b*, *col2a1a* and *col2a1b* expression in control and ATG mo morphants (2.0 dpf). NS, not significant; Student's *t*-test ($n = 13$).

obvious in morphants (data not shown). The expression of *sox9b*, *col2a1a* and *col2a1b* was not also affected in ATG mo morphants (Fig. 3E,F). This was in agreement with morphants not exhibiting head deformity. On the other hand, the ATG mo morphants exhibited a significantly higher incidence of developmental delay (Fig. 3A) and were significantly smaller in size (Figs 3D, S5B) compared to the control and E2I2 mo morphants at 2 dpf. The ATG mo morphants also exhibited significantly higher incidence of body axis abnormality than the control or E2I2 mo morphants (Fig. 3B). The incidence of heart abnormality was similar between the ATG mo morphants and control, but was threefold higher in E2I2 mo morphants compared to the control

(Fig. 3C), suggesting that the phenotypes observed in R80S homozygous mutant fish are due to the loss-of-function effect of *ESCO2*. The phenotypes of ATG mo morphants and E2I2 mo morphants were different. The maternal and embryonic *ESCO2* are targets of ATG mo, and E2I2 mo may inhibit *ESCO2* after midblastula transition. Therefore, the difference of phenotype is attributable to the difference of timing for inhibition. Lastly, we crossed mutants (F4) of the original strain from the southern population, K-Cab, with another strain from the northern population, K-Kaga. The homozygous mutants (*ESCO2*^{R80S/R80S}) of the offspring (F'2) showed similar head deformities and developmental arrest by 2 dpf. Taken together, these results