D．考察

iPS細胞において、導入した遺伝子(Sox2, Oct3/4, Klf4, c-Myc)が高発現しているものは多分化能、自己複製能に乏しいことが先行研究により示されている。樹立したiPS細胞において、今後は導入した遺伝子発現量をRT-PCRにより比較し高発現しているものを除去する必要がある。また未分化マーカー(Nanog, Tra1, Rexなど)の発現、iPS細胞を免疫不全マウスに移植し奇形腫形成能を評価し、多分化能をもつiPS細胞lineを選出する必要がある。iPS細胞をBMPシグナル、TGF-βシグナルを阻害する環境で培養することで神経堤細胞となることが知られている（Lee G, et al. Nature Protocol 2010)。この方法を用いてiPS細胞を神経堤細胞へ分化誘導した。誘導したCHAGE症候群-iPS細胞由来神経堤細胞でCHD7遺伝子変異に伴い神経堤細胞の移動能が阻害されているかどうかを評価中である。

E．結論

CHARGE症候群患者由来のiPS細胞を作成した。Lorenz Studerらの方法をもとに、iPS細胞より神経堤細胞（CD57/CD271 (+/+)）を得た。これらの神経堤細胞について、コントロール-iPS細胞由来、CHARGE-iPS細胞由来のものが同等にSOX10を発現すること、多分化能をもつことを確認した。現在、これらを用いて遊走能についてin vitro, in vivo解析を行っている。今後、CHARGE症候群の発症機転の検討や候補薬剤のスクリーニングに用いる。計画である。それにより患者自身の細胞から正常細胞を作製することが可能になる。CHARGE症候群では感覚器(視覚、聴覚、嗅覚)の異常を多くみとめるが、細胞移植による治療を検討することで患者および家族のQOLの向上が期待できる

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G．知的財産権の出願・登録状況

1. 特許取得

【海外】

（1）発明の名称 神経幹細胞製造方法
出願番号 アメリカ 13/127, 566
出願日 2011年5月4日
出願人 学校法人慶應義塾
発明者 岡野栄之、赤松和土

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

メチマゾールによるCHARGE症候群モデルの作成

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研究要旨

甲状腺機能亢進症の治療薬であるメチマゾールへの胎児期の曝露により、後鼻腔閉鎖および乳頭低形成を発症する症例が報告されている。また研究代表者の小崎らはメチマゾール曝露後の虹彩・網膜欠損を見出した。このように、メチマゾール曝露後の先天異常は、CHARGE症候群に極めて類似する。本研究では、ゼブラフィッシュの受精卵をメチマゾールに曝露後、表現型を詳細に観察し、メチマゾールへの曝露が胚発生に与える影響を検討することにより、CHARGE症候群の発症機序の解明を試みた。

A．研究目的

メチマゾールが催奇性を有する可能性は長らく示唆されてきており、曝露後に発症する先天異常はCHARGE症候群に極めて類似しているが、メチマゾールの曝露が胎児発生に与える影響とその発症機序は明らかでない。その一因として、至適なモデル動物による実験系の不備が挙げられる。そこで、本研究ではゼブラフィッシュをモデル動物として用い、受精卵に対するメチマゾール曝露が発生に与える影響についての検討を行い、CHARGE症候群の発症機序解明のためのモデル系を作成することを目的とした。

B．研究方法

ゼブラフィッシュの成魚は、産卵盛期の日照サイクルである 14 時間明/10 時間暗の長日条件、28.5℃の最適水温下で飼育し、繁殖ストレスのかからないよう各個体一週間に一回以下の頻度で自然交配を行い、受精直後の卵を採取した。採取した卵は、顕微鏡観察下で正常な受精卵を選別後、六穴プレートに移し、さまざまな濃度のメチマゾールを含む飼育水を直ちに加え、28.5℃に設定した湿潤気相インキュベーター内で飼育し曝露した。曝露した受精卵は生存数を随時計数しながら、正常胚が自然孵化し始める受精後 48 時間まで飼育した。実体顕微鏡による外表的な表現型の観察を行い、特徴的な異常を抽出し、各異常の発現頻度を算出した。同様の実験を独立に 3 回繰返して行った。さらに詳細な表現型観察のため、パラフィン包埋試料の横断切片を作成し、ヘマトキシリンーエオシン染色を施した。また、細胞死の種類を判定するため TUNEL 試験を行った。なお本研究は、東京女子医科大学設置の「動物実験委員会」および「動物実験倫理委員会」の定める規定を遵守して行った。

C．研究結果

まず、メチマゾールの至適曝露濃度の範囲を特定するため、1 M から 1 nM の広範囲にわたって曝露を行った。その結果、1 mM 以下の濃度では外表的な表現型は観察されず、100 mM を超える濃度では胚発生早期に死滅した。さらに詳細に曝露濃度の範囲を検討した結果、曝露した胚が孵化まで生育し、かつ表現型が顕著に観察されるのは 2 mM から 10 mM の範囲であることが明らかになった。

次に、受精卵を上記のメチマゾール曝露条件下において、受精後 48 時間まで飼育し、外表的な表現型を実体顕微鏡にて観察した。その結果、体表面および網膜のメラニン色素の減少、前脳の細胞死と後脳の低形成、脊索の、特に末端部の形態異常、体節形成異常を伴う体幹の変形が確認された。その出現頻度は濃度依存的であり、高い再現性が確認された。

さらに、外表的表現型の原因となる内部構造の異常を確認するため、メチマゾール曝露胚の横断切片にヘマトキシリンーエオシン染色を施し、組織形態学的に観察を行った。その結果、神経菅背側の消失と神経菅不閉鎖を伴う極端な脳の低形成、脊索の形態異常とそれに起因すると考えられる体節形成異常が顕現した。また、この観察により初めて咽頭閉塞が示唆された。さらに、TUNEL 試験により、前脳背側部に限定的にアポトーシスによる細胞死が観察された。

D．考察

ゼブラフィッシュの受精卵をメチマゾールに曝露することにより、さまざまな異常が生じた。それらのうち、咽頭閉鎖を示唆する表現型は、胎児期のメチマゾール曝露後に生じる代表的な先天異常である後鼻腔閉鎖と直接関連付けられることから、ゼブラフィッシュをモデル動物として用いて、メチマゾール曝露が発生に与える影響について検討する、という本研究の方法は妥当であると考えられる。

メチマゾール曝露により異常を呈する器官がゼブラフィッシュ胚、ヒト胎児ともに多岐に及ぶことから、影響を受ける発生イベントがそれぞれに複数ずつ存在する可能性が示唆される。その一方で、一見無関係に見える種々の表現型が、発生初期のある共通のイベントに生じた異常に起因し、その結果が多様な器官で表出した可能性も同様に示唆される。異常を呈している各器官の正常発生に必須なイベントと遺伝子群の詳細な検討を行うことにより、これら二つの可能性の峻別とともに、メチマゾール曝露後に生じる先天異常の発症機序の解明が可能になる

と考えられる。CHARGE症候群とメチマゾール母体曝露で生じる先天異常が強い類似性を示していることから、両者では同じ発生イベントが影響を受けている可能性が高いと考えられる。従って、本研究でモデル系を作成したことは、CHARGE症候群の発症機序解明に向けて一定の成果を挙げたと考えられる。

また、従来のげっ歯類に代表される有胎盤類を用いた研究では困難であった母体曝露を再現する実験系において、ゼブラフィッシュが有用なモデル動物となり得ることを示した、という点も本研究の重要な成果であると考えられる。

E．結論

ゼブラフィッシュの受精卵をモデル系として用い、メチマゾールへの曝露が胚発生に与える影響を検討することにより、CHARGE症候群の発症機序の解明を試みた。メチマゾールに曝露したゼブラフィッシュ胚では、さまざまな異常が出現したが、CHARGE症候群とメチマゾール曝露後の先天異常とに共通の表現型と直接関連付けしうる異常も観察され、ゼブラフィッシュモデルの有用性が示された。

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G．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

〔Ⅳ〕

刊行に関する一覧表

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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研究成果の刊行物・別冊

RESEARCH ARTICLE

AMERICAN JOURNAL OF
medical genetics

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 (%)			
Findings	Bilateral	Unilateral	Total	Number of 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 κ 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

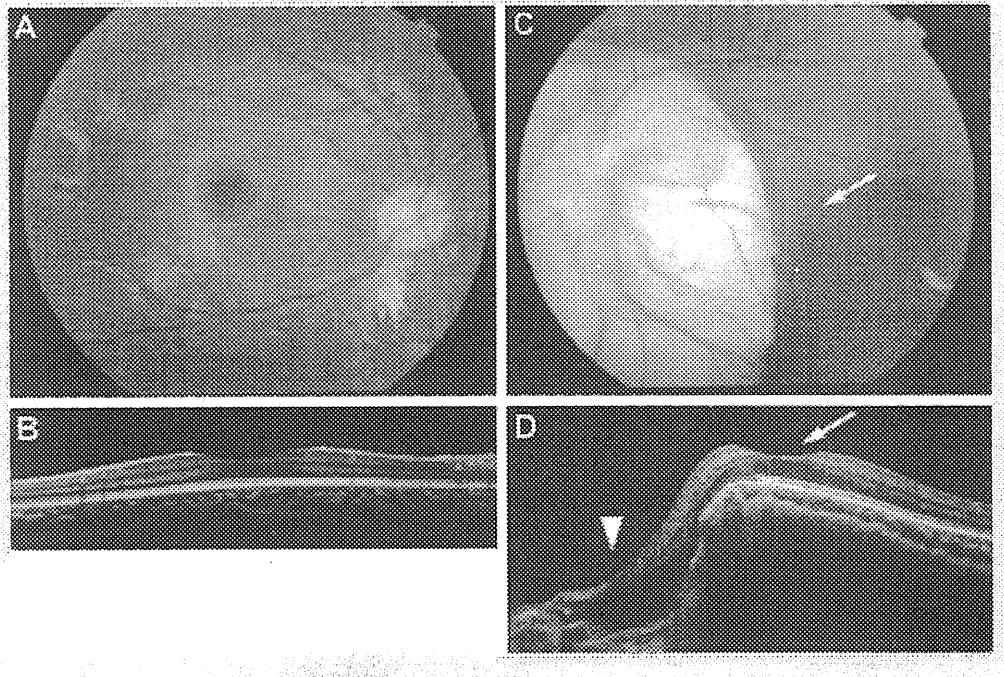

FIG. 1. Fundus photographs and spectral domain optical coherence tomography (SD-OCT) scan of the retina in the right eye (A,B) and the left eye (C,D) in a 6-year-old girl. A: Retinochoroidal colobomata inferior to the optic disk is visible in the right eye. B: The SD-OCT shows a good macular formation 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 ambiyopia treatment.

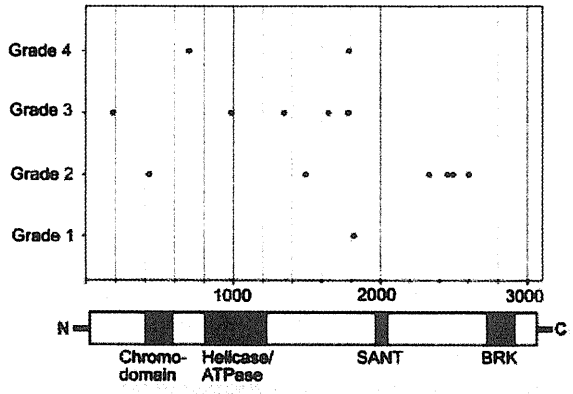

FIG. 2. The correlation between the anatomical severity of the eyes in an individual and the amino acid position where the truncation of the CHD7 protein occurred in the same individual. Horizontal axis indicates amino acid position of the CHD7 protein together with the domains of the protein. Vertical 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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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

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

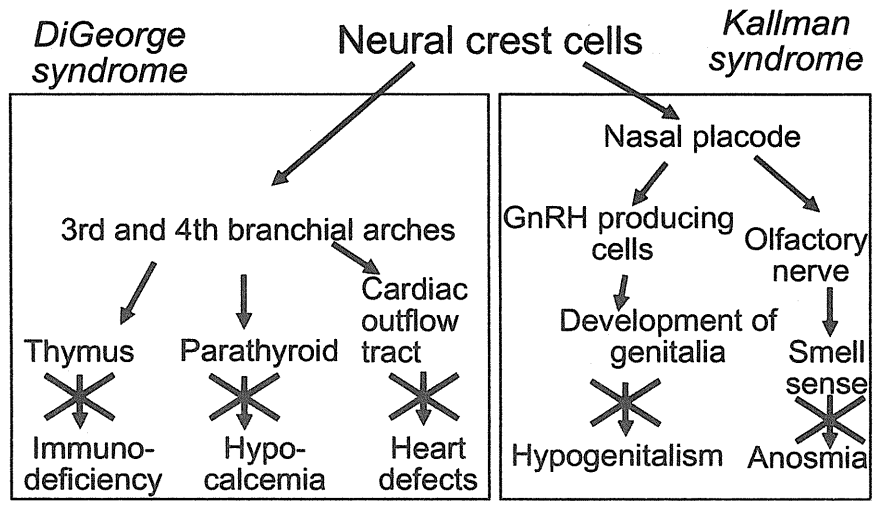

Fig. 1 Two developmental pathways affected in DiGeorge syndrome phenotype and Kallman syndrome phenotype are depicted. The two pathways share a common feature: the involvement of the neural crest.

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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CLINICAL INVESTIGATION

Survey of microphthalmia in Japan

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Abstract
Purpose To report the current status of patients with microphthalmia based on a cross-sectional survey of patient hospital visits.
Methods A questionnaire was sent to the departments of ophthalmology in 1,151 major Japanese hospitals to survey the following: the number of patients with microphthalmia who visited the outpatient clinics between January 2008 and December 2009; gender; age; family history; associated ocular anomalies; complications and systemic diseases; surgical treatment; vision and management. A retrospective quantitative registry of 1,254 microphthalmic eyes (851 patients) from 454 hospitals (39.4%) was compiled.
Results Of the patients for whom data were available, 50% ranged in age from 0 to 9 years. The major ocular findings were nanophthalmos, coloboma, and vitreoretinal malformations. Ocular complications frequently developed, including cataracts, glaucoma, and retinal detachment. Surgery was performed in 21.4% of all cases, and systemic diseases were present in 31% of all cases. The vision associated with microphthalmia exceeded 0.1 in about 30% of the eyes. Glasses and low vision aids were used by 21.6% of patients.
Conclusions Patients with microphthalmia often have ocular and systemic anomalies. Early assessment and preservation of vision and long-term complication management are needed.

Keywords Microphthalmos · Epidemiology · Survey · Intractable disease

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Introduction

Microphthalmos is defined as the arrested development of all global dimensions and is often is associated with other ocular and systemic anomalies [1]. Chromosomal disorders, genetic syndromes, and environmental factors, such as maternal infection and exposure to X-rays or drugs, are reported as causes [2]. However, in most cases the precise pathogenesis is unknown although some causative genes (*SOX2* and *PAX6*) have been identified [2–4].

Previous studies conducted in the UK report that the prevalence rates of microphthalmia, anophthalmia, and typical coloboma are 10–19 per 100,000 births [4–7]. Microphthalmia is rare, and only a few disease, genetic, and epidemiologic studies and a few reports on the practical patient status have been published. The condition generally causes substantial visual impairment, but standard management and treatments have not been established.

We conducted a cross-sectional national survey to investigate the current status of patients with microphthalmia, focusing especially on ocular associations,

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