FGFR2 mutations and associated clinical observations in two Chinese patients with Crouzon syndrome

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Abstract. The aim of the present study was to identify mutations in the fibroblast growth factor receptor 2 (*FGFR2*) gene in patients with Crouzon syndrome and characterize the associated clinical features. A total of two Chinese patients diagnosed with Crouzon syndrome underwent complete examinations, including best-corrected visual acuity, slit-lamp, examination, fundus examination, optical coherence tomography and computed tomography of the skull. Genomic DNA was extracted from peripheral blood samples collected from the patients, as well as their family members and 200 unrelated control subjects from the same population. Exons 8 and 10 in the *FGFR2* gene were amplified by polymerase chain reaction and directly sequenced. Patient #1 had a heterozygous missense mutation (c.1025G>A, p.C342Y) in exon 10 of *FGFR2*. Patient #2 had a heterozygous mutation (c.1084+1 G>T; IVS10+1G>T) in intron 10. The mutations were not present in any of the unaffected family members or unrelated control subjects. These findings expand the mutation spectrum of *FGFR2*, and are valuable for genetic counseling in addition to prenatal diagnosis in patients with Crouzon syndrome.

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Introduction

Craniosynostosis is characterized by premature fusion of one or more cranial sutures, resulting in an abnormal growth pattern of the skull (1). Craniosynostosis can be clinically manifested as Crouzon syndrome, Jackson-Weiss syndrome, or Pfeiffer syndrome. Crouzon syndrome, first reported by Louis Edouard Octave (2-5) in 1912, is recognized as one of the most common craniosynostosis syndromes (6). The prevalence of Crouzon syndrome is between 1/60,000 to 1/1,000 live birth, depending on race, region, and ethnicity (7-9). Crouzon syndrome is typically characterized by craniosynostosis, exorbitism, hypertelorism, midface hypoplasia, hooked nose, thin vermilion of the upper lip, and mandibular prognathism (10,11). Unlike Pfeiffer syndrome that can present hand abnormalities, such as wide and deviated thumbs, or Jackson-Weiss syndrome that can present broad great toes with medial deviation and tarsal-metatarsal coalescence, Crouzon syndrome usually does not present limb abnormalities (1,9). Most patients with Crouzon syndrome present altered ocular appearance such as ocular proptosis, and initially seek medical care from neurosurgeons or ophthalmologists, rather than orthopedists (8,12,13). Since Crouzon syndrome is a relatively rare syndrome, and is usually not easy to diagnose, molecular diagnosis will provide useful information for the disease diagnosis and genetic counseling.

Craniosynostosis is generally associated with abnormal function of fibroblast growth factor receptors (FGFRs) (14,15). To date, more than 50 distinct mutations in the *FGFR2* gene have been linked to Crouzon syndrome. Approximately 95% of patients have a mutation in either 8 (IIIa) or exon 10 (IIIc), which encode the extracellular immunoglobulin-like III (IgIII) domain of the receptor (14,16). Growth factors, such as FGF and TGF, play pivotal roles for controlling cell growth and differentiation (17-23). Mutations in *FGFR2* can lead to increased ligand affinity and altered ligand specificity, disrupting the differentiation of mesenchymal stem cells, and

therefore causing developmental defects (24,25). Although Crouzon syndrome is often inherited as an autosomal dominant trait, *de novo* mutations at *FGFR2* can also result in sporadic cases (3,5,26). Here, we report the results of a mutational analysis of two sporadic patients with Crouzon syndrome from two unrelated Chinese families.

Materials and methods

Patient recruitment and clinical evaluations. All experimental protocols and methods which were carried out in accordance with the guidelines were approved by the Ethics Committee of Zhongshan Ophthalmic Center. Informed consents were obtained from all participating subjects in accordance with the Declaration of Helsinki. The following series of ophthalmic tests were performed in patients and their family members. Visual acuity was examined using the Early Treatment Diabetic Retinopathy Study (EDTRS) chart (Precision Vision, LaSalle, IL, USA). Anterior segment photographs were captured by a BX 900 slit lamp (Haag-Streit AG, Köniz, Switzerland). Anterior segment measurements were obtained by a Pentacam HR version 70700 (OCULUS Optikgeräte GmbH, Wetzlar, Germany). Optical coherence tomography (OCT) was carried out by Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, CA, USA). Computed tomography (CT) and physical examinations, including blood examination, urinalysis, electrocardiogram, chest X-ray, blood biochemistry, blood lipid, and blood coagulation tests, were conducted to exclude systemic diseases.

Sample collection and mutational screening. Genomic DNA samples were extracted from peripheral blood leucocytes of the patients and their relatives with the Qiagen kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were measured by NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). In addition, DNA samples collected from 200 subjects from the same population without diagnostic features of Crouzon syndrome were used as controls. Exons 8 and 10 in the *FGFR2* gene were amplified using polymerase chain reaction (PCR) as described previously (24,25,27). Primers were obtained from the Beijing Genomics Institute (Guangzhou, China). The sequences of the primers are listed in Table I. All reagents used for the PCR reactions were purchased from Takara Bio Inc. (Tokyo, Japan). The amplification included a single 5-min step at 94°C; followed by 40 cycles of 94°C for 45 sec, 61°C for 45 sec, and 72°C for 45 sec; and finally a 10-min step at 72°C. The PCR products were sequenced in both directions using an ABI3730 Automated Sequencer (PE Biosystems, Foster City, CA, USA). The sequencing results were analyzed using SeqMan (version 2.3; Technelysium Pty, Ltd., Brisbane, QLD, Australia), and compared against reference sequences obtained from the National Center for Biotechnology Information (NCBI) database (accession no. NC_000010) (28).

Results

Clinical presentations. We diagnosed two patients of two unrelated families from the southern region of China. Systemic diseases were excluded upon examination.

Patient #1 was a two-year-old girl and was the only child of two healthy parents (Fig. 1). She was referred by her local pediatrician at two months of age due to concerns about an elongated head shape and the possible diagnosis of sagittal synostosis. Until this point, the patient's development was otherwise unremarkable, with normal feeding and steady weight gain after birth. Examination of this patient revealed shallow orbits and ocular proptosis, accompanied by midface hypoplasia, craniosynostosis, a curved beak-like nose (Fig. 1A), and clinically normal hands and feet. An approximately 2 mm gap was observed when she attempted to close her eyelids (Fig. 1B). The patient presented with exotropia in both eyes, but the corneas were transparent with normal size. Also, the lenses were transparent and normally positioned (Fig. 1C and D). Fundus examination showed normal retinas (Fig. 1E and F). Because of the patient was young, we were unable to measure visual acuity, but the child had normal visual tracking and the results of the optometry were +3.0 D (OD) and +3.25 D (OS). CT scan revealed shallow orbits and exotropia in both eyes (Fig. 1G). Both parents had normal visual acuity and unremarkable eye examinations, and all family members had no known history of learning difficulties or genetic problems.

Patient #2 was a 21-year-old woman and was also the only child of two healthy parents (Fig. 2). She presented with midface hypoplasia and craniosynostosis (Fig. 2A). She had normal visual acuity. Her hands and feet had normal flexibility. Radiography showed no obvious carpal fusion (Fig. 2B and C). No abnormalities were detected in the cornea or lens (Fig. 2D and E). CT scan revealed shallow orbits (Fig. 2F). OCT revealed normal retina in both eyes (Fig. 2G and H). In general, the clinical manifestations of this patient were less severe than patient #1.

Mutational screening. Patient #1 carried a heterozygous missense mutation (c.1025G>A; p.C342Y) in exon 10 of the FGFR2 gene (Fig. 3A). Patient #2 carried a heterozygous mutation (IVS10+1G>T; c.1084+1 G>T) in intron 10 of the FGFR2 gene (Fig. 3B). This mutation is located at a splicing site. Both mutations were not presented in any of the unaffected family members or unrelated controls, therefore are considered *de novo* mutations.

Clinical manifestations and mutational screening results of the two patients in this study are summarized in Table II.

Discussion

Crouzon syndrome is a common autosomal dominant form of craniofacial complexes, characterized by premature craniosynostosis, orbital proptosis, and midface hypoplasia (7). Both patients we reported here do not present limb malformations, which differentiates Crouzon syndrome from other types of craniosynostosis (1,9).

In patient #1, the c.1025G>A mutation causes a cysteine-to-tyrosine substitution at amino acid 342 in FGFR2. The loss of this cysteine residue is one of the most frequent mutations in Crouzon syndrome patients and has been reported in French, British, and German populations (5,6,29-32). Therefore, the amino acid C342 in FGFR2 is considered as a mutation 'hotspot'. Several identified mutations at this position are C342R (c.1024T>C), C342Y

Table I. Summary of the primers and product length used for the amplification of the exons of FGFR 2.

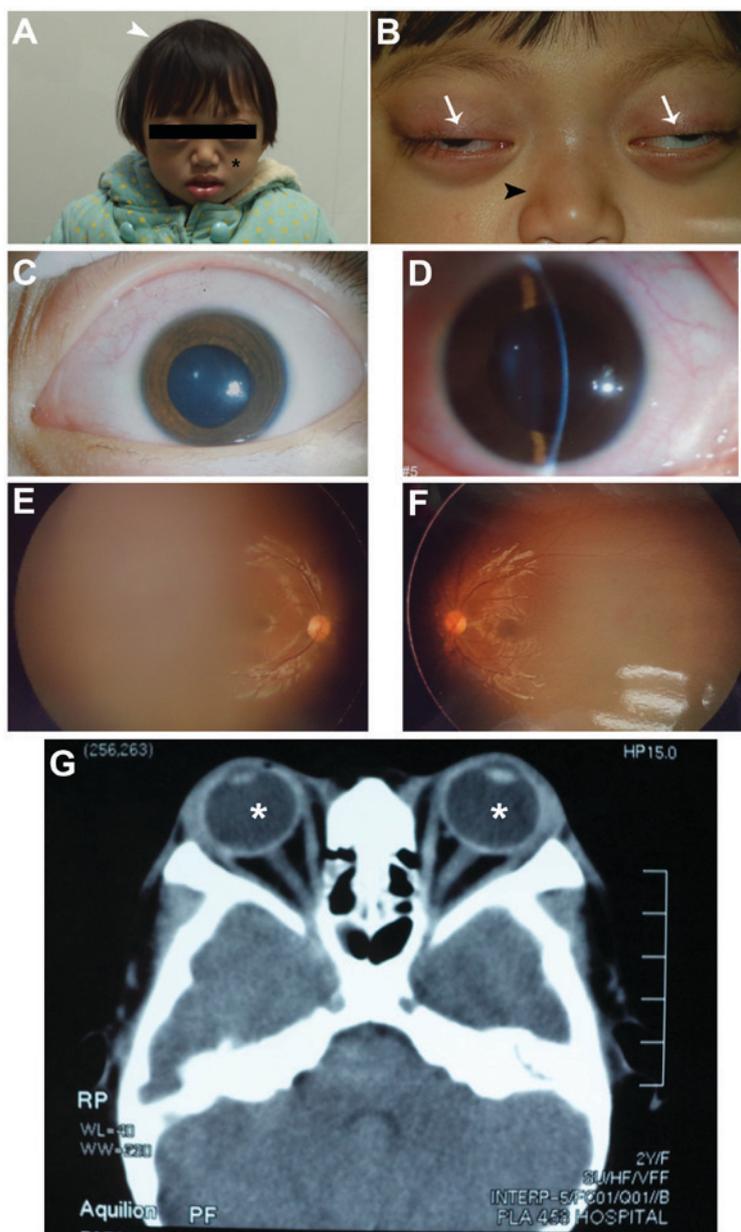| Exon	Forward (5'-3')	Reverse (5'-3')	Product size (bp)	Annealing temperature (˚C)
FGFR2-8 (IIIa)	GGTCTCTCATTCTCCCATCCC	CCAACAGGAAATCAAAGAACC	325	61
FGFR2-10 (IIIc)	CCTCCACAATCATTCCTGTGTC	ATAGCAGTCAACCAAGAAAAGGG	257	61

Figure 1. Clinical manifestations of patient #1. (A and B) The patient presented with ocular proptosis (white arrows), extropia, midface hypoplasia (black asterisk), craniosynostosis (white arrowhead), and a curved, beak-like nose (black arrowhead). An approximately 2 mm gap was observed when she attempted to close her eyelids. (C and D) The corneas of both eyes were transparent with normal size, and the lenses are clear and normally postioned. (E and F) Fundus examination showed normal retina in both eyes. (G) A CT scan reveals shallow orbits and exotropia in both eyes (white asterisks).

(c.1025G>A), C342S (c.1025G>C), C342F (c.1025G>T), and C342W (c.1026C>G) (30,32,33). Mutations at C342 can cause Crouzon syndrome as well as Pfeiffer syndrome (34). Studies have shown that C342 is part of the disulfide bridge that stabilizes the IgIII loop in all FGFR proteins and is the most conserved extracellular amino acid in the Ig superfamily. The loss of C342 leaves an unbridged C278, which may cause the ligand-independent dimerization of receptor molecules, leading to constitutive receptor activation (35,36).

Table II. Summary of clinical manifestations and mutational screening results of the two patients.

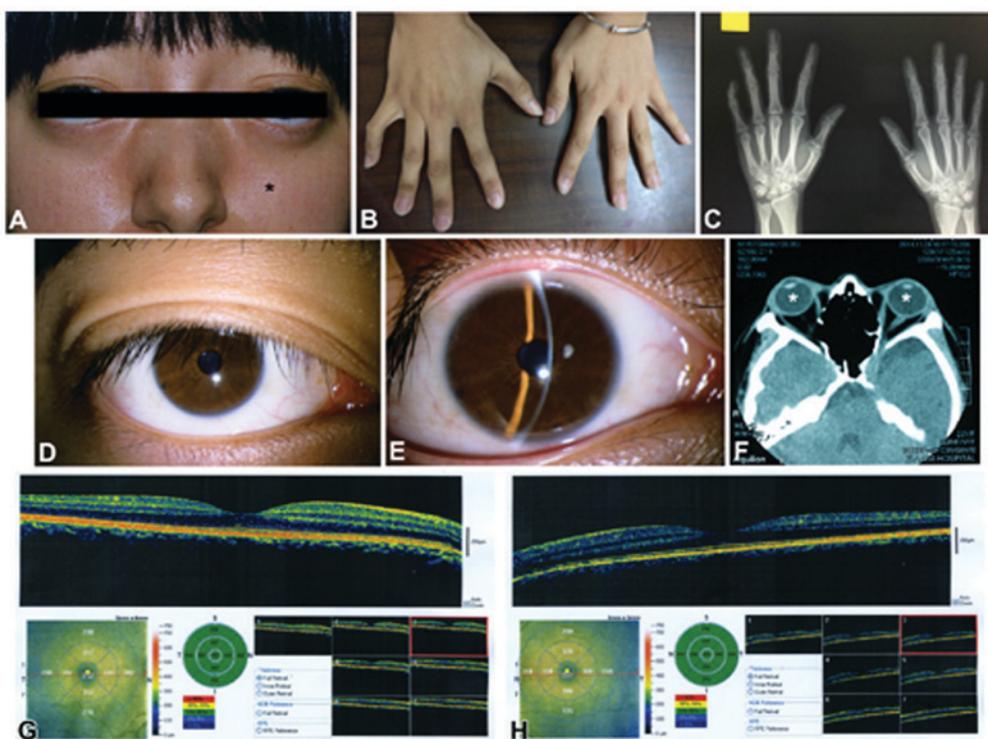| Patient (#) | Gender | Age | Clinical manifestations | | | | | Mutation |
			Facial characteristics	Limbs	Lens/cornea	Fundus	CT scan	
1	Female	2	Midface hypoplasia, craniosynostosis, curved beak-like nose, lagophthalmus	Normal	Normal	Normal	Shallow orbits, proptosis, exotropia	c.1025G>A
2	Female	21	Midface hypoplasia, craniosynostosis	Normal	Normal	Normal	Shallow orbits	c.1084+1 G>T, IVS10 +1G>T;

Figure 2. Clinical manifestations of patient #2. (A) The patient presented with midface hypoplasia (black asterisk). (B) Clinically normal hands. (C) Radiography revealed no obvious carpal fusions in this patient. (D and E) The cornea and lenses were normal. (F) CT scan revealed shallow orbits (white asterisks). (G and H) OCT examination revealed normal retina in both eyes.
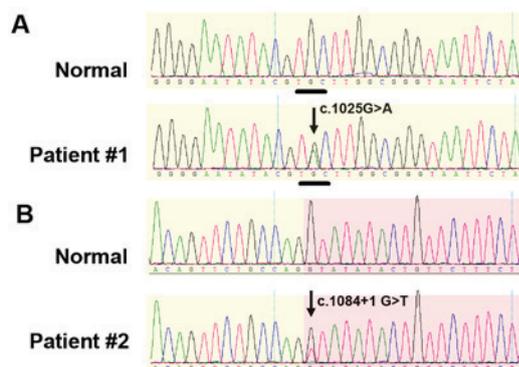

Figure 3. Mutational screening results of the patients. (A) A heterozygous missense mutation (c.1025G>A; p.C342Y) in exon 10 of the *FGFR2* gene was identified in patient #1. (B) A heterozygous mutation (IVS10+1 G>T; c.1084+1 G>T) in intron 10 of the *FGFR2* gene was identified in patient #2. Yellow boxes denote exon regions. Pink boxes denote intron regions.

The fidelity of the splice site sequence, particularly the first two nucleotides in the donor site, is essential for accurate splicing. The presence of a guanine base at the +1 position at the intron-exon boundary of *FGFR2* gene is essential for splice site recognition. In patient #2, the splicing site mutation (c.1084+1 G>T) can cause alternative splicing, disrupt the third immunoglobulin-like domain of FGF2, and generate pathogenic protein isoforms. However, compared to the cysteine mutation in Patient #1, mutations affecting FGFR2 pre-mRNA splicing usually cause relatively mild clinical manifestations (37-39). Interestingly, a similar mutation in the *FGFR2* gene at the same position (c.1084+1 G>A) can cause mild bicoronal synostosis (38).

Craniosynostosis may be complicated with other ophthalmic anomalies. For example, some craniosynostosis patients with *FGFR2* mutation can also present with Peters anomaly (a rare

form of anterior segment dysgenesis), optic nerve hypoplasia, scleralization of the cornea, and corectopia (13). In this study, patient #1 also had strabismus, which expands the list of clinical manifestations associated with Crouzon syndrome.

In summary, we identified two distinct mutations in the *FGFR2* gene in two Chinese patients with Crouzon syndrome from unrelated families. These findings expand the mutational spectrum of *FGFR2*, and provide valuable information for genetic counseling and prenatal diagnosis in families with Crouzon syndrome. Although our understanding of the function of FGFR is still limited, the discovery of these mutant variants provides an opportunity and rationale for in-depth mechanistic studies, and may help to reveal critical pathophysiology underlying related skull development disorders in general.

Acknowledgements

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