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A Novel FGFR2 Mutation in Tyrosine Kinase II Domain, L617F, in Crouzon Syndrome

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ABSTRACT

The purposes of this study were to find a novel mutation of *FGFR2* in Korean Crouzon syndrome patients and to identify the functional consequences of this mutation. The samples consisted of 16 Crouzon patients. Peripheral venous blood was collected from the patients. *FGFR2* mutation screening was performed by direct PCR sequencing of all exons and part of the introns. Restriction fragment length polymorphism (RFLP) analysis was performed to confirm the novel mutation. For functional studies, we performed luciferase assay for *Runx2* transcriptional activity, real-time PCR for the bone markers (osteocalcin and alkaline phosphatase), and Western blot for phosphorylated *FGFR2* and *ERK1/2-MAPK* protein. Among 16 patients, 10 showed *FGFR2* mutations that had already been reported elsewhere. A novel *FGFR2* mutation associated with tyrosine kinase II (TK-II) domain, L617F, was found in one Crouzon syndrome patient by direct PCR sequencing. Presence of this mutation was confirmed using RFLP analysis. *Runx2* transcriptional activity and expression of osteocalcin and alkaline phosphatase significantly increased in L617F-transfected cells compared to wild-type cells. *FGFR2* autophosphorylation in L617F-transfected cells increased in 1% serum, but *ERK1/2-MAPK* protein was not activated. The *FGFR2*-L617F mutation associated with the TK domain is potentially related to premature suture closure in Crouzon syndrome patient. J. Cell. Biochem. 115: 102–110, 2014.

KEY WORDS: NOVEL FGFR2 MUTATION; L617F; TYROSINE KINASE DOMAIN; CROUZON SYNDROME

Craniosynostosis is a congenital defect with premature fusion of one or more sutures of the skull. Crouzon syndrome, the most common syndrome that has craniosynostosis, shows deformation of the skull, exorbitism, maxillary hypoplasia, parrot-beaked nose, pseudoschisis and ogival palate, and skeletal Class III malocclusion [Carinci et al., 1994; Lajeunie et al., 1995]. The fibroblast growth factor receptor 2 (*FGFR2*) gene is known to be responsible for most of these conditions [Bonaventure and El Ghouzzi, 2003; Wilkie et al., 2006]. It belongs to a family of transmembrane tyrosine kinase and maps to 10q26. It consists of three extracellular immunoglobulain-like (Ig) domains (Ig-I, Ig-II, and Ig-III), a single transmembrane segment, and a split tyrosine kinase (TK-I/TK-II) domain [Fujisawa et al., 2002]. This gene is involved in intracellular signaling. If *FGF* binds to its receptor, the receptor autophosphorylates and there is an increase in tyrosine phosphorylation of several intracellular proteins [Mohammadi et al., 1996].

To date, more than 40 mutations have been found in Crouzon syndrome alone, most of which are localized in the extracellular Ig-III domain and adjacent linker regions (IIIa and IIIc) [Zhang et al., 1999; Wong et al., 2001; Kan et al., 2002; Lajeunie et al., 2006]. Mutations in

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these domains are known to cause a conformational change in the extracellular *FGF* binding site, resulting in activation of the receptor [Mai et al., 2010].

However, Kan et al. [2002] first reported novel mutations in other regions of the receptor including the Ig-II loop and tyrosine kinase (TK) domains. Several studies have reported that *FGFR2* mutations in the TK-I and TK-II domains are associated with Crouzon and Apert syndromes (K526E, N549H, E565G, N549H in the TK-I domain [Kan et al., 2002; de Ravel et al., 2005; Lajeunie et al., 2006]; K641R, K659N, G663E, R678G in the TK-II domain [Kan et al., 2002; Lajeunie et al., 2006]).

To our knowledge, there have been only four reports dealing with genetic aspects of Korean craniosynostosis patients: three case reports about Crouzon syndrome (Cys342Ser) [Bae et al., 1998], Beare–Stevenson syndrome (Tyr375Cys) [Eun et al., 2007], and Pfeiffer syndrome (Cys278Leu) [Lee et al., 2010], and one genetic analysis of craniosynostosis patients [Park et al., 2012]. Although Park et al. [2012] found a novel mutation, E731K, in the TK-II domain, the number of craniosynostosis patients was only six. Therefore, the sample size needs to be increased in order to investigate other novel mutations in Korean craniosynostosis patients. In this study, we collected 16 Korean Crouzon patients. The purposes of this study were to find a novel mutation in *FGFR2* in Korean Crouzon patients and to identify the functional consequences of this mutation.

MATERIALS AND METHODS

SUBJECTS

The samples consisted of 16 Crouzon patients [2 families (mother, brother, and sister; and 2 sisters, respectively)]. Diagnosis of craniosynostosis was made through clinical inspections by well-trained medical doctors and dentists. For mutation searches, peripheral venous blood of patients was collected at Seoul National University Dental Hospital (SNUDH).

ETHICS STATEMENT

The study protocol was reviewed and approved by the institutional review board at Seoul National University Dental Hospital (SNUDH IRB CRI-G07002). Informed consent was received from each subject or parents before the sampling.

FGFR2 MUTATION SCREENING

Genomic DNA (gDNA) was extracted using G-DEX IIb genomic DNA extraction kit (iNtron, Seongnam, Korea). After the polymerase chain reaction (PCR) primers were designed (Table I), PCR for the target sequence was performed with sensi 5× PCR premix (Lugen, Seoul, Korea). After the sequencing process at Solgent (Daejeon, Korea), acquired sequences were analyzed using the BioEdit program (Carlsbad, CA).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

The gDNA was amplified with PCR using Pfu polymerase (Thermo Scientific, Vilnius, Lithuania). The restriction enzyme CviKI-I (New England Biolabs, Ipswich, MA) was added to approximately 1 U per 1 μg of PCR products to generate DNA fragments. Both amplified products and enzyme-treated products were loaded on a 10% polyacrylamide gel and run at 50 mA in 0.5% TBE buffer. The resolved DNA band was visualized under ultraviolet light after ethidium bromide staining.

PLASMID CONSTRUCTS

Full-ORF length *FGFR2*-3x Flag was used as a wild-type construct for mutagenesis. The L617F mutation, a novel mutation found in this study, was synthesized by cloning PpuMI/BstZ17I fragments of *FGFR2*-L617F into the PpuMI/BstZ17I site of *FGFR2*-3x Flag. Primers for this process were designed with the primerX program (Bioinformatics Organization, MA; Forward 5′-CATGGAG-TACTTGGCTTGC CAAAAATGTATTCATC-3′, Reverse 5′-GATGAA-TACATTTTTGGCAAGCCAAGTACTCCATG-3′). The point mutation created by mutagenesis was confirmed with direct sequencing.

CELL CULTURE

C2C12 cells and HEK293T cells were maintained in Dulbecco′s modified Eagle′s medium (DMEM, Hyclon, Logan, UT), containing 5% fetal bovine serum (FBS, Hyclon), 1% antibiotics, and 10 ng/ml of *FGF2* (R&D Systems, Minneapolis, MN) at 37°C in a humidified

TABLE I. Sequence of FGFR2 PCR Primers and the Expected Sizes of PCR Products

Primer name	Size	Forward primer	Reverse primer
Exon 1-2	384	CACTTGGGCTGGAGTGATTT	TTAACAATCTGCCCCCAGAC
Exon 2-3	397	CGTTCTCTCCTCTCCCTCCT	CCTTTTCACTTGGCCAAAAA
Exon 3-1	242	CCTGGGTTGTTGACTTTGCT	CAGAACTTCCCTCCATGCTC
Exon 4-4	321	TTTACTCATGGAGGGGAAGC	CGAGACTCCATCGCAAAAA
Exon 5-1	250	GAAAGCACAGTACTTGGTAT	AACGAGTCAAGCAAGAATGG
Exon 6-1	339	AGCCCTCTGGACAACACAAC	AAGAACCTGTGGCCAAACC
Exon 7-2	248	CCACAATCATTCCTGTGTCG	CAGTCAACCAAGAAAAGGGAAA
Exon 8-3	378	GCGTCAGTCTGGTGTGCTAA	GCACATGGAAGCTCACAGAA
Exon 9-1	295	GATACTCTGGCTGGGCTCTG	CCAATATCCCCATTTATAGCTGA
Exon 10-1	186	ACCCCATCACCAGATGCTAT	TTCACATGCCACAAAAGGAA
Exon 11-3	248	ACAGTAGCTGCCCATGAGTT	GGAAGCCCAGCCATTTCTA
Exon 12-5	340	GTTTTGCTGAATTGCCCAAG	AGCATGTCCAAATTGCCTGT
Exon 13-1	238	CTTTTGTTCTGGCGGTGTT	GGAACATTCTGAGCCTCACC
Exon 14-2	244	ACAGGGCATAGCCCTATTGA	GCAGCAGCCACTAAAGAAGG
Exon 15-2	294	AGCTGGGCGTGTTTAGGTTT	GGGCCTTCAAAAACGAGATA
Exon 16-1	243	CACGTCCCCATATTGCCTAT	GCATGTCTCACAAGACAACCA
Exon 17-1	404	TCCTGTCCCACGTCCAATAC	ATGGTCTCCCTGCTCAGTGT

atmosphere of 5% CO_2 and 95% air. The medium was changed every 3 days.

LUCIFERASE ASSAY FOR *RUNX2* TRANSCRIPTIONAL ACTIVITY

After transient transfection with the Polyjet™ in vitro DNA transfection reagent (Signagen, Rockville, MD), a luciferase assay was performed using a Luciferase Reporter Assay kit (Promega, Fitchburg, WI) according to the manufacturer's recommendations (200 ng expression plasmid/96 well, culture for 48 hr). The Enhanced Green Fluorescent Protein (pEGFP)-N1 plasmid was included as a negative control.

REAL-TIME PCR FOR OSTEOCALCIN (OC) AND ALKALINE PHOSPHATASE (ALP)

The mRNA levels of bone marker genes such as OC and ALP between controls and FGFR2-L617F were compared by real-time PCR. cDNA was synthesized from 1 μg of total RNA using ImpromII™ reverse transcription system (Promega). Real-time PCR was performed using SYBR premix Ex Taq™ (TaKaRa, Shiga, Japan) and an AB 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA). Real-time quantification was performed and a fluorescence threshold value was calculated using iCycle iQ system software (BioRad, Hercules, CA), and target genes were normalized to the reference housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Relative fold differences were calculated for each experimental group using normalized C_t values for the control. The primer sequences of genes for real-time PCR are described in Table II.

IMMUNOPRECIPITATION

Expression constructs pcDNA, *FGFR2*-WT, *FGFR2*-L617F, and *FGFR2*-S354C were transfected into HEK293 cells using Polyjet™ in vitro DNA transfection reagent (Signagen). After 24 hr, low-serum media (1% FBS) or high-serum media (10% FBS) were added. After an additional 24 hr, the cells were harvested with lysis buffer containing 50 mM NaF, 2 mM Na_2VO_4, 1× phosphatase inhibitor cocktail, 1× protease inhibitor, and 0.2% SDS. Anti-Flag M2 affinity gel beads (Sigma, St. Louis, MO) were used for immunoprecipitation. After the reaction between beads and protein was completed, the beads were washed three times with 1× HEPES lysis buffer. Proteins were separated from the beads by vigorous shaking for 10 min in non-reducing sample buffer. Immunoprecipitation samples were obtained by adding β-mercaptoethanol to the supernatant obtained from the centrifuge of the beads. Then, the samples were subjected to immunoblot analysis and autophosphorylated *FGFR* (phospho-*FGFR*) protein was detected (Cell Signaling Technology, Danvers, MA).

TABLE II. The Primer Sequences of Genes for Real-Time PCR

Bone marker	Primer	Primer sequence
ALP	Forward	5′-CCAACTCTTTTGTGCCAGAGA-3′
	Reverse	5′-GGCTACATTGGTGTTGAGCTTTT-3′
GAPDH	Forward	5′-CATGTTCCAGTATGACTCCACTC-3′
	Reverse	5′-GGCCTCACCCCATTTGATGT-3′
OC	Forward	5′-ATCTCACCATTCGGATGAGTCT-3′
	Reverse	5′-TCAGTCCATAAGCCAAGCTCTCA-3′

IMMUNOBLOT

Protein samples were made by mixing the same amount of the sample and 4× sample buffer. The samples were heated at 80°C for 5 min, then transferred to 10% sodium dodecyl sulfate polyacrylamide gel to detect the protein. As primary antibodies, phosphor-*MAPK* (*ERK1/2*) and *MAPK* (*ERK1/2*), phosphor-*FGFR* (Cell Signaling Technology) were used at a 1:1,000 dilution. As secondary antibodies, goat anti-rabbit antibodies (Sigma) were used to visualize the result at a 1:5,000 dilution. β-actin was used as a loading control.

RESULTS

A NOVEL L617F MUTATION WAS IDENTIFIED IN *FGFR2* TK-II DOMAIN

Among 16 patients, 10 showed *FGFR2* mutations that had already been reported elsewhere (Table III): (1) 6 subjects with a mutation in Ig-IIIa domain: C278F, Q289P, W290R, and K292E; (2) 4 subjects with a mutation in Ig-IIIc domain: Y328C and C342Y [Jabs et al., 1994; Gorry et al., 1995; Steinberger et al., 1997; Hatch et al., 2006; Mai et al., 2010; Park et al., 2012]. A novel *FGFR2* mutation associated with the TK-II domain, L617F was found by direct PCR sequencing (Table III). The 617th leucine (TTG) was substituted to phenylalanine (TTC) in the TK-II domain (Fig. 1). Mutation of this site was confirmed using RFLP by CviKI-1 enzyme (Fig. 2). No mutation in *FGFR2* gene was identified in the other five patients (Table III).

CLINICAL AND CEPHALOMETRIC EVALUATION OF CASE WITH THE NOVEL MUTATION *FGFR2*-L617F

A 5-year-old boy visited for a periodic check of his craniofacial anomalies and growth observation. In his facial and intraoral photographs, he showed a mild Crouzon syndrome phenotype including exophthalmos, hypertelorism, retruded maxilla, relatively prognathic mandible, and anterior crossbite (Fig. 3). In his lateral and posteroanterior cephalograms, digital markings, mildly pointed calvaria, and a narrowed airway were found (Fig. 4A). There were no dental anomalies found in his orthopantomogram (Fig. 4B). His hands had no anomalies, either clinically or radiographically (Fig. 4C). His parents did not show any kinds of craniofacial or limb anomalies.

The patient also showed retruded maxilla (smaller SNA) and skeletal Class III pattern (smaller ANB and greater Wits appraisal) (Table IV). Although the ramus height and mandibular body length were normal, the mandible was also posteriorly positioned (SNB). These results were concordant with those from a report of Kreiborg et al. [1999]. In addition, the ramus appeared to be inclined slightly inward, forming a greater angle with the cranial base as reported by Boutros et al. [2007] (Table IV).

RUNX2 TRANSACTIVATION ACTIVITY WAS MILDLY ENHANCED IN FGFR2-L617F TRANSFECTED CELLS

Runx2 activity of *FGFR2*-L617F was compared to that of the negative controls (*pEGFP* and *FGFR2*-WT) and positive control (*FGFR2*-S354C). Regardless of the concentration of FBS (1% or 10% serum

TABLE III. Mutation Analysis Data of the Craniosynostosis Syndrome Patients

	Gender	Mutation	Domain	Refs.
1	M	C278F	Ig-IIIa	Hatch et al. [2006]
2	M	Q289P	Ig-IIIa	Gorry et al. [1995]
3	M	W290R	Ig-IIIa	Mai et al. [2010]
4	F	W290R	Ig-IIIa	Mai et al. [2010]
5	F	K292E	Ig-IIIa	Steinberger et al. [1997]
6	F	K292E	Ig-IIIa	Steinberger et al. [1997]
7	F	Y328C	Ig-IIIc	Jabs et al. [1994]
8	F	C342Y	Ig-IIIc	Gorry et al. [1995]
9	M	C342Y	Ig-IIIc	Gorry et al. [1995]
10	F	C342Y	Ig-IIIc	Gorry et al. [1995]
11	M	L617F	TK-II	Novel mutation
12	F	NF		–
13	F	NF		–
14	M	NF		–
15	M	NF		–
16	F	NF		–

NF represents that mutation was not found in *FGFR2*.

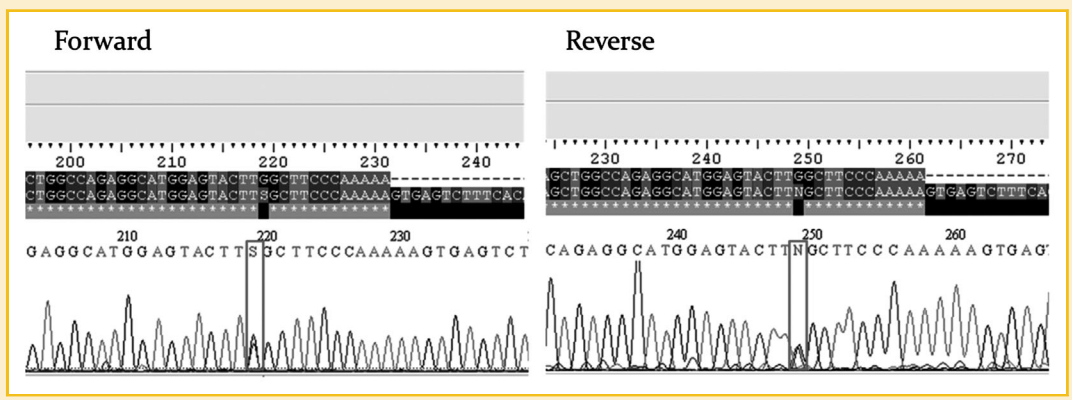

Fig. 1. Identification of a novel FGFR2-L617F mutation. A novel mutation in TK-II domain. The 617th leucine (TTG) was substituted with phenylalanine (TTC). The mutation was identified through both forward and reverse gDNA sequencing. Arrow indicates the substituted nucleotide.

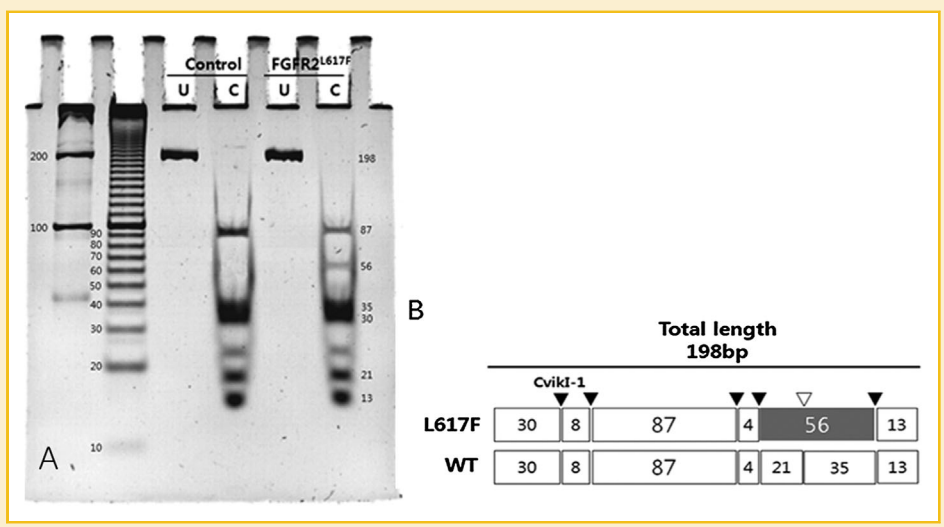

Fig. 2. Confirmation of the mutation by restriction fragment length polymorphism (RFLP). A: Result of RFLP analysis. The PCR product was digested with CviKI-1 restriction endonuclease. B: A diagram of RFLP. The CviKI-1 restriction enzyme cut six sites and made seven fragments with different sizes in the PCR products of patient's gDNA while L617F mutant had five cutting sites and resulted in six fragments. Fragment size of 56 base pairs was generated in the mutant. U: gDNA PCR product was uncut with enzyme, C: gDNA PCR product was cut with CviKI-1.

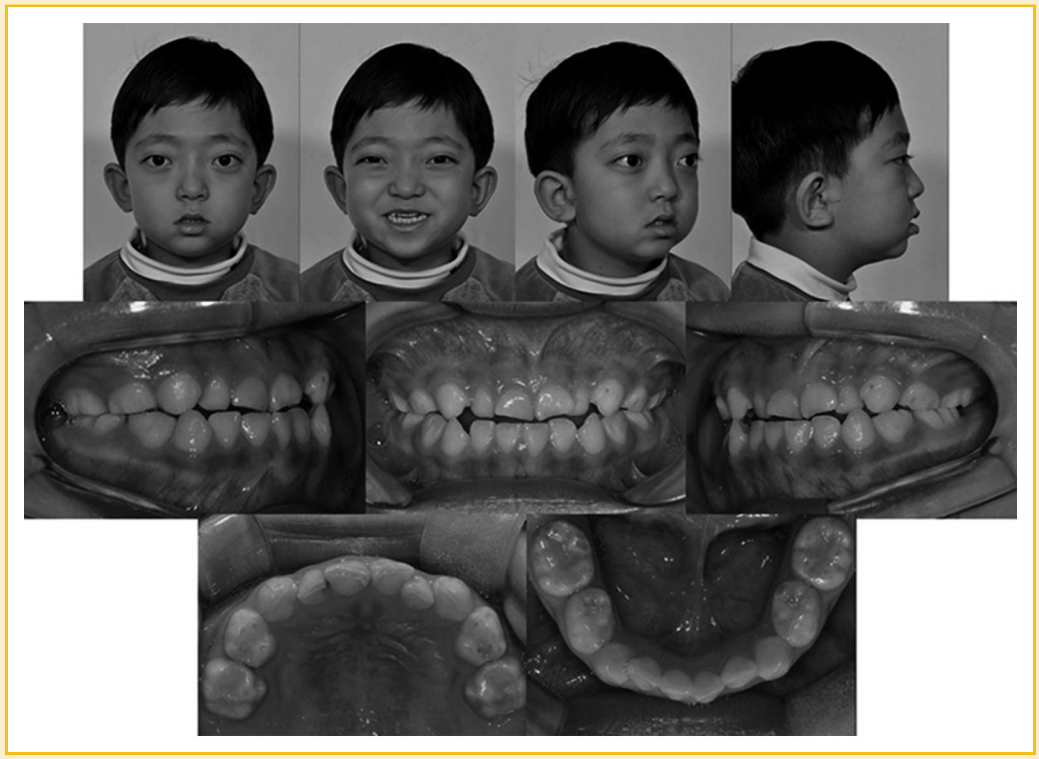

Fig. 3. Facial and intraoral photographs. Characteristics of mild Crouzon syndrome such as exophthalmos, hypertelorism, relatively retruded maxilla and anterior crossbite are shown.

media) in C2C12 cell culture, *FGFR2*-L617F stimulated *Runx*2 activity compared to that of negative controls (Fig. 5), which means that receptors with L617F mutation were activated without *FGF* ligands binding. This result was supported by the positive control, S354C mutation, in which *Runx2* transactivation activity was much more stimulated than that of L617F.

OC AND *ALP* MRNA LEVEL WAS INCREASED BY L617F MUTANT TRANSFECTION

In previous studies, both *OC* and *ALP* are target genes of *Runx2* transcription factor. Compared to those of EGFP and *FGFR2*-WT transfected cells, mRNA levels of *OC* and *ALP* in *FGFR2*-L617F and *FGFR2*-C342Y (positive control) were significantly stimulated (Fig. 6), which means that the L617F is a gain-of-function mutation of *FGFR2*.

PHOSPHORYLATION OF FGFR2 PROTEIN WAS ENHANCED IN GAIN-OF-FUNCTION MUTANTS EVEN IN THE LOW SERUM STIMULATION

When *FGFR2* was phosphorylated, it activated the *ERK1/2-MAPK* pathway and caused osteoblast differentiation [Boutros et al., 2007; Kim et al., 2003]. Autophosphorylated *FGFR2* was detected by immunoprecipitation, and activated *ERK1/2-MAPK* was detected by immunoblot. In 1% serum, *FGFR2*-L617F showed an activation band of p-*FGFR* and the band was stronger in *FGFR2*-S342C (Fig. 7). In 10% serum, the activation pattern of *FGFR2*-L617F appeared to be similar to that of wild-type (Fig. 7).

DISCUSSION

This study was performed to find a novel mutation in *FGFR2* in Crouzon patients and to analyze its functional outcomes.

FUNCTIONAL ANALYSIS OF THE NOVEL MUTATION

In a luciferase assay, *Runx2* transcription activity was increased in the cells with a L617F mutation (Fig. 5). Since *Runx2* is an essential transcription factor for osteoblast differentiation from the early commitment step to final differentiation, Kim et al. [2004] suggested that the use of a stable cell line that carries the 6xOSE2-Luciferase reporter vector could be a good evaluation system to determine *Runx2* transcriptional activity.

Park et al. [2012] insisted that the E731K mutation in the TK-II domain of *FGFR2* increased phosphorylation of *FGFR2* and *ERK-MAP* kinase, stimulation of *Runx2* transcriptional activity, and enhancement of osteogenic marker gene expression. In this study, similar results were obtained through real-time PCR of OC and ALP in the cells with L617F mutation, which is also in the TK-II domain (Fig. 6). However, compared to the *Runx2* stimulatory effect of E731K or S352C mutants, L617F mutant showed stimulating effect on the *Runx2* transactivation activity.

In immunoprecipitation and immunoblot analysis, although the L617F mutation caused phosphorylation of *FGFR2* in the low (1%) serum level, this effect has been neutralized in the high (10%) serum level (Fig. 7). The level of *ERK/MAPK* phosphorylation was found to

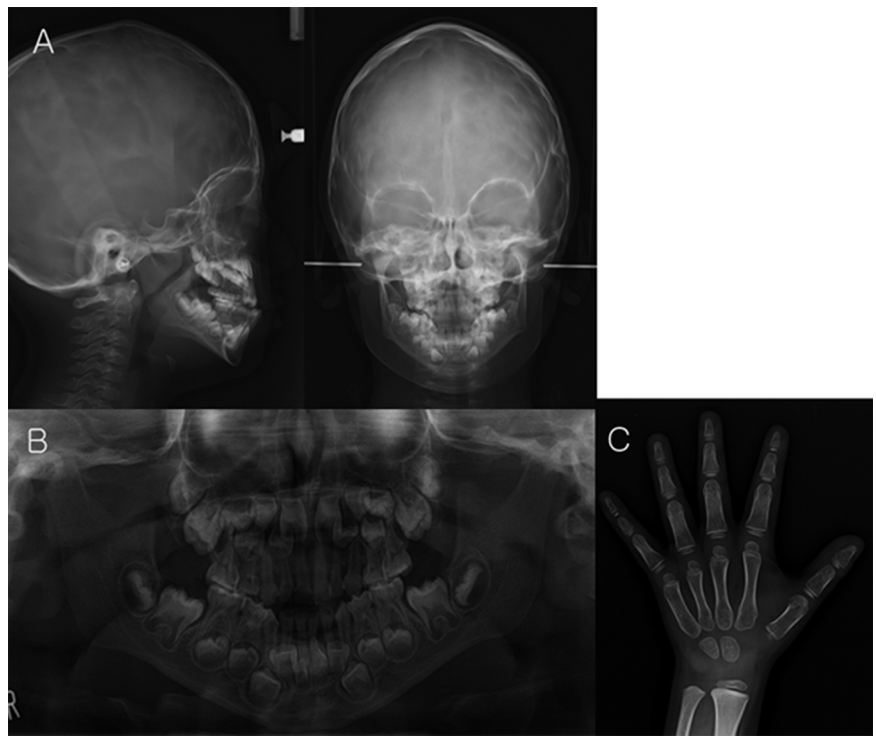

Fig. 4. A: Lateral and posteroanterior cephalograms. B: Orthopantomogram. C: Hand–wrist radiograph. Digital markings of calvaria and retruded maxilla were found. There were no dental or hand anomalies.

be similar to that of the wild-type (Fig. 7). These findings together with *Runx2* transactivation activity can explain the mild phenotype of the Crouzon patient with this mutation (Figs. 3 and 4).

MUTATIONS IN THE TK DOMAINS

This study is the fifth report about *FGFR2* mutation in the TK domain. In the first report, Kan et al. [2002] found two TK-I and four TK-II mutations (N549H, E565G and K641R, K659N, G663E, R678G; respectively) in Crouzon syndrome, Pfeiffer syndrome, and unclassified syndrome. Zankl et al. [2004] insisted that the K526E mutation

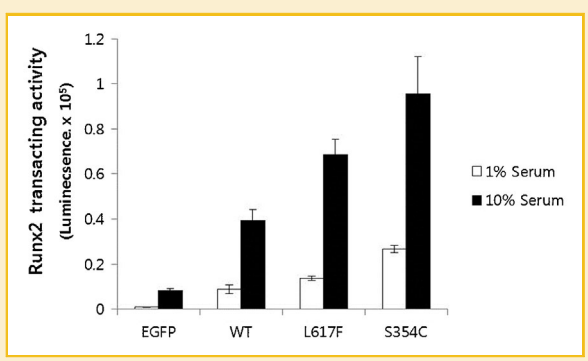

Fig. 5. *Runx2* transacting activity was enhanced by overexpression of *FGFR2*-L617F. C2C12 cells were co-transfected with 6xOSE2-Luc reporter vector with or without *FGFR2* expression vectors (*FGFR2*-WT, *FGFR2*-L617F, or *FGFR2*-S354C). Twenty-four hours after transfection, cells were cultured with 1% or 10% FBS containing medium for additional 24 hr. Cells were harvested and luciferase activity was analyzed.

TABLE IV. Skeletal and Dental Measurements of the Patient #11 Lateral and Posteroanterior Cephalograms

Measurement	Mean	SD	Patient #11
Lateral cephalogram			
Skeletal			
A-P relation			
SNA	80.67	2.68	69.63
SNB	76.54	2.91	67.25
ANB	4.43	1.48	2.38
Wits appraisal	−2.24	0.3	0.29
Vertical relation			
FMA	30.64	3.1	38.34
SN-GoMe	35.93	3.92	45.91
Dental			
U1-FH	96.61	5.42	92.45
IMPA	84.06	5.43	95.88
Interincisal angle	150.17	5.65	133.33
Soft tissue			
Upper lip–E line	1.06	1.39	1.99
Lower lip–E line	1.37	1.33	3.5
Lateral and posteroanterior cephalograms			
Mandibular morphology			
Ramus height	36.52	3.02	36.16
Mandibular body length	54.78	2.71	57.99
Bicondylar width	87.44	3.43	86.50
Bigonial width	74.91	10.07	77.90
Bicondylar/bigonial ratio	1.15	0.05	1.11
Ramus to intercondylar plane angle			
Left	83.2	2.88	84.7
Right	82.6	2.97	85.6

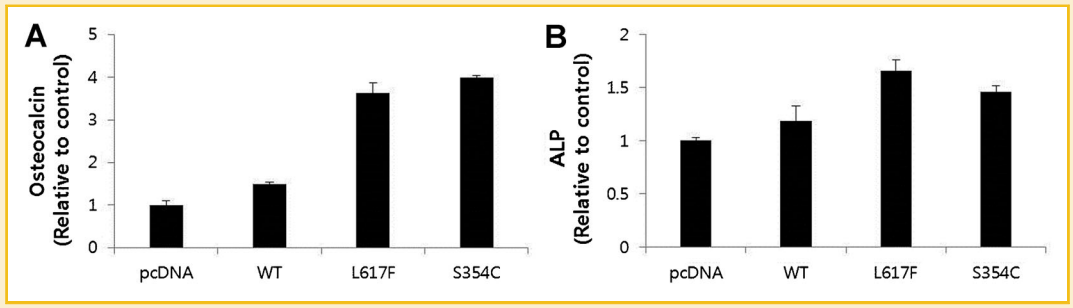

Fig. 6. Bone marker gene expression was enhanced by *FGFR2*-L617F. A: Osteocalcin (OC). B: Alkaline phosphatase (ALP). C2C12 cells were transfected with each represented construct. After 48 hr, the mRNA was extracted from the cells to measure expression level of OC and ALP by quantitative real-time PCR analysis.

was related to mild Crouzon syndrome. Lajeunie et al. [2006] reported one TK-I (N549H) and two TK-II mutations (K641R and K659N) in Crouzon and Pfeiffer phenotypes. Interestingly, N549H mutation had both Crouzon and Pfeiffer phenotypes [Lajeunie et al., 2006]. Park et al. [2012] reported E731K mutation in a Korean Apert syndrome patient.

Since the Leucine at the 617th amino acid is highly conserved among several species such as humans, mice, and chickens (http://www.uniprot.org), and the sequences around this amino acid also show high homology with those of *FGFR1* and *FGFR3* (Fig. 8), this domain could be thought of as a functionally important site. For the pathogenic mechanism of the TK domain mutation, Robertson et al. [1998] assumed that if unpaired cysteine residues were not involved, the loop in the protein-binding domain would be relatively integrated. This might explain the cause of the mild phenotype in the patient with the L617F mutation. Since the parents of the patient with the L617F mutation did not show any kind of syndromes or phenotypes, this mutation might have occurred de novo. However, since the patient with E731K showed the Apert phenotype [Park et al., 2012], further studies are needed to elucidate the mechanism by which a mutation in the TK domain causes craniosynostosis.

THE OTHER KOREAN CRANIOSYNOSTOSIS PATIENTS

In this study, based on *FGFR2* screening, mutations were detected in exons IIIa, IIIc, and TK (Table III). Except for the TK domain mutations, all the other mutations occurred in the mutation hotspots of *FGFR2*. Since five craniosynostosis patients did not show *FGFR2* mutations (Table III), these patients might have mutations in other genes like *FGFR1*, *FGFR3*, and *TWIST*.

There were two families with Crouzon syndrome, and they shared a mutation (K292E for Patients #5 and #6; C342Y for Patients #8, #9, and #10; Table III). This is concordant with the fact that Crouzon syndrome is autosomal dominant [Carinci et al., 1994].

In this study, 16 Crouzon patients were included as subjects. As far as we know, this is the largest sample size in studies dealing with Korean craniosynostosis patients. However, further investigations

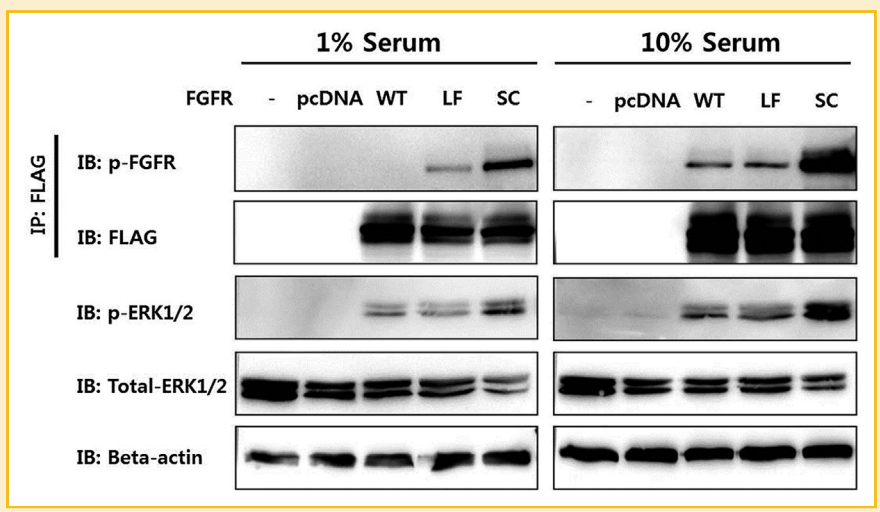

Figure 7. *FGFR2*-L617F increased *FGFR* autophosphorylation. HEK293 cells were transfected with pcDNA, *FGFR2*-WT(WT), *FGFR2*-L617F(LF), or *FGFR2*-S354C(SC). Twenty-four hours after transfection, cells were cultured in the 1% serum media or 10% serum media for additional 24 hr. Autophosphorylated *FGFR* and activated *ERK1/2* were detected. IP, immunoprecipitation; IB, immunoblot; LF, L617F; SC, S342C.

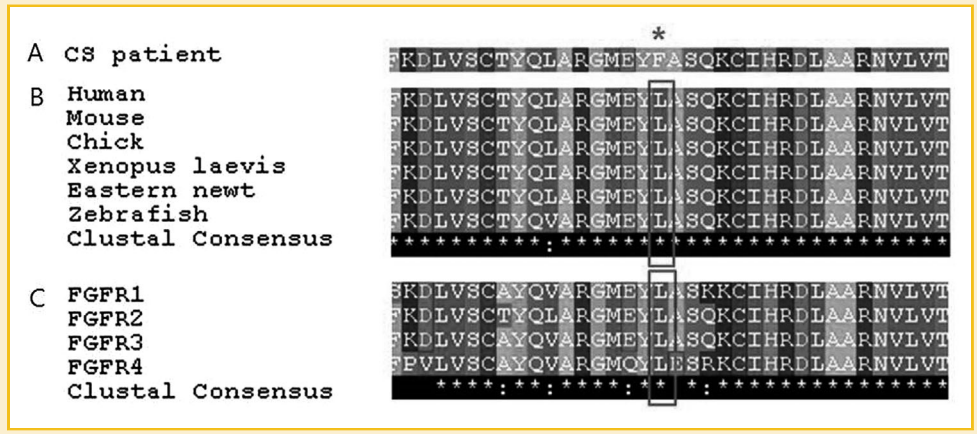

Fig. 8. Amino acid sequence of *FGFR2* around L617F. A: The patient with *FGFR2*–L617F mutation. B: Amino acid sequence in species of the vertebrates. C: Amino acid sequence in *FGFR* family (http://www.uniprot.org).

into other genes such as *FGFR1*, *FGFR3*, and *TWIST* are needed to find mutations in the other five patients without mutations in *FGFR2*. And it is also necessary to unravel the mechanism of molecular changes caused by TK domain mutations.

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