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Functional Characterization of a Novel FGFR2 Mutation, E731K, in Craniosynostosis

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ABSTRACT

Craniosynostosis is a condition in which some or all of the sutures in the skull of an infant close prematurely. Fibroblast growth factor receptor 2 (FGFR2) mutations are a well-known cause of craniosynostosis. Many syndromes that comprise craniosynostosis, such as Apert syndrome, Crouzon syndrome, and Pfeiffer syndrome, have one of the phenotypes that have been reported in FGFR2 mutant patients. FGFRs have been reported in four types (FGFR1–4), and upon binding with FGF ligands, signal transduction occurs inside of cells. Activated FGFR stimulates an osteogenic master transcription factor, Runx2, through the MAP kinase and PKC pathways. We obtained a genetic analysis of six Korean patients who have craniosynostosis as a phenotype. All of the patients had at least one mutation in the FGFR2 gene; five of those mutations have already been reported elsewhere, while one mutation is novel and was hypothesized to lead to Apert syndrome. In this study, we reported and functionally analyzed a novel mutation of the FGFR2 gene found in a craniosynostosis patient, E731K. The mutation is in the 2nd tyrosine kinase domain in the C-terminal cytoplasmic region of the molecule. The mutation caused an enhanced phosphorylation of the $FGFR2^{E731K}$ and ERK-MAP kinase, the stimulation of transcriptional activity of Runx2, and consequently, the enhancement of osteogenic marker gene expression. We conclude that the substitution of E731K in FGFR2 is a novel mutation that resulted in a constitutive activation of the receptor and ultimately resulted in premature suture obliteration. J. Cell. Biochem. 113: 457–464, 2012.

KEY WORDS: CRANIOSYNOSTOSIS; APERT SYNDROME; FGFR2; PHOSPHORYLATION; ERK PATHWAY; Runx2

Craniosynostosis is a congenital anomaly characterized by the premature fusion of one or more sutures and is known to be caused by genetic and environmental factors [Wilkie, 1997]. Mutations in the MSX2, fibroblast growth factor receptor (FGFR) 1, FGFR2, FGFR3, and TWIST genes are causally related to craniosynostosis [Howard et al., 1997; Anderson et al., 1998; Ornitz and Marie, 2002]. Among these genes, FGFR2 mutations have been the most frequently recognized genetic cause of the malformation [Reardon et al., 1994]. A great many distinct genetic syndromes involving FGFR2 mutations, including Apert syndrome, Crouzon syndrome, and Pfeiffer syndrome, have been defined. Craniosynostosis is a common phenotype in these genetic syndromes, while each syndrome has its own specific phenotype. For example, Apert syndrome is characterized by bony syndactyly of the hands and feet [Wang et al., 2005], whereas the main clinical features of Pfeiffer syndrome are broad thumbs and large toes. In contrast, Crouzon syndrome does not show any abnormality in the limbs, but demonstrates craniosynostosis [Rutland et al., 1995; Bellus et al., 1996]. Even though craniosynostosis can be classified into several different syndromes by its clinical phenotypes, the correlation between genotype and phenotype does not appear to be strong [Wilkie et al., 1995; Meyers et al., 1996; White et al., 2005; Baroni et al., 2005a].

FGFR2 protein consists of three extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain, and two tyrosine kinase domains in the cytoplasmic region [Anderson et al., 1998].

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FGFR2 is activated by ligands binding to the Ig-like domains. Until recently, at least 23 fibroblast growth factors (FGF1–FGF23) have been identified [Anderson et al., 1998; Ornitz and Itoh, 2001]. Specifically, FGF2 forms a low affinity with heparan sulfate and forms a very specific and high affinity complex with the 2nd and 3rd immunoglobulin-like ligand-binding domains of FGFR2 [Plotnikov et al., 2000]. FGFs binding to FGFR2 affect the regulation of cell growth, differentiation, embryogenesis, and angiogenesis by receptor dimerization and subsequent tyrosine autophosphorylation and phosphorylation of the target substrate [Chaudhary and Avioli, 1997; Schlessinger, 2000; Debiais et al., 2001; Eswarakumar et al., 2005]. Autophosphorylation of receptor tyrosine molecules provides binding sites for signaling proteins that include Src homology2 (SH2) domains. The binding of SH2 domain-containing proteins has been proven for the activation of downstream signaling cascades, such as mitogen-activated protein (MAP) kinase pathways [Larsson et al., 1999]. Based on genetic studies of FGFR mutations in craniosynostosis patients, most of the mutations are constitutive activation mutations, some of which enhance receptor dimerization, enhance ligand binding affinity, or cause receptor tyrosine kinase activation [Spivak-Kroizman et al., 1994; Rice et al., 2000; Eswarakumar et al., 2005; Ornitz, 2005]. In some cases, the functional changes of FGFR2 mutations still remain to be classified.

Runx2 is a transcription factor that has an indispensable role in the regulation of bone cell differentiation and bone mineralization [Komori et al., 1997]. Our previous report indicated that FGF binding to FGFR stimulated Runx2 gene expression and Runx2 protein activity through the activation of protein kinase C [Kim et al., 2003a] or several MAP kinases [Kim et al., 2003b]. In addition, the $FGFR2^{C342Y}$ mutation, which causes the premature fusion of cranial sutures in mice, stimulates Runx2 expression [Eswarakumar et al., 2004]. The $FGFR2^{P253R}$ and $FGFR2^{S252W}$ mutations increased Runx2 expression in human calvarial osteoblasts from Apert syndrome patients [Tanimoto et al., 2004; Baroni et al., 2005b]. In this study, we have screened FGFR2 mutations from six Korean craniosynostosis patients. Six mutations from five patients were known mutations of the FGFR2 molecule and clinically diagnosed as Crouzon syndrome, but a novel mutation was determined in an Apert syndrome patient. The mutation was a single nucleotide substitution and caused a missense mutation, E731K, in the 2nd tyrosine kinase domain of the FGFR2. In this study, we attempted to identify the functional consequence of this mutation in osteogenic cell differentiation and provide evidence that E731K constitutively activates FGFR2, leading to premature suture obliteration.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), modified Eagle's medium (MEM), and fetal bovine serum (FBS) were obtained from Hyclon (Logan, UT). Trypsin–EDTA and other cultural reagents were obtained from Gibco Invitrogen (Carlsbad, CA). Recombinant human FGF2 was purchased from R&D Systems (Minneapolis, MN). The RNase Plus kit for total RNA extraction, i-star Taq™ DNA polymerase for polymerase chain reaction (PCR), Maxime RT Premix kit for cDNA synthesis in quantitative real-time PCR, and WEST-ZOL (plus) for Western blot detection were purchased from iNtRON (Seoul, Korea). Primers for the real-time PCR were synthesized by CosmoGene (Seoul, Korea). SYBR Premix EX Taq™ for quantitative real-time PCR was purchased from TaKaRa (Shiga, Japan). The cell count kit was purchased from Dojindo (Kamimashiki-gun, Japan). Phosphatase inhibitor cocktail I and II were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Anti-phospho-ERK, anti-ERK, anti-phospho-FGF receptor, and anti-phospho-tyrosine antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-FLAG M2 monoclonal antibody was purchased from Sigma. Anti-actin, immunopure goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated, goat anti-rabbit IgG HRP-conjugated, and bovine anti-goat IgG HRP-conjugated antibodies were all purchased from Cell Signaling Technology (Denver, MA). WelFect-Ex™ Plus for transient transfections for immunoprecipitation and immunoblotting was from WelGENE (Seoul, Korea). The luciferase assay system was purchased from Promega (Madison, WI).

IDENTIFICATION OF KINDRED AND ENROLLMENT OF HUMAN SUBJECTS

This study was reviewed and approved by the Institutional Review Board at the Seoul National University Hospital. The experiments were undertaken with the understanding and written consent of each subject according to the Declaration of Helsinki.

FGFR2 MUTATION SCREENING

Genomic DNA was isolated from peripheral whole blood using the QuickGene DNA whole blood kit S with QuickGene-Mini80 equipment (Fujifilm, Tokyo, Japan). The purity and concentration of the DNA was measured by spectrophotometry, according to the OD_{260}/OD_{280} ratio. We performed a mutational analysis of the exons and flanking intron sequences of the *FGFR2* gene for the patients according to previous reports [Zhang et al., 1999] using the HiPi DNA polymerase premix (ElpisBio, Deajun, Korea). The sequences of the primers used are shown in Table I. To confirm the DNA sequence of the PCR products, the PCR products were purified with a PCR Purification Kit (ElpisBio) and sequenced at Macrogen (Seoul, Korea).

PLASMID CONSTRUCTS

Full-length FGFR2-3xFlag [Tanimoto et al., 2004] was used as a template. The craniosynostosis-associated E731K mutation was introduced by site-directed mutagenesis using the following primer sets; forward 5′-CCA ACT GCA CCA ACA AAC TGT ACA TGA TGA TG-3, reverse 5′-CAT CAT CAT GTA CAG TTT GTT GGT GCA GTT GG-3′, forward 5′-GGT GAG GAT AAC ACG C-3′, and reverse 5′-GGA TCA CTA CTT GTC ATC GTC A-3′. The site-directed mutagenesis of the point mutation was confirmed by direct sequencing. The E731K mutation was constructed by cloning *Xho*I/*Bam*HI fragments of $FGFR2^{E731K}$ into *Xho*I/*Bam*HI sites of FGFR2-3xFlag. The introduced point mutation was reconfirmed by DNA sequencing.

CELL CULTURE

C_2C1_2 cells and HEK293T cells were maintained in DMEM in 10% FBS and 1% antibiotics at 37°C in 95% humidified air plus 5% CO_2.

TABLE I. Sequences of FGFR2 PCR Primers and the Expected Size of PCR Products

Primer name	Size	Forward primer	Reverse primer
2	384	CACTTGGGCTGGAGTGATTT	TTAACAATCTGCCCCCAGAC
3	397	CGTTCTCTCCTCTCCCTCCT	CCTTTTCACTTGGCCATGCTC
4	242	CCTGGGTTGTTGACTTTGCT	CAGAACTTCCCTCCATGCTC
5	321	TTTACTCATGGAGGGAAGC	CGAGACTCCATCGCAAAAA
6	250	GAAAGCACAGTACTTTCA	CCTTTTCACCTGCCCAATTA
7	237	TCCTGTTCTCCCATAAGTTTCA	CCTTTTCACCTGCCCAATTA
8	339	AGCCCTCTGCAACACAAC	AAGAACCTGTGCCAAACC
9	296	GGTGGGACCATAGACAATGC	TCTCCCAAAGCACCAAGTCT
10	248	CCACAATCATTCCTGTGTCG	CAGTCAACCAAGAAAAGGGAAA
11	378	GCGTCAGTCTGGTGTGCTAA	GCACATGGAAGCTCACAGAA
12	295	GATACTCTGGCTGGGCTCTG	CCAATATCCCCATTTATAGCTGA
13	186	ACCCCATCACCAGATGCTAT	TTACATGCCACAAAAGGAA
14	248	ACAGTAGCTGCCATGAGTT	GGAAGCCCAGCCATTTCTA
15	340	GTTTTGCTGAATTGCCCAAG	AGCATGTCCAAATTGCCTGT
16	238	CTTTTGTTCTGGCGGTGTT	GGAACATTCTGAGCCTCACC
17	244	ACAGGGCATAGCCCTATTGA	GCAGCACCCACTAAAGAAGG
18	294	AGCTGGGCGTGTTTAGGTTT	GGGCCTTCAAAAACGAGATA
19	243	CACGTCCCCATATTGCCTAT	GCATGTCTCACAAGACAACCA
21	404	TCCTGTCCCACGTCCAATAC	ATGGTCTCCCTGCTCAGTGT

To construct stable cell lines, the C_2C1_2 cells were counted and transfection was performed with FGFR2-3xFlag and FGFR2^{E731K}-3xFlag using electroporation (NanoEn Tek, Inc., Seoul, Korea). The transfected C_2C1_2 cells were selected by 2 mg/ml G418 for 14 days. Next, the stable cells were maintained with DMEM in 10% FBS, antibiotics, and 200 μg/ml of G418. For differentiation, C_2C1_2 cells were cultured with DMEM supplemented with 5% FBS, antibiotics, and 10 ng/ml of FGF2.

CELL PROLIFERATION ASSAY

MC3T3-E1 cells were counted and transfection was performed with electroporation. In each transfection, 200 ng of expression plasmid (pCMV-Flag, FGFR2-3xFlag, and FGFR2^{E731K}-3xFlag) were used for each well of a 96-well culture plate. When duplicated cells were plated in 96-well plates, the density was 3×10^3 and 6×10^3/well. Sixteen hours after the transfection, the medium in one set was changed, and the medium of another set remained as MEM in 10% FBS and antibiotics. After 12 h, cell proliferation was estimated with a Cell Count Kit-8 (Gaithersburg, MD) according to the manufacturer's instructions.

LUCIFERASE REPORTER ASSAY

C_2C1_2 cells were counted and transfection was performed with electroporation. In each transfection, 100 ng of expression plasmid (pCMV-Flag, FGFR2-3xFlag, and FGFR2^{E731K}-3xFlag) and 200 ng of reporter plasmid (6xOSE2-luc) were used for each well. When the cells were plated in 96-well plates, the density was 80% confluent. Twenty-four hours after the transfection, the medium was changed to serum-free medium treated with 10 ng/ml of FGF2, and the cells were cultured for an additional 18 h. The cells were harvested and luciferase activity was determined by using a luminescent luciferase assay kit and a luminometer GloMax-Multi detection system (Promega).

QUANTITATIVE REAL-TIME PCR

C_2C1_2 cells and stable C_2C1_2 cells (FGFR2-3xFlag, FGFR2^{E731K}-3xFlag) cells were plated at a density of 5×10^5 cells/cm^2 in 60 mm tissue culture dishes. When the cell population reached 80% confluency in the maintenance medium, the culture medium was changed to DMEM supplemented with 5% FBS and antibiotics. For the quantitative real-time PCR, cDNA was synthesized from 2 μg of total RNA and total a volume of 20 μl, containing 10 μl SYBR Premix Ex Taq™, 0.2 μM primers, 0.4 μl ROX™ Reference dye, and 1.5 μl cDNA. Real-time PCR analysis was carried out with the US/7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Each sample was analyzed in triplicate, and the target genes were normalized to the reference housekeeping gene, GAPDH. The sequences of primers used were as follows: forward: 5′-GGC TAC ATT GGT CTT GAG CTT TT-3′ and reverse: 5′-CCA ACT CTT TTG TGC CAG AGA for alkaline phosphatase (ALP), forward: 5′-TTC TCC AAC CCA CGA ATG CAC-3′ and reverse: 5′-CAG GTA CGT GTG GTA GTG AGT-3′ for Runx2, forward: 5′-GCT GCC TTC TAC GCA CCT G-3′ and reverse: 5′-GCC GCT GTA ATC CAT CAT GC-3′ for MyoD, and forward: 5′-CAT GTT CCA GTA TGA CTC CAC TC and reverse: 5′-GGC CTC ACC CCA TTT GAT GT-3′ for GAPDH. Fold differences were calculated for each treatment group using the normalized Ct values for the control.

IMMUNOBLOTS

The cells were washed twice with phosphate-buffered saline (PBS) and lysed in RIPA buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1% NA-40, 2% SDS, 1% sodium deoxycholate, phosphatase inhibitor cocktail I&II, and protease inhibitor). Protein concentration was measured with BCA reagents. Fifty micrograms of protein were mixed with 2X Laemmli sample buffer (60 mM Tris–HCl [pH 6.8], 2% SDS, 25% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol), separated on 10% SDS–polyacrylamide gels and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with a blocking buffer containing 5% skim milk in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) for 1 h. The appropriate primary antibodies were used at a 1:1,000 dilution in blocking buffer and the membrane was incubated with primary antibody at 4°C overnight. After washing three times with TBS-T, the membrane was incubated with HRP-conjugated secondary

antibodies at 1:4,000 dilutions for 1 h. Immune complexes were visualized with WEST-ZOL (iNtRON Biotec. Seongnam-si, Korea).

IMMUNOPRECIPITATION

Cells were washed with ice-cold PBS twice and lysed in ice-cold 1X HEPES lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 10 mM sodium butyrate, 1% NP-40, sodium deoxycholate, 10% glycerol, phosphatase cocktail I & II, and protease inhibitor). Protein was measured, and 1 mg of protein from each sample was used for an immunoprecipitation with the appropriate primary anti-Flag antibody. The binding reactions were performed for 4 h at 4°C with continuous rotation. The beads were collected and washed three times with 1× HEPES lysis buffer. Bound proteins were eluted by boiling in 1× Laemmli sample buffer with 1 M DTT, separated by SDS–PAGE, and subjected to immunoblot analysis.

RESULTS

CLINICAL DIAGNOSIS AND GENETIC ANALYSIS

Subjects for this study included six patients with craniosynostosis, all of whom were Koreans who had visited the Department of Pediatric Surgery, Seoul National University Hospital. Craniosynostosis was diagnosed by conventional X-ray and three-dimensional computerized tomography (3D-CT) of head. Cranial, palatal, hand, and foot developments were also examined clinically and radiographically. The patients were clinically diagnosed with Crouzon syndrome (Cases 1 and 2) or Apert syndrome (Cases 3, 4, 5, and 6).

FGFR2 mutation screening was performed by direct PCR sequencing of all exons and a part of the introns, including exon–intron junctional sequences. The PCR primers for this experiment are in Table I. All of the patients had at least one mutation in FGFR2 coding sequences. Mutational analysis of the FGFR2 gene identified two missense mutations (c.1025C > G, p.C342S; c.1040C > G, p.S347C) in exon 10 (Fig. 1, Cases 1 and 2, respectively) of two Crouzon syndrome patients. Case 1 and 2 mutations are located in the third immunoglobulin domain, which has been known to play a critical role in FGF ligand-binding. We found the same mutation (c.557T > C, p.M186T) in two unrelated cases. The mutation was a single nucleotide change in exon 5 (Fig. 1, Cases 3 and 4), which resulted in an amino acid substitution in the second immunoglobulin domain. A previous report indicated that the domain also plays a part in ligand binding [Kan et al., 2002]. In Case 3, there was an additional mutation in an intron (data not shown). In Case 5, there was a single substitution mutation in exon 8 (c.755C > G, p.S252W) (Fig. 1, Case 5) in the linker region of IgII/IgIII. Genetic analysis of Case 6 demonstrated a novel mutation in exon 18 (c2191G > A, p.E731K), which is translated into the tyrosine kinase II domain of FGFR2 (Fig. 1). The 731st glutamate (GAA) was substituted to lysine (AAA) in the tyrosine kinase II domain.

The amino acid sequence comparison of human and other FGFR2 showed that the E731 and surrounding sequences are well conserved from rodents to humans (Fig. 2A). Moreover, sequence alignment of other FGFRs revealed that the amino acid is highly conserved in the other FGFRs, except for FGFR4 (Fig. 2B). The high conservation of glutamate in the 2nd tyrosine kinase domain indicated that E731 may have a critical role in the FGFR function. Upon the base of this background, we started a functional study of the E731K mutant protein.

FGFR2 AUTOPHOSPHORYLATION WAS INCREASED BY E731K

Mohammadi et al. [1996] discovered six novel autophosphorylation sites, Y-463, Y-583, Y-585, Y653, Y654, and Y-730, on Fgfr1 and elucidated their important roles for signal transduction in FGFR. The authors also explicated the most impressive autophosphorylation sites, Y-653 and Y-654, which were conserved among all FGFRs. Because the E731K mutation was located on the tyrosine kinase II domain, we determined whether the E731K mutant could alter the

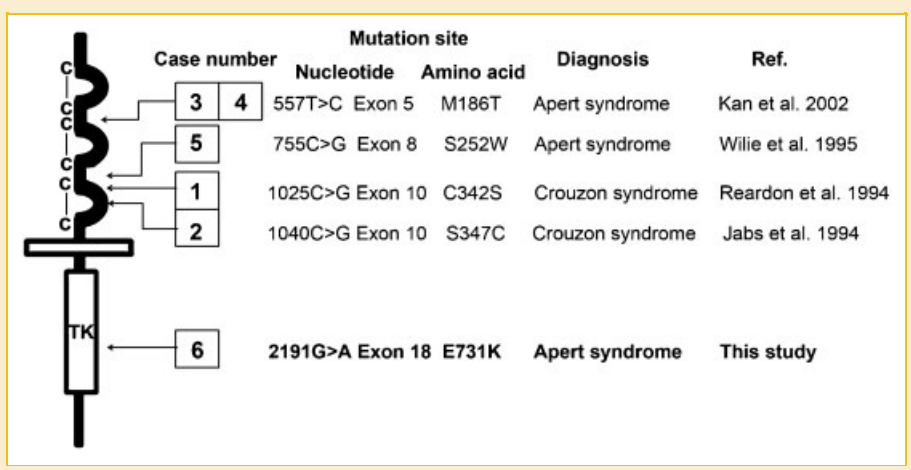

Fig. 1. FGFR2 mutations identified in six patients with syndromic craniosynostosis. The mutation site of each patient is indicated. Mutations found in five patients have already been reported (references were indicated). Only one patient showed a novel mutation, which is in exon 18, in the C-terminal cytoplasmic tyrosine kinase 2 domain of the molecule.

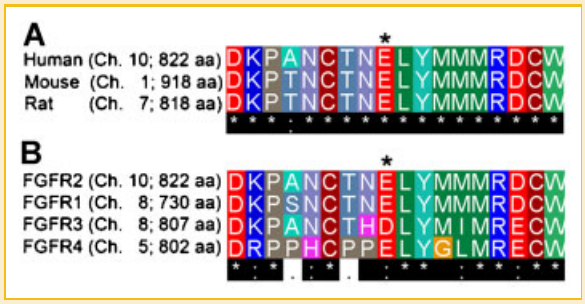

Fig. 2. Amino acid sequences of FGFR2 around E731K was highly conserved. A: The sequences were highly conserved in humans, mice, and rats. B: The sequences also showed high homology with FGFR1 and FGFR3. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

autophosphorylation of FGFR2. Y-730, one of the autophosphorylated sites on FGFR1, was the site for Y-733 on FGFR2, which was extremely close to the 731st glutamate. Therefore, the difference of autophosphorylation between $FGFR2^{WT}$ and $FGFR2^{E731K}$ was investigated by immunoprecipitation and immunoblot analysis. As shown in Figure 3, the autophosphorylation of Y-653/Y654 on $FGFR2^{E731K}$ was dramatically elevated even in the absence of FGF2 treatment, and the phosphorylation state of $FGFR2^{E731K}$ remained high for a longer time than that of $FGFR2^{WT}$. Flag antibody labeling indicated that the overexpressed wild type and E731K FGFR2 protein amount were comparable (Fig. 3). This result suggests that E731K is constitutively activated and can transduce signals to an intracellular site by autophosphorylation even in the absence of FGF ligands.

ERK1/2 ACTIVATION WAS INCREASED BY E731K

When FGFR2s are exposed to FGF ligands, they form dimers, and their tyrosine kinase domains are activated by autophosphorylation and reciprocally phosphorylated by their dimerized counterpart. The phosphorylation of Y653/654 could be the hallmark of FGFR2 activation, which subsequently activates intracellular events. One known pathway activated by FGFR2 is the ERK1/2 pathway, and activated ERK1/2 stimulates osteoblast differentiation [Kim et al., 2003c; Ahmed et al., 2008; Yin et al., 2008]. Because $FGFR2^{E731K}$ was highly autophosphorylated, we investigated the activation of ERK1/2 signaling as the next step. Similar to the autophosphorylation level of FGFR2, ERK-MAP kinase was activated in $FGFR2^{E731K}$ transfected cells even in the absence of ligand stimulation. No increase of ERK phosphorylation was observed in the WT transfected cells. Interestingly, the FGF2 treatment did not alter ERK phosphorylation in $FGFR2^{E731K}$ overexpressed cells (Fig. 4). These data suggested again that $FGFR2^{E731K}$ might be a constitutively active form.

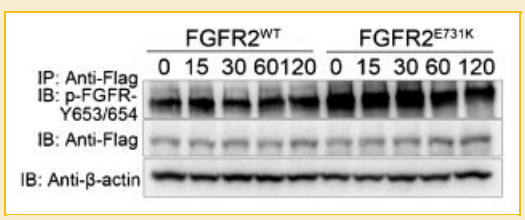

Fig. 3. Autophosphorylation of $FGFR2^{WT}$ and $FGFR2^{E731K}$ was compared. HEK293T cells were transfected with pCMV-Flag, $FGFR2^{WT}$, or $FGFR2^{E731K}$. Twenty-four hours after the transfection, the transfected cells were serum-starved for 16 h and treated with FGF2 (10 ng/ml) for the indicated period. Autophosphorylated FGFR2 was detected by immunoprecipitation (IP) with an anti-Flag and subsequent immunoblot (IB) with p-tyrosine and phospho-FGFR Y-653/654 antibody.

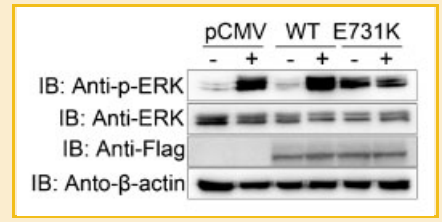

Fig. 4. $FGFR2^{E731K}$ enhanced ERK pathway. Transiently transfected HEK293T cells were serum-free starved for 16 h after the transfection. FGF2 was added to the cells for 30 min. Phospho-Erk was detected by immunoblotting.

THE NOVEL MUTATION, E731K, AND ENHANCED CELL PROLIFERATION

FGF2 is involved in cell proliferation and the early differentiation of osteoblasts and stimulates the regeneration of skeletal tissue as an effectual growth factor [Zellin and Linde, 2000]. Therefore, cell proliferation by the mutation E731K was examined. The cells expressing $FGFR2^{E731K}$ exhibited increased levels of proliferation compared to the $FGFR2^{WT}$ transfected control (Fig. 5). This result indicates that E731K can promote cell proliferation.

TRANSCRIPTIONAL ACTIVITY OF Runx2 AND BONE MARKER GENES WERE ENHANCED BY E731K

Runx2 is a member of the *runt*-family transcription factors that acts as a master switch for osteoblast differentiation. Therefore, detection

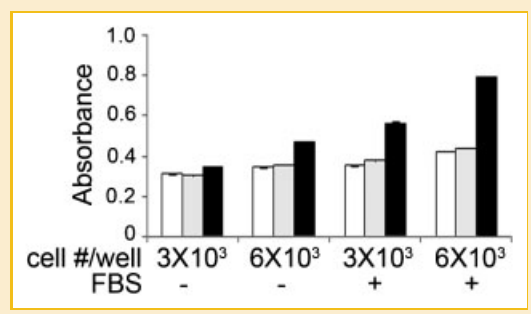

Fig. 5. Cell proliferation was accelerated by $FGFR2^{E731K}$. MC3T3E1 cells were transfected with pCMV-Flag (○), $FGFR2^{WT}$ (■), or $FGFR2^{E731K}$ (▲). When duplicated cells were plated in 96-well plates, the density was in 3×10^3, 6×10^3, 1.2×10^4/well. Sixteen hours after the transfection, the medium in one set was changed. After 12 h, cell proliferation was measured.

of Runx2 activity would be the next step to discern whether $FGFR2^{E731K}$ has a constitutively active form. Using the reporter vector, 6xOSE2-Luc, the altered Runx2 activity by $FGFR2^{E731K}$ was compared with that by $FGFR2^{WT}$ with a luciferase assay. FGF2 treatment increased the 6xOSE2-reporter activity by approximately fivefold in the absence of FGFR overexpression. $FGFR2^{WT}$ overexpression was sufficient to stimulate the reporter activity, but the treatment of FGF2 of the transfected cells further stimulated the activity by approximately fivefold. The overexpression of $FGFR2^{E731K}$ stimulated Runx2 activity by approximately eightfold compared to that of the pCMV vector transfected culture. The FGF2 treatment of the $FGFR2^{E731K}$ further stimulated the reporter activity by approximately threefold (Fig. 6A). These results suggest that through autophosphorylation and ERK1/2 pathway activation, $FGFR2^{E731K}$ stimulated Runx2 activity and osteoblast differentiation.

ALP, osteocalcin, and Runx2 are well-known osteoblast differentiation makers. We compared the mRNA level of these marker genes between $FGFR2^{WT}$ and $FGFR2^{E731K}$ transfected cultures. C_2C1_2 cells were stably transfected with $FGFR2^{WT}$ and $FGFR2^{E731K}$ and the confluent cells were further cultured in the presence or absence of FGF2. As shown in Figure 6B, C_2C1_2 cells without FGFR2 overexpression differentiated into myoblasts showing well developed myotubes (Cont), while overexpression of $FGFR2^{WT}$ or $FGFR2^{E731K}$ did not show myotube formation. ALP and Runx2 mRNA levels were elevated by the overexpression of $FGFR2^{WT}$ and further increased by the overexpression of $FGFR2^{E731K}$. MyoD is expressed in early myoblast differentiation and controls the differentiation. The MyoD mRNA level was decreased by $FGFR2^{WT}$ overexpression and further decreased by $FGFR2^{E731K}$ overexpression (Fig. 6C).

DISCUSSION

In this study, we report the first description of a novel mutation, E731K, in the tyrosine kinase domain of the FGFR2 gene and underwent the functional study of this mutation. To find the FGFR2 mutation, we carried out mutation screening in six patients with craniosynostosis. As a result of the screening, mutations were detected in exon 5, exon 8, exon 10, and exon 18, all of which were due to a single amino acid substitution, which were M186T, S252W, C342S, S347C, and E731K, respectively. Cases 1 and 2 were already known Crouzon syndrome mutations [Jabs et al., 1994; Reardon et al., 1994]. Cases 3 and 4 were already reported genetically in craniosynostosis patients [Kan et al., 2002] and were diagnosed as Apert syndrome according to the clinical observation. Case 5 was one of the most popular mutations in Apert syndrome [Wilkie et al., 1995]. However, E731K on exon 18 has never been reported in craniosynostosis patients.

There have been a few longitudinal studies of suture pattern concerning craniosynostosis. In the case of Crouzon syndrome, most of the calvarial sutures become fused before 1 or 2 years of age, and there is no midline defect. However, in the case of Apert syndrome, both coronal sutures are synostosic. Most of the skull base synchondrosis is fused early years of life, and there are midline bony defects [Cohen and Kreiborg, 1993]. Our Crouzon syndrome cases followed the same pattern. In all of the Apert syndrome cases, bilateral coronal synostosis was observed at the first visit. However, in Cases 3 and 4, only the left lambdoidal suture became fused at 2 years of age. In Cases 5 and 6, the sagittal and lambdoidal sutures were patent until 10 years old, and there were also midline bony defects. Lemonnier et al. [2001] reported that the Apert $FGFR2^{S252W}$ mutation induces premature osteoblast apoptosis in the human suture and the

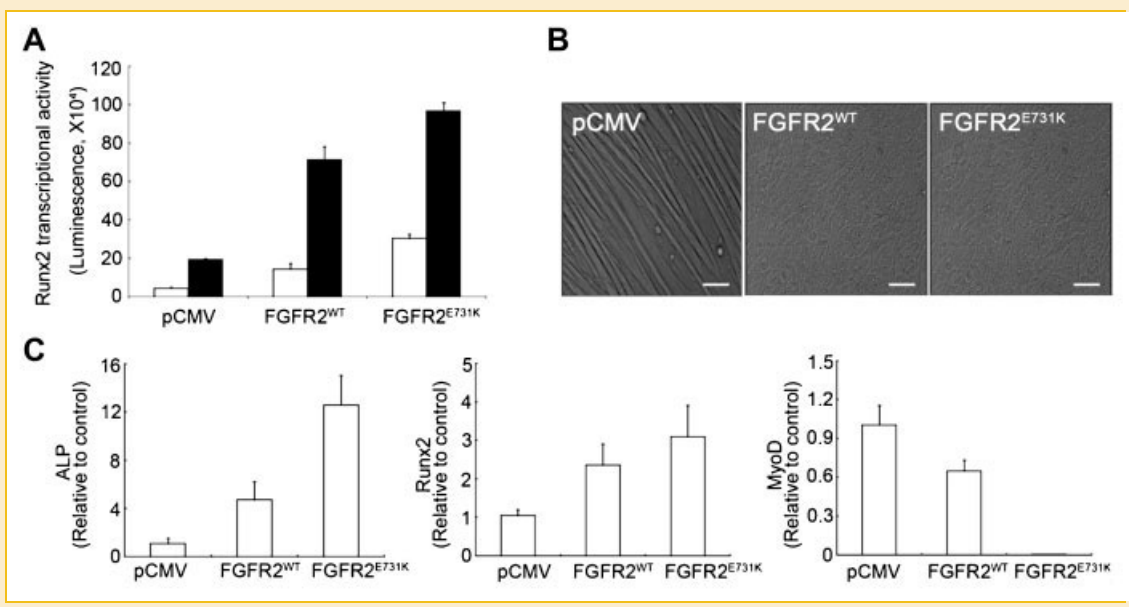

Fig. 6. Transcriptional activity of Runx2 and its downstream bone marker gene expression were enhanced by $FGFR2^{E731K}$. A: C_2C1_2 cells were co-transfected with 6xOSE2-Luc reporter vector and pCMV-Flag, FGFR2, or $FGFR2^{E731K}$. After 24 h, the cell treated with vehicle (white bar) and FGF2 (black bar) for 16 h and luciferase activity was measured using luminometry. B: Stable transfection of C_2C1_2 cells with the indicated constructs were examined for cell morphology on day 3 (40×). C: Quantitative real-time PCR analysis showed the mRNA levels of alkaline phosphatase (ALP), Runx2, and MyoD. Bars represent relative levels.

FGF signaling mechanism distinctively affects mature and immature calvaria osteoblasts. These results combined with our observations suggest that the midline bony defect may result from early premature osteoblast apoptosis and suture area bone formation defects in early postnatal life. Apert syndrome patients of almost 10 years of age (Cases 5 and 6) revealed a still-patent sagittal suture and lambdoidal suture. There may be different regulation mechanisms between sutures, although the reason is still unclear.

FGFR activates multiple signal transduction pathways, including ERK-MAP kinase pathways, through receptor dimerization and intrinsic tyrosine phosphorylation [Kuo et al., 1997; Maher, 1999]. Therefore, the effect of FGFR mutations on the alternation of phosphorylation and ERK-MAP kinase pathways has been studied. The most common mutations of Apert syndrome, S252W and P253R on FGFR2, have been studied for clear molecular mechanisms that lead to the syndrome. Profound changes of phosphorylation and downstream MAP kinase activity were observed in S252W and P253R mutations compared to the FGFR2 wild type [Ahmed et al., 2008]. Signaling by a mutation of FGFR2 alters the entire bone formation process. Mohammadi et al. [1996] identified specific, essential autophosphorylated amino acids, which were Y-653/Y-654. Based on knowledge regarding the molecular mechanisms for Apert syndrome mutations, signaling by a novel FGFR2 mutation, E731K, could be also confirmed by altered autophosphorylation and downstream ERK activity. As found in a P253R mutation, phosphorylation of $FGFR2^{E731K}$ was dramatically increased even on basal level, and the ERK activation level was augmented (Figs. 3 and 4). In this study, we were the first to identify E731K as a novel mutation resulting in Apert syndrome.

The ERK-MAP kinase pathway induces cell proliferation in mesenchymal cells [Miraoui et al., 2008]. The FGF2 signal is a well-known factor in promoting cell proliferation; therefore, the cell proliferation rate by the expression of $FGFR2^{E731K}$ should be investigated. The present data show that over-expressed $FGFR2^{WT}$ is substantial to increase cell proliferation; however, $FGFR2^{E731K}$ showed a much higher cell proliferation rate (Fig. 5).

Having observed altered autophosphorylation and ERK signaling, changes of Runx2 activity were investigated based on the 6XOES2-Luc reporter system, which has six tandem Runx2 binding sites for the detection of Runx2 activity [Kim et al., 2004]. More recently, we also reported that FGF-induced ERK activation enhanced Runx2 phosphorylation and acetylation, which strongly upregulated Runx2 transcriptional activity [Park et al., 2010]. Hence, the increase of Runx2 activity by $FGFR2^{E731K}$ transfected cells must result from the FGFR2-ERK signaling pathway. Similarly, $FGFR2^{E731K}$ also stimulated the expression of bone maker genes (Fig. 6C). These results indicate that a novel FGFR2 mutation, $FGFR2^{E731K}$, has similar functions to previously found Apert syndrome mutations and induces abnormal osteoblast differentiation and bone formation.

One key aspect of the point mutation in the receptor is the enhanced basal phosphorylation levels in the absence of ligand. According to a previous report [Ahmed et al., 2008], P253R was highly phosphorylated, whereas S252W was significantly less phosphorylated. Like P253R, E731K was also highly phosphorylated. Ahmed et al. [2008] interprets the highly phosphorylated P253R to indicate that P253R has more of the intermediate form of the receptors, whereas S252W has a more matured form due to an apparently higher molecular mass. A remarkable feature of $FGFR2^{E731K}$ was the exceedingly high basal phosphorylation of the receptor itself and its downstream ERK-MAP kinase. On the contrary, in response to FGF2 treatment, the ERK phosphorylation by $FGFR2^{E731K}$ was less elevated than that by $FGFR2^{WT}$ (Fig. 4). The mechanisms are unclear why ERK phosphorylation by $FGFR2^{E731K}$ is not prominently altered by FGF2. The activation of FGFRs by ligand engagement or other mechanism influences the intracellular sorting of FGFRs. In this study, several results indicated that $FGFR2^{E731K}$ would be a constitutively active mutant. The activated receptors may be internalized, while some parts undergo degradation by ubiquitination and another part stay in cytosol for recycling. Thus, less receptors must be exposed in the $FGFR2^{E731K}$ overexpressed cell surface to accept new ligand. Moreover, the exposed receptors are already active form so that they may not be able to respond to FGF2 treatment as does $FGFR2^{WT}$.

In summary, novel $FGFR2^{E731K}$ is a ligand-independent constitutively active form to convey FGF signaling in the absence of FGF ligand and consequently to express the phenotype of Apert syndrome. However, further studies remain to illuminate the subsequent molecular mechanism involving the profound molecular changes caused by $FGFR2^{E731K}$ mutation.

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