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Leucine-rich repeat, immunoglobulin-like and transmembrane domain 3 (LRIT3) is a modulator of FGFR1

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ABSTRACT

Fibroblast growth factor receptors (FGFRs) play critical roles in craniofacial and skeletal development via multiple signaling pathways including MAPK, PI3K/AKT, and PLC-γ. FGFR-mediated signaling is modulated by several regulators. Proteins with leucine-rich repeat (LRR) and/or immunoglobulin (IG) superfamily domains have been suggested to interact with FGFRs. In addition, fibronectin leucine-rich repeat transmembrane protein 3 (FLRT3) has been shown to modulate the FGFR-mediated signaling via the fibronectin type III (FNIII) domain. Therefore proteins with LRR, IG, and FNIII are candidate regulators of the FGFRs. Here we identify leucine-rich repeat, immunoglobulin-like and transmembrane domain 3 (LRIT3) as a regulator of the FGFRs.

1. Introduction

Fibroblast growth factor (FGF)-signaling plays crucial roles in cell proliferation and differentiation. FGFs activate the different isoforms of the FGF receptors (FGFR1, 2, 3 and 4) [1]. All FGFRs are type I membrane proteins that are synthesized in the endoplasmic reticulum (ER). Heparin or heparan sulfate forms a bridge between FGF and FGFR and is necessary for efficient FGF-signaling [2]. Upon exposure to FGF, FGFR dimerizes, resulting in activation of the tyrosine kinase (TK) activity and transautophosphorylation of tyrosine residues on the intracellular portion of the receptor [3]. Phosphorylated FGFR activates a variety of cellular signaling pathways including the mitogen-activated protein kinase (MAPK), PLC-γ, and PI3K/AKT [1,4,5].

Missense mutations or amplification of FGFR have been implicated in cancer as well as in other developmental diseases including craniosynostosis (CS), or premature fusion of cranial sutures [1,6]. Especially, gain-of-function mutations of FGFR cause syndromic craniosynostosis (SC) where CS is associated with other developmental anomalies and inherited in a Mendelian fashion [7–9]. However, SC only accounts for a small fraction of CS cases and the majority of CS cases occur as sporadic findings without other associated anomalies [10]. Considering the major impact of aberrant FGF-signaling in SC, it is possible that some NSC cases are caused by alterations in proteins that regulate or mediate FGF-signaling.

Multiple cellular factors such as Sprouty (SPRY), MAP kinase phosphatase 3 (MKP3), Similar Expression to FGFs (SEF), and fibronectin-leucine-rich transmembrane protein 3 (FLRT3) have been shown to regulate the FGF-signaling pathway [11–13]. In addition, a recent study has shown that proteins with leucine-rich repeat (LRR) and/or immunoglobulin (IG) superfamily domains may interact with FGFRs [14]. In the case of FLRT3, interaction with FGFR1 is via its fibronectin type III (FNIII) domain and activation of the MAPK signaling pathway [13]. Thus, families of proteins with FNIII, LRR, and IG domains may serve as regulators of FGFRs and other growth factor receptors.

We sought to identify new regulators of FGFR1. We focused on fibronectin leucine-rich repeat transmembrane protein 3 (LRIT3) as it contains the suggested domains for FGFR interaction. Our results suggest that LRIT3 regulates maturation and signaling of FGFR1.

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2. Materials and methods

2.1. Antibodies

The following antibodies were used for immunoblotting: Mouse anti-Myc (Millipore, USA; 1/1000), rabbit anti-beta-tubulin (Cell signaling Tech, USA, 1/1000), rabbit anti-phospho-ERK (Cell signaling Tech, USA, 1/1000), rabbit anti-ERK (Cell signaling Tech, USA, 1/1000), mouse anti-LRIT-3 (Novus Bioscience, USA, 1/1000), rabbit anti-phospho-AKT (Cell signaling Tech, USA, 1/1000), rabbit anti-AKT (Cell signaling Tech, USA, 1/1000), rabbit anti-PLC-γ (Cell signaling Tech, USA, 1/1000), rabbit anti-phospho-PLC-γ (Cell signaling Tech, USA, 1/1000), anti-rabbit IgG conjugated with horse radish peroxidase (Amersham Bioscience, USA, 1/5000), rabbit anti-FGFR1 (Cell signaling Tech, USA, 1/1000).

2.2. Subjects and clinical data

Informed consent was obtained from all patients and/or their parents. This study was approved by the Institutional Review Boards of the University of California, Davis, and was conducted in accordance with institutional guidelines.

2.3. PCR, DNA sequencing, and sequence analyses

A total of 431 individuals with non-syndromic craniosynostosis were selected for sequencing of LRIT3. Peripheral blood or saliva samples were collected from individuals, and genomic DNA was isolated as per conventional protocols with PureGene (5 Prime Inc.) or Oragene (Nalgene). *LRIT3* exons were amplified by polymerase chain reactions. PCR products were purified with Shrimp Alkaline Phosphatase and Exonuclease I (USB Corporation, Cleveland, OH). PCR primers are available in Table S1. Purified DNA fragments were sent to UC Davis Sequencing Facility and electropherograms were analyzed with VectorNTI™ Version 11 computer program. The 5′- and 3′-untranslated regions of LRIT3, as well as at least 100 base pairs of flanking intronic sequence for each exon were included in the sequencing analysis. The observed variants were confirmed by independent PCRs and sequencing of the reverse DNA strands. Parental samples (when available) were sequenced. Single nucleotide polymorphisms (SNPs) were considered novel if not described in the NCBI SNP database.

2.4. Taqman assays

Five Custom TaqMan® SNP Genotyping Assays manufactured by Applied Biosystems were designed to detect the novel polymorphic variants on Human Random Control DNA Panels 1–5 (European Collection of Cell Cultures, kind gifts from Michael L. Cunningham, University of Washington) using the ABI 7900HT QPCR machine. QPCR primers, probes, and conditions are available upon request. Allelic Discrimination was performed to classify the zygosities of the targeted templates by analyzing the fluorescence signals in each reaction well.

2.5. Construction of a human calvarial osteoblast cDNA library

Total RNA from human calvarial osteoblasts was isolated using Trizol reagent (Invitrogen, USA) RNA extraction reagent. cDNA was synthesized from 1 μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The DNA fragment containing the new exon 1 and a part of the previously known exon 1 of human *LRIT3* was amplified using osteoblast cDNA library with following synthetic oligonucleotide pairs (forward, 5′-ATGCATCTC TTTGCATGTCTGTGC-3′; reverse, 5′-CACGGGGAGGTTCGTAGGCAGC TCGTTCATATC-3′). The PCR product was confirmed by DNA sequencing.

2.6. Cell culture and transient transfection

The HEK 293T cells were cultured in DMEM media containing 10% fetal bovine serum and maintained in a water-jacketed incubator at 37 °C with 5% CO_2 enrichment (Boyd et al., 2006). Sub-cultured cells were maintained in DMEM media with 10% fetal bovine serum and split 1:5 weekly or when confluent. The plasmid DNAs were transiently transfected into HEK 293T cells using Lipofectamine and Plus according to the manufacturer's protocol (Invitrogen, USA).

2.7. Immunoblotting

Cells were washed in cold PBS and lysed in radioimmunoprecipitation assay buffer (25 mM Tris–HCl, pH7.6, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate and 5 mM EDTA) containing protease inhibitors (Roche, USA). The proteins concentration of cell lysates was determined with a bicinchroninic acid assay according to the manufacturer's protocol (Pierce, USA). Protein lysates were resolved in SDS–PAGE, transferred to PVDF membrane, probed with primary antibodies, incubated with secondary antibodies conjugated with horse radish peroxidase (HRP), and visualized with ECL plus.

2.8. Site-directed mutagenesis and plasmid construction

The human LRIT3 coding region was amplified from pCR-Blunt II-LRIT-3 (Open Biosystems, USA) using synthetic oligonucleotides pairs (5′-GGCTAACTAGAGAACCCACTG-3′ and 5′-GATTCTAGATTAC AGGTCCTCCTCTGAGAT-3′). The amplified fragments were digested with *Nae* I and *Xba* I and inserted into mammalian expression vector pCMV-SPORT6 (Invitrogen, USA). The resulting plasmid has a Myc-tag at the C terminus. The mutagenic primers for LRIT3 (T53M, S494T, and C592Y) were as follows: sense LRIT3 T53M, 5′-CCCGCTAGCATGGATATGAACGAGCTGCCTATGAACCTC-3′; antisense LRIT3 T53M, 5′-GAGGTTCATAGG CAGCTCGTTCATATCCATGC-TAGCGGG-3′; sense LRIT3 S494T, 5′-GCAATAGAAAACCTCAGGGT GGTCACTGAGACTAAAG-3′; antisense LRIT3 S494T 5′-CGTCAATGT-CACACTCT CTTTAGTCTCAGTGACCAC-3′; sense LRIT3 C592Y 5′-GACCAG ACTGCCTATGTTGTTATC-3′; antisense LRIT3 C592Y 5′-GATAACAACATA GGCAGTACTGGTC. To incorporate a signal sequence for LRIT3, an oligonucleotide (5′-ATGCATCTCTTTGCATGTC TGTGCATTGTCCTTAGCTTTTTGGAAGGAGTGGGCTGTTTGTGTCCTTC ACAGTGCACCTGTGATTATCACGGCAGAAATGACGGCTCAGGATCAAG GTTGGTGCTATGTAATGAC-3′) was used. The sequence was confirmed by DNA sequencing.

2.9. Deglycosylation experiments

Cleared cell lysates (30 μg) obtained from transiently transfected cells with either LRIT3 or FGFR1 or both were heat treated in 1× Glycoprotein Denaturing Buffer at 100 °C for 10 min according to the manufacturer's instructions (New England Biolabs Inc, Beverly, MA, USA). The denatured proteins were treated with peptide N glycosidase F (PNGase F) or endoglycosidase H (Endo H) at 37 °C 1 h. The resulting proteins were analyzed by immunoblotting after separation with 10% SDS–PAGE.

3. Results

When we compared the amino terminal sequence of human *LRIT3* with those of the piscine and rodent *LRIT3*, it was noted that human *LRIT3* lacked a signal sequence and its immediate flanking