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A Novel Skeletal Dysplasia with Developmental Delay and Acanthosis Nigricans Is Caused by a Lys650Met Mutation in the Fibroblast Growth Factor Receptor 3 Gene

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Summary

We have identified a novel fibroblast growth factor receptor 3 (FGFR3) missense mutation in four unrelated individuals with skeletal dysplasia that approaches the severity observed in thanatophoric dysplasia type I (TD1). However, three of the four individuals developed extensive areas of acanthosis nigricans beginning in early childhood, suffer from severe neurological impairments, and have survived past infancy without prolonged life-support measures. The FGFR3 mutation (A1949T: Lys650Met) occurs at the nucleotide adjacent to the TD type II (TD2) mutation (A1948G: Lys650Glu) and results in a different amino acid substitution at a highly conserved codon in the kinase domain activation loop. Transient transfection studies with FGFR3 mutant constructs show that the Lys650Met mutation causes a dramatic increase in constitutive receptor kinase activity, approximately three times greater than that observed with the Lys650Glu mutation. We refer to the phenotype caused by the Lys650Met mutation as "severe achondroplasia with developmental delay and acanthosis nigricans" (SADDAN) because it differs significantly from the phenotypes of other known FGFR3 mutations.

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Introduction

Fibroblast growth factor receptors (FGFRs) comprise a family of structurally related tyrosine kinases (FGFRs 1–4) encoded by four distinct genes. These receptors interact with a large group of ligands known as fibroblast growth factors (FGFs), leading to receptor activation and initiation of several different intracellular signaling pathways. FGF/FGFR signaling is known to play important roles in many diverse biological processes during embryonic development and in mature organisms (for reviews, see Basilico and Moscatelli 1992; Johnson and Williams 1993). Recent genetic studies have established that missense mutations in FGFRs 1, 2, and 3 cause several autosomal dominant human skeletal dysplasias and craniosynostosis syndromes and, in some of these cases, dermatological disorders.

Recurrent mutations in the FGFR3 gene were discovered first in achondroplasia, the most common genetic form of short-limbed dwarfism in humans (Rousseau et al. 1994; Shiang et al. 1994). Subsequent work has shown that several other human skeletal dysplasias with more-severe (thanatophoric dysplasia types I and II [TD1 and TD2][Tavormina et al. 1995*b*]) or less-severe (hypochondroplasia [Bellus et al. 1995*b*]) phenotypes are also caused by specific point mutations in the FGFR3 gene. Common phenotypic features observed in all these disorders include short stature with rhizomelic shortening of the extremities, brachydactyly, macrocephaly, short vertebral pedicles, and narrowing of lumbar interpedicular distances (Rimoin and Lachman 1993). Although it first appeared that all FGFR3 mutations were associated with skeletal dysplasia, subsequent work has identified other missense mutations that result in craniosynostosis with normal stature (i.e., Crouzon syndrome with acanthosis nigricans (Meyers et al. 1995)

and Muenke craniosynostosis (Bellus et al. 1996*a*; Muenke et al. 1997).

TD is a sporadically occurring lethal skeletal dysplasia, which, based on clinical and molecular criteria, has been further divided into two types—TD1 and TD2 (Langer et al. 1987; Tavormina et al. 1995*b*). TD1 is characterized by short curved femurs with variable (but usually absent) craniosynostosis and severe disturbances of the growth plates. TD2, in contrast, is characterized by straight femurs, invariant craniosynostosis (often cloverleaf skull), and relative preservation of the growth plate (Langer et al. 1987; Norman et al. 1992). Further genetic studies have revealed that TD1 may be caused by as many as 10 distinct FGFR3 missense mutations. Five TD1 mutations result in specific cysteine substitutions in the extracellular domain of the receptor (Tavormina et al. 1995*a,* 1995*b;* Rousseau et al. 1996). The other five mutations eliminate the stop codon and result in translation of a 141–amino acid C-terminal extension (Rousseau et al. 1995; Bellus et al. 1997). In contrast, FGFR3 mutations in TD2 (Tavormina et al. 1995*a*), achondroplasia (Rousseau et al. 1994; Shiang et al. 1994; Bellus et al. 1995*a*) and hypochondroplasia (Bellus et al. 1995*b,* 1996*b;* Prinos et al. 1995) all result from mutations at specific nucleotides leading to unique amino acid substitutions in the receptor protein.

Recent studies have shown that the FGFR3 mutations causing achondroplasia (Gly380Arg), TD2 (Lys650Glu), and TD1(Arg248Cys) lead to ligand-independent, constitutive activation of the FGFR3 receptor and that the degree of activation correlates directly with the severity of the associated skeletal phenotype (Naski et al. 1996; Webster and Donoghue 1996; Webster et al. 1996). These data are supported by the phenotype of the FGFR3 knockout mouse, which exhibits overgrowth of the proximal long bones and vertebral bodies (Deng et al. 1996; Colvin et al. 1996), and are consistent with clinical observations that individuals with homozygous achondroplasia and compound heterozygosity for achondroplasia and hypochondroplasia have more-severe skeletal phenotypes than simple heterozygotes (Hall et al. 1969; McKusick et al. 1973). Together, these data suggest that a normal function of FGFR3 is to inhibit bone growth and that increasing amounts of inappropriate FGFR3 activity lead to more-severe skeletal dysplasia.

Genetic analyses have been performed on many patients with these forms of skeletal dysplasia, but mutations have not been found for all affected individuals (Bellus et al. 1995*a;* Tavormina et al. 1995*b*) and rare patients have phenotypes of intermediate severity between the recognized disorders (Bellus et al. 1995*a;* Nishimura et al. 1995; Superti-Furga et al. 1995). Such cases may represent variable expressivity of a common mutation or may represent novel uncommon mutations such as the FGFR3 Gly375Cys mutation in achondroplasia with atypical radiological findings (Nishimura et al. 1995; Superti-Furga et al. 1995). We describe here the discovery of a unique recurrent FGFR3 mutation in four unrelated individuals with previous diagnoses of severe achondroplasia or TD1. None exhibit craniosynostosis, and three survived past infancy without the need for prolonged ventilator support. The surviving patients have severe developmental delay and developed acanthosis nigricans as infants or toddlers. The mutation (A1949T: Lys650Met) occurs at the nucleotide adjacent to the TD2 mutation (A1948G: Lys650Glu) within the receptor tyrosine kinase domain activation loop and predicts a lysine-to-methionine substitution at codon 650. Using in vitro transfection studies, we demonstrate that the Lys650Met mutation results in strong constitutive activation of FGFR3 receptor protein kinase activity.

Material and Methods

Patients and Cell Lines

Three of the four patients were evaluated either at the medical genetics clinics at Johns Hopkins University Hospital or at the University of Utah Health Sciences Center. Informed consent for participation in genetic testing was obtained through the parents of each affected individual. Fibroblast and lymphoblast cell lines from the fourth patient were provided by the International Skeletal Dysplasia Registry.

PCR Amplification

DNA was isolated from blood leukocytes or lymphoblast and fibroblast cell lines derived from affected individuals or their parents by standard methods. PCR amplification of FGFR3 sequence was carried out as follows. In three patients, a 334-bp amplicon containing FGFR3 exon 15, intron 15, and flanking regions of intron 14 and exon 16 was defined by the forward primer 5′-AGGTGTGGGTGGAGTAGGCTG-3′ and the reverse primer 5′-CTCAGGCGCCATCCACTTCAC-3′. PCR reactions were performed in a total volume of 50 μl containing 1X PCR buffer (Perkin Elmer: 10 mM Tris, pH 8.3, 50 mM KCl), 10% dimethyl sulfoxide, 250 μM each nucleotide (Pharmacia), and 2 U *Taq* DNA polymerase (Perkin Elmer). PCR reactions were done in a Hybaid Omnigene thermocycler with the following thermal cycling program: 94° for 5 min; 35 cycles of 94° for 30 s, 64° for 45 s, and 72° for 45 s; and a final cycle of 72° for 10 min. DNA from a lymphoblast cell line derived from the other patient was PCR amplified with primer sequences and cycling conditions described elsewhere, to amplify FGFR3 exon 15 sequence (Tavormina et al. 1995*b*).

Restriction Enzyme Analysis and Sequencing

PCR products were digested with the restriction enzyme *Bbs*I (New England Biolabs), according to the manufacturer's instructions. Digested DNA was analyzed by electrophoresis on 2% agarose or 6% polyacrylamide gels using Tris-borate buffer and visualized by staining with ethidium bromide. DNA sequencing was performed on purified PCR products (Promega Wizard spin columns or electroelution) by means of the fluorescent dideoxyterminator method of cycle sequencing on a PE/ABI 373a automated DNA sequencer or by the PCR products sequencing kit (USB), as described by the manufacturer.

Construction of FGFR3 Mutants

Pairs of annealed oligonucleotides encoding each Lys650 substitution (Lys650Glu and Lys650Met) were inserted between the *Eco*RV site at amino acid 641 and the *Age*I site at amino acid 658 of the FGFR3 derivative described elsewhere (Webster et al. 1996). All constructs were confirmed by sequencing.

Immunoprecipitation and In Vitro Kinase Assays

National Institutes of Health (NIH) 3T3 cells were transfected with 10 μg of each expression construct, by means of a modified calcium phosphate transfection method (Chen and Okayama 1987). Four hours after refeeding, the medium was changed to serum-free Dulbecco's minimal essential medium. Twenty-four hours later, cells were lysed in Nonidet P (NP)-40 lysis buffer (20 mM Tris, pH 7.5; 137 mM NaCl; 1% NP-40; 5 mM EDTA; 10% glycerol; 10 mg/ml aprotinin; 1 mM sodium orthovanadate) and immunoprecipitated with antiserum specific for the C-terminus of FGFR3 (Santa Cruz Biotechnology). Immune complexes were washed once in 20 mM Tris, pH 7.5, and subjected to in vitro kinase reactions in the presence of 40-μl kinase buffer (20 mM Tris, pH 7.5; 10 mM $MnCl_2$; 5mM $MgCl_2$, 5 μCi $\gamma[^{32}P]$-ATP), for 15 min at 37°C. Products were washed extensively with NP-40 lysis buffer, electrophoresed through a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and exposed to x-ray film. In vitro kinase activity was quantitated by laser scanning densitometry of the autoradiograms. Similar quantitative results were obtained from multiple experiments.

Immunoblotting

Filters prepared as described above were incubated with anti-FGFR3 antiserum (Santa Cruz Biotechnology) and horseradish peroxidase–conjugated goat antirabbit IgG and developed by enhanced chemiluminescence (Amersham), as per the manufacturer's instructions.

Results

Identical mutations were discovered independently in two unrelated patients in different laboratories. Patient 1 originally carried a diagnosis of achondroplasia and was the only patient of 154 previously described, unrelated individuals with achondroplasia who did not have an FGFR3 Gly380Arg mutation (Bellus et al. 1995*b*). It was noted in that report that this patient had skeletal changes that were more severe than normally observed in achondroplasia and exhibited other unusual skeletal features (e.g., reverse bowing of the tibia and fibula). An extensive search for FGFR3 mutations in this patient, using exon-specific PCR primers covering 95% of the coding sequence and heteroduplex analysis, was unsuccessful. Because of her severe skeletal phenotype, we next considered the possibility that she was a somatic mosaic for a TD mutation. DNA was isolated from both lymphoblast and fibroblast cell lines derived from this patient and screened for the known TD1 and TD2 mutations by means of PCR and digestion with specific restriction enzymes. TD2 is caused by an A→G transition at position 1948 that predicts a glutamic acid for lysine substitution at codon 650 and disrupts a unique *Bbs*I restriction site in exon 15. A mutation disrupting this *Bbs*I site was found to be present in DNA isolated from fibroblasts and lymphocytes from this patient and was not present in either of her parents (fig. 1). Sequencing

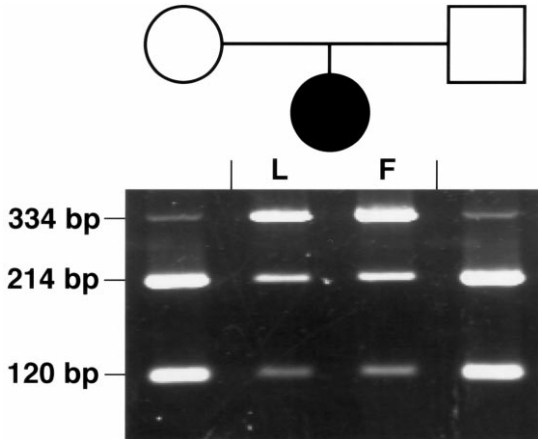

Figure 1 An affected individual is heterozygous for a sporadic mutation disrupting a unique *Bbs*I restriction site in FGFR3 exon 15. PCR amplification of FGFR3 exon 15 was performed on DNA isolated from fibroblasts and lymphoblast cell lines derived from patient 1 and from lymphoblast cell lines derived from both her parents. Products were digested with the restriction enzyme *Bbs*I, analyzed on 2% agarose gels, and visualized by ethidium bromide staining. *Bbs*I digests essentially all the 334-bp amplicon derived from the four parental FGFR3 alleles but does not digest one allele of the amplicon derived from the patient's DNA. L = patient lymphoblast cell line; F = patient fibroblast cell line.

of the FGFR3 exon 15 PCR amplicon derived from this patient revealed that she was heterozygous for an A→T transversion at position 1949, predicting a methionine for lysine substitution at codon 650 (fig. 2). The FGFR3 A1949T (Lys650Met) mutation is unlikely to represent a polymorphism, since it was not found on screening >150 normal chromosomes by digestion with *Bbs*I (data not shown).

The other patient had phenotypic features consistent with TD1 and died shortly after birth. A lymphoblastoid cell line was made from a blood sample from this patient, submitted to the International Skeletal Dysplasia Registry, and included in a large cohort of patients being screened for FGFR3 mutations. Of 84 patients with a clinical diagnosis of TD1 in whom an FGFR3 mutation was found, this patient was the only one found to have lost the FGFR3 exon 15 *Bbs*I site on one allele (data not shown). DNA sequencing demonstrated that the mutation disrupting this restriction site in this patient was also an A→T transversion at position 1949, predicting a Lys650Met mutation (data not shown). Subsequently, two additional unrelated patients with phenotypes similar to that of the first patient were identified, screened by *Bbs*I digestion and sequencing, and found to be heterozygous for the Lys650Met mutation (data not shown).

FGFR3 Lys650Met Phenotype

The clinical and radiographic features of these four individuals with FGFR3 Lys650Met mutations fit within the spectrum of the other FGFR3 skeletal dysplasias and include several unique features. Detailed case reports and a more extensive description of the clinical features are to be reported elsewhere (G.A. Bellus, M.J. Bamshad, K. Przylepa, J. Dorst, R. Lee, O. Hurko, E.W. Jabs, C.J.R. Curry, W.R. Wilcox, R.S. Lachman, D.L. Rimoin, C.A. Francomano, unpublished data). Although one patient had skeletal features similar to TD1 and died shortly after birth, the other three patients survived and have not needed prolonged ventilator support. Continued mechanical ventilation has been required in the few reported cases of TD1 with extended survival (MacDonald et al. 1989; Baker et al. 1997). The overall appearance of the surviving patients with the FGFR3 Lys650Met mutation is similar to that of patients with achondroplasia, because of rhizomelia and megalencephaly. However, there is a much greater restriction in growth, with stature being less than the 5th percentile for achondroplasia (fig. 3*a*). Redundant skin folds of the arms and legs, which are commonly seen during infancy in achondroplasia, persisted into late childhood. Craniosynostosis was not observed, and the skull shape is reminiscent of achondroplasia and TD1 with severe midface hypoplasia and frontal bossing (fig. 3*a* and *b*). Long-

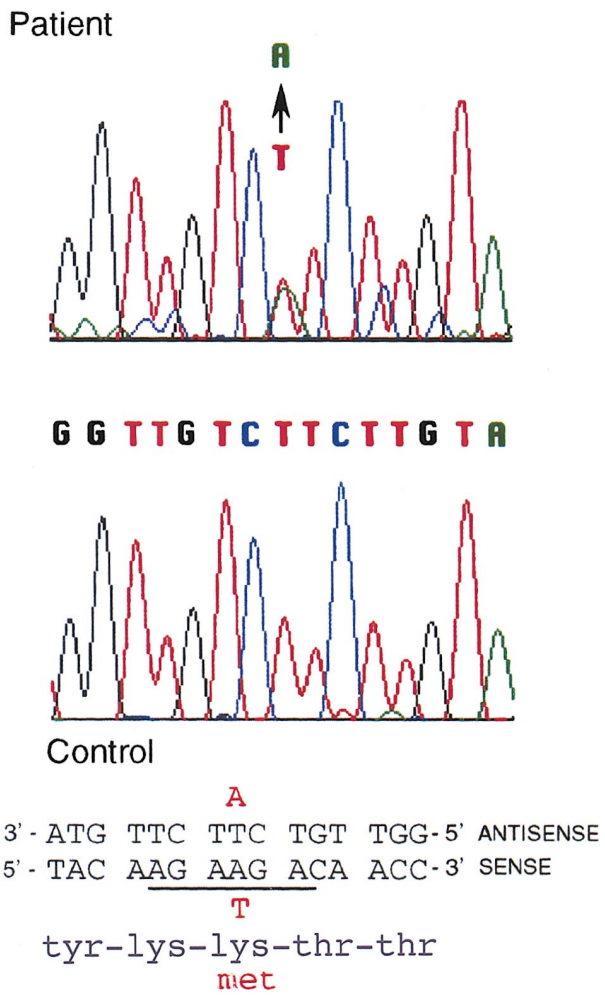

Figure 2 Mutation disrupting the *Bbs*I site—an A→T transversion at position 1949, predicting a Lys650Met substitution. The FGFR3 PCR amplicon from patient 1 and one of her parents (control) was sequenced with fluorescent dideoxynucleotides. The sequence of the antisense strand is shown, and the nucleotide change in the patient's DNA is indicated. The predicted amino acid substitution in the sense strand (lysine to methionine) is shown below, and the *Bbs*I recognition sequence is underlined.

bone growth is severely limited and there is femoral bowing similar to that observed in TD1. In addition, three of the four patients exhibited an unusual bowing of the tibias, which occurs in the direction opposite that of the femoral bowing (fig. 3*a* and *c*). Platyspondyly was observed but was generally less severe than that observed in TD1 or TD2 (fig. 3*d*).

During infancy, all the surviving patients developed generalized seizures, which have been reasonably well controlled with standard anticonvulsant medications. Two of the surviving patients have undergone ventriculostomy placements for hydrocephalus and surgery to relieve spinal and foramen magnum stenosis. The three

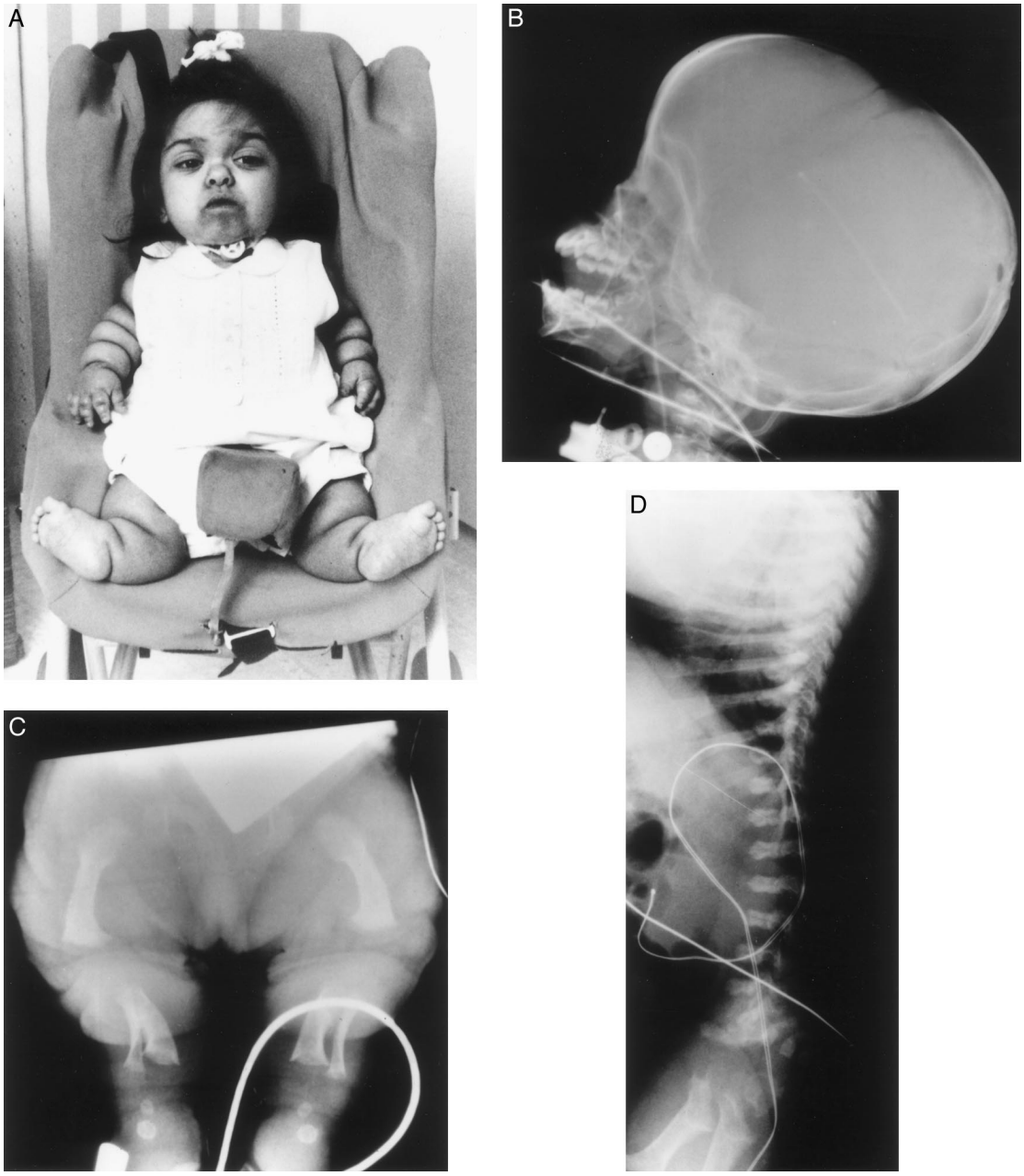

Figure 3 Clinical and radiographic features of a patient with an FGFR3 Lys650Met mutation. *A*, Patient 1: age 4 years. *B*, Patient 1: age 1 year. The lateral skull is shown. Note severe midface hypoplasia. *C*, Patient 1: age 1 d. The lower extremities are shown. Note severe shortening of the long bones as well as femoral and tibial bowing. *D*, Patient 1: age 1 d. The lateral spine is shown. Note moderately severe platyspondyly.

surviving patients exhibit severe mental retardation and developmental delays. Magnetic resonance imaging evaluation of the brain has revealed several structural abnormalities that include thinning of the corpus callosum, paucity of white matter with generalized brain atrophy, and an abnormally large cerebellum.

Finally, during or shortly after infancy, the three surviving patients developed acanthosis nigricans, which has progressively spread over large areas of the body surface. Insulin levels were tested and found to be normal in all three patients, indicating that the acanthosis nigricans is not due to insulin resistance.

Lys650Met Mutation Activates FGFR3 Kinase Activity

The phenotype observed in our patients is different from TD2 despite the fact that both mutations lead to different amino acid substitutions at the same codon in

the activation loop of the FGFR3 tyrosine kinase domain. It was therefore of interest to determine how the Lys650Met mutation compared with the Lys650Glu mutation in terms of its ability to activate the receptor kinase. To assess the effect of the Lys650Met substitution on FGFR3 activity, constructs encoding wild-type FGFR3, the TD2 mutation (Lys650Glu), or the Lys650Met mutation were transiently transfected into NIH 3T3 cells under the control of the cytomegalovirus promoter. FGFR3 proteins were immunoprecipitated from lysates of serum-deprived cells and subjected to in vitro kinase assays in the presence of $\gamma[^{32}P]$-ATP. As shown previously (Webster et al. 1996) (fig. 4), there is a dramatic increase in the ligand-independent kinase activity of the Lys650Glu mutant receptor compared with the wild-type receptor, which is essentially inactive in this assay. Scanning densitometry of the autoradiograms reveals that the Lys650Glu mutation results in an ~100-fold stimulation of FGFR3 kinase activity over wild-type FGFR3 and a 20-fold stimulation over that observed with the Gly380Arg mutation found in achondroplasia (Webster et al. 1996). The Lys650Met receptor exhibits a further threefold increase in autophosphorylation activity compared with the Lys650Glu receptor. This greater activity does not occur because of increased expression of mutant receptor, since immunoblotting of immunoprecipitates used in the kinase assay with FGFR3-specific antiserum shows that similar amounts of FGFR3 protein are present in each assay.

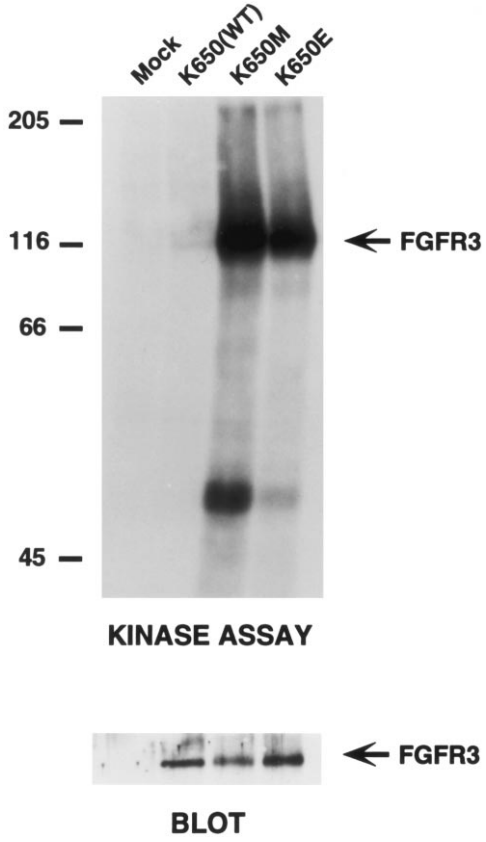

Figure 4 FGFR3 Lys650Met results in strong activation of kinase activity. NIH 3T3 cells were transfected with 10 μg of each expression construct, as described in the Material and Methods section, and immunoprecipitated with FGFR3 antibody. Kinase assays were performed on the immune complexes with $\gamma[^{32}P]$-labeled ATP, and the products were analyzed by electrophoresis on a 7.5% SDS-PAGE gel followed by transfer to nitrocellulose and exposure to x-ray film. The phosphorylated FGFR3 protein is indicated by the arrow. Background FGFR3 activity of untransfected NIH 3T3 cells is shown in the lane labeled "mock." The identity of the lower-molecular weight–labeled product is not currently known. To control for the amount of receptor loaded, duplicate filters prepared as above were incubated with anti-FGFR3 antiserum and horseradish peroxidase–conjugated goat anti-rabbit IgG and visualized by enhanced chemiluminescence, as described in Material and Methods.

Discussion

We describe here a novel FGFR3 mutation (A1948T: Lys650Met) causing a pleiotrophic phenotype that includes skeletal dysplasia, CNS dysfunction, and acanthosis nigricans. This mutation occurs within the FGFR3 tyrosine kinase domain activation loop and affects the same codon altered in TD2 (Lys650Glu) but results in a phenotype that is significantly different from TD2. The FGFR3 Lys650Met mutation is compatible with survival in three of the four patients we have identified and, therefore, calling this disorder a variant of TD seems inappropriate. Instead, we propose that the phenotypic syndrome associated with the FGFR3 Lys650Met mutation be referred to as "severe achondroplasia with developmental delay and acanthosis nigricans" ("SADDAN"). SADDAN represents the seventh distinct genetic syndrome caused by an FGFR3 missense mutation (fig. 5).

Four phenotypic features (skeletal dysplasia, craniosynostosis, acanthosis nigricans, and CNS malformation) are associated with FGFR3 missense mutations in humans, and some or all of these features may occur, depending on the specific mutation. All four may occur in TD1, although acanthosis nigricans is a late finding and is observed only in rare cases with extended survival (Baker et al. 1997). CNS malformations, including abnormal sulcation, polymicrogyria, neuronal heterotopia, nuclear dysplasia, and abnormal neuronal bundles, have been documented in TD with and without cloverleaf skull (Wongmongkolrit et al. 1983; Ho et al. 1984; Shigematsu et al. 1985). Of the four features, three are present in SADDAN (Lys650Met) (skeletal dysplasia, CNS malformation, and acanthosis nigricans) and three are present in TD2 (Lys650Glu) (skeletal dysplasia, CNS malformation, and craniosynostosis). However, extended survival has not been reported in TD2, and it is not known whether acanthosis nigricans would develop later in these individuals. Two features (craniosynostosis and acanthosis nigricans) are present in Crouzon syn-

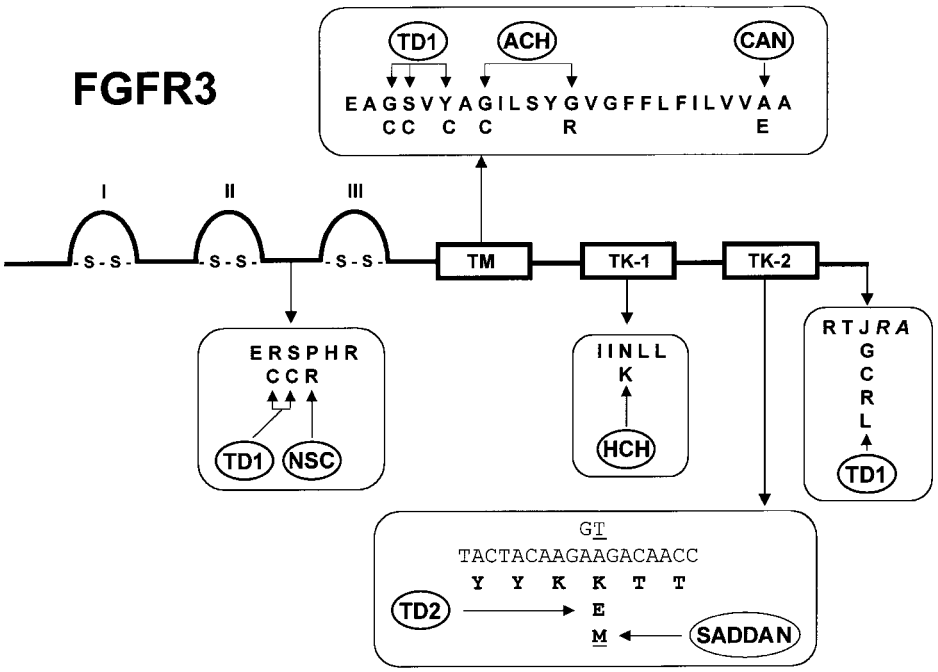

Figure 5 FGFR3 mutations associated with human genetic disorders. A schematic diagram of the FGFR3 protein is presented and the locations of the known human missense mutations are indicated. Immunoglobulin-like loops in the extracellular domain are labeled I–III. Standard single-letter amino acid abbreviations are used to indicate the location and nature of the mutations associated with each human disorder. The nucleotide and amino acid sequence of part of the kinase activation loop is shown, and the locations of the nucleotide substitutions in TD2 and SADDAN are indicated. The nucleotide and amino acid substitutions in SADDAN are underlined. ACH = achondroplasia; CAN = Crouzon syndrome with acanthosis nigricans; NSC = nonsyndromic craniosynostosis; HCH = hypochondroplasia; TM = transmembrane domain; TK-1 = proximal tyrosine kinase domain (ATP binding); and TK-2 = distal tyrosine kinase domain (substrate binding and catalytic). References for the mutations listed are given in the Introduction.

drome with acanthosis nigricans (Ala391Glu), and only one feature is associated with achondroplasia (Gly380Arg) (skeletal dysplasia) and with hypochondroplasia (Asn540Lys) (skeletal dysplasia), although it is suspected that individuals with hypochondroplasia may have a higher incidence of mild CNS dysfunction (Walker et al. 1971; G. A. Bellus, unpublished data). Craniosynostosis and distal skeletal anomalies are variable in the syndrome defined by the Pro250Arg mutation (Muenke et al. 1997).

Why certain amino acid substitutions in the FGFR3 protein lead to different combinations of these four phenotypic features is not well understood. The degree of long-bone dysplasia observed in SADDAN is variable but is always more severe than typically seen in achondroplasia or TD2 and approaches the severity observed in TD1. On the other hand, dysplasia of the axial skeleton (i.e., platyspondyly) in SADDAN appears to be less severe than in TD1 or TD2. Our results from in vitro transfection assays demonstrate that the SADDAN FGFR3 Lys650Met mutation leads to higher levels of ligand-independent FGFR3 autophosphorylation than the TD2 Lys650Glu or achondroplasia Gly380Arg mutations and are consistent with other in vitro studies that have shown that FGFR3 mutations associated with more-severe disturbances of long-bone growth result in larger increases in constitutive receptor activity (Naski et al. 1996; Webster et al. 1996). Considering these data in conjunction with the FGFR3 knockout mouse, which exhibits a phenotype of endochondral bone overgrowth (Colvin et al. 1996; Deng et al. 1996), one may conclude that FGFR3 may indeed function normally as a negative regulator of endochondral bone growth and that increasing amounts of FGFR3 tyrosine kinase activity lead to progressively more-severe disturbances in growth. The FGFR3 Lys650Glu mutation has been associated with activation of STAT1 and expression of $p21^{waf1/cip1}$ in chondrocytes and may initiate inappropriate cell cycle arrest (Su et al. 1997). The FGFR3 Lys650Met mutation may act in a similar manner and may lead to higher levels of $p21^{waf1/cip1}$ expression in endochondral growth plates, resulting in more-complete cell cycle arrest. Alternatively, increased intensity or duration of FGFR3 signaling by the Lys650Met mutation may cause activation or inhibition of other signaling pathways controlling endonchondral bone growth.

Insights into the mechanisms of other associated FGFR3 phenotypic features (craniosynostosis, CNS malformations, and acanthosis nigricans) are less apparent. Synostosis of multiple sutures (cloverleaf skull deformity) is seen uniformly in TD2, occasionally in TD1, and not, as yet, in SADDAN. Acanthosis nigricans is observed in conjunction with craniosynostosis, but not skeletal dysplasia, in Crouzon syndrome with acanthosis nigricans, and in conjunction with skeletal dysplasia, but not craniosynostosis, in SADDAN. Malformations of the CNS are severe in TD1 and TD2 yet compatible with survival in SADDAN. FGFR3 is constitutively activated in all these disorders, but there may be differences among these mutations in the mechanism and intensity of receptor activation and/or activation of downstream signaling molecules. The TD1 extracellular domain mutations (e.g., Arg248Cys) create unpaired cysteines that most likely function to induce ligand-independent dimerization of mutant receptors permitting *trans* and/or *cis* autophosphorylation of critical intracellular tyrosine residues (Naski et al. 1996). These mutant receptors are fully active and cannot be stimulated further by ligand. In contrast, the TD2 Lys650Glu and achondroplasia Gly380Arg mutations result in moderate levels of FGFR3 kinase activation and can be stimulated further by addition of FGF ligands (Naski et al. 1996). It is also possible that the FGFR3 TD2 Lys650Glu and SADDAN Lys650Met mutations may not require receptor dimerization to activate the tyrosine kinase. The ability of a *Xenopus* TD2 equivalent mutation (XFGFR1: Lys562Glu) to induce mesoderm in *Xenopus* embryos is not inhibited by FGFR-dominant negative constructs (which act by blocking dimerization of functional receptors), although mutations of XFGFR1 homologous to TD1 and achondroplasia mutations are inhibited by dominant negative constructs (Neilson and Freisel 1996).

X-ray crystallographic studies of the FGFR1 kinase domain (Mohammadi et al. 1996*b*) and FGFR3 mutagenesis studies (Webster et al. 1996) provide some additional insights into the effects of the FGFR3 Lys650Glu and Lys650Met mutations on receptor kinase activity. The FGFR3 Lys 650 codon is located within the kinase activation loop and must play a critical role in maintaining the unligated receptor kinase in a quiescent state. Mutations that convert adjacent codons to glutamate codons do not activate the kinase, and two highly conserved adjacent tyrosine residues (Tyr 647 and Tyr 648), whose equivalents are normally critical for ligand-dependent activation of the FGFR1 kinase domain, are not required for activation of the FGFR3 kinase when a Lys650Glu mutation is present (Webster et al. 1996). Mohammadi et al. (1996*a*) proposed that the FGFR3 Lys650Glu substitution may stabilize the kinase activation loop in an active conformation, thereby permitting substrate interaction with the kinase-active site. Our results suggest that a methionine substitution may have a more dramatic effect than the glutamate substitution in either stabilizing the active conformation or destabilizing the quiescent conformation of the kinase activation loop.

A striking and unexplained difference between TD2 and SADDAN is the common occurrence of severe craniosynostosis in the former and not in the latter. It is possible that there is a threshold effect and that craniosynostosis does not occur if there is too much or too little constitutive activation of the FGFR3 kinase. An alternate explanation is that FGFR3 receptors (or translation products of FGFR3 mRNA splicing isoforms) with either mutation differentially activate other FGFRs or lead to activation or inhibition of different downstream signaling pathways. Different amino acid substitutions at this critical residue may alter the binding affinity of other substrate-signaling molecules so as to phosphorylate an inappropriate substrate or fail to phosphorylate an appropriate one. Another possibility is that these mutations may differentially interfere with the ability of tyrosine phosphatases to down-regulate receptor signaling.

Much still needs to be learned before we fully understand the molecular and developmental effects of these and other FGFR3 mutations. More patients with Lys650Met mutations will need to be evaluated to determine the full spectrum of the SADDAN phenotype. Further studies with transfected mutant constructs and transgenic mice will be helpful in sorting out FGFR3 signaling pathways and how they are affected by specific FGFR3 mutations.

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