

MUTATION IN BRIEF

Identification of a Novel Mutation of *SH3BP2* in Cherubism and Demonstration that *SH3BP2* Mutations Lead to Increased NFAT Activation

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We describe a novel missense mutation (Aspartic acid to Asparagine, p.D419N (g.1371G>A, c.1255G>A) within exon 9 of *SH3BP2* in a patient with cherubism, an autosomal dominant syndrome characterized by excessive osteoclastic bone resorption of the jaw. Two siblings and the father were carriers but lacked phenotypic features. Transient expression of p.D419N (c.1255G>A), as well as three previously described exon 9 mutations from cherubism patients (p.R415Q (c.1244G>A), p.D420E (c.1259G>A), and p.P418R (c.1253C>G)) increased activity of NFAT (nuclear factor of activated T-cells), an osteoclastogenic mediator, indicating that cherubism results from gain of function mutations in *SH3BP2*. Published 2006 Wiley-Liss, Inc.

KEY WORDS: *SH3BP2*; cherubism; NFAT; osteoclastogenesis; giant cell tumor; bone; bone resorption

INTRODUCTION

Cherubism (MIM# 118400) is a rare autosomal dominant disorder with incomplete penetrance characterized by extensive pathological bone remodeling within the mandible or maxilla due to heterozygous germline mutations in the gene that encodes the adapter protein *SH3BP2* (Src Homology-3 Binding Protein-2; MIM# 602104). (Ladhani et al. 2003; Lannon et al. 2001; Lo et al. 2003; Petschler et al. 2003; Schultze-Mosgau et al. 2003; Ueki et al. 2001) The onset of cherubism typically coincides with eruption of secondary teeth, age 2-5 years, at which time patients develop characteristic giant cell lesions in the maxilla and mandible, substantial facial swelling and cervical lymphadenopathy. (Schultze-Mosgau et al. 2003; Ueki et al. 2001) The bone lesions and facial swelling are progressive until puberty, at which time the swelling regresses but the osseous defects persist until old age. (Lannon et al. 2001; Schultze-Mosgau et al. 2003) The radiological appearance of the jaw in patients with cherubism is one of intense bone resorption, and histologically there are numerous giant cells that express the osteoclast marker tartrate-resistant acid phosphatase (TRAP). (Lannon et al. 2001; Schultze-Mosgau et al. 2003)

SH3BP2 has 13 exons, but all *SH3BP2* mutations described thus far in patients with cherubism occur within a six amino acid region (amino acids 415-420) encoded by exon 9. (Imai et al. 2003; Lo et al. 2003; Ueki et al. 2001) Loss of function was previously reported for some *SH3BP2* mutations (p.R413Q, p.P416H, and G418R) in mast

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cells.(Miah et al. 2004) These investigators created the *SH3BP2* mutations in the mouse SH3BP2 cDNA and the mouse SH3BP2 protein (GenBank RefSeq-file accession number AAH85497) is shortened (by two amino acids) compared to the human form and has other dissimilarities. The wild-type mouse SH3BP2 in our experience does increase NFAT relative to the human wild-type SH3BP2 (data not shown) and might indeed lead to different results. Moreover we feel that comparisons of the human wild-type and human mutant SH3BP2 cDNA's are more relevant to the human condition of cherubism. Further, although the specific function of the six amino acid region in SH3BP2 in which missense mutations occur in patients with cherubism remains unclear, at least two observations make it unlikely that these mutations result in a loss of function. First, *SH3BP2* has been localized to chromosome 4p16.3, a region that is frequently deleted in patients with Wolf-Hirschhorn syndrome(Bell et al. 1997). Despite haploinsufficiency of *SH3BP2*, patients with Wolf-Hirschhorn syndrome have distinctive facial and developmental features that show no resemblance to those of cherubism. Secondly, the discrete clustering of the missense mutations in cherubism within a six amino acid domain of the protein suggests that these mutations affect a specific function of the SH3BP2 protein.

MATERIALS AND METHODS

Materials

Thermosequenase, USB, Swampscott, MA (www.amersham.com); Vent polymerase, NEB, Beverly MA (www.neb.com); Perkins Elmer dNTPs Boston, MA (www.perkinelmer.com); Primers idtdna, Coralville, Iowa (www.idtdna.com); Gel extraction kit Qiagen, Valencia, CA (www.qiagen.com); Actin antibodies, Sigma St Louis, MO (www.sigmaaldrich.com); Secondary anti-mouse and anti-rabbit, Caltag, Burlingame, CA (www.caltag.com); Luminometer Zylux, 144 Ridgeway Center Oak Ridge, TN (www.zylux.com); Dual Luciferase Assay, Promega, Madison WI (www.promega.com); 6 well dishes, Corning, Acton, MA (www.corning.com).

Patient analysis

We evaluated a patient with Cherubism and his family (5 family members total). The proband was 5 years old and presented with facial swelling. The protocol was approved by the institutional review board and written consent was obtained from the parents and children.

Genomic DNA was isolated directly from blood with the use of commercial reagents (QIAamp DNA MiniKit, Qiagen, Valencia, CA). We used the polymerase chain reaction (PCR) to amplify exon 9 of the *SH3BP2* gene from the samples in a final volume of 50 μ l that contained 10 picomoles each of sense (5-CCGCCCCGTGTCTGACAGTGAAAT-3) and antisense (5-TTCCCACCACCTGTCCACCTACTG-3) primers (idtdna, Coralville, IA), 2 units of *Taq* polymerase (Perkins Elmer) and standard buffer conditions. After an initial denaturation at 94^o C for 3 minutes, samples were amplified by 35 cycles at 94^o C for 30 seconds, 60^o C for 30 seconds, and 72^o C for 40 seconds, followed by a final extension at 72^o C for 5 minutes. PCR products were isolated and sequenced by Thermosequenase terminator cycle sequencing (USB, Swampscott, MA, www.amersham.com) with the sense primer and a 60^o C annealing temperature.

Site directed mutagenesis

We obtained a hSH3BP2 cDNA in the pCMV10 plasmid as a generous gift from Dr. Bruce Mayer (University of Connecticut) (GenBank RefSeq-file accession number BC022996). We created the novel mutation that we discovered (p.D419N) as well as the most common mutations previously described in other cherubism patients (p.R415Q, p.D420E, and p.P418R)(Ueki et al. 2001) using site-directed mutagenesis with overlapping primers. The sequence was confirmed by direct sequencing of PCR products. We then cloned the wild-type and mutant SH3BP2cDNA's into the EcoR1/Sal1 site of the pMT3 vector (a generous gift of Dr. Marcel Deckert)(Deckert et al. 1998; Foucault et al. 2003).

Expression and analysis of SH3BP2

Wild-type or mutant SH3BP2 cDNA's were transiently transfected into Jurkat TAg cells in the presence of a luciferase reporter gene NFAT-luc, that contains 3 copies of the minimal IL-2 promoter that includes an NFAT binding site and a control reporter gene that constitutively expresses Renilla luciferase (Graef et al. 2001) (Promega, Madison, WI). Cells were transfected by electroporation using a nucleofector (Amaxa, Cologne,

Germany), and conditions were optimized to the T01 program using fluorescent microscopy to evaluate the percent of cells that expressed green fluorescent protein reporter plasmid (Promega). Cells were lysed 24 hours after transfection and luminescence was evaluated using a Zylux luminometer (10 second reading time) according to the Dual Luciferase Reporter assay system (Promega).

Immunoblot analysis

Whole cell lysates were assayed for total protein using BCA reagent (Bio-Rad, Hercules, CA) and equal amounts of protein were electrophoresed through 4-12% bis-tris polyacrylamide gels at 200 volts for 1 hour in MOPS buffer (Invitrogen, Carlsbad, CA). The gel was stained with Coomassie blue stain to verify lane loading. The resolved proteins were electrophoretically transferred from the polyacrylamide gel to a PVDF membrane, and non-specific binding was blocked by overnight incubation with 5% non-fat dried milk (Carnation) at 4°C. PVDF membranes were incubated with a specific polyclonal antiserum to SH3BP2 (Rockland Immunochemicals, Gilbertsville, PA, 1:2000) in 5% non-fat dried milk, and with an antiserum to β actin (1:2000 in 5% nonfat dried milk) as a control to evaluate protein loading. Antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham, Piscataway, N.J.). The image was imported into the ImageJ program (Bethesda, MD, <http://rsb.info.nih.gov/ij>) and each band was analyzed for intensity.

RESULTS

We identified a novel guanine to adenine transition that predicts the non-conservative replacement of aspartic acid (GAT) with asparagine (AAT) at codon 419 of *SH3BP2* (p.D419N) in a patient with typical features of cherubism (Fig. 1). This mutation occurs within the six amino acid domain in exon 9 of *SH3BP2* in which all previously described mutations in patients with cherubism have been identified. Molecular genetic analysis of the proband's parents and two siblings demonstrated that the father and both sibs were carriers of the g.1371G>A *SH3BP2* allele but lacked clinical features of cherubism, consistent with incomplete penetrance or variable expressivity of the mutation (Fig. 1c). However, as we were unable to obtain radiographs of the jaws of these carriers, it is not possible to exclude variable expressivity of the mutation and it is conceivable that these carriers manifest very mild features of cherubism. Nevertheless, the difference in the clinical presentation between the proband and his carrier father and siblings is striking.

To analyze the effect of the cherubism mutations on SH3BP2 function we transiently co-expressed in Jurkat TAG cells cDNA's encoding wild-type human SH3BP2 (GenBank number BC022996) or four different mutant forms of human SH3BP2 (p.D419N, p.R415Q, p.D420E, and p.P418R) plus NFAT-luc, a reporter gene in which expression of luciferase is under the control of a selective NFATc1/c2 binding site, and Renilla-luc, a reporter that constitutively expresses Renilla luciferase and therefore can serve as a control for transfection efficiency. (Deckert et al. 1998 ;Foucault et al. 2003) We assessed luciferase activity and SH3BP2 protein expression 24 hours after transfection. Mutant SH3BP2 proteins consistently induced 2-3 fold greater luciferase activity than wild-type SH3BP2 (Fig. 2) in four separate experiments but immunoblot analysis showed similar levels of the wild-type and mutant SH3BP2 recombinant proteins (Fig. 2). These results provide strong evidence that the *SH3BP2* mutations are activating, and presumably result in enhanced dephosphorylation and translocation of the NFATc1/c2 protein(s) to the cell nucleus (Hirovani et al. 2004).

DISCUSSION

SH3BP2 binds to a variety of cytosolic proteins, including LAT, Syk and I κ B, and is an integral component of a signaling pathway that activates NFAT. (Foucault et al. 2003) The NFATc1 isoform (also termed NFAT2) has been proposed to be the master transcription factor for osteoclastogenesis and experimental evidence supports a critical role for the protein in osteoclast development. For example, overexpression of a constitutively active form of NFATc1 in RAW264.7 cells induces osteoclastogenesis. (Hirovani et al. 2004) Moreover, NFATc1 is the most highly induced gene after treatment of murine bone marrow-derived monocyte/macrophage precursor cells with RANKL (receptor activator of NF κ B ligand), which stimulates osteoclastogenic differentiation. (Takayanagi et al. 2002) Although it is still unclear which cell (or cells) is responsible for the resorptive giant cell lesions in cherubism, we evaluated SH3BP2 activity in Jurkat TAG cells, as T cells utilize NFAT signaling and provide an important model for understanding SH3BP2 action. (Foucault et al. 2003) Moreover, T cells secrete RANKL (Kong

et al. 1999 ;Kong et al. 1999), indicating that these cells and this molecule are important for coordination of the interplay between the fields of bone biology and immunology (*i.e.*, osteoimmunology)(Takayanagi,2005).

In contrast to our results, Miah et al. (Miah et al. 2004) found that overexpression of murine SH3BP2 R413Q, P416H and G418R (which correspond to R415Q, P418H, and G420R in human SH3BP2) in ret RBL-2H3 mast cells led to suppression of degranulation, decreased activation of Vav1 and Rac1 and suppression of cytokine synthesis and Lyn activation. These authors thus concluded that *SH3BP2* mutations in patients with cherubism result in a loss of function. It is conceivable that these missense mutations in fact have both activating and inhibiting effects depending upon the cell type or biological effect examined. It is also conceivable that there is species variability not only in sequence but also in function between the mouse SH3BP2 cDNA that they used and the human SH3BP2 cDNA that we used. Nevertheless, our results using human SH3BP2 support a gain of function that leads to osteoclast activation, and are consistent with the lesions that produce the clinical phenotype of cherubism. Although our studies show that *SH3BP2* mutations in cherubism behave as activators of NFAT, it remains unclear how *SH3BP2* mutations cause such site-specific development of giant cell tumors in cherubism, or why the phenotype regresses after puberty. Finally, we found evidence for incomplete penetrance or variable expressivity of the *SH3BP2* mutation g.1371G>A which suggests that unidentified genes or other factors may modulate the effect(s) of this mutation on the development of cherubism. Further genotype-phenotype associations in extended cherubism pedigrees may disclose the basis for the variability in the bone lesions and may provide important new insights into the molecular biology of bone resorption.

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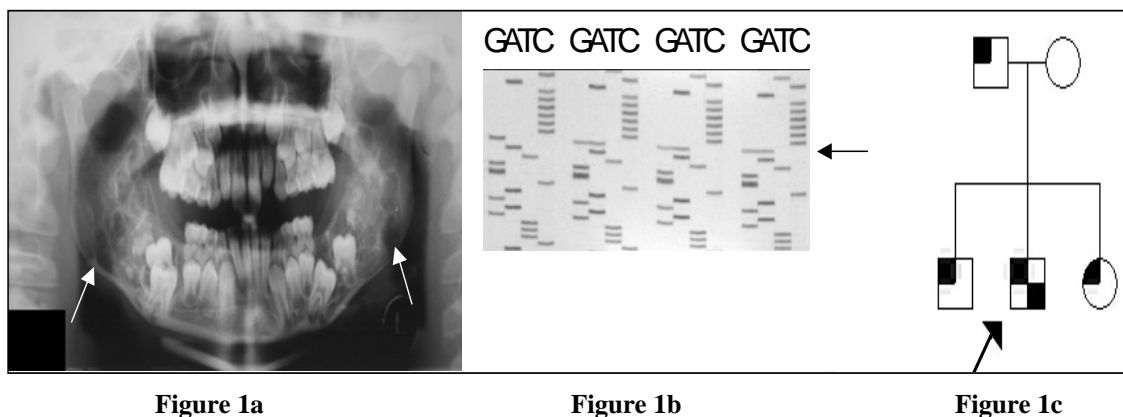


Figure 1. **a:** Panorex radiograph of the proband's mandible and maxilla showing typical features of the symmetrical osteolytic bone lesions (arrows) seen in patients with cherubism. **b:** Sequence analysis of exon 9 of the *SH3BP2* gene. The guanine to adenine transition is in one of two alleles in lanes 2, 3, and 4. The arrow in lane 4 indicates the proband's mutation. Lanes 2 and 3 represent DNA sequence from two unaffected family members who carry the same mutation, whereas lane 1 represents DNA sequence from the proband's unaffected mother. **c:** Pedigree analysis. The family pedigree demonstrates incomplete penetrance (or variable expressivity) of the *SH3BP2* mutation g.1371G>A with only the proband manifesting clinical features of cherubism. The institutional review board approved this protocol and written informed consent was obtained from the parents and children. The *SH3BP2* g.1371G>A mutation is represented by \square . The cherubism phenotype is represented by \blacksquare . The proband is indicated by \nearrow .

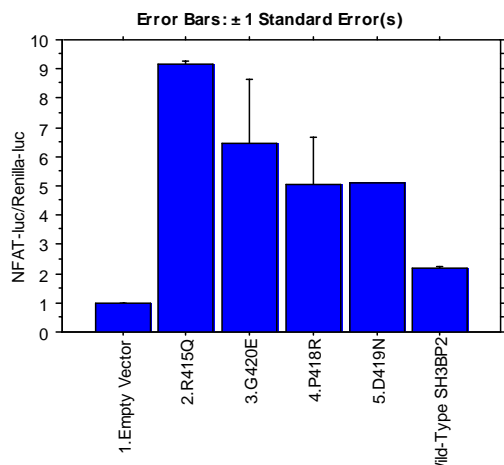


Figure 2a

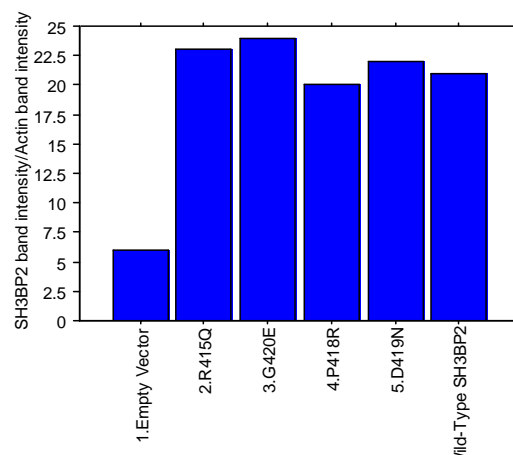


Figure 2b

Figure 2. SH3BP2 proteins with the mutations found in cherubism patients are more active, in terms of NFAT activation, than the wild-type SH3BP2 protein. Figure 2a shows the image analysis of the band intensity of the various forms of SH3BP2 (normalized by β -actin) from an immunoblot of total cell lysate from co-transfected Jurkat TAg cells. β -actin, was used as a control for loading equivalence. Figure 2b shows that SH3BP2 p.D419N and other human SH3BP2 mutants stimulate transcriptional activity of the NFAT-luc reporter gene to a greater extent than the wild-type human SH3BP2 or the empty vector. These results are representative of four separate experiments and the error bars represent standard error of the mean. The graph demonstrates relative luciferase activity in Jurkat T Ag cells after co-transfection using nucleofection with an NFAT-luciferase reporter gene plus cDNA's encoding either wild-type (GenBank number BC022996) or mutant forms of SH3BP2. Legend: EV represents the empty vector, R415Q represents the p.R415Q (c.1244G>A) SH3BP2 cDNA, G420E represents the p.G420E (c.1259G>A) SH3BP2 cDNA, P418R represents the p.P418R (c.1253C>G) SH3BP2 cDNA, D419N represents the p.D419N (c.1255G>A) SH3BP2 cDNA, and WT represents the wild-type SH3BP2 cDNA.

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