Case Report

Multiple Fractures in a Patient with Mutations of *TWIST1* and *TNSALP*

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Abstract Hypophosphatasia is a rare inherited disorder characterized by defective skeletal mineralization and low alkaline phosphatase activities in the serum. The genetic cause of hypophosphatasia is believed related to inactivating mutations in the *TNSALP* gene, encoding tissue-nonspecific alkaline phosphatase. Another rare inheritable disease, Saethre-Chotzen syndrome, leads to premature fusion of the cranial sutures caused by heterozygous mutations of the human *TWIST1* gene. Because the two disorders apparently are not genetically related (only reported individually) yet both involve defective skeletal formation, we believe it is important to report our findings on a patient harboring mutations of *TNSALP* and *TWIST1*.

Introduction

Bone matrix formation and mineralization is a coordinated process involving the activity of several gene products expressed by osteoblasts [9]. Research using genetically

modified mouse models and the molecular analysis of inherited human diseases has uncovered an increasing number of genes whose mutation can result in skeletal defects [13]. These include the transcription factor *TWIST1*, a negative regulator of bone formation, and tissue-nonspecific alkaline phosphatase (*TNSALP*), a positive regulator of bone matrix mineralization [7, 12, 19]. Although the physiologic importance of both molecules has been shown through the identification of disease-causing mutations leading to Saethre-Chotzen syndrome and hypophosphatasia, respectively, their molecular mechanisms of action have been revealed mostly through combining the respective mouse deficiency models with other mutant mouse strains.

For instance, the antiosteogenic function of *Twist1* could be explained by an inhibitory interaction with the transcription factor *Runx2*, which is considered the master regulator of osteoblast differentiation [15]. Likewise, while *Twist1* +/– mice display craniosynostosis and *Runx2* +/– mice show delayed closure of the fontanels, the compound heterozygosity of both genes leads to normal calvarial development [4]. In the case of *Tnsalp*, a molecular mechanism of action involving the cleavage of pyrophosphate was uncovered by the observation that the bone mineralization defects of the *Tnsalp*-deficient mice were normalized by the additional absence of either *Enpp1* or *Ank*, two enzymes raising the extracellular concentrations of pyrophosphate [10, 11]. These examples show a combination of two genetic defects affecting osteoblast differentiation or function can be informative, especially when the observed phenotypes are not fully penetrant with one gene defect alone. Therefore, we report the case of a patient with combined mutations of *TWIST1* and *TNSALP*, because these mutations have been described only separately.

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Each author certifies that his or her institution has approved the reporting of this case report, that all investigations were conducted in conformity with ethical principles of research, and that informed consent for participation in the study was obtained.

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Case Report

A 33-year-old man was admitted to our clinic after a periimplant fracture of his right femoral shaft distal to the tip of an implanted intramedullary nail (Fig. 1A). To prevent muscle atrophy and other complications we extracted all the metal from the right femur in one session and implanted a long intramedullary nail for internal fixation (Fig. 1B). A previously recognized pseudofracture of the left femoral neck (Fig. 2A–C) was stabilized using two cannulated screws (Fig. 2D). The patient was discharged after 3 weeks and sent to a rehabilitation center.

The patient was referred to our clinic owing to multiple fractures. The patient had a history of 18 pathologic fractures since his childhood. Because of swelling of the fontanel, he was examined by cranial radiography at the age of 1 year, which led to the diagnosis of craniosynostosis. Although the sagittal suture of the cranium was surgically reopened to lower the intracranial pressure at that point, the turricephalus of the patient was still prominent when he came to our clinic at the age of 33 years. Four years previously, radiographs of the right hip were obtained because of persisting pain. This revealed the presence of Looser's zones in the right femoral neck (Fig. 3A). Consequently, bone scintigraphy was performed to check the rest of the skeleton. It showed Looser's zones in the left subtrochanteric femur, the right tibial shaft, and the right ankle, none of

which were treated surgically (Fig. 3B–D). To decrease the fracture risk, the patient was treated with ibandronate thereafter, which led to an increase of bone mineral density from 0.73 to 0.793 (representing improvement of the T-score from -3.3 to -2.7) within the first 2 years, without occurrence of additional fractures. Because the medication had been stopped approximately 2 months before the subtrochanteric fracture of the right proximal femur, we recommended restarting aminobisphosphonate. On examination, he had a high forehead with a narrow sloping vertex region and a minor ptosis (Fig. 4A). Moreover, the brow ridges were atrophied, and the lid gaps pointed in a caudal-lateral direction, as described for Saethre-Chotzen syndrome [7, 12]. The patient was 1.68 m tall, and both tibiae displayed valgus angulation in the frontal plane and a curved deformity in the sagittal plane, thereby resulting in impaired walking ability (Fig. 4B–C).

Because the skeletal abnormalities of the patient had never been analyzed histologically, we did a bone biopsy during the surgical procedure. The specimen was obtained from a nonweightbearing part of the trochanteric region of the right proximal femur, where no lesions were observed on the preoperative radiographs. After fixation in buffered formalin, the specimen was embedded undecalcified in methylmethacrylate [1]. Sections of 5- μ m thickness were analyzed using von Kossa/van Gieson or Goldner staining [2]. With both stains, we observed an increase in osteoid volume (14.5%) and osteoid surface (45.3%) (Fig. 5).

To determine whether the observed defects of bone mineralization were caused by impaired activity of alkaline phosphatase (AP), we measured the total AP activity and the bone-specific AP activity (ostase) in the serum of the patient and found both parameters were low (Table 1). Accordingly, serum concentrations of pyridoxalphosphate, a physiologic substrate of AP [20], were abnormally high in the patient, suggesting hypophosphatasia as a cause of the skeletal mineralization defects. In contrast, the serum concentrations of osteocalcin, parathyroid hormone, 1,25-dihydroxycholecalciferol, and 25-hydroxycholecalciferol were in the normal range (Table 1).

Based on the examinations described to this point, we reasoned the patient might have an unusual combination of two inherited diseases, Saethre-Chotzen syndrome and hypophosphatasia. Because the affected genes have been identified in both cases, we performed sequence analysis of the coding regions of the genes encoding the basic helix-loop-helix transcription factor *TWIST1* and tissue-nonspecific alkaline phosphatase (*TNSALP*), respectively. Genomic DNA was extracted from peripheral blood leukocytes derived from the patient and an unrelated healthy individual. The coding region of the *TWIST1* gene, located on chromosome 7, was amplified by polymerase chain reaction (PCR) (for primer sequences) (Table 2). To

Fig. 1A–B (A) The patient sustained a fracture distal to the tip of an implanted femur nail (gamma nail). (B) Therefore, our primary surgical intervention led to internal fixation using a long intramedullary femur nail.

Fig. 2A–D A radiographic analysis over time shows the history of a pseudo-fracture in the patient's left femoral neck. **(A)** An earlier radiograph shows no pseudo-fracture. **(B)** A radiograph (anteroposterior view) taken 14 months before admission shows the first signs of a pseudo-fracture (white arrow) in the left femoral neck. **(C)** A radiograph (anteroposterior view) taken after admission shows the pseudo-fracture (white arrow) now affecting the whole diameter of the left femoral neck. **(D)** The pseudo-fracture was stabilized using cannulated screws.

improve the sequencing results, the PCR products were subcloned into the plasmid pCR2.1-TOPO (Invitrogen Corp, Carlsbad, CA), thereby enabling us to analyze individual clones using common sequencing primers (M13 forward and reverse). Whereas all clones derived from the healthy individual corresponded to the wild-type *TWIST1* sequence, we observed a 3-bp insertion of GGC in 50% of the clones derived from the patient, indicative of a heterozygous mutation of the *TWIST1* gene as a cause of the craniosynostosis (Fig. 6). In the case of the *TNSALP* gene, located on chromosome 1, direct sequencing of PCR products was possible because only point mutations were observed. These were found in exon 6 and exon 9, where the patient showed heterozygous transitions from G to A and from A to G, respectively (Fig. 7).

Discussion

We describe a patient with a combination of hypophosphatasia and Saethre-Chotzen syndrome. The patient had a diagnosed craniosynostosis at 1 year of age. The severity

of the patient's disease is reflected by his history of at least 18 pathologic fractures since childhood. Our examination revealed several aspects of hypophosphatasia, ie, a pathologic enrichment of nonmineralized osteoid, low serum activities of total and bone-specific AP, and high circulating levels of pyridoxalphosphate. Moreover, the patient's head was cylindrically shaped, and he had a remarkable exophthalmia of both eyes with additional ptosis. The suspected combination of hypophosphatasia and Saethre-Chotzen syndrome was confirmed genetically by detection of three heterozygous mutations in the genes encoding *TNSALP* and *TWIST1*, respectively. To our knowledge, these two inherited diseases have been described only individually so far, and in both diseases, several mutations have been identified, thereby explaining variability of the clinical expression.

The first mutation (E174 K) we found in our patient was located in the active site of *TNSALP*. Although the E174 K mutation is actually the most frequent *TNSALP* mutation known in the Caucasian population, it is found only in moderate forms of hypophosphatasia [16]. This is explained by the finding that the E174 K mutation only

Fig. 3A–D The comparison of radiographs from selected regions of the skeleton ((A) proximal femur, (B) tibia, (C) distal fibula) with the results of the (D) bone scintigraphy revealed several pseudofractures in the patient before admission to our clinic.

leads to a marginal decrease of the enzymatic activity of *TNSALP* [21]. Moreover, the sequence comparison of *TNSALP* and placental alkaline phosphatase (PLAP), another member of the AP family, revealed glutamate 174 is not conserved in PLAP, where an aspartate residue is located in the corresponding position [17]. However, the combination of the heterozygous E174 K mutation with another heterozygous *TNSALP* mutation (M45I) has been found in a case of childhood hypophosphatasia [18]. Therefore, we reasoned the childhood hypophosphatasia in our patient might have been caused by the combination with the second mutation (M278V) found in exon 9.

Although the M278V mutation has been described in one previous report, its contribution to the clinical outcome of the disease has not been analyzed [17]. However, mutations leading to replacement of the adjacent amino

Fig. 4A–C (A) The patient was characterized by a turriccephalus indicative of craniosynostosis. Moreover, the (B) a.p.-radiograph and the (C) lateral radiograph of the left lower leg revealed obvious skeletal abnormalities such as a saber-shaped tibia.

acid aspartate 277 by alanine or tyrosine (D277A and D277Y) have been associated with severe forms of hypophosphatasia [17]. The region surrounding M278 has been suggested to serve as a binding site for calcium ions that is critical for the enzyme activity. This again was based on the sequence comparison between *TNSALP* and PLAP, because a crystal structure was available for the latter [14]. Moreover, the analysis of the *TNSALP* activity in vitro revealed the D277A mutation, although not influencing the cleavage of the artificial substrate p-nitrophenylphosphate, led to a marked reduction of the pyrophosphatase activity [5]. Because aspartate 277 and methionine 278 are conserved between *TNSALP* and PLAP, it is likely the heterozygous M278V mutation found in our patient, together with the E147K mutation, caused his childhood hypophosphatasia.

Because the second aspect of the patient's disease was premature fusion of the cranial sutures, we reasoned this might have been caused by heterozygous mutations of the human *TWIST1* gene, indicative of Saethre-Chotzen syndrome [7, 12]. Moreover, because the patient had been screened for *FGFR1* and *FGFR2* mutations previously and because we also could rule out the presence of the Pro250Arg mutation in the *FGFR3* gene, which also can result in autosomal dominant craniosynostosis [3], we believe identification of a *TWIST1* mutation in the patient provides the required genetic evidence for the diagnosis of a Saethre-Chotzen syndrome. The *TWIST1* gene encodes a transcription factor with 202 amino acids harboring a basic helix-loop-helix DNA-binding motif in the C-terminal part of the protein, whereas the N-terminal part of the molecule

Fig. 5A–D Von Kossa/van Gieson and Goldner staining of undecalcified sections from a bone biopsy specimen taken from the right greater trochanter revealed a severe mineralization defect. The amount of osteoid (stained in red) is pathologically enriched (A: Stain, von Kossa/van Gieson; original magnification, $\times 12.5$; B: Stain, Goldner; original magnification, $\times 12.5$; C: Stain, von Kossa/van Gieson; original magnification, $\times 200$; D: Stain, Goldner; original magnification, $\times 200$).

Table 1. Serum levels of bone metabolism parameters

Parameter	Patient	Normal Range
Total alkaline phosphatase (U/L)	24*	40–129
Bone-specific alkaline phosphatase ($\mu\text{g/L}$)	3.1*	8.0–16.8
Pyroxidalphosphate (ng/mL)	40.0*	5.0–20.0
Osteocalcin ($\mu\text{g/L}$)	5.7	3.1–13.7
Parathyroid hormone (ng/L)	23	15–65
1,25-Dihydroxycholecalciferol ($\mu\text{g/L}$)	55	20–67
25-Hydroxycholecalciferol ($\mu\text{g/L}$)	18	10–68
Serum calcium (mg/dL)	9.1	8.5–10.2
Serum phosphorus (mg/dL)	3.2	2.1–4.1

* Indicates patient values different from normal range.

Table 2. Primers used for polymerase chain reaction amplification

Gene	Amplified region	Primer sequences
<i> Twist1 </i>	Exon 1	5'-AATGACACTGCTGCCCCAAAC-3' 5'-GCTGACAAAACGGTCCTTACCC-3'
<i> TNSALP </i>	Exon 6	5'-TTGTGACCACCACGAGAGTGAAC-3' 5'-CCAACCGCAAATCCCCTAATG-3'
<i> TNSALP </i>	Exon 9	5'-GCCACAAAAATCACCCAGATAAG-3' 5'-CCGTCTCCTTCCACAACCTATTC-3'

has been shown to interact with the histone acetyltransferase p300 [8]. A study using a haploinsufficient *Twist1*^{+/-} mouse model has established an antiosteogenic function of Twist1, which is mediated through inhibition of the osteoblast-specific transcription factor Runx2 [4].

Fig. 6 Sequencing of the *Twist1* gene revealed 50% of the PCR products amplified from the patient's genomic DNA had an insertion of GGC resulting in an additional glycine residue. Blue line = cytosine (C); red line = thymine (T); black line = guanine (G); green line = adenine (A).

Fig. 7 Two heterozygote point mutations were found in the patient's *TNSALP* gene. The first in exon 6 leads to replacement of glutamate by lysine and the second in exon 9 to replacement of methionine by valine. Blue line = cytosine (C); red line = thymine (T); black line = guanine (G); green line = adenine (A).

The patient we describe harbors a 3-bp insertion that affects a glycine-rich sequence, (Gly)₅Ala(Gly)₅, of as-yet-unknown function, representing the amino acids 82–92. In fact, the mutation found in the patient leads to an extension of this sequence, (Gly)₆Ala(Gly)₅, and therefore represents a novel mutation of the *TWIST1* gene that is associated with Saethre-Chotzen syndrome. However, the same mutation on the protein level was found in a nonaffected individual, and because other mutations in the glycine-rich sequence are not consistently associated with the disease, it was concluded rearrangements of the glycine-rich sequence of the *TWIST1* protein are only weakly pathogenic [6].

Taken together, our data provide the first reported example for a patient with combined hypophosphatasia and Saethre-Chotzen syndrome, based on clinical findings and genetic analysis. Although this observation likely reflects only a rare coincidence, we believe it is important to report these findings because it is possible the combination of two gene defects might result in additional phenotypes that are not observed by the absence of either gene alone. It is possible the severe phenotype of the patient, especially his craniosynostosis, might be caused by the coexistence of *TWIST1* and *TNSALP* mutations because the extension of the glycine-rich region in the *TWIST1* protein should, if anything, result in a mild form of Saethre-Chotzen syndrome. Mechanistically, one could imagine a defect of bone mineralization in the craniofacial skeleton could trigger an additional increase of bone matrix deposition and thereby amplify the craniosynostosis phenotype. Currently, however, we can only speculate whether the two genetic defects influence each other, but clarification probably would be possible by combining the corresponding mouse deficiency models.

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References

1. Amling M, Herden S, Pösl M, Hahn M, Ritzel H, Delling G. Heterogeneity of the skeleton: comparison of the trabecular microarchitecture of the spine, the iliac crest, the femur, and the calcaneus. *J Bone Miner Res*. 1996;11:36–45.
2. Amling M, Priemel M, Holzmann T, Chapin K, Rueger JM, Baron R, Demay MB. Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. *Endocrinology*. 1999;140:4982–4987.
3. Bellus GA, Gaudenz K, Zackai EH, Clarke LA, Szabo J, Francmano CA, Muenke M. Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. *Nat Genet*. 1996;14:174–176.
4. Bialek P, Kern B, Yang X, Schrock M, Sosic D, Hong N, Wu H, Yu K, Ornitz DM, Olson EN, Justice MJ, Karsenty G. A twist code determines the onset of osteoblast differentiation. *Dev Cell*. 2004;6:423–435.
5. Di Mauro S, Manes T, Hessle L, Kozlenkov A, Pizauro JM, Hoylaerts MF, Millán JL. Kinetic characterization of hypophosphatasia mutations with physiological substrates. *J Bone Miner Res*. 2002;17:1383–1391.
6. Elanko N, Sibbring JS, Metcalfe KA, Clayton-Smith J, Donnai D, Temple IK, Wall SA, Wilkie AO. A survey of *TWIST* for mutations in craniosynostosis reveals a variable length polyglycine tract in asymptomatic individuals. *Hum Mutat*. 2001;18:535–541.
7. el Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Bourgeois P, Bolcato-Bellemin AL, Munnich A, Bonaventure J. Mutations of the *TWIST* gene in the Saethre-Chotzen syndrome. *Nat Genet*. 1997;15:42–46.
8. Hamamori Y, Sartorelli V, Ogryzko V, Puri PL, Wu HY, Wang JY, Nakatani Y, Kedes L. Regulation of histone acetyltransferases

- p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell*. 1999;96:405–413.
9. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature*. 2003;423:349–255.
 10. Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millán JL. Concerted regulation of pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. *Am J Pathol*. 2004;164:1199–1209.
 11. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-non-specific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci USA*. 2002;99:9445–9449.
 12. Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz de Luna RI, Garcia Delgado C, Gonzalez-Ramos M, Kline AD, Jabs EW. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nat Genet*. 1997; 15:36–41.
 13. Kornak U, Mundlos S. Genetic disorders of the skeleton: a developmental approach. *Am J Hum Genet*. 2003;73:447–474.
 14. Le Du MH, Stigbrand T, Taussig MJ, Menez A, Stura EA. Crystal structure of alkaline phosphatase from human placenta at 1.8 Å resolution: implication for a substrate specificity. *J Biol Chem*. 2001;276:9158–9165.
 15. Lian JB, Stein GS. Runx2/Cbfa1: a multifunctional regulator of bone formation. *Curr Pharm Des*. 2003;9:2677–2685.
 16. Mornet E. Hypophosphatasia: the mutations in the tissue-nonspecific alkaline phosphatase gene. *Hum Mutat*. 2000;15:309–315.
 17. Mornet E, Stura E, Lia-Baldini AS, Stigbrand T, Menez A, Le Du MH. Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization. *J Biol Chem*. 2001;276:31171–31178.
 18. Taillandier A, Sallinen SL, Brun-Heath I, De Mazancourt P, Serre JL, Mornet E. Childhood hypophosphatasia due to a de novo missense mutation in the tissue-nonspecific alkaline phosphatase gene. *J Clin Endocrinol Metab*. 2005;90:2436–2439.
 19. Whyte MP. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. *Endocr Rev*. 1994;15:439–461.
 20. Whyte MP, Landt M, Ryan LM, Mulivor RA, Henthorn PS, Fedde KN, Mahuren JD, Coburn SP. Alkaline phosphatase: placental and tissue-nonspecific isoenzymes hydrolyze phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5'-phosphate: substrate accumulation in carriers of hypophosphatasia corrects during pregnancy. *J Clin Invest*. 1995;95: 1440–1445.
 21. Zurutuza L, Muller F, Gibrat JF, Taillandier A, Simon-Bouy B, Serre JL, Mornet E. Correlations of genotype and phenotype in hypophosphatasia. *Hum Mol Genet*. 1999;8:1039–1046.