An Unbalanced Rearrangement of Chromosomes 4:20 is Associated with Childhood Osteoporosis and Reduced Caspase-3 Levels

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Abstract

The purpose of this study was to investigate the association of a chromosome 4:20 imbalance with osteoporosis in three related children. Bone biochemistry, bone turnover markers, and dual-energy X-ray absorptiometry (DXA) scanning were performed in all three cases and bone biopsy and histomorphometry in one. The chromosome imbalance was delineated by array comparative genomic hybridization (aCGH) and analyzed for candidate genes. A potential candidate gene within the deleted region is caspase-3, previously linked to low bone mineral density (BMD) in heterozygous mice thus caspase-3 activity was measured in cases and controls. Routine bone biochemistry and markers of bone turnover did not reveal any abnormality. DXA showed reduced total and lumbar spine bone mineral content. aCGH showed an 8 megabase (Mb) deletion of terminal chromosome 4q incorporating a region previously linked to low BMD and a 15 Mb duplication of terminal chromosome 20p. Bone biopsy showed a high bone turnover state, trabecularisation of cortical bone and numerous small osteoclasts coupled with normal bone formation. Basal serum caspase-3 activity was lower in cases compared with controls. We conclude that the early-onset osteoporosis with low basal levels of caspase-3 and abnormal osteoclasts is a feature of this chromosomal translocation. Further investigation of the role of the deleted and duplicated genes and especially caspase-3 is required.

Keywords

- ▶ osteoporosis
- ▶ chromosome translocation
- ▶ bone histomorphometry
- ▶ osteoclast
- ▶ caspase

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Introduction

Although there has been significant progress in identifying genes that contribute to the variation in bone mineral density (BMD) in the population, it is likely that a large number remain unidentified.¹ Studying individuals with early onset osteoporosis and chromosomal aberrations may provide further etiological insight. We describe three children with osteoporosis and an unbalanced chromosomal translocation resulting in a 4q deletion and a 20p duplication. The aim of the study was to understand the nature of the bone pathology in this syndrome and investigate whether it was related to a disorder of bone formation or resorption to elucidate new mechanisms important in bone homeostasis. Ultimately, this may allow development of targeted drugs for the treatment of osteoporosis. Bone histomorphometry showed a state of high bone turnover with normal osteoblasts but an increase in immature osteoclasts. The caspase-3 gene lies within the deleted chromosome region, of note, because heterozygous caspase-3 deficient mice have low BMD.² The affected children showed a functional deficiency of caspase-3 activity, a state of high bone turnover with normal osteoblasts and an increase in immature osteoclasts thus highlighting the role of caspase-3 in bone development.

Methods

Case History

Case 1—A 12-year-old girl had a fracture of the radius at the age of three years and six subsequent fractures. A lumbar scoliosis and osteopenia were noted on X-ray at the age of 10 years. A leg length inequality necessitated epiphysiodesis and bone biopsy was performed.

Case 2—An 18-year-old girl, who is the older sister of case 1, sustained a fracture of the femur at the age of three years and four further fractures with minimal trauma. She was treated with weekly oral risedronate 35 mg for 2 years from the age of 11 years.

Case 3—A 17-year-old boy, who is the cousin of cases 1 and 2, sustained his first fracture of the radius and ulna at the age of three years and three further forearm fractures.

All three children had vesicoureteric reflux, moderate learning disability, and sensorineural deafness and the two sisters had ventricular septal defects. Clinical features are summarized in ► **Table 1**.

Table 1 Table summarizing clinical features of subjects

Clinical features	Patient 1	Patient 2	Patient 3
Ventricular septal defect	+	—	+
Vesico-ureteric reflux	+	+	+
Deafness	—	+	+
Learning difficulty	+	+	+
Recurrent fractures	+	+	+
Risedronate treatment	+	—	—

Biochemical Assessment

A blood sample was obtained from the three cases for determination of serum calcium, phosphate, alkaline phosphatase, parathyroid hormone, 25-hydroxy vitamin D levels, and markers of bone turnover including bone-specific alkaline phosphatase (BALP), osteocalcin, and C-terminal telopeptide of type I collagen (CTX). Markers of bone turnover were measured by enzyme-linked immunosorbent assay using commercially available kits (Immunodiagnostic Systems Ltd, Boldon, United Kingdom). A random urine sample was obtained for determination of urinary calcium/creatinine ratio.

Bone Densitometry

Dual-energy X-ray absorptiometry (DXA) scan (Lunar Prodigy) was performed to assess bone density. Bone mineral content (BMC) values were calculated for bone area, as previously described.³ Tibial volumetric BMD and surrogate markers of bone strength were determined by peripheral quantitative computed tomography (pQCT) using the Stratec XCT-2000 equipment (Stratec, Burton upon Trent, United Kingdom) at the 4% and 66% site and stress-strain index (SSI; mm³) at the 38% site.

Bone Biopsy and Histomorphometry

Iliac crest bone biopsy was performed on case 1 at the time of a clinical epiphysiodesis. Separate stains were performed with tartrate resistant acid phosphatase (TRAP) counterstained with aniline blue and van Gieson/von Kossa. Bone histomorphometry parameters were measured using custom software and compared with reference values for pediatric samples.^{4,5} Ultrastructural studies on iliac crest trephine biopsy were performed and the sections examined for abnormalities in osteoblasts and osteoclasts.

Genetic Analysis

Array comparative genomic hybridization was performed using a Bluegenome Cytochip v1.1 bacterial artificial clone array with confirmation of deletion/duplication using fluorescent in-situ hybridization (FISH).

Caspase-3 Protein Levels and Activity

Caspase-3 protein and activity levels were measured in patients and age-matched controls in triplicate. Cells remained untreated or were treated with doxorubicin (Calbiochem) to induce apoptosis and caspase-3 activity. Total protein was isolated from cases and controls pretreatment and poststimulation. Total caspase-3 protein levels were analyzed by Western blotting and caspase-3 activity measured using Apo-One Caspase-3/7 activity kit (Promega, Madison, United States) and results adjusted for total protein.

Results

Biochemistry and Bone Densitometry

Biochemical analysis revealed no abnormality of bone mineral homeostasis. Analysis of markers of bone turnover showed that bone formation (BALP and osteocalcin) and bone resorption (CTX) was suppressed in case 2, previously treated with

Table 2 Table showing markers of bone turnover and DXA scan results

	Patient 1	Patient 2	Patient 3
Bone ALP ($\mu\text{g/L}$) (age matched 10 th -90 th centile)	66 (32–118)	20 (32–118)	109 (61–86)
Osteocalcin ($\mu\text{g/L}$) (age-matched 10 th -90 th centile)	66 (80–191)	23 (80–191)	81 (62–131)
CTX ($\mu\text{g/L}$) (age-matched 10 th -90 th centile)	1.9 (2.0–3.7)	0.5 (2.0–3.7)	2.9 (1.6–2.8)
DXA TB BMC SDS for bone area	–0.7	–0.2	–0.6
Z score age-matched total body less head	–1.6	0.6	–0.7
DXA LS BMC SDS for bone area	–0.1	–1.3	–1.2
Z score age-matched lumbar spine	–1.6	–1.5	–1.9
pQCT vBMD SDS – 4% site	–1.5	–1.5	–2.5
pQCT vBMD SDS – 66% site	–1.8	0.2	–3.6

bisphosphonates. In the other two cases, there was no consistent abnormality. DXA analysis revealed low BMC standards deviation score (SDS), marked in cases 2 and 3, and low Z scores. PQCT analysis of volumetric BMD at the 4% site reflecting trabecular bone was reduced in all 3 cases. In cases 1 and 3, volumetric BMD was reduced at the 66% site reflecting loss of cortical bone. This reduction was not apparent in case 2 after 2 years of risedronate treatment (►Table 2.).

Bone Histology and Histomorphometry

Osteoclast numbers were analyzed on sections stained for TRAP. These revealed many small osteoclasts lining the trabeculae, accompanied by osteoblasts producing osteoid (►Fig. 1A, B). Histological analysis suggested decreased trabecular connectivity and cortical trabecularisation. Histomorphometry parameters showed the most marked changes being osteoclast surface/bone surface (Oc.S/BS), osteoclast surface/tissue volume (Oc.S/TV), and number of osteoclasts/bone perimeter (N.Oc/B.Pm) markedly increased compared with the reference range (►Table 3). The osteoclasts appeared to be small, most showing fewer than three nuclei. Ultrastructural analysis showed osteoclasts with normal cytoplasmic features, secretory granules, (►Fig. 1C–E) and normal ruffled borders. Osteoblast ultrastructure and the appearance of the osteoid and bone matrix were normal (►Fig. 1F). Overall, the biopsy indicated low bone volume with an increased number of small osteoclasts accompanied by normal osteoblast appearance and bone formation parameters at the upper end of the normal range.

Genetic Analysis

Array comparative genomic hybridisation in case 2 showed an unbalanced chromosome complement with a 15 Mb duplication of chromosome 20p13–12.1 containing 112 genes and an 8 Mb deletion of chromosome 4q34.3–35.2 containing 37 genes. This was confirmed in all three cases by FISH. The deleted and duplicated genes are shown in ►Figs. 2A, 2B. The father of cases 1 and 2 and the mother of case 3 (themselves siblings) were shown by FISH to carry a balanced reciprocal translocation of chromosome 4:20.

Caspase-3 Activity Assay

In all three cases, basal caspase-3 activity was reduced compared with controls with an average 17.4% reduction (►Fig. 3A). Caspase-3 protein levels from controls had an average 4.5-fold higher level compared with cases (►Fig. 3B). However, the caspase-3 activity levels from cases recovered to similar levels to controls after both low (►Fig. 3C) and high dose (►Fig. 3D) stimulation with the apoptotic agent doxorubicin with no subsequent statistically significant difference.

Discussion

Although the children reported here have several abnormalities, we have concentrated on trying to identify genes causative of their low BMD and fracture susceptibility. Bone biopsy showed low bone volume/tissue volume (BV/TV) and an increased number of small osteoclasts and disconnected trabeculae in the presence of normal osteoblasts and relatively high indices of bone formation.

A review of individuals with similar chromosome 4q deletions highlighted no problems with fractures.⁶ The deleted region on chromosome 4q contains 37 genes, none to date associated with osteoporosis. Of the deleted genes, *CASP3* at 4q35 stood out as a good candidate as homozygous caspase-3 deficient mice show severe bone formation defects while heterozygous caspase-3 deficient mice show decreased BMD.² In addition, the administration of caspase-3 inhibitor led to accelerated bone loss in ovariectomized wild-type mice. Caspase-3 is required for the final execution of apoptosis and maturation and differentiation of numerous cell types, including those involved in bone development and remodeling.⁷ Caspase-3 expression levels in different mouse strains show a positive correlation with osteoblast apoptosis rate and negative correlation with osteoblast differentiation and bone formation. Caspase-3 heterozygous mice show a reduced level of bone osteogenic differentiation and proliferation, decreased BV/TV, lower calcium accumulation, and bone formation. In addition, mouse lines expressing different levels of caspase-3 have variable levels of osteoblast differentiation,

Fig. 1 Bone histomorphometry (A, B) Methylmethacrylate sections stained for tartrate resistant acid pPhosphatase and counterstained with hamatoxylin. Note the thin and disconnected trabeculae (B) Trabecularisation of the cortex. Osteoclasts (marked by asterisks) are abundant and generally small. Osteoid is indicated by arrow (C) Semi-thin section of Epon-embedded bone stained with toluidine blue. Bone formation with an active seam of osteoblasts (arrow) on a trabecular surface (D) Methyl-methacrylate section stained with von Kossa/van Gieson. Von Kossa/van Gieson staining indicating the mineralized bone in black and the osteoid (inset, arrow) indicating the relatively wide osteoid seams € Electron micrograph of an actively resorbing osteoclast, marked OC. No obvious abnormalities were seen (F) Detail of ruffled border and active bone resorption in the osteoclast shown in 1E.

Table 3 Table showing bone histomorphometry parameters

	Patient	Reference range age 11–14 years	Unit
BV/TV	20.54	18.0–32.0	(%)
BS/BV	19.80	11.0–20.8	mm ² /mm ³
BS/TV	3.23	2.67–4.55	mm ² /mm ³
Tb.Th	105.80	102–193	µm
Tb.N	1.85	1.26–2.14	mm ⁻¹
Tb.S	434.84	318–651	µm
Oc.S/BS	3.11	0.40–1.79	(%)
Oc.S/TV	0.10	0.01–0.06	mm ² /mm ³
N.Oc/B.Pm	0.92	0.13–0.65	(mm ⁻¹)
ES/BS	9.88	8.5–30.1	(%)
OS/BS	36.40	12.8–36.5	(%)
OV/BV	4.25	0.79–4.13	(%)
O.Th	11.82	4.4–10.0	(µm)

apoptosis, ability to differentiate osteoblasts, and bone formation rates.^{2,8} Haploinsufficiency of caspase-3 in mice is associated with decreased BV/TV, attributed to reduced proliferation and differentiation of bone marrow stromal stem cells.² In vitro studies suggested that the functional osteoclast defects seen in-vivo in mice with reduced caspase-3 levels were attributed to dysfunctional osteoblasts.² Others have reported that reduced cell life span is a likely cause of the reduced osteoclast numbers.⁹ We have demonstrated low basal serum levels of caspase-3 in our patients compared with age-matched controls, suggesting *CASP3* may be involved in the phenotype. On patient biopsy, however, our findings differ from those seen in the mouse models as, by contrast, we found an increased number of small osteoclasts with apparently normal resorptive function accompanied by normal osteoblasts and high normal osteoblast parameters. Our bone biopsy results showed an increased number of osteoclasts which may be due to a decrease in apoptosis as a result of lower caspase-3 levels, but could also be due to an increase in differentiation as noted in mice that express lower levels of

Fig. 2 (A, B) Genes within deleted and duplicated regions. Schematic representation of deleted and duplicated regions showing genes within these regions of chromosome 4 and 20 respectively.

caspase-3. Further investigations are required to assess a possible role of caspase-3 in this osteoporotic phenotype.

Other candidate genes within the deleted region should be considered. These include *Claudin-22* which encodes a tight junction protein known to be expressed in osteoblasts and thought to be involved in ion transport across the bone membrane and trafficking of bone matrix proteins.¹⁰

These cases also have a 15 Mb terminal duplication of chromosome 20p13–12.1 involving 112 genes. Osteopenia and intrauterine fractures have previously been reported in trisomy

and tetrasomy 20p suggesting that loci for BMD lie within this region.^{11–13} A meta-analysis of genome-wide linkage studies for BMD showed linkage to 20pter–20p12.3 refined to 20p12 at the *JAG1* locus.^{14–16} *JAG1* is known to be expressed in osteoblasts and its activation associated with increased bone deposition.¹⁷ *Bone morphogenetic protein-2* (*BMP2*) lies within the duplicated region, is expressed in hypertrophic chondrocytes and induces endochondral bone formation through osteoblast differentiation.¹⁸ Polymorphisms in *BMP2* have been associated with osteoporosis adding support that it plays a role in bone health.¹⁹

Fig. 3 Caspase studies (A) Caspase-3 activity was measured in both case and age-matched control blood samples prestimulated with the apoptosis inducing agent doxorubicin. An increase in caspase-3 activity was noted in the controls but this was not statistically significant ($p = 0.0591$). These data are representative of two independently analyzed blood samples from each case paired with an age-matched control. Each blood sample was analyzed in triplicate for caspase-3 activity (B) Caspase-3 protein levels in whole blood as determined by densitometry post western blotting analysis. Total protein was extracted from whole blood samples and quantified before equal concentrations were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Post western blotting, ImageJ analysis was used to compare the ratio of caspase-3 protein present in the case samples and each age-matched control. The reduction in caspase-3 levels noted in each case was graphed as the fold change in caspase-3 level compared with the control (C) Caspase-3 activity in blood samples post-stimulation with the apoptosis inducing agent doxorubicin (100 nm). No statistical significance was noted between case and age-matched control samples at 2, 4, or 6 hours post treatment with doxorubicin (D) Caspase-3 activity in blood samples post stimulation with the apoptosis inducing agent doxorubicin (1 μ M). No statistical significance was noted between case and age-matched control samples at 2, 4, or 6 hours posttreatment with doxorubicin.

In summary, we describe a familial case of early osteoporosis associated with an unbalanced rearrangement of chromosomes 4 and 20, an increased number of small osteoclasts on bone biopsy and low basal caspase-3 levels. Given the large number of deleted or duplicated genes in these patients, it is likely that there are multiple contributory factors. However, we propose that the phenotype may be linked to the reduction in caspase-3 activity and the role of the caspase family in determining bone mass needs further study.

Patient Consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

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