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HAJDU CHENEY SYNDROME; REPORT OF A NOVEL *NOTCH2* MUTATION AND TREATMENT WITH DENOSUMAB

Adami Giovanni¹, Rossini Maurizio¹, Gatti Davide¹, Orsolini Giovanni¹, Idolazzi Luca¹, Viapiana Ombretta¹, Scarpa Aldo², and Canalis Ernesto³

¹The Rheumatology Unit, Department of Medicine, University of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy

²ARC-Net Research Centre, University and Hospital Trust of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy

³Departments of Orthopaedic Surgery and Medicine, and the UConn Musculoskeletal Institute, UConn Health, Farmington, CT, 06030

Abstract

Notch receptors play a central role in skeletal development and homeostasis. Hajdu Cheney Syndrome (HCS) is a rare disease associated with mutations of *NOTCH2* that lead to the translation of a truncated, presumably stable, NOTCH2 protein. As a consequence, a gain-of-NOTCH2 function is manifested. We report a subject presenting with HCS and her child, both harboring a new heterozygous mutation in Exon 34 of *NOTCH2* upstream of the PEST domain. The subject presented with osteoporosis, fractures, acroosteolysis and splenomegaly but did not have neurological complications, cardiovascular defects or polycystic kidneys. Sequencing of genomic DNA revealed a previously unreported mutation at nucleotide 6667C>T leading to a Gln2223Ter protein product in the subject and her son. Preclinical studies have demonstrated that the bone loss in HCS is secondary to enhanced osteoclastogenesis and bone resorption, and the same mechanism may operate in humans. Accordingly, the case we report was treated and responded to therapy with denosumab with an increase in bone mineral density (BMD). However, acroosteolysis progressed and was not modified by denosumab. In conclusion, we report a case of HCS associated with a novel mutation in *NOTCH2* and its response to denosumab on BMD.

Keywords

Notch; bone remodeling; fractures; acroosteolysis; denosumab; splenomegaly

Corresponding author: Giovanni Adami, Rheumatology Unit, Department of Medicine, University of Verona, Piazzale L. Scuro 2, 37134 Verona, Italy, gio90it90@gmail.com.

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INTRODUCTION

Hajdu Cheney Syndrome (HCS) is a rare genetic disorder characterized by acroosteolysis, severe osteoporosis with fractures and craniofacial dysmorphism[1,2]. Individuals with HCS also present with short stature, wormian bones, platybasia, neurological complications, cardiovascular septal and valvular defects and polycystic kidneys[3–5]. The disorder was originally described by Hajdu in 1948 and reported as a syndrome by Cheney in 1962[6,7]. HCS is inherited as a dominant genetic disorder, although many sporadic cases occur. HCS is associated with mutations in exon 34 of *NOTCH2* upstream the proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) domain, resulting in the expression of a NOTCH2 truncated protein[8–10]. Because the PEST domain is necessary for the ubiquitination and degradation of NOTCH2, the protein is presumably stable so that the mutation causes a gain of NOTCH2 function.

Notch 1 to 4 are transmembrane proteins that determine cell fate and function, and play a critical role in embryonic development and cell renewal[11–13]. Notch receptors are activated following interactions with their ligands, and Jagged 1 and 2 and Delta-like 1, 3 and 4 are considered canonical Notch ligands. Although Notch 1 through 4 have structural similarities, their functions are not necessarily redundant. In skeletal cells, Notch activation inhibits chondrogenesis and osteoblastogenesis and causes severe osteopenia[13,14]. However, the effects of Notch are cell context dependent and when activated in osteocytes, Notch1 causes osteopetrosis[15,16]. Whereas Notch1 seems to have an inhibitory function in osteoclastogenesis, in part by inducing osteoprotegerin, Notch2 induces osteoclast maturation by interacting with Nuclear factor KappaB and inducing the transcription of nuclear factor of activated T cells 1[17,18].

Mutations in Notch receptors, their ligands or various components of the Notch signaling pathway are associated with a variety of developmental skeletal disorders[19]. Recently, a mouse model replicating the human mutation of HCS was reported[20]. A point mutation creating a STOP codon was introduced into exon 34 of *Notch2* upstream sequences coding for the PEST domain by homologous recombination. The *HCSNotch2* mutant mouse exhibited osteopenia secondary to increased osteoclast number and bone resorption. There was an increase in the preosteoclast cell pool as well as in the osteoclastogenetic response to Receptor activator of nuclear factor KappaB ligand (Rankl). In addition, osteoblasts and osteocytes expressed increased levels of Rankl. These observations indicated that the osteopenic phenotype in the *HCSNotch2* mouse mutant was secondary to an increase in osteoclastogenesis. If these observations were to apply to the human condition, the osteoporosis of HCS could be ameliorated by the use of agents that inhibit osteoclastogenesis and bone resorption.

Because of the limited number of individuals affected by HCS, there are no controlled trials on therapeutic interventions. Occasional patients have been treated with bisphosphonates or teriparatide in an effort to lessen the osteoporosis but evidence of a beneficial response is limited[21,22].

In the present work, we describe a mother and son presenting with HCS and harboring a novel mutation in exon 34 of *NOTCH2*. We also report the skeletal response of the adult subject with HCS to treatment with denosumab, an agent known to block RANKL and inhibit osteoclastogenesis and bone resorption.

CASE REPORT

In October 2013, a 33-year-old female patient was seen at the Rheumatology Unit of the University of Verona (Italy) with a history of generalized bone pain, particularly in both hands, hips and back, and severe osteoporosis with fractures. She had suffered multiple vertebral fractures from 1999 to 2013 between T6 and L3 as well as atraumatic fractures of the left knee, right tibia, right fibula and 4th metatarsal bone of the right foot. From 2011 to 2013, the subject suffered two vertebral fractures (T11 and T12). The subject described progressive shortening of the middle finger of the right hand and of the 2nd and 5th finger bilaterally. The patient suffered from congenital right hip dysplasia treated with several surgical procedures and eventually with a total hip replacement. There was no history of neurological or cardiovascular complications or urinary symptoms. The subject's son suffered a low trauma fracture of the right clavicle at 5 years of age. The report of the case was approved by the Verona Medical School Institutional Review Board (IRB) and both mother and son provided written consent and assent, respectively, for the publication of this report.

Prior to her visit to the Rheumatology Unit of the University of Verona, the subject of study had been treated with vitamin D supplementation, pamidronate a 30 mg IV infusion once in 2008, clodronate intramuscularly 100 mg twice in 2009 and with strontium ranelate 2 g daily for five months in 2010. On physical exam the patient's height was 155 cm and weight was 63 kg. She displayed a prominent forehead, hypertelorism and micrognathia, finger deformities and hallux valgus, and her hands were short and stubby. There were no signs of local inflammation in either fingers or toes. The spleen was palpable and no abnormalities were noted on cardiovascular and neurological exams.

Complete blood count, serum electrolytes, liver and renal function tests were within the normal range. Serum, alpha-1-antitrypsin, glucose, haptoglobin, blood iron, ferritin, transferrin, growth hormone, insulin-growth factor 1, adrenocorticotrophic hormone, thyroid stimulating hormone, luteinizing hormone, prolactin, tryptase, anti-nuclear antibodies, anti-extractable nuclear antigens antibodies, anti-double-strand DNA antibodies, anti-citrullinated peptide antibodies, anti-neutrophil cytoplasmic antibodies and complement were within the normal range. Serum calcium, phosphorous, parathyroid hormone (PTH), 25-hydroxy vitamin D (25OHD), osteocalcin, C-telopeptide of type I collagen (CTX), bone specific alkaline phosphatase (BSAP), and procollagen I intact N-terminal peptide (PINP) were within the normal range (Table 1), CTX, BSAP and PINP were measured by the IDS-ISYS Multi-Discipline Automated Analyzer (Immunodiagnostic System, Boldon, UK). Serum RANKL was elevated at 0,451 pmol/L and osteoprotegerin (OPG) was decreased at 1,244 pmol/L, when compared to values obtained in ten age matched female controls. RANKL and OPG were measured by enzyme-linked immunosorbent assay (Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria). All laboratory tests were conducted in

the clinical laboratories of the University of Verona Hospital. Studies in normal volunteers were approved by the Verona Medical School IRB and written consent was obtained from volunteers prior to blood drawing.

In October 2013, prior to initiation of therapy with denosumab, dual-energy X-ray absorptiometry (DXA) performed with a QDR Hologic Delphy instrument, revealed a bone mineral density (BMD) at the lumbar spine (L1–L4) of 0,810 g/cm² and a T-score of –3,1 (Z-score –2,9), a BMD at the left femoral neck of 0,848 g/cm² and a T-score of –1,4 (Z-score –1.0) and at the total left hip of 0,870 g/cm² and a T score of –1.1 (Z-score –0.8).

Chest X-ray revealed normal lungs and multiple vertebral fractures (Figure 1). Skull X-rays revealed anteroposterior diameter augmentation, paranasal sinus hypoplasia and opened lambdoid suture. Hand X-rays showed acroosteolysis involving the distal phalanx of the 2nd and 5th fingers of both hands (Figure 2). X-rays of both feet showed diffuse demineralization and severe deformities of the first metatarsophalangeal joint bilaterally (Figure 2).

Spine Magnetic Resonance Imaging (MRI) demonstrated severe scoliosis, “Biconcave lens” vertebral deformities from T6 to L3, associated with angiomatous formation in the vertebral bodies of T3 and L5 (Figure 3). Abdominal ultrasound revealed an enlarged spleen of 14,5 cm in length and 8 cm in width, and no evidence of polycystic kidneys (not shown). Nailfold hand capillaroscopy, performed for the morphologic assessment of the microcirculation, showed increased capillary branching.

To establish the diagnosis of HCS, DNA sequence analysis of the *NOTCH2* coding sequence was performed on genomic DNA extracted from peripheral leucocytes at the ARC-NET Research Centre, University and Trust of Verona (Verona, Italy)[23]. A custom panel to analyze the coding sequence of *NOTCH2* was designed using Ion AmpliSeq Designer software. A suitable library for samples from mother and son was obtained and analyzed on Ion Torrent PGM and loaded on chip 318v2 (Thermo Fisher, Waltham, MA). Data analysis, including alignment to the hg19 human reference genome and variant calling, was performed using the Torrent Suite Software v.5.0 (Thermo Fisher). Filtered variants were annotated using a custom pipeline based on vcflib (<https://github.com/ekg/vcflib>), SnpSift, the Variant Effect Predictor (VEP) software and NCBI RefSeq database[24,25]. DNA sequence analysis of the subject of this report revealed a heterozygous nonsense (NP_077719.2:p.Gln2223Ter; NM_024408.3:c.6667C>T) mutation in exon 34 of *NOTCH2* not described previously (Figure 4). The 6667C>T mutation in exon 34 of *NOTCH2* leads to the creation of a Stop codon and a predicted truncated protein product of 2222 amino acids.

The subject initiated treatment with denosumab, 60 mg subcutaneously every 6 months and vitamin D3 supplementation at an oral dose of 10,000 IU weekly. Serum RANKL decreased following the administration of denosumab (Table 1). Serum CTX declined from 225 pg/ml to 63 pg/ml 3 days after denosumab was administered, but serum CTX levels were within the normal range prior to each subsequent dose of denosumab. Serum markers of bone formation, including BSAP and osteocalcin did not change with the treatment. A progressive increase in lumbar spine BMD was observed after treatment with denosumab; BMD

increased by 6.8% at L1–L4 and by 2.6% at the femoral neck two years after the initiation of therapy (Figure 5). The subject did not report any non-vertebral fractures or symptoms suggestive of vertebral fractures while receiving treatment with denosumab. Absence of new vertebral fractures was verified by DXA morphometric vertebral fracture assessment (VFA) obtained following therapy with denosumab (Figure 6) and comparison with DXA VFA, vertebral XRays and MRI obtained prior to therapy.

During the treatment period, the patient suffered from fluctuating pain in the 3rd finger of the right hand. A radiograph of the hands was obtained after two years of treatment with denosumab, and revealed progression of acroosteolysis and newly discovered involvement of the distal phalanx of the 3rd finger of the right hand (Figure 7). Treatment with denosumab was not associated with adverse events.

The study subject's son was assessed at 5 years of age following a low trauma fracture of the clavicle. His weight was 24 kg and his height was at the 25th percentile and, on examination, he was on stage Pubic (P)1 and Genitals (G)1 of Tanner pre-puberal scale. On physical examination, he presented micrognathia, but no other signs of HCS. DXA revealed a lumbar spine BMD of 0,633 g/cm² (Z score -0,1) and total left hip BMD of 0,832 g/cm² (Z score +1,7), left femoral neck BMD of 0,808 g/cm² (Z score +1,3). X-rays of the hands did not reveal any abnormalities. Sequence analysis of genomic DNA obtained from peripheral blood revealed a 6667C>T (Gln2223Ter) mutation in exon 34 of *NOTCH2*.

DISCUSSION

We report a mother and her child affected by HCS associated with a novel 6667C>T (Gln2223Ter) *NOTCH2* heterozygous mutation. The subject presented with a variety of skeletal abnormalities including osteoporosis with fractures, scoliosis and acroosteolysis. Polycystic kidneys and cardiovascular developmental anomalies, including patent ductus arteriosus, atrial and ventricular septal defects and valve defects can be clinical manifestations of HCS[4,26,27]. The subject of this report did not present with any cardiovascular or renal defects, but had splenomegaly, a possible consequence of increased NOTCH2 activity in the marginal zone of the spleen and not previously reported in HCS[28]. Work conducted in murine models of *Notch2* global and conditional inactivation in cells of the hematopoietic or the B-cell lineage has demonstrated that Notch2 is indispensable for marginal zone B-cell development[29,30]. Moreover, the constitutive activation of Notch2 in the B-cell lineage results in an increase in marginal zone B-cells of the spleen[28]. HCS is an autosomal dominant inherited disorder, although sporadic cases may occur. DNA sequencing information was not available and could not be obtained from the subject's parents since the subject had lost contact with them. Consequently, we could not document the type of inheritance in the subject of this report with certainty. DNA sequence analysis indicated a heterozygous mutation which is in accordance with previously reported cases of HCS[8–10]. It is possible that homozygous mutations result in embryonic lethality.

Previous reports have demonstrated the presence of point mutations or short deletions in exon 34 of *NOTCH2*, upstream the PEST domain in families affected by HCS[8,9]. The

mutations create a stop codon and the premature termination of the protein product leading to the accumulation of a stable protein and presumably persistent NOTCH2 signaling[8,9]. The individual we report had a novel mutation 6667C>T in *NOTCH2* creating Gln2223Ter at the amino acid level. In accordance with previously described subjects afflicted by HCS, the mutation was in exon 34 upstream the PEST domain. Recently, we conducted studies in a mouse model carrying a 6955C>T *Notch2* corresponding to a 6949C>T mutation in *NOTCH2* found in a subject affected by HCS[20]. The *Notch2* mutation in this mouse model is in exon 34 upstream the PEST domain, and leads to the translation of a truncated protein of 2318 amino acids and enhanced Notch2 signaling in skeletal cells[20]. There was higher expression of Notch target genes (*Hes*, *Hey*) in osteoblasts and osteoclasts as well as in bone extracts from mice carrying the *HCSNotch2* mutation. Mice harboring *HCSNotch2* mutations exhibit cancellous and cortical bone osteopenia, and bone histomorphometric analysis showed increased osteoclast number and bone resorption[20]. *In vitro* studies demonstrated that osteoclastogenesis and the bone resorptive capacity of osteoclasts were enhanced. These observations were attributed to a direct effect of Notch2 on osteoclast precursors as well as to an induction of Rankl in osteoblasts and osteocytes[18,20]. In line with these observations, the individual we report had increased serum levels of RANKL and a favorable BMD response to denosumab, an agent known to inhibit osteoclastogenesis. There was a progressive increase in BMD and the subject was free of fractures during the two years of therapy as demonstrated by DXA VFA, whereas she had suffered multiple fractures in the preceding years. Although the patient presented with multiple vertebral fractures and these might have affected the DXA values, the degree of deformity in the L1–L4 region was relatively modest so that it is reasonable to believe that the changes in vertebral BMD represent a response to the therapeutic intervention. Moreover, an increase in BMD, although modest, also was observed in the femoral neck.

Clinical studies have offered limited information on the pathogenesis of the acroosteolysis of HCS. It is accompanied by neovascularization, inflammation and fibrosis, but the underlying mechanism is not clear and appears to be unrelated to the one responsible for the bone loss and fractures[31,32]. In accordance with this hypothesis, the acroosteolysis of the individual we report progressed while the osteoporosis improved with denosumab.

It is of interest that somatic *NOTCH2* mutations causing loss of the PEST domain exhibit enhanced NOTCH activation and have been identified in B-cell lymphoma and lymphomas of the marginal zone of the spleen[33–35]. There is no apparent increase in the incidence of B-cell lymphoma in HCS. However, the individual we report had splenomegaly, a manifestation of the disease not reported previously. Splenomegaly can be explained by the fact that Notch2 is required for the formation of the marginal zone of the spleen, and overexpression of Notch2 in B-cells results in increased cellularity of the marginal zone[34,35]. It is not known, however, whether Notch activation in the marginal zone plays any role in the skeletal disease of subjects affected by HCS.

In conclusion, we report a novel *NOTCH2* mutation in an individual with HCS and her child. The individual presented with significant osteoporosis and a favorable response to treatment with denosumab.

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HIGHLIGHTS

- Hajdu Cheney Syndrome (HCS) is associated with *NOTCH2* mutations
- A novel 6667C>T mutation in *NOTCH2* is reported in a case of HCS
- Subject with HCS presented with osteoporosis, acroosteolysis and splenomegaly
- Bone mineral density, but not acroosteolysis, responded to denosumab

Figure 1.
X-rays showing normal heart and lungs and vertebral deformities (pointed by the arrows)
prior to treatment with denosumab.

Figure 2.

X-rays of hands showing acroosteolysis of distal phalanges of the 2nd and 5th finger of both hands, pointed by the arrows, and X-rays of both feet showing hallux valgus and deformities in a subject with HCS.

Figure 3. Magnetic Resonance Imaging of the spine showing scoliosis and vertebral biconcave lens deformities in a subject with HCS. Arrows point to deformities.

Figure 4.

Genomic DNA from blood samples of the subject with HCS was sequenced by next generation sequencing on Ion Torrent (Thermo Fisher) for the coding sequence of the NOTCH2 gene. The representation of the reads aligned to the reference genome, as provided by the Integrative Genomics Viewer software (IGV v 2.1, Broad Institute), shows a 1:1 signal ratio of C (blue) to T (red) at nucleotide 6667 demonstrating the presence of a heterozygous 6667C>T mutation.

Figure 5.

Bone mineral density (BMD) of the lumbar spine and left femoral neck obtained prior to (October 2013) and following therapy with 5 doses of denosumab, 60 mg given subcutaneously every 6 months through October 2015 in a subject with HCS. Note that scale for y-axis for lumbar and femoral neck BMD is different.

Figure 6. Morphometric vertebral fracture assessment by densitometry (DXA) of the spine of a subject with HCS obtained before (A) and following (B) therapy with 5 doses of denosumab, 60 mg, given subcutaneously every 6 months from October 2013 through October 2015.

Figure 7. X-rays of hands showing acroosteolysis of the 2nd and 5th finger of both hands as well as the 3rd finger of the right hand in a subject with HCS following two years of treatment with denosumab.

Table 1

Biochemical Parameters on a Subject with HCS

	Baseline (8/2013)	3 days after 1st denosumab (10/2013)	5 days before 2nd denosumab (4/2014)	1 day before 3rd denosumab (12/2014)	1 day before 4th denosumab (7/2015)	5 days after 5th denosumab (10/2015)	Last follow-up (3/2016)
Calcium (mg/dL) (8.9–10.1)	9.2	8.4	9.9	9.6	9.8	9.0	9.2
Phosphorous (mg/dL) (2.4–4.1)	3.1	2.3	3.0	3.0	3.4	2.7	3.0
PTH (pg/mL) (10–55)	29	33	28	31	25	64	19
25OHD (ng/mL) (>30)	34.6	31.4	40.0	30.5	31.4	32.1	24
CTX (pg/mL) (100–670)	225	63	220	189	277	33	416
BSAP (U/L) (6–23)	11	11	9	10	9	9	9
PINP (ng/mL) (19.3–76.3)	20.5	21.1	/	16.0	20.0	11.2	34.3
RANKL (pmol/L) (0.022–0.282) *	0.451	0.008	/	0.292	0.269	0.008	0.328
OPG (pmol/L) (4.850–6.840) *	1.244	1.021	/	1.411	1.539	1.422	1.092
RANKL/OPG ratio (0.004–0.041)	0.362	0.007	/	0.206	0.174	0.005	0.300

PTH= Parathyroid hormone, 25OHD= 25 hydroxy Vitamin D, CTX= C-Telopeptide of type I collagen, BSAP= Bone specific alkaline phosphatase, PINP= Procollagen I intact N-terminal peptide, RANKL= Receptor activator of nuclear factor Kappa-B ligand, OPG= Osteoprotegerin; Normal range values are in parenthesis.

* Normal range was established by measuring serum RANKL and OPG in **ten age-matched female normal volunteers**.