

Truncating mutations in the last exon of *NOTCH2* cause a rare skeletal disorder with osteoporosis

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Hajdu-Cheney syndrome is a rare autosomal dominant skeletal disorder with facial anomalies, osteoporosis and acro-osteolysis. We sequenced the exomes of six unrelated individuals with this syndrome and identified heterozygous nonsense and frameshift mutations in *NOTCH2* in five of them. All mutations cluster to the last coding exon of the gene, suggesting that the mutant mRNA products escape nonsense-mediated decay and that the resulting truncated *NOTCH2* proteins act in a gain-of-function manner.

Hajdu-Cheney syndrome (HCS; MIM#102500) is a rare skeletal disorder characterized by the association of facial anomalies, radiological findings (acro-osteolysis, general osteoporosis and insufficient ossification of the skull) and periodontal disease (premature loss of permanent teeth) (Fig. 1)¹. Other features include cleft palate, congenital heart defects, polycystic kidneys, orthopedic problems and anomalies of the genitalia, intestines and eyes. Although most cases are sporadic, transmission in a few families suggests autosomal dominant inheritance (Supplementary Fig. 1)¹. The low number of cases and multiplex families has impaired the identification of mutations underlying HCS by conventional genetic approaches.

We sequenced the exomes of six unrelated individuals with Hajdu-Cheney syndrome. Clinical features of these individuals are presented in Table 1 and Supplementary Table 1. We obtained informed consent from each family member for genetic analyses according to local ethical guidelines from all participating institutions. We performed enrichment by hybridizing shotgun fragment libraries to SureSelect Human All Exon Agilent microarrays followed by massively parallel sequencing (Supplementary Methods). On average, we generated 3.02 Gb of sequence per sample to achieve $\times 45$ coverage of the mappable, targeted exome (38 Mb total).

We focused our analyses on non-synonymous variants located in coding sequences, anticipating that synonymous variants in coding sequences and variants in 5' untranslated regions (UTR), 3'UTR and intronic sequences were far less likely to be pathogenic (Supplementary Table 2). Because of the probable dominant nature of the disease, we hypothesized that variants causing HCS should be absent from control populations and thus from all public datasets, including dbSNP129 and the 1000 Genomes Project. We identified 78 genes that were affected by distinct missense variants in three unrelated cases (Supplementary Tables 2 and 3). However, based on a thorough literature review, none of these genes appeared to be a good candidate.

In contrast, only five genes (*ARSD*, *NOMO3*, *NOTCH2*, *OR2T35* and *CDK18*) were inactivated by nonsense mutations in two unrelated cases, among which *NOTCH2* appeared to be a good functional candidate (Supplementary Table 4). By checking unfiltered sequencing data for any missed genetic variants within *NOTCH2*, we identified an additional nonsense mutation in a third affected individual. This mutation had previously been overlooked because of low coverage (Supplementary Fig. 2). Next, we searched for frameshift mutations specifically in *NOTCH2* and identified small insertions and deletions

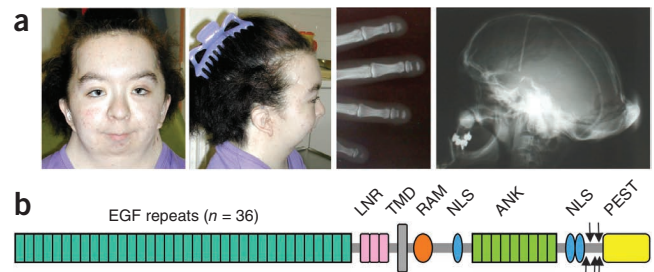


Figure 1 Identification of *NOTCH2* mutations in individuals with Hajdu-Cheney syndrome. **(a)** Facial dysmorphism of individual F2-II-2 with micrognathia, thick eyebrows, long philtrum, hypertelorism, and low-set and posteriorly rotated ears. We obtained written consent to publish photographs of this individual. Phalanges radiograph of individual F5-II-2 showing acro-osteolysis of all distal phalanges. Skull radiograph of individual F4-II-1 showing characteristic findings of HCS, including platybasia, thickened occipital bone, open sutures and wormian bones. **(b)** Predicted protein domains of *NOTCH2*. The black arrows indicate the location of the truncating mutations identified in the five families with HCS. EGF, epithelial growth factor; LNR, cysteine-rich Lin12-Notch repeats; TMD, transmembrane domain; NLS, nuclear localizing domain; ANK, ankyrin repeats; PEST, domain containing conserved proline-glutamic acid-serine-threonine-rich motifs.

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Table 1 Clinical features of individuals with Hajdu-Cheney syndrome carrying *NOTCH2* mutations

| Individual ID | F1-II-1 | F2-II-1 | F3-II-1 | F4-I-1 | F4-II-1 | F4-II-5 | F4-III-1 | F5-II-2 | F5-III-1 |
|-------------------------------------------|-----------------|---------------|------------------|------------|-------------------------------------|------------|-------------|------------|------------|
| Gender | M | F | M | M | M | F | M | F | F |
| Age (yrs) at time of last report | 19 | 25 | 57 | 54 | 35 | 36 | 16 | 31 | 3 |
| Craniofacial features | | | | | | | | | |
| Facial dysmorphism ^a | + | + | + | + | + | + | + | + | + |
| Microretrognathism/Pierre-Robin sequence | - | + | + | - | + | - | + | + | - |
| Periodontal disease | + | - | + | + | + | + | + | + | + |
| Cognitive and sensory function | | | | | | | | | |
| Developmental delay | - | - | - | - | - | - | - | - | - |
| Neurologic symptoms | + | + | + | - | + | - | + | - | - |
| Hearing deficit | + | + | + | + | + | + | + | + | + |
| General physical features | | | | | | | | | |
| Short stature | + | + | + | - | - | - | - | - | - |
| Congenital heart defect | PDA, VSD | PDA | - | - | - | - | - | - | - |
| Polycystic kidneys | - | + | + | - | - | - | - | - | - |
| Joint hyperlaxity | + | + | + | - | - | - | - | + | + |
| Radiographic abnormalities | | | | | | | | | |
| Acro-osteloyosis | + | + | + | + | + | + | + | + | - |
| Osteoporosis | + | + | + | + | + | - | + | + | - |
| Wormian bones | + | + | + | + | + | + | + | + | + |
| Bowing of the fibula | - | - | - | - | - | - | - | + | - |
| Vertebral compression/fracture | - | - | + | + | + | - | - | - | - |
| Platybasia/basilar impression | + | - | + | + | + | - | + | - | - |
| Additional features | | | | | | | | | |
| | - | Iris coloboma | Umbilical hernia | - | Small right kidney, inguinal hernia | - | Hypospadias | - | - |
| <i>NOTCH2</i> mutations identified | | | | | | | | | |
| cDNA (NM_024408.2) | c.6427_6428insT | c.6853C>T | c.6449_6450delCT | c.7119T>G | c.7119T>G | c.7119T>G | c.7119T>G | c.6949C>T | c.6949C>T |
| Protein (NP_077719.2) | p.Glu2143X | p.Gln2285X | p.Pro2150ArgfsX2 | p.Tyr2373X | p.Tyr2373X | p.Tyr2373X | p.Tyr2373X | p.Gln2317X | p.Gln2317X |

F, female; M, male; PDA, patent ductus arteriosus; VSD, ventricular septal defect.

^aFacial dysmorphism features include hypertelorism, thick eyebrows, low-set ears, malar hypoplasia, prognathism, long philtrum, small mouth.

(indels) in two additional cases (**Supplementary Table 5**). In the remaining case, we could not detect any mutation in the coding regions of *NOTCH2* or in the exon-intron boundaries by exome sequencing. Although the phenotypes of this individual and his affected father were similar to that of carriers of *NOTCH2* mutations, we could not find any potentially deleterious mutations in genes of the NOTCH pathway in his exome.

Sanger sequencing confirmed the presence of heterozygous *NOTCH2* mutations in the five affected individuals (**Supplementary Fig. 1**). We tested inheritance in families 1 and 2 and showed that both mutations had occurred *de novo*. We confirmed parental origin in all available case-parent trios by microsatellite marker analysis. By capillary sequencing, we found that the *NOTCH2* mutations were carried by four additional affected relatives in families 4 and 5, whereas we identified only wild-type alleles in healthy individuals in these families. We found none of the *NOTCH2* mutations in 152 ethnically-matched control individuals. These results indicate that mutations in *NOTCH2* are the principal cause of HCS.

The NOTCH signaling pathway is critical for cell-fate determination². In humans, there are four NOTCH receptors (NOTCH1–NOTCH4) and at least five NOTCH ligands. NOTCH proteins are single-pass type I membrane proteins with an extracellular domain consisting mainly of multiple epidermal growth factor-like (EGF) repeats and an intracellular domain (NICD) containing ankyrin repeats, nuclear localization sequences (NLS) and a conserved proline-glutamic acid-serine-threonine-rich (PEST) domain at the C terminus, which regulates the stability of the NICD. NOTCH signaling is activated through cell-cell contact: ligand binding induces cleavage of NOTCH and translocation of

the NICD to the nucleus where it regulates gene expression in association with transcriptional cofactors. The clustering of the five observed truncating mutations in a 236-amino-acid interval (~9.5% of the *NOTCH2* coding sequence) is striking. The interruption of the *NOTCH2* coding sequence caused by the mutations preserves the ankyrin-repeat domain and the last NLS but removes the PEST domain, which is implicated in the regulation of ubiquitination and proteasomal degradation of proteins carrying such domains² (**Fig. 1** and **Supplementary Fig. 3**). In addition, as these mutations create a premature stop codon located in the last exon of *NOTCH2*, they likely escape nonsense-mediated mRNA decay³. The mutant mRNA is expected to be stable and the resulting truncated protein to be functional but with a constitutively active intracellular domain.

Components of the NOTCH signaling pathway have been implicated in common human pathologies, including cancer, stroke and Alzheimer's disease. Loss of NOTCH signaling causes monogenic disorders affecting skeletal tissue, such as spondylocostal dysostosis (MIM#277300) and Alagille syndrome (MIM#118450). Most cases of Alagille syndrome are caused by mutations leading to haploinsufficiency of the NOTCH ligand *JAG1* (ref. 4). In addition, one missense and one splice mutation in *NOTCH2* have been identified in two families with Alagille syndrome with no *JAG1* mutations⁵. Notably, the splice mutation removes exon 33 and results in a premature stop codon in exon 34; thus, the aberrant transcripts escape nonsense-mediated mRNA decay. However, in contrast to the *NOTCH2* mutations in HCS, the mutation removes three of seven ankyrin repeats and the last NLS, in addition to the PEST domain, and likely results in a loss-of-function or dominant-negative mutation. This difference between the effects of *NOTCH2* mutations in HCS and Alagille syndromes is a rare example in

which notably different phenotypes caused by allelic truncating mutations depend on their exact location. Interestingly, frameshift mutations affecting the PEST domain of *NOTCH1* contribute to human T-cell acute lymphoblastic leukemia through a gain-of-function mechanism⁶, highlighting the critical role of the PEST domain. Similarly, activating somatic mutations in *NOTCH2* have recently been observed in a minority of large B-cell lymphomas⁷.

In mouse, homozygosity for a targeted *Notch2* hypomorphic allele (which produces two different in-frame transcripts deleted for EGF repeats) results in perinatal lethality with prominent defects in the kidney and developmental anomalies affecting the heart and eye but with no reported skeletal anomalies, whereas heterozygous mice appeared normal and were fertile⁸. However, conditional knockout of both *Notch1* and *Notch2* in early limb mesenchyme resulted in skeletal anomalies and a complex age-related bone phenotype with increased bone mass in young mice and osteopenia in aged mice⁹.

In vitro and *in vivo* studies support a role for the Notch pathway in osteoblastogenesis, osteoclastogenesis and bone formation^{9–12}. However, the direction (inhibitory or stimulatory) of the effect of Notch signaling on osteoblastogenesis is controversial. Loss of Notch signaling in osteoblasts may lead to osteoporosis through activation of osteoclastogenesis^{10,11}. In contrast, suppression of Notch signaling by a selective γ -secretase inhibitor or Notch2 short hairpin RNA suppresses RANKL-induced osteoclastogenesis, whereas ectopic expression of intracellular Notch2 promotes osteoclastogenesis¹². Interestingly, a genome-wide association study identified *JAG1* as a candidate gene for bone mineral density regulation and a potential risk factor for fracture¹³. These findings are concordant with the increased risk for pathologic fractures observed in individuals with Alagille syndrome¹⁴. Further investigations are needed to understand the mechanisms by which acro-osteolysis and osteoporosis—two cardinal features of HCS—may be caused by NOTCH2 dysfunction.

The five nonsense or frameshift mutations causing HCS that we found were invariably located within the last coding exon of *NOTCH2*, distal to the ankyrin repeats and NLS necessary for the transcriptional activity of NOTCH2 intracellular domain, and delete the PEST domain. We propose that all mutations result in increased NOTCH2 signaling. In contrast, more proximal mutations may result in a loss-of-function (or dominant-negative) effect as suggested by the two *NOTCH2* mutations previously reported in Alagille syndrome⁵. The rarity of HCS may thus be explained by the very small target size for mutations leading to this particular phenotype. This mutational mechanism also suggests that decreasing NOTCH2 signaling using selective γ -secretase inhibitors may have therapeutic benefit in this severe and progressive disease¹⁵. Finally, our findings establish an important role

for NOTCH2 signaling in bone homeostasis that may lead to better understanding of the mechanisms contributing to osteoporosis.

URLs. Open-source software in the Google-code repository used to filter exome data, <http://code.google.com/p/code915/source/browse/trunk/hc/src/fr/inserm/umr915/hc/HCAlyser.java>.

Accession codes. The reference sequences in this study are available from GenBank under the following accession codes: *NOTCH2* cDNA, NM_024408.2 and NOTCH2 protein, NP_077719.2.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We are grateful to the affected subjects and their families who participated in this study. We thank F. Gros, H. Eldjouzi, A. Briand, C. Beneteau and S. Lecointe for technical assistance, and R. Houlgatte and C. Chevalier from Biogenouest de Nantes. This research was funded by grants from Inserm, Fondation pour la Recherche Médicale, Fédération Française de Cardiologie and Région Pays-de-la-Loire. P.L. is supported by the Direction Hospitalière de l'Organisation des Soins (DHOS). S.J. is funded by the "bourse de relèvement académique de la Faculté de Biologie et Médecine de l'Université de Lausanne."

AUTHOR CONTRIBUTIONS

C.L.C., B.I., S.B., V.C.-D., L.F. and A.D. conceived the project and planned the experiments. B.I., V.C.-D., L.F., M.L.M., S.J., D.M.-C., C.T.-R. and A.D. clinically characterized the HCS cases and collected blood samples. O.P. performed validation experiments. P.L., C.D., R.R., B.I., J.-L.M. and C.L.C. analyzed and interpreted the exome data. C.L.C., B.I., R.R., J.-L.M. and S.J. wrote the manuscript. All authors contributed to the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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