

Mutations in *NOTCH2* cause Hajdu-Cheney syndrome, a disorder of severe and progressive bone loss

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We used an exome-sequencing strategy and identified an allelic series of *NOTCH2* mutations in Hajdu-Cheney syndrome, an autosomal dominant multisystem disorder characterized by severe and progressive bone loss. The Hajdu-Cheney syndrome mutations are predicted to lead to the premature truncation of *NOTCH2* with either disruption or loss of the C-terminal proline-glutamate-serine-threonine-rich proteolytic recognition sequence, the absence of which has previously been shown to increase Notch signaling.

Hajdu-Cheney syndrome (HCS; MIM102500) is characterized by progressive focal bone destruction, including acro-osteolysis and generalized osteoporosis, together leading to characteristic radiographic abnormalities¹. Additional and variable manifestations include craniofacial anomalies and renal cysts². To date, approximately 50 cases of HCS have been reported, with many identified as a sporadic occurrence. Within multiplex kindreds, disease segregation supports autosomal dominant inheritance¹.

Osteoporosis is the most common metabolic bone disease and is a major public health problem, yet the molecular genetic basis of osteoporosis remains poorly understood². Researchers in recent genome-wide association studies have identified common variants of small effect size that contribute to osteoporosis risk³. However, the identification of mutations of large effect in monogenic disorders such as HCS have the capacity to provide substantial insight into the pathogenic mechanisms of osteoporosis and thereby identify potential targets for future therapeutic intervention. The relatively small

number of reported cases and the paucity of large multiplex kindreds have hindered efforts to uncover HCS gene mutations.

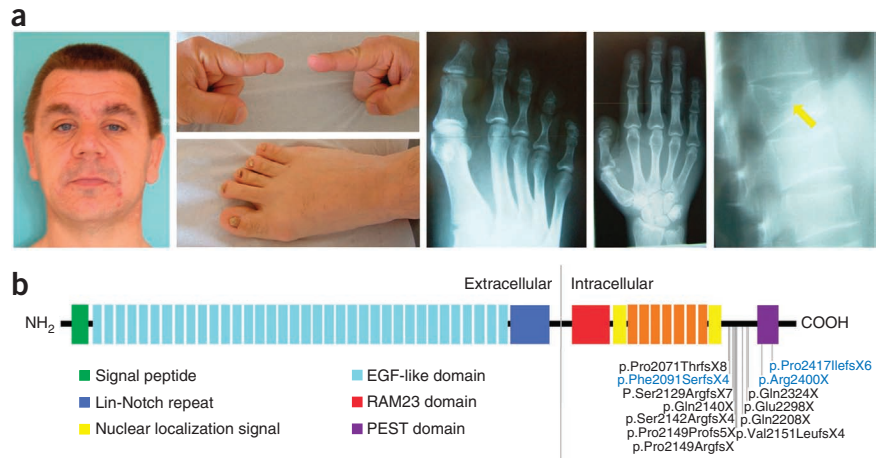
To search for disease alleles in HCS, we performed whole-exome sequencing of three unrelated affected individuals of European origin: two sporadic cases and one individual with a multigenerational history of the disorder (**Supplementary Fig. 1**). All affected individuals had characteristic clinical and radiographic features of HCS (**Fig. 1a** and **Supplementary Fig. 2**). We performed exome capture by in-solution hybridization followed by massively parallel sequencing (**Supplementary Methods**). We generated over 3.5 Gb of sequence for each subject such that >75% of the coding bases of the exome defined by the Consensus Coding Sequence (CCDS) Project were represented by at least 20 reads (**Supplementary Table 1**). We generated variant profiles using an in-house variant-calling pipeline⁴ (**Supplementary Table 2**) and compared the three exome variant profiles using a model of a rare autosomal dominant disorder requiring at least one previously unobserved heterozygous non-synonymous or splice site substitution or a coding insertion or deletion in the same gene in all three individuals. This process identified *NOTCH2* as the only candidate gene matching these criteria (**Supplementary Methods** and **Supplementary Table 3**).

The three *NOTCH2* variants are all located in exon 34, corresponding to the terminal exon of the 11.4-kb transcript. Each was predicted to lead to the premature termination of the protein product with a high likelihood of functional impact (**Fig. 1b**), and we confirmed each by Sanger sequencing of the proband and all available relatives consistent with inheritance. Specifically, the two genetic variants identified in the individuals with sporadic disease (HSC-02 and HCS-03) were shown to have arisen *de novo* and the third (HSC-01) was transmitted across generations between affected subjects within the kindred (**Table 1**).

We next addressed the hypothesis that further mutant *NOTCH2* alleles may explain additional cases of HCS. We performed Sanger sequencing of exon 34 and its associated acceptor splice site in 12 additional independent HCS kindreds (10 European and 2 South American kindreds). We identified heterozygous variants in 11 kindreds, including one (p.Gln2208X) that we observed in two unrelated families (HCS-09 and HCS-14). All identified variants are predicted to lead to premature termination (**Table 1**). We confirmed mutations as *de novo* events in six of the seven cases without a family history of HCS (parental samples for HCS-11 were not available). We confirmed transmission and co-segregation with the disease phenotype in the four kindreds with multiple affected individuals (**Table 1** and **Fig. 1b**). We detected none of the observed mutations in 300 unrelated,

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Figure 1 Clinical features of Hajdu-Cheney syndrome and a summary of *NOTCH2* mutations. (a) Clinical images of the individual exome sequenced from pedigree HCS-01 (marked with an asterisk in **Supplementary Fig. 1**). Images show characteristic coarse facies, digital clubbing and consequences of acro-osteolysis in the hands and feet, further shown in the radiographic images that also show a vertebral compression fracture in the lumbar spine (yellow arrow). All images are published with permission from each subject. (b) Location of the nonsense, insertion and deletion mutations identified in the 14 HCS families. We identified the three mutations in blue by exome sequencing.



ethnically matched control chromosomes. We sequenced all remaining coding exons and associated splice sites of *NOTCH2* in the single subject in whom we did not detect any variation in exon 34, but we did not observe any additional variants within this gene nor did we detect evidence of abnormal copy number of or within *NOTCH2* as assessed by quantitative PCR (data not shown).

In total, we investigated 15 HCS kindreds and identified mutations in 14 of them (~93%). The affected individual in whom we did not identify a *NOTCH2* protein-altering variant may be considered atypical, with no evidence of acro-osteolysis at seven years of age. This case may therefore represent a phenocopy of HCS, be explained by locus heterogeneity in HCS or be caused by an HCS allele comprising a noncoding or undetected coding variant in *NOTCH2*.

Four Notch receptors have been described in humans. All are single-pass transmembrane receptors that have high levels of structural similarity, share multiple ligands and have been shown to play a role in development, including skeletogenesis⁵. Following stimulation of Notch receptors, the Notch intracellular domain (NICD) and extracellular domain are cleaved. The NICD translocates to the nucleus where it mediates transcriptional activation and repression⁶. The C terminus of the *NOTCH2* intracellular domain contains a PEST sequence known to mediate proteosomal destruction of the protein⁷. Importantly, all identified HCS alleles are predicted to lead to premature termination of the protein product before the complete translation of the PEST domain. Sequencing of the *NOTCH2* transcript in the probands from pedigrees HCS-01 and HCS-04 (heterozygous for c.6272delT and c.6387delT, respectively) confirmed the presence of both mutant and

wild-type transcripts. We used a semiquantitative RT-PCR approach to show that the level of expression for two of the HCS alleles was comparable to that of wild-type alleles and that total abundance of the *NOTCH2* transcript was equivalent to that in wild-type controls (**Supplementary Fig. 3**). We interpreted these findings to be in keeping with the established notion that nonsense mutations in the terminal exon of many genes have reduced capacity to activate the process of nonsense-mediated decay⁸. Indeed, we were able to detect a protein of the size of the predicted truncated *NOTCH2* intracellular domain in primary skin fibroblasts from HCS-01 heterozygous for the mutation resulting in p.Phe2091SerfsX4 (**Supplementary Fig. 3**). Taken together, these findings suggest that HCS alleles generate a mature *NOTCH2* protein product that contains a disrupted or absent proteolytic PEST sequence. Notably, prior *in vitro* studies using a Notch-sensitive luciferase reporter assay have shown that *NOTCH2* receptors with partial or complete deletion of the PEST domain generated an elevated level of Notch signaling compared to wild-type constructs⁹. PEST domains are known to promote the degradation of proteins that contain them, with the elevated activity of Notch mutants lacking the PEST domain resulting from an increase in the half-life of the NICD, leading to a persistence of the Notch intracellular signal¹⁰.

Mutations in *NOTCH2* have previously been observed in two kindreds with Alagille syndrome¹¹ (ALGS, MIM610205), a multisystem monogenic disorder in which the majority of affected subjects harbor mutations in the gene encoding the Notch ligand *JAG1*. Skeletal manifestations, typically vertebral deformities, are often observed in individuals with ALGS, although none of these features were reported in the individuals with ALGS with *NOTCH2* mutations. It is noteworthy that renal cysts, typically a minor feature of ALGS but commonly seen in HCS, were observed in all individuals with ALGS with *NOTCH2* mutations. The ALGS alleles of *NOTCH2* are predicted to have distinct functional consequences to the HCS alleles we now report. That is, these two ALGS mutations are predicted to inhibit the activity of *NOTCH2* signaling, a mechanism consistent with reduced Notch signaling that results from *JAG1* alleles that also underlie ALGS^{11,12}.

NOTCH2 pays an essential role during development. It is ubiquitously expressed in human embryonic tissues (**Supplementary Fig. 3**),

Table 1 Mutations in *NOTCH2* in individuals with Hajdu-Cheney syndrome

Pedigree	Affected	Nucleotide mutation	Protein alteration	Exon	Inheritance
HCS-01 ^a	3	c.6272delT	p.Phe2091SerfsX4	34	Transmitted
HCS-02 ^a	1	c.7249-7250delCC	p.Pro2417IlefsX6	34	<i>de novo</i>
HCS-03 ^a	1	c.7198C>T	p.Arg2400X	34	<i>de novo</i>
HCS-04	1	c.6387delT	p.Ser2129ArgfsX7	34	<i>de novo</i>
HCS-05	2	c.6208-6209delAGinsTCAACAC	p.Pro2071ThrfsX8	34	Transmitted
HCS-06	1	c.6418C>T	p.Gln2140X	34	<i>de novo</i>
HCS-07	1	c.6424-6427delTCTG	p.Ser2142ArgfsX4	34	<i>de novo</i>
HCS-08	3	c.6460delT	p.Val2151LeufsX4	34	Transmitted
HCS-09	3	c.6622C>T	p.Gln2208X	34	Transmitted
HCS-10	1	c.6450delT	p.Pro2149Profs5X	34	<i>de novo</i>
HCS-11	1	c.6986G>T	p.Glu2298X	34	n/a
HCS-12	1	c.6973C>T	p.Gln2324X	34	<i>de novo</i>
HCS-13	1	c.6449-6450delICT	p.Pro2149ArgfsX	34	<i>de novo</i>
HCS-14	2	c.6622C>T	p.Gln2208X	34	Transmitted

^aProband from these three pedigrees had their exomes sequenced in the primary analysis. n/a, parental samples were not available for mutation analysis.

and homozygous deletion of mouse *Notch2* leads to early embryonic lethality¹³. This widespread expression pattern has the potential to explain the multisystem nature of the HCS phenotype. However, the characteristic and most debilitating clinical features of HCS are the skeletal abnormalities. There is growing evidence for the importance of NOTCH2 in the development and maintenance of the skeleton⁵, and this protein has been shown to modulate RANKL-induced osteoclastogenesis¹⁴. Overactivation of this pathway by HCS alleles may be responsible for the progressive bone loss observed in HCS.

In summary, we have shown that a restricted range of mutations in the terminal exon of *NOTCH2* underlie HCS, a disorder associated with severe and progressive osteoporosis. These findings show the critical role of NOTCH2 signaling in the regulation of bone mass, a position further supported by the recent association of common variants in *JAG1* with bone mineral density and osteoporotic fractures¹⁵. Although additional studies will be required to explore the consequences of HCS alleles on osteoblast and osteoclast activity, these findings highlight the Notch pathway as a potential target for therapeutic intervention in osteoporosis.

URLs. Consensus Coding Sequence Project project, <http://www.ncbi.nlm.nih.gov/projects/CCDS/>; dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>; 1000 Genomes Project, <http://www.1000genomes.org/>.

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

The authors thank the families for participating in this study. This work was supported by grants from the British Heart Foundation (BHF) to R.C.T. and

from Cure Kids New Zealand to S.P.R. and M.J.G. The authors also acknowledge support from the UK Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's and St. Thomas' National Health Service (NHS) Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. Grateful acknowledgment is also made to I. Marik, Chief of the Centre for Patients with Locomotor Defects, Prague, Czech Republic for assistance in recruitment to this study.

AUTHOR CONTRIBUTIONS

R.C.T., M.A.S. and M.D.I. conceived and designed the experiments. M.A.S., E.A., M.J.G. and D.D. performed the experiments. M.A.S. performed statistical analysis. M.D.I., F.V.E., S.M., S.E.H., C.E.B., M.H.-E., S.P.R., W.M.D., M.J.G., B.K.B., K.H.K., S.A., H.S., C.A.K. and R.M.P. contributed reagents, materials and analysis tools. R.C.T., M.A.S. and M.D.I. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturegenetics/>.

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