LETTER

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Nonsense mutation in the *LGR4* gene is associated with several human diseases and other traits

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Low bone mineral density (BMD) is used as a parameter of osteoporosis. Genome-wide association studies of BMD have hitherto focused on BMD as a quantitative trait, yielding common variants of small effects that contribute to the population diversity in BMD[1–7]. Here we use BMD as a dichotomous trait, searching for variants that may have a direct effect on the risk of pathologically low BMD rather than on the regulation of BMD in the healthy population. Through whole-genome sequencing of Icelandic individuals, we found a rare nonsense mutation within the leucine-rich-repeat-containing G-protein-coupled receptor 4 (*LGR4*) gene (c.376C>T) that is strongly associated with low BMD, and with osteoporotic fractures. This mutation leads to termination of LGR4 at position 126 and fully disrupts its function. The c.376C>T mutation is also associated with electrolyte imbalance, late onset of menarche and reduced testosterone levels, as well as an increased risk of squamous cell carcinoma of the skin and biliary tract cancer. Interestingly, the phenotype of carriers of the c.376C>T mutation overlaps that of *Lgr4* mutant mice.

We selected individuals with standardized BMDs below minus one standard deviation (−1 s.d.) for inclusion in the low BMD group. As controls we combined those who had a measured BMD above −1 s.d. and, for increased power, individuals who had not had their BMD measured. The low BMD group included 4,931 individuals and the control group comprised 69,034 individuals. We then tested for associations between 34.2 million sequence variants and low BMD. The variants were identified through whole-genome sequencing of 2,230 Icelanders and then imputed into the entire sample set; 95,085 Icelanders genotyped with single nucleotide polymorphism (SNP) chips and 296,526 close relatives of those who were chip genotyped through familial imputation (Supplementary Fig. 1 and Supplementary Methods).

The two most significant associations with low BMD ($P < 5 \times 10^{-8}$) were with common variants at 13q14 that have previously been reported to be associated with BMD, represented by rs8001611 (odds ratio (OR) = 1.21; $P = 1.6 \times 10^{-12}$)[1,6], and a novel association with a group of correlated rare variants at 11p14 (Supplementary Fig. 2 and Supplementary Table 1). This group includes 16 variants with $P < 10^{-9}$, all with population frequencies of between 0.14% and 0.18%. Among the rare variants that associate with BMD at 11p14 is a SNP (University of California Santa Cruz (UCSC) Genome Browser position hg18_chr11:27369242_A) that introduces a nonsense codon (c.376C>T; in the Refseq NM_018490 transcript) into exon 4 of the *LGR4* gene. None of the other rare variants at 11p14 were significant after accounting for the effect of c.376C>T (Supplementary Table 1), nor did any other coding variant in *LGR4* associate with BMD (Supplementary Table 2). We validated, and improved, the imputation of c.376C>T by direct genotyping (Supplementary Information); this resulted in a slightly stronger association with low BMD (OR = 4.30 and $P = 1.3 \times 10^{-10}$) (Table 1).

A common variant at this same locus, rs10835187 (minor allele frequency (MAF) 45%), was previously reported to associate with spine BMD ($P = 4.9 \times 10^{-8}$) but not with hip BMD ($P = 0.03$) in a large meta-analysis of 80,000 individuals[7]. rs10835187 is not correlated with c.376C>T ($r^2 < 0.001$) and its effect on low BMD is much weaker (OR = 1.06, $P = 0.031$). Testing the association of c.376C>T with BMD-related traits conditional on the effect of rs10835187 has very little impact on the results, and the association of rs10835187 with BMD is not changed if we adjust for c.376C>T (Supplementary Table 3). Thus, the two variants represent two independent BMD association signals in the 11p14 region. Because the association of rs10835187 with spine BMD is weak in our data set ($\beta = -0.036$ and $P = 0.0042$) we are not well powered to refine the rs10835187 association signal. However, we notice two variants in the 3′ untranslated region (UTR) region of lin-7 homologue C (*LIN7C*) at 11p14, rs3209593 and rs1140711, that are highly correlated with rs10835187 ($r^2 > 0.9$) and whose association with spine BMD is indistinguishable from that of rs10835187. The expression of *LIN7C* in blood and adipose tissue has previously been shown to be highly correlated with rs10835187. Another variant, rs61888800 ($r^2 = 0.31$ with rs10835187), located in the 5′ UTR region of the brain-derived neurotrophic factor (*BDNF*) gene could also explain the association of rs10835187 with BMD. No variant in *LGR4* can explain the rs10835187 association (Supplementary Table 4). Our data, although not conclusive, indicate that rs10835187 acts through *LIN7C* or *BDNF* rather than *LGR4* (Supplementary Information).

We examined the association of the *LGR4* c.376C>T variant with low BMD measures at separate sites, and with other osteoporosis-related traits (Table 1). All three low BMD measures associate with c.376C>T; the strongest site-specific association is with low whole-body values, with OR = 6.45 ($P = 5.2 \times 10^{-8}$). An association is also observed with risk of osteoporotic fractures (OR = 3.12, $P = 0.00013$) and with the BMD definition of osteoporosis[8] (OR = 3.27,

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Table 1 | Associations of the LGR4 c.376C>T nonsense mutation with osteoporotic traits

Phenotype	OR (95% CI)	*P* value	*N* (N_{wg})* Cases	*N* (N_{wg})* Controls
Low BMD†				
Hip, spine or whole body	4.30 (2.76–6.71)	1.3×10^{-10}	4,931 (4,188)	69,034 (58,632)
Whole body	6.45 (3.30–12.62)	5.2×10^{-8}	1,206 (1,159)	73,566 (70,699)
Hip	3.30 (1.89–5.76)	2.6×10^{-5}	2,804 (2,349)	70,100 (58,725)
Spine	3.81 (2.25–6.46)	6.9×10^{-7}	3,015 (2,538)	69,345 (58,374)
Osteoporosis BMD‡	3.27 (1.84–5.79)	5.0×10^{-5}	2,615 (2,162)	65,375 (54,050)
Any osteoporotic fractures	3.12 (1.74–5.59)	0.00013	2,668 (2,532)	72,036 (68,364)

Phenotype	β§	*P* value§	*N* (N_{wg})*
BMD			
Whole body	−0.75 (0.16)	1.6×10^{-6}	7,359 (6,896)
Hip	−0.49 (0.12)	6.2×10^{-5}	21,024 (17,171)
Spine	−0.52 (0.15)	3.8×10^{-5}	21,001 (17,193)

The results shown are for the A allele of the reference plus strand (hg18_chr11:27369242_A), presenting the nonsense T mutation of *LGR4* at codon 126. CI, confidence interval.
* *N* is the total number of individuals included in the analysis, and N_{wg} is the number of included individuals who have been chip typed.
†The low BMD phenotypes are defined as those BMD values that are below −1 s.d. from the mean.
‡World Health Organization definition of osteoporosis BMD; <−2.5 s.d. in young women at the spine or hip, uncorrected for age or weight[8].
§The effect β (the regression coefficient), and the standard error (s.e.) and *P* values from a linear regression of the age, sex and weight-adjusted BMD values on the mutation status. All *P* values are corrected for relatedness using the method of genomic controls (see Supplementary Information).

$P = 0.000050$). Furthermore, the association becomes stronger if a more extreme cut-off is used to define low BMD (Supplementary Table 5). Comparison of carriers and non-carriers of c.376C>T shows that the mean BMD value is 0.49–0.75 s.d. lower in carriers than in non-carriers, depending on site (Table 1 and Supplementary Table 6).

Analysis of 5,835 individuals with repeated BMD measurements did not show an association of *LGR4* c.376C>T with age-related changes in BMD (Supplementary Table 5), suggesting that the effect of the mutation is to reduce peak bone mass rather than increasing the rate of age-related bone loss. This is further supported by our observation that the strongest site-specific association is with low whole-body BMD, which is the site with the lowest remodelling rate. However, our data do not indicate that the mutation primarily associates with cortical rather than trabecular bone (Supplementary Table 5).

The *LGR4* gene encodes a cell-surface receptor that directly binds R-spondins[9–11], secreted agonists of the Wnt pathway[12]. The Wnt signalling pathway has been shown to regulate bone mass[13,14] and to be essential for differentiation of osteoblasts both in skeletogenesis and in the maintenance of the adult skeleton[15]. Furthermore, mutations in the *LRP5* and *SOST* genes, which take part in the Wnt pathway, have previously been identified in families with rare bone disorders[13], and common variants with small effects on BMD at these loci have been identified in genome-wide association studies[2,3,7]. On the basis of LGR4 potentiation of Wnt signalling, the lack of LGR4 should be reflected in less Wnt signalling in osteoblasts. Indeed, $Lgr4^{-/-}$ mice have delayed osteoblast differentiation and mineralization during embryonic bone formation. Postnatal bone remodelling (bone density, bone formation rate, osteoid formation) is also significantly impaired in these mice[16]. The *LGR4* c.376C>T mutation is predicted to truncate the LGR4 protein, normally composed of 951 amino acids, at position 126, thus fully disrupting its function (Supplementary Figs 3 and 4). We detected reduced levels of mutated *LGR4* messenger RNA isolated from white blood cells and adipose tissue of heterozygous c.376C>T carriers (Fig. 1a), consistent with a nonsense-mediated decay of transcripts containing nonsense mutations[17](Fig. 1b and Supplementary Fig. 5).

To investigate whether the *LGR4* c.376C>T nonsense mutation is present in other populations, we screened the public Exome Variant Server database[18] (EVS), and directly genotyped the variant in two sample sets of Northern European descent; the PERF study of 3,032 Danish postmenopausal women[19] and 1,393 individuals in the Australian DOES study[20]. The mutation was neither present in the EVS database nor in the Australian samples. One carrier of the nonsense mutation was found in the Danish sample, who was subsequently shown to be of Icelandic origin (Supplementary Information). Investigation of the size of the haplotype background shared by the carriers indicates that the mutation was introduced into the Icelandic gene pool about 400 years ago (Supplementary Information). Although the c.376C>T mutation seems to be specific to the Icelandic population, two truncating (frameshift) mutations in *LGR4* that could mediate similar effects to c.376C>T were found in the EVS database. The combined frequency of these two truncating mutations in the EVS is similar to that of the c.376C>T mutation in Iceland. We genotyped these two truncating mutations in the PERF and DOES samples by Sanger sequencing. Neither mutation was found to be present in these samples.

Various abnormalities other than those related to bone formation and remodelling have been reported in *Lgr4* mutant mice[10,21–28]. To get a more detailed picture of the overall effect of the c.376C>T mutation, we investigated its effect on a variety of human diseases and other traits for which we have genotype and phenotype information (Supplementary Information). This revealed a complex pattern of similarities between phenotypes displayed by *Lgr4* mutant mice and the carriers of the c.376C>T mutation (Supplementary Table 7): reduced birth size (weight $P = 0.00026$, length $P = 0.0055$); lower weight ($P = 0.0064$); electrolyte disturbances (with reduced plasma sodium ($P = 0.00014$) and bicarbonate levels ($P = 0.0017$)); elevated potassium levels ($P = 0.00063$) (Table 2); and normal serum levels of calcium ($P = 0.25$) and phosphate ($P = 0.90$). Furthermore, c.376C>T associates with reduced testosterone levels ($P = 0.00022$) and late onset of menarche ($P = 0.0018$),

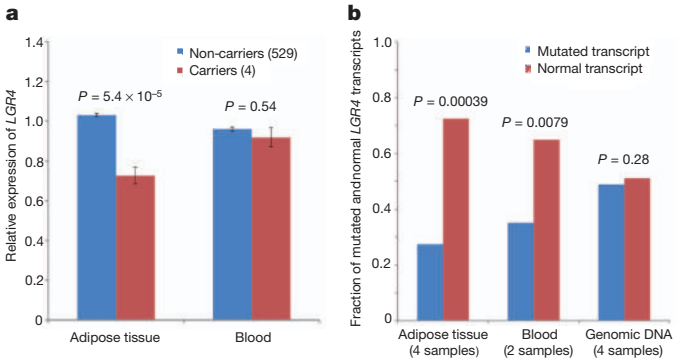

Figure 1 | Reduced expression of the *LGR4* transcript containing the c.376C>T mutation. **a**, Expression of *LGR4* in adipose tissue and blood in c.376C>T carriers and non-carriers, shown as 10 to the power of average mean log expression ratio (MLR). *P* values are from regression of the MLR on the carrier status, adjusting for age and sex, and differential cell counts for blood. Error bars show standard error of the mean. **b**, Proportion of mutated and normal *LGR4* transcripts in adipose tissue and white blood cells from heterozygous carriers of c.376C>T based on complementary DNA sequencing. Proportion of the two alleles determined by sequencing genomic DNA from heterozygous carrier is also shown. The *P* values are from a one-sample *t*-test.

Table 2 | The *LGR4* c.376C>T mutation is associated with a wide range of phenotypes

Phenotype	β (s.e.)	*P* value	Effect*	N (N_{wg})
Birth length	−0.799 (0.29)	0.0055	−0.03 cm	12,661 (1,485)
Birth weight	−1.046 (0.29)	0.00026	−0.46 kg	12,678 (1,485)
Weight	−0.237 (0.09)	0.0064	−3.0 kg	76,499 (65,461)
Sodium	−0.18 (0.05)	0.00014	−0.71 mmol l^{-1}	69,597 (63,194)
Potassium	0.16 (0.05)	0.00063	0.061 mmol l^{-1}	70,799 (64,152)
Bicarbonate	−0.34 (0.11)	0.0017	−1.2 mmol l^{-1}	14,563 (13,783)
Testosterone	−1.17 (0.32)	0.00022	−4.0 pg ml^{-1}	2,781(2,556)
Age at menarche	0.38 (0.12)	0.0018	0.48 years	39,574 (35,757)

Phenotype	OR (95% CI)	*P* value	N (N_{wg}) Cases	N (N_{wg}) Controls
Gallbladder and biliary duct cancer	9.85 (2.61–37.2)	0.00074	320 (99)	39,680 (12,276)
SCC	2.81 (1.29–6.12)	0.0092	1,508 (958)	144,768 (91,968)

Association of the c.376C>T mutation with phenotypes in deCODE's database. Included is, for each trait, the effect estimate β and s.e. from linear regression of the trait on the number of copies of the mutation, or the OR and the 95% CI from logistic regression for a case–control analysis, and the corresponding *P* values. All *P* values have been adjusted for relatedness using the method of genomic control. For detailed comparison, see Supplementary Table 7.
*The estimated difference in age- and gender-adjusted trait values between carriers and non-carries of the mutation.

possibly reflecting defects in the development of the male reproductive tract[21] and developmental delays[26], respectively, as has been described for *Lgr4* mutant mice. Many other abnormalities have been described for *Lgr4* mutant mice[10,22,24,27,28], warranting further investigation of the *LGR4* c.376C>T carriers.

In addition to the phenotypes previously described for *Lgr4* mutant mice, we found the *LGR4* c.376C>T nonsense mutation to associate with an increased risk of squamous cell carcinoma of the skin (SCC) (OR = 2.81, $P = 0.0092$), and cancers of the gallbladder and biliary tract (OR = 9.85, $P = 0.00074$) (Table 2). The loss-of-function nature of the c.376C>T mutation suggests a tumour suppressor role for *LGR4* in these cancers. This is further supported by a predisposition to SCC in homozygous carriers of a loss-of-function mutation in the LGR4 ligand, R-spondin 1 (ref. 29). *Lgr4*$^{-/-}$ mice lack gallbladders[23], demonstrating an essential role for *LGR4* in the development of this organ. Classical tumour suppressor genes are characterized by loss of the wild-type allele in tumours of heterozygous carriers (loss of heterozygosity (LOH)). We tested for LOH at the *LGR4* locus in DNA isolated from biliary tract tumours of four carriers of the c.376C>T mutation (Supplementary Information and Supplementary Fig. 6). The results indicate LOH in three out of the four carriers, suggesting that *LGR4* acts as a tumour suppressor gene in biliary tract cancers. Increased cancer risk has not been described in *Lgr4*$^{-/-}$ mice; however, these mice have a short lifespan and the oldest mice analysed so far have not been older than 24 weeks, which may not be sufficient time for cancer development.

Through whole-genome sequencing of Icelanders, we have found a rare nonsense mutation, c.376C>T, within the *LGR4* gene that leads to termination of the LGR4 protein at position 126. Carriers of this mutation are at increased risk of various conditions, similar to the phenotypes that are observed in *Lgr4* mutant mice (Table 3). Our results highlight the value of human genome sequence information in the context of rich phenotypic information from which the effects of rare deleterious mutations can be directly assessed in humans, creating a human model of physiological disturbance or disease.

Table 3 | Phenotypic similarities between human carriers of c.376C>T and *Lgr4* mutant mice

Human c.376C>T carriers	*Lgr4* mutant mice
Low BMD	Low BMD[16]
Osteoporotic fractures	Defective bone formation and remodelling[16]
Reduced birth size	Reduced birth size[16,26]
Reduced weight	Reduced weight[16,26]
Elevated serum potassium	Elevated serum potassium[25]
Reduced serum sodium	Reduced serum sodium[25]
Reduced serum bicarbonate	Reduced serum bicarbonate[25]
Serum calcium unchanged	Serum calcium unchanged[16]
Serum phosphate unchanged	Serum phosphate unchanged[16]
Late age at menarche	Developmental delay[16,25,26]
Reduced free testosterone	Defective male reproductive tract[21]
Gallbladder and biliary duct cancers	Lack of gallbladder and cystic duct[23]

For a more detailed comparison between the phenotypes shown by *Lgr4* mutant mice and carriers of c.376C>T, see Supplementary Table 7.

METHODS SUMMARY

The Icelandic, Danish and Australian BMD samples were described previously[3,19,20]. In the discovery analysis, cases were defined as those with adjusted BMD levels less that −1 s.d. from the mean at the hip (total hip), lumbar spine (L2–L4) or whole body, whereas the control group comprised individuals with BMDs above −1 s.d. and those with no BMD information available. Serum electrolytes and all other trait measurements were normalized to a standard normal distribution and adjusted for sex, year of birth and age at measurement. SCC and biliary tract cancer cases were identified in the nationwide Icelandic Cancer Registry, and self-reported information on age at menarche and menopause was retrieved from the Icelandic Cancer Registry.

About 34.2 million sequence variants (30.6 million SNPs and 3.6 million indels) identified through whole-genome sequencing (to an average sequencing depth of >10×) of 2,230 Icelanders using Illumina GAIIx and HiSeq2000 instruments, were imputed into 95,085 Icelanders genotyped with Illumina SNP chips and phased using long-range phasing. Familial imputation methods were used to impute the variants into 296,526 ungenotyped relatives of the 95,085 individuals using the nationwide Icelandic genealogy database. Association testing for case–control analysis was performed using logistic regression, matching controls to cases based on how informative the imputed genotypes were, whereas quantitative traits were tested using a generalized form of linear regression. Single SNP genotyping was carried out by the Centaurus (Nanogen) platform and deletions identified from the EVS were genotyped by Sanger sequencing.

Differences in expression of *LGR4* mRNA in blood and adipose tissue between carriers and non-carriers of c.376C>T were tested using multiple regression, adjusting for age and gender, and for differential cell counts in blood. Differences in the normalized amount of mutated versus wild-type *LGR4* transcripts in carriers of c.376C>T were tested using one-sample *t*-test.

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Supplementary Information is available in the online version of the paper.

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Author Contributions The study was designed and results were interpreted by U.S., G.T., D.F.G., P.S., A.K., U.T. and K.S. Sequence data analysis, imputation and association analysis was carried out by G.T., P.S., D.F.G., O.T.M., M.L.F., A.K. and G.M. Subject recruitment, phenotype analysis and biological material collection was organized and carried out by G.B.W., J.R.C., T.V.N., J.A.E., C.C., J.G.J., L.T., G.I.E., A.T., T.J., T.I., I.O., T.R. and G.S. Sequencing and genotyping were supervised by O.T.M. and U.T. Sanger sequencing and Centaurus genotyping was carried out and analysed by H.T.H. and H.J. Expression experiments were carried out and analysed by G.T., A.S., Aslaug J., Adalbjorg J., K.B., M.H.O. and E.S. Multiple alignment and topology analysis of LGR4 was performed by A.O. The age of the *LGR4* mutation in the population gene pool was estimated by A.H. The paper was drafted by U.S., G.T., U.T. and K.S. All authors contributed to the final version of the paper.

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