ORIGINAL ARTICLE

A genome-wide copy number association study of osteoporotic fractures points to the 6p25.1 locus

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ABSTRACT

Background Osteoporosis is a systemic skeletal disease characterised by reduced bone mineral density and increased susceptibility to fracture; these traits are highly heritable. Both common and rare copy number variants (CNVs) potentially affect the function of genes and may influence disease risk.

Aim To identify CNVs associated with osteoporotic bone fracture risk.

Method We performed a genome-wide CNV association study in 5178 individuals from a prospective cohort in the Netherlands, including 809 osteoporotic fracture cases, and performed in silico lookups and de novo genotyping to replicate in several independent studies.

Results A rare (population prevalence 0.14%, 95% CI 0.03% to 0.24%) 210 kb deletion located on chromosome 6p25.1 was associated with the risk of fracture (OR 32.58, 95% CI 3.95 to 1488.89; $p=8.69\times10^{-5}$). We performed an in silico meta-analysis in four studies with CNV microarray data and the association with fracture risk was replicated (OR 3.11, 95% CI 1.01 to 8.22; p=0.02). The prevalence of this deletion showed geographic diversity, being absent in additional samples from Australia, Canada, Poland, Iceland, Denmark, and Sweden, but present in the Netherlands (0.34%), Spain (0.33%), USA (0.23%), England (0.15%), Scotland (0.10%), and Ireland (0.06%), with insufficient evidence for association with fracture risk.

Conclusions These results suggest that deletions in the 6p25.1 locus may predispose to higher risk of fracture in a subset of populations of European origin; larger and geographically restricted studies will be needed to confirm this regional association. This is a first step towards the evaluation of the role of rare CNVs in osteoporosis.

INTRODUCTION

Osteoporosis is a major public health problem in a rapidly aging population. This systemic skeletal disease is characterised by reduced bone mass and microarchitectural deterioration of bone tissue. The disease progresses 'silently' until the increase in bone fragility leads to increased fracture risk.[1 2] The importance of genetic variation in the regulation of bone mass and bone turnover was first highlighted by linkage analysis in severe Mendelian disorders such as osteoporosis-pseudoglioma syndrome and high bone mass syndrome.[3] In the case of non-Mendelian forms of osteoporosis, common genetic variants have been found to be associated with fracture risk in well powered candidate gene settings.[4] Meta-analysis of single nucleotide polymorphism (SNP) genome-wide association studies (GWAS) for bone mass have identified more than 56 loci independently associated with normal variation of bone mineral density, and some of these studies also found associations with fracture risk.[5–12]

In addition to SNPs, copy number variants (CNVs) have shown associations with complex phenotypes such as schizophrenia, autism, and obesity.[13–16] A study in Chinese individuals suggested an association of a common CNV with osteoporotic fractures[17]; however, the same variant was not replicated in a follow-up study of individuals of European origin,[18] potentially showing population specific effects. Most of the common CNVs are well tagged by common SNPs,[19] and thus are easy to identify with a SNP based GWAS. On the other hand, rare CNVs are difficult to tag and rare and large CNVs have been found to be associated with different diseases.[20] Nevertheless, it is not known whether rare CNVs play a significant role in fracture risk. Thus, we conducted a genome-wide CNV association study on a discovery dataset of 809 fracture cases and 4369 controls drawn from a prospective cohort study. We further looked for in silico replication of the CNV region showing the most significant association in 1096 fracture cases and 47 340 controls from four independent studies with CNV microarray data. Finally, using a breakpoint specific genotyping assay we evaluated the association of this deletion in an additional 9760 fracture cases and 16 542 controls.

METHODS

Subjects

All studies were approved by the institutional ethics review committees of the respective organisations, and all participants provided written informed consent. The Rotterdam Study (RS-I) is a prospective population based cohort study of chronic disabling conditions in Dutch individuals aged 55 years or above (http://www.epib.nl/ergo.htm).[21–23] The Rotterdam Study II (RS-II) is an extension of the Rotterdam Study, which started in 1999 and used the same inclusion criteria and design as the original cohort. Briefly, 3011 individuals (response rate 67%) who had turned 55 years of age or had moved into the study district of Ommoord, Rotterdam, since the start of the original study in 1990 were included in the extension cohort. The Icelandic deCODE Genetics (dCG) study comprises a population based sample to identify the genetic basis of complex diseases.[10] The Framingham Osteoporosis Study (FOS) is embedded in the Framingham Heart Study, a community based, longitudinal, prospective cohort comprising three generations of individuals in multigenerational pedigrees and additional unrelated individuals (http://www.framinghamheartstudy.org/). The PROSPER study is a randomised controlled clinical trial to test the effect of pravastatin on cardiovascular outcomes in the elderly at risk. In addition, we performed de novo genotyping —that is, targeted locus assessments because no CNV microarray data are available—in 15 studies with a variety of epidemiological designs that are part of the GENOMOS DNA collection (http://www.genomos.eu) across Canada, Europe, and Australia. Given the rarity of this deletion event, we pursued genotyping only in those largest GENOMOS studies having at least 200 fracture cases and a total sample size of at least 1000 subjects with phenotype information concerning the fracture status. More information can be found in online supplementary tables S1 and S2. All study participants included were of Caucasian ancestry.

Fracture definition

Fracture cases were defined as fractures at any skeletal site (except fingers, toes, and skull) occurring after age 18 years assessed by x-ray screening, clinical radiographic report, clinical record, clinical interview, and/or questionnaire. High trauma fractures were excluded whenever possible, for example, motor vehicle accidents or falls from greater than standing height. Controls were defined as individuals without a history of fracture. Additional information for each study is available in online supplementary tables S3 and S4.

GWAS genotyping

The four studies were genotyped using the Illumina Infinium HumanHap550 Beadchip (RS-I, RS-II), Quad660 (PROSPER), the HumanCNV370 Beadchip (dCG) or the Affymetrix Dual NspI/StyI GeneChip 2×250 K with 50 K gene centred MIP set (FOS), all according to manufacturer's protocols and quality control standards. The exclusion/filtering criteria for individuals are described in online supplementary table S1A.

CNV analysis of microarray data

Studies used either QuantiSNP[24] or PennCNV[25] to segment CNVs as described below. Quality control (QC) steps for RS-I, RS-II, PROSPER, and FOS are summarised in online supplementary figure S1.

RS-I: Log R ratio (LRR) signal intensity and B allele frequency (BAF) were extracted from 5974 samples using BeadStudio 3.1.3. A Hidden-Markov model, implemented in the software QuantiSNP, was used to make CNV calls. A measure of confidence, log Bayes factor, was computed for each CNV call. A correction for local difference in GC content is implemented in the algorithm to adjust for irregularities in signal intensity. We excluded 547 samples with a mean autosomal LRR SD >0.3 or a BAF SD >0.15. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=305 475). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb or CNVs with less than two consecutive SNPs in the CNV event. This filter effectively reduces the majority of false positive calls, although it has the disadvantage that many putatively real CNV calls might be lost (QC2 nCNVs=58 866). Finally we removed 249 samples with an excess of CNV calls (expressed as upper quartile + 1.5 × (IQR))=20 CNVs (QC3, nCNVs=49 229 in 5178 samples).

RS-II: 2157 samples were used for CNV analyses using QuantiSNP; 154 of these samples with a mean LRR SD >0.35 or a BAF SD >0.15 were excluded. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=129 941). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb, or only one consecutive SNP in the CNV event (QC2 n=15 266). For samples with a LRR SD between 0.3 and 0.35, we applied a stricter threshold of log Bayes factor=15. Thirty-eight samples with >20 CNVs were excluded (QC3, 13 038 CNVs in 1965 samples).

dCG: Illumina BeadStudio (V.2.0) was used to call genotypes, normalise the signal intensity data, and establish the LRR and BAF at every SNP according to standard Illumina protocols. All samples passed a standard SNP based QC procedure with an SNP call rate >0.97. PennCNV was used for detection of CNVs. The input data for PennCNV are LRR and BAF. PennCNV employs a hidden Markov model to analyse the LRR and BAF values across the genome. CNV calls are made on the basis of the probability of a given copy state at the current marker, as well as on the probability of observing a copy state change from the previous marker to the current one.

FOS: A total of 8734 Framingham participants with genome-wide genotypes using Affymetrix 550 k chips were used for CNV calling on autosomal chromosomes. The raw Affymetrix CEL files were read and normalised with Affymetrix power tools to estimate the LRR and BAF at every SNP probe. All samples passed a standard SNP based QC procedure with average genotype call rate >0.95. We excluded SNP probes with

call rate <0.97. We first used PennCNV package (a hidden Markov model) to segment CNV and define the boundaries of a CNV on autosomal chromosomes. The estimated CNVs were then confirmed by another software package, GoldenHelix SVS, with an optimal segmenting algorithm (the Copy Number Analysis Method, CNAM). A principal component analysis was applied to correct for batch effects. We applied several QCs to filter out low quality or questionable quality samples as follows: average LRR SD value >0.35; high total length (10% per chromosome) of CNV; and high total number of CNV (CNV counts >50 per sample). A total of 1300 samples were excluded. In addition, we excluded CNV with less than three consecutive SNPs; CNV with length <1 kb; CNV in the regions of high GC content (80%); and CNVs in the immunoglobulin regions. Among 7434 high quality genotyped samples, 112 746 CNVs were assigned. Fracture data were available for 3529 of these FOS samples.

PROSPER: LRR and BAF measurements were extracted from 5244 samples using GenomeStudio V2009.1. QuantiSNP v2 was used to make CNV calls. We excluded 446 samples with a mean autosomal LRR SD>0.25 or a BAF SD>0.08. We also excluded CNV calls that spanned the centromere (QC1, nCNVs=2 683 302). We discarded all CNV calls with a log Bayes factor value <10, a CNV size <1 kb or CNVs with less than two consecutive SNPs in the CNV event (QC2 nCNVs=1 228 214). Finally, we removed 89 samples with an excess of CNV calls (N>344 CNVs, QC3, nCNVs=1 195 162 in 4709 samples).

Association analysis

The genome-wide association analysis on the discovery cohort was carried out using the rare CNV module implemented in Plink V.1.0.5 on binary copy number differences (deletion vs no deletion between cases and controls). Ten million permutations were performed to assess the significance of the genome-wide association results. Each study provided counts for case–control status among carriers and non-carriers. Odds ratio, confidence intervals and p values were calculated using study counts in an exact Cochran-Mantel-Haenszel exact test statistic implemented in the *stats* package within the R statistical framework.

Quantitative PCR analysis

We validated the deletion that was found to be associated with fractures using quantitative PCR (qPCR) in 12 RS samples where this deletion was found. The primers for the real-time qPCR experiments were as follows (5′–3′ direction):

A. forward primer: GGCAGACAGAGAAAAATGTGGC
B. reverse primer: TGTCAGCTTGATGGATTTGTCC

qPCR assays were validated by demonstrating linearity over three orders of magnitude and by observation of a single melt peak by plotting relative fluorescence units (RFU) data with time (T) (−d(RFU)/dT) on the y-axis as a function of temperature on the x-axis. Reactions contained 200 nM primer; 1X KAPA SYBR FAST qPCR Master Mix and 5 ng genomic DNA. All reactions were performed as triplicates on an Applied Biosystems 7300 Real-Time PCR System cycle conditions: 94°C 3 min initial denaturation followed by 30 cycles 5 s 94°C denaturation and 30 s 60°C primer annealing, extension and RFU data collection. Two reference targets were used normalising on genomic DNA obtained from healthy individuals.

SEL1L reference

Feb. 2009 (GRCh37/hg19) Assembly chr14:81952705-81952790 86 bp

5′-GAATGTATGTGAACGAGGCCGttggtctgaaaggcttatgactgc-ctataacagctataaagatgGCGATTACAATGCTGCAGTGA-3′

RBM11 reference

Feb. 2009 (GRCh37/hg19) Assembly chr21:15587866 +15587951 86 bp

5′-ACAAAACTGGCTCACTCTCACCagtatatcccttggtatttgctttc-tcaagttccttttggagtCCACTTAAAACCTCTGCGACC-3′

Data were analysed using Applied Biosystems RQ Study Software V1.2.3. A fold-change <0.7 (deletion) or >1.25 (duplication) was considered to constitute a true event. For two subjects the assay was inconclusive (DNA amount was not sufficient for full qPCR cycles).

Determination of the deletion breakpoints

Sanger sequencing was used to map the breakpoints of the 6p25 deletion in a population-control cohort. DNA was obtained from one individual from the SAGE cohort that was genotyped with the Illumina 1M array and was identified as a 6p25 deletion carrier (see online supplementary table S5). The cohort and array/CNV analysis have been described previously.[26] The exact breakpoints were found at chromosome 6 in the positions: 4 198 453 and 4 418 843 (NCBI36 hg18).

De novo genotyping

The deletion was genotyped in 15 GENOMOS studies by K-Biosciences (http://www.kbioscience.co.uk) using a competitive allele specific PCR (KASPar) assay designed to identify those individuals with a different sequence at the breakpoint identified by sequencing. Allele specific sequencing to design the probe was set as follows: allele X: 5′-AGGAAAAAAA CATGTTAGCAGGCTTCT-3′; allele Y: 5′-GGAAAAAAACATG TTAGCAGGCTTCC-3′. Given the low frequency of this variant, three positive controls were included in each plate before genotyping. All genotyped plates were evaluated to show signals for the positive controls.

CNV population database query

We queried available CNV population databases to increase the precision for the prevalence estimate of the deletion on a general population level and to check for population specific differences. The resources available were SAGE, OHI, PopGen, WTCCC2, CHOP, the Pharmacogenomics and Risk of Cardiovascular Disease study (PARC), the National Institute for Neurological Disorders and Stroke (NINDS), and the Human Genome Diversity Panel (HGDP), which altogether included 13 441 individuals of European, African American, and Asian descent from various regions throughout the world (see online supplementary table S5).[19 20 26–30]

RESULTS

We obtained the normalised intensity data on a discovery cohort composed of 5974 northwestern European individuals from the RS-I, a population based cohort of individuals aged 55 years and over who had been genotyped with the Illumina 550 K Array (see online supplementary table S1). After QC (see Methods), 49 229 CNVs (mapping to 26 162 genomic locations) were identified in 5178 individuals using a hidden Markov model method to segment CNV regions from microarray DNA intensity data. As expected, we found an inverse correlation between the size and number of CNV events (figure 1). Nevertheless, 90% of the analysed subjects presented with at least one large CNV at some position in the genome (length >100 kb).

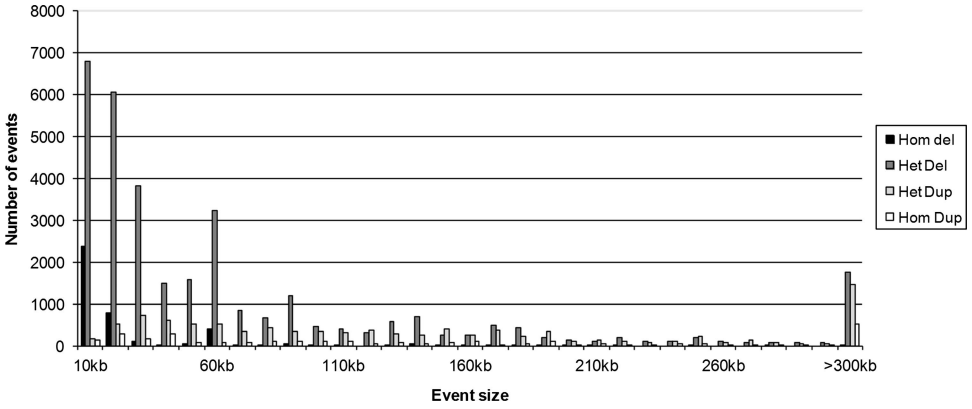

Figure 1 Copy number variant (CNV) type, length, and frequency. CNVs were plotted according to event type (colour), length (x-axis), and frequency in the Rotterdam Study (y-axis, number of samples n=5178).

First, we investigated if there was a difference in the global burden of CNV events between cases with fractures and controls in the RS-I study. Out of 5178 individuals, with a mean follow-up of 7.7 years, 809 subjects presented at least one osteoporotic fracture, of which most were fractures of the hip, spine, and wrist. While no difference was found in the overall burden of CNV events between fracture cases and controls, the proportion of fracture cases with at least one or more rare (frequency <1%) deletions was significantly higher compared to controls (OR 1.04, p=0.03).

Next, we tested the association of segmental rare deletions across the genome with fracture risk in the RS-I study. A rare (population prevalence 0.14%, 95% CI 0.03% to 0.24%) 210 kb deletion located on chromosome 6p25.1 (figure 2A) was the only significantly associated locus with fracture risk after adjusting for multiple testing based on permutations of individual level data (OR 32.58, 95% CI 3.95 to 1488.89; $p=8.69\times10^{-5}$; permuted p=0.027).

We then attempted to replicate this association of the 210 kb deletion on 6p25.1 with fracture in four additional cohort studies with CNV microarray data: the RS II (n=2157, 161 cases), FOS (n=3513, 367 cases), deCode Genetics Study (dCG, n=38 250, 178 cases), and a multicentre randomised clinical trial entitled the PROspective Study of Pravastatin in the Elderly at Risk Study (PROSPER_SC, PROSPER_IR, PROSPER_NL, n=4708, 390 cases). In FOS, we found four cases and 12 non-fracture controls with one copy deletion in the 6p25.1 region. Among them, two cases and six controls had a smaller deletion (~26 kb) inside the same 6p25.1 region (figure 2A). The remaining two cases and six controls had exactly the same size of deletion in the 6p25.1 region. Both CNVs were aggregated in families and segregated from parents to offspring in FOS. We included samples with either one of the CNVs in the CNV-fracture association analyses.

The increased prevalence of this 6p25.1 deletion in fracture cases was replicated in RS-II (1.24% in cases, 0.17% in controls) and FOS (1.09% in cases, 0.38% in controls) studies (table 1). The 6p25.1 deletion was present in three controls from the PROSPER study, but we did not find the 6p25.1 deletion either in cases or in controls of the dCG study (table 1). Combining the data from the in silico replication studies (RS-II, FOS, dCG, and PROSPER) using a Cochran-Mantel-Haenszel test yielded a significant threefold increase in the risk of fracture (OR 3.11, 95% CI 1.01 to 8.22; p=0.02) (table 1).

We validated the presence of this variant with qPCR (see Methods) in 12 6p25del carriers of the RS-I and RS-II cohorts. Ten of them showed clear evidence for deletion (figure 2B). The microarray data in these 12 samples suggested a common breakpoint for all carriers (figure 2A). To identify the breakpoint at a base pair resolution, we sequenced one sample in which the 6p25del had been identified (see Methods). Validation of the sequence level PCR gel to detect this deletion in the same 12 deletion carriers and 12 controls showed perfect assignment of carrier status as determined from the microarray data (figure 2B). Thus, we can conclude that all 12 carriers from the Rotterdam Study share exactly the same breakpoint at sequence level.

We then designed a Kaspar genotyping assay using the sequence level breakpoint information to perform de novo genotyping of this deletion in an additional set of 9760 fracture cases and 16 542 controls from 15 independent studies across Europe, Australia, and Canada (figure 2C and online supplementary table S1). Despite having a large sample size, we could only detect 21 additional 6p25del carriers (frequency <0.1% in both cases/controls) with no significant association with fracture risk (p=0.81) (table 1).

We queried six available CNV population databases for the prevalence of this deletion (see online supplementary table S5; SAGE n=1287 European, 495 African Americans from USA; OHI n=1234 European from Canada; PopGen n=1123 European from Germany; WTCCC2 n=4783 European from UK; CHOP n=1320 European, 694 African American, 12 Asian from USA; a combined dataset from PARC, NINDS, and the HGDP n=2493 individuals from different ethnicities and countries of origin).[19 20 26–30] The deletion was identified in five out of six studies: SAGE (1/1287), OHI (5/1234), PopGen (0/1123), WTCCC2=7/4783, CHOP (4/1320), PARC (2/936), NINDS (2/671), HGDP (0/886). All carriers were found in samples of European ancestry. The deletion was not found in 886 samples from the Human Genome Diversity Project (51 different world populations) or in two studies of African ancestry (see online supplementary table S5).

DISCUSSION

We report here a genome-wide scan for CNVs and risk of fracture assessed in the RS-I cohort. A microdeletion in 6p25.1 was found to be associated with increased risk of fracture and remained significant after permutation testing. The deletion was validated with qPCR and was also replicated in silico in two additional studies: RS-II and FOS; the deletion was only found in three controls of the PROSPER study and it was not found in the deCode study. Combining all four in silico replication studies, the deletion is associated with a threefold higher risk of

bone fracture in individuals of mainly Dutch and US American ancestry. Additional replication was pursued using breakpoint genotyping in 15 studies; however, the deletion was only found in six of the 15 studies with no replication of association with fracture risk.

The frequency of this deletion showed regional variation, being present in studies of the Netherlands (0.34%), Spain (0.33%), USA (0.23%), England (0.15%), Scotland (0.10%), and Ireland (0.06%) (Table 1). The deletion seems to be absent or in lower frequency in certain populations such as Iceland (dCG) and Sweden (UFO) where, despite having assessed more than 30 000 and 4000 subjects for each population, respectively, no additional carriers were found. Founder effects can effectively remove rare variants from the gene pool in a population. These population effects could explain why we did not detect the 6p25.1 microdeletion in 30 000 individuals from a population with relatively similar genetic background (Iceland) as the one in which we found the microdeletions (northwestern European from Netherlands and USA).

While replication of the association with fracture risk in two cohorts was achieved using in silico data, the meta-analysis of de novo genotyped studies was not statistically significant. There are several potential explanations for the lack of replication in this subset of studies. First, limitations in study power could make it difficult to identify a significant association (at $p<0.05$) with a variant of such low frequency. Considering an OR=3, and a minor allele frequency (MAF)=0.05% in controls, almost 18 000 cases and an equal number of controls would be needed to reach 80% power. Secondly, it is possible that while 12 of the microarray based carriers in the Rotterdam Study share the same breakpoint, other 6p25del carriers may have different breakpoints (such as the eight carriers with a smaller 26 kb deletion detected in FOS) which were not detected by our specially designed genotyping probe. Thirdly, it may be possible that a two-hit model involving a yet unknown genetic variant is affecting the predisposition of 6p25del carriers to an increased risk for fracture. Finally, some degree of misclassification may have occurred, as carriers currently classified as controls may eventually develop a fracture later in life; this could have potentially affected our results.

The deletion is located in an intergenic region in the subtelomeric region of chromosome 6p (figure 2A) in the proximity of the *peroxisomal D3,D2-enoylCoA isomerase* (*PECI*) gene which codes for an enzyme relevant for the metabolism of fatty acids. *PECI* was first cloned by using pooled antisera from autoimmune diabetes patients.[31] Hence, it is possible that even though the 6p25.1 microdeletion is 200 kb away from *PECI*, this region may be regulating the expression of *PECI*. Both type

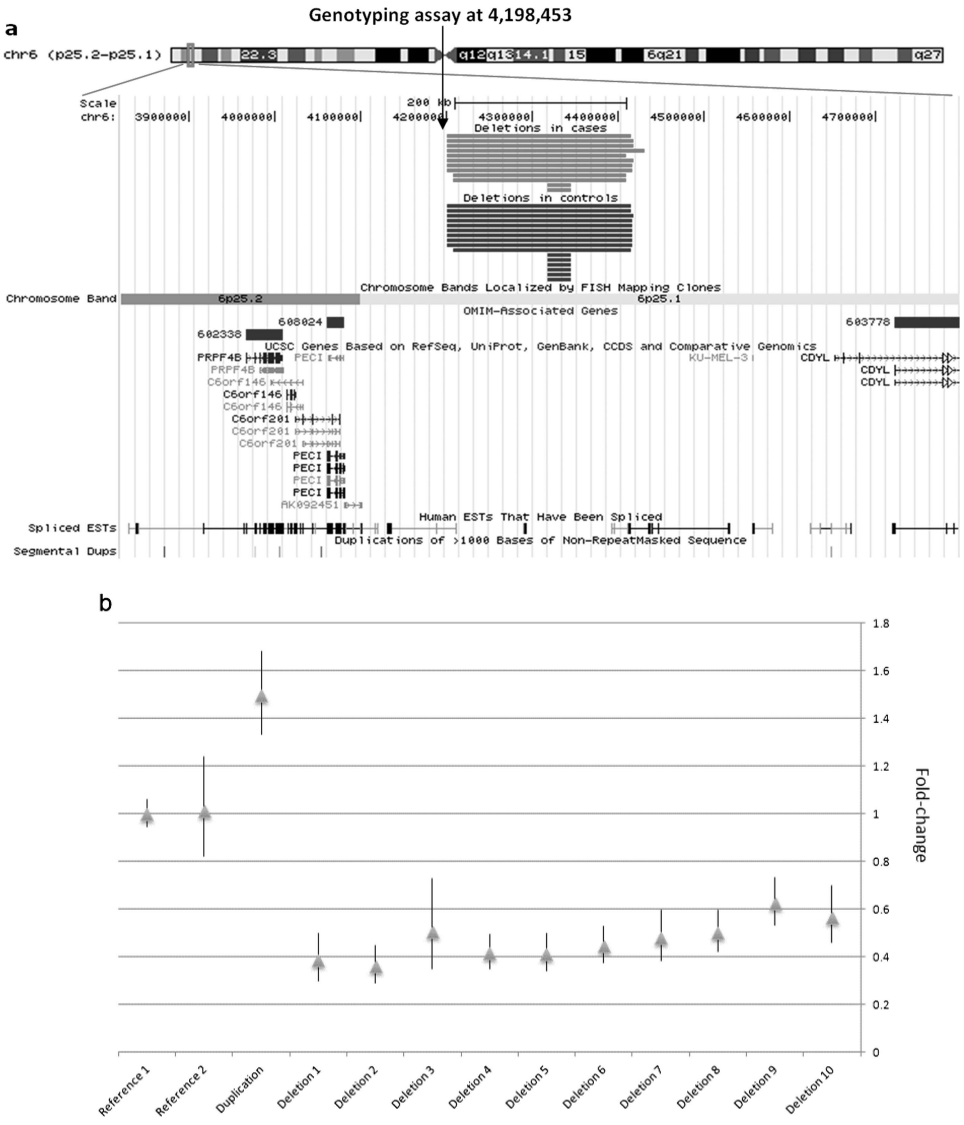

Figure 2 Identification and validation of deletions at 6p25.1. (A) 210 kb deletions were first identified in six fracture cases and one control from the Rotterdam Study I (RS-I). Additional carriers from the RS-II and Framingham Osteoporosis Study are also depicted. Refseq genes and OMIM associated genes are depicted. (B) Quantitative PCR validation of 12 carriers in the RS (for two samples the assay failed). The third sample, labelled as 'Duplication', is a FISH (fluorescence in situ hybridisation) validated complete 6p arm duplication. (C) Validation of a sequence based breakpoint detection of the 6p25.1 deletion. Twelve deletion carriers (wells A1, C1, E1, G1, A2, C2, E2, B2, B1, C1, E1, H1) show amplified PCR product exactly with the same length as the sequenced controls.

Figure 2 (Continued)

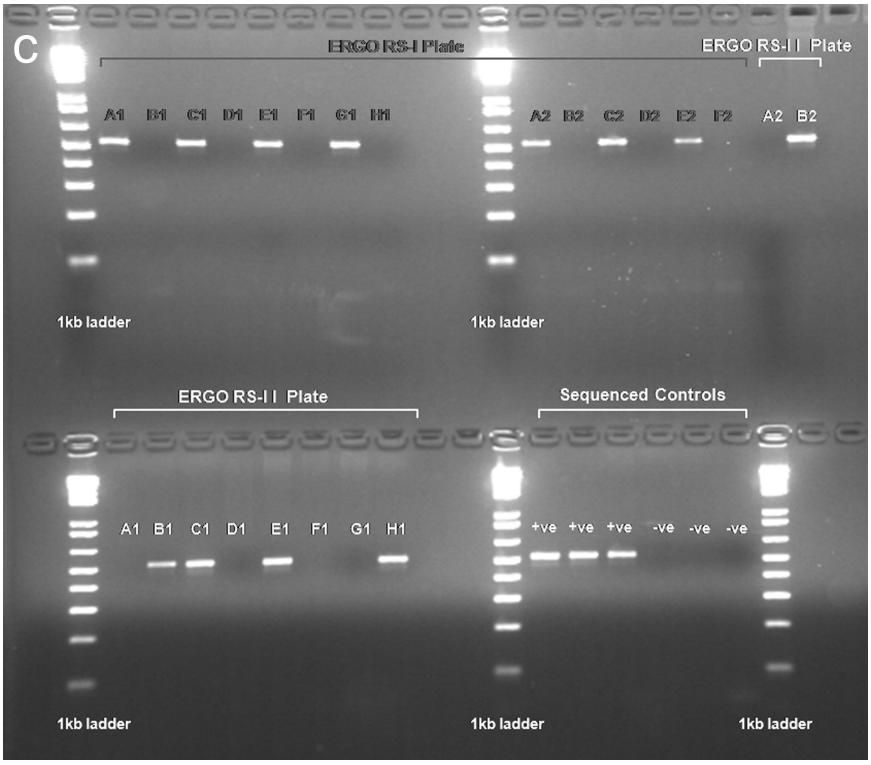

Table 1 Association results of 6p25.1 deletion with increased fracture risk

Stage	Study	Country	Deletions/total cases	Frequency in cases (%)	Deletions/total controls	Frequency in controls (%)
Discovery	RS-I	Netherlands	6/809	0.74	1/4369	0.02
	Frequency (95% CI)		0.74% (0.15 to 1.33)		0.02% (0 to 0.20)	
	OR (95% CI)		32.58 (3.95 to 1488.89) $p=8.69\times10^{-5}$			
In silico	dCG	Iceland	0/178	0	0/38 072	0
Replication	FOS	USA	4/367	1.09	12/3146	0.38
	PROSPER_IR	Ireland	0/141	0	1/1650	0.06
	PROSPER_NL	Netherlands	0/57	0	0/756	0
	PROSPER_SC	Scotland	0/192	0	2/1912	0.10
	RS-II	Netherlands	2/161	1.24	3/1804	0.17
	Total		6/1096	0.55	18/47 340	0.04
	Frequency (95% CI)		0.18% (0.11 to 0.98)		0.04% (0.02 to 0.05)	
	OR (95% CI)		3.11 (1.01 to 8.22) p=0.02			
Breakpoint	APOSS	Scotland	0/531	0	0/2129	0
Genotyping	CABRIO-C	Spain	1/327	0.31	3/1018	0.29
Replication	CABRIO-CC	Spain	4/1023	0.39	3/1104	0.27
	CAIFOS	Australia	0/736	0	0/581	0
	CAMOS	Canada	0/235	0	0/1732	0
	DOPS	Denmark	0/410	0	0/1242	0
	EDOS	Scotland	0/1500	0	0/193	0
	EPICNOR	England	0/227	0	1/1127	0.09
	EPOLOS	Poland	0/231	0	0/446	0
	EPOS	England	1/686	0.15	2/1289	0.16
	HCS	England	0/339	0	3/2308	0.13
	LASA	Netherlands	0/313	0	3/562	0.53
	MANMC	Canada	0/750	0	0/0	0
	NOSOS	Scotland	0/342	0	0/740	0
	UFO	Sweden	0/2110	0	0/2071	0
	Total		6/9760	0.06	15/16 542	0.09
	Frequency (95% CI)		0.06% (0.01 to 0.11)		0.09% (0.04 to 0.14)	
	OR (95% CI)		0.78 (0.24 to 2.24) p=0.81			

p, p value computed with an exact Cochran-Mantel-Haenszel test statistic.
dCG, Icelandic deCODE Genetics Study; FOS, Framingham Osteoporosis Study; RS, Rotterdam Study

1 and type 2 diabetes are associated with higher risk of fracture, even though bone density is not low.[32–36] Thus, the increased risk we see with individuals with the 6p25del may be mediated by comorbidity with diabetes.

Another candidate hypothesis is that microdeletions in 6p25.1 are disrupting an unidentified gene in the critical region. A similar mechanism was shown in which microdeletions of 1q21.1 disrupted an expressed sequence tag (EST) that was in fact an unknown gene which subsequently increased the risk for neuroblastoma.[37] There are two spliced ESTs that map to the 6p25 region covered by the microdeletions (figure 2A). One of the ESTs, AL121205.1, shares 33% of its structure with the *KREMEN1* gene. *KREMEN1* encodes a high affinity dickkopf homolog 1 (*DKK1*) transmembrane receptor that cooperates with DKK1 to block Wnt/β-catenin signalling, which is an important pathway in bone biology.[38] The second EST, DB318881.1, shares 38% structural similarity with *WDR66*, which does not have any clear connection to our findings. Further analyses are required to test whether the deletion of those ESTs are indeed related to the increased risk of fracture.

Other individuals have been reported with the same microdeletion in different populations of Caucasian origin.[20 29] These reports provide further evidence of the existence of this rare microdeletion in other populations. Similarly, it has been reported that patients with 6p25 microdeletions present a variety of phenotypes such as ocular dysgenesis, hearing impairment, and craniofacial, skeletal, cardiac, and renal malformations.[39–42] However, these deletions are much larger (from 1 to 13 Mb in size), and cover many genes. Therefore, the relation of those larger events with the association we found between the 6p25.1 microdeletion and fractures is not direct.

Our study has three particular strengths: (1) samples from the discovery and in silico replication sets were drawn from cohort studies where cases and controls were genotyped at random in the same laboratory. This is important for avoiding the biases that occur when cases are genotyped at different time points or centres than controls are; (2) DNA was extracted from blood for samples used for the discovery of this CNV—the use of other DNA sources such as cell lines can introduce noise in CNV analysis; (3) our discovery sample size was large enough to detect rare variants (~1%) with large effects (OR >3) and we also replicated the association in the independent studies in silico; (4) we validated the microdeletion using different technologies (qPCR, Sanger sequencing and Kaspar).

Performing a genome-wide scan for CNVs has limitations. Because the SNP arrays that we used for the discovery phase were not designed to evaluate CNVs, many CNV enriched regions were not covered in this study, and may not have been identified in our scan. Also, to minimise the rate of false positive CNV calls, we used stringent QC thresholds which may have filtered out real CNV calls.

In summary, we have shown that a microdeletion of 6p25.1 is associated with an increased risk of fracture in a group of populations mostly of Dutch origin. Further studies are needed to replicate this variant in populations of similar ancestral background and to identify the specific gene or genes in the region for which this deletion contributes to an increased risk for fracture. Although this event is rare, the effect on fracture risk was substantially greater than the effects usually observed for SNPs. If rare CNVs have similar degrees of effect as the one detected here, it might be possible to identify them with better powered genome scans, not only for fracture risk but also for other human traits and diseases.

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