

## ELECTRONIC LETTER

# Familial isolated hyperparathyroidism is linked to a 1.7 Mb region on chromosome 2p13.3–14

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**Background:** Familial isolated hyperparathyroidism (FIHP) is an autosomal dominantly inherited form of primary hyperparathyroidism. Although comprising only about 1% of cases of primary hyperparathyroidism, identification and functional analysis of a causative gene for FIHP is likely to advance our understanding of parathyroid physiology and pathophysiology.

**Methods:** A genome-wide screen of DNA from seven pedigrees with FIHP was undertaken in order to identify a region of genetic linkage with the disorder.

**Results:** Multipoint linkage analysis identified a region of suggestive linkage (LOD score 2.68) on chromosome 2. Fine mapping with the addition of three other families revealed significant linkage adjacent to D2S2368 (maximum multipoint LOD score 3.43). Recombination events defined a 1.7 Mb region of linkage between D2S2368 and D2S358 in nine pedigrees. Sequencing of the two most likely candidate genes in this region, however, did not identify a gene for FIHP.

**Conclusions:** We conclude that a causative gene for FIHP lies within this interval on chromosome 2. This is a major step towards eventual precise identification of a gene for FIHP, likely to be a key component in the genetic regulation of calcium homeostasis.

Primary hyperparathyroidism is a relatively common endocrine disorder caused by abnormal regulation and hyper-secretion of parathyroid hormone (PTH) from the parathyroid glands. Its clinical features include renal calculi and calcification, bone loss, neuromuscular dysfunction, and acute hypercalcaemic crisis.<sup>1</sup> Biochemically it is characterised by persistent hypercalcaemia in the presence of inappropriately normal or elevated PTH.<sup>2</sup> Eighty per cent of surgically proven cases of primary hyperparathyroidism are associated with a solitary parathyroid adenoma.<sup>1</sup>

Familial isolated hyperparathyroidism (FIHP) is an autosomal dominantly inherited form of primary hyperparathyroidism. It is thought to comprise about 1% of cases of primary hyperparathyroidism with age of onset at least one decade earlier than that of the commoner sporadic form.<sup>3</sup>

While sporadic primary hyperparathyroidism is typically associated with a solitary parathyroid adenoma, in FIHP solitary or multiple adenomata, hyperplasia of multiple parathyroid glands, or parathyroid carcinoma<sup>4</sup> may be found.

FIHP is known to be a genetically heterogeneous condition. In a study of 22 unrelated subjects with the FIHP phenotype, five were found to have mutations in *MEN1* and four in *CASR*,<sup>4</sup> which more commonly give rise to the multiple endocrine neoplasia type 1 (MEN1) and familial hypocalcaemic hypercalcaemia (FHH) phenotypes, respectively.

Mutations in *HRPT2*, the hyperparathyroidism-jaw tumour syndrome (HPT-JT) gene, have also been observed in a few pedigrees with FIHP.<sup>5–6</sup> However, the genetic basis for most cases of FIHP is not known.

Whilst recognising the possibility that mutations in a single gene may not account for all remaining cases, we undertook a genome-wide screen of DNA from families with FIHP in an attempt to identify a region of genetic linkage with the disorder. The value of identifying another causal gene for FIHP extends beyond the obvious diagnostic benefit for affected families. Investigation of its function has the potential to substantially improve understanding of parathyroid physiology and pathophysiology and calcium metabolism in general. This may, in turn, lead to improvements in the management of calcium metabolic disorders.

## METHODS

### Subjects

Nine pedigrees with FIHP were ascertained from families previously reported.<sup>4</sup> One other (fig 1, pedigree D), a family with multiglandular primary hyperparathyroidism, was ascertained after that report. Causative mutations in *MEN1*, *CASR*, and *HRPT2* had been excluded by demonstration of wild type sequence of these genes in at least one affected individual from each kindred.

Ethics approval for the study was obtained from the University of Queensland and Princess Alexandra Hospital human research ethics committees. Written informed consent was obtained from each individual and peripheral blood obtained for biochemical analysis and DNA extraction. Affection status was determined from an individual's medical history, serum biochemistry, and age. Subjects were considered affected if they had a history of hyperparathyroidism, confirmed surgically, or had persistent hypercalcaemia with inappropriately normal or elevated PTH<sup>2</sup> and normal renal function at the time of ascertainment. The affection status of individuals with normal serum calcium and PTH levels was considered "unknown" for those under the age of 40 years while those over the age of 40 were regarded as unaffected. Although the average age of onset of FIHP is not known, 40 years was chosen as the age cut off for affection status classification because in the largest report on the clinical and genetic characteristics of probands with FIHP, the average age at non-prospective diagnosis was 40 ± 3 years (n = 28).<sup>3</sup> As primary hyperparathyroidism has a preclinical phase of asymptomatic hypercalcaemia, the onset of hypercalcaemia in FIHP can be predicted to occur before the age of 40 and, therefore, prospective diagnoses should be possible by the age of 40.

**Abbreviations:** AGRF, Australian Genome Research Facility; FIHP, familial isolated hyperparathyroidism; HPT-JT, hyperparathyroidism-jaw tumour syndrome; MEN1, multiple endocrine neoplasia type 1

Figure 1 shows the 10 pedigrees studied. A total of 59 individuals were available for genotyping. Twenty two were affected, 24 unaffected, and 13 of unknown affection status (age <40 years). One family was Polynesian, the remainder Caucasian. Affection status of deceased pedigree members was determined from medical records where possible. Where these were not available and offspring were confident of their knowledge, affection status was ascribed from reports of offspring. In any instances of uncertainty, "unknown" status was attributed to the individual.

### Biochemistry

Serum ionised calcium, total calcium, albumin, creatinine, and urea were quantitated using standard automated techniques at nationally accredited laboratories within Australia. Plasma intact PTH levels were determined by immunoradiometric or immunochemiluminometric assays.

### Genotyping

DNA was extracted from peripheral blood leukocytes using a published method.<sup>7</sup>

A genome-wide screen of the 22 autosomes, using 382 microsatellite markers with an average spacing of 10 cM (ABI Medium Density Linkage Mapping Set Version II), was performed at the Australian Genome Research Facility (AGRF), Melbourne. Fine mapping of the FIHP locus was also performed at the AGRF using microsatellite markers identified from the UCSC Genome Browser (<http://www.genome.ucsc.edu/cgi-bin/hgGateway?org=human>). The order and position of each marker were verified and its heterogeneity obtained using the MAP-O-MAT v1.1 web-based linkage mapping server (<http://compgen.rutgers.edu/mapomat>).<sup>8</sup> Markers with heterogeneity  $\geq 0.7$  in locations providing even coverage across the locus were then genotyped.

### Linkage analysis

Prior to undertaking the genome-wide screen, simulation analysis was performed using the SLINK package<sup>9, 10</sup> to estimate the probability of detecting linkage with the available pedigrees. Genotypes were simulated for two highly polymorphic markers with eight alleles of equal frequency. The disease gene was assumed to lie equidistant between the two markers spaced 10 cM apart (akin to the worst case scenario when performing a genome screen at 10 cM resolution). All the families were considered homogeneous and simulations were carried out for 200 replicates. Penetrance models were developed assuming an autosomal dominant mode of inheritance with a disease allele frequency of 0.00001, phenocopy rate of 0.001, and a penetrance of 0.90. The exact penetrance of the FIHP trait is not known and published pedigrees<sup>3, 4</sup> are too small to provide conclusive evidence. A high penetrance rate of 0.9 was therefore chosen as the "best guess" from the pedigrees in this study and published penetrance data concerning the related MEN1 and HPT-JT.<sup>11-13</sup>

This analysis indicated that three pedigrees had low power to demonstrate linkage. These (fig 1A,F,H) were set aside for later inclusion in fine mapping. The 382 marker screen was performed on DNA from the remaining seven families.

Genotyping data were analysed for pedigree relationship errors using PREST software.<sup>14</sup> Two point LOD score analysis was carried out using MLINK from the FASTLINK 4.1P suite of linkage programs.<sup>15</sup> Multipoint LOD scores were calculated using GeneHunter 2.1.<sup>16</sup> Heterogeneity (het) multipoint LOD scores were also calculated with GeneHunter to account for possible genetic heterogeneity of FIHP in these pedigrees. All these analyses assumed the same linkage parameters as were applied to the simulation analysis. SIB-PAIR (<http://www.qimr.edu.au/davidD/davidd.html>) was used to

construct locus and pedigree files in LINKAGE format, to calculate allele frequencies, and correct for genotyping errors.

### Sequencing candidate genes

Protein coding regions with splice junctions of protein phosphatase 3 (formerly 2B), regulatory subunit B, 19 kDa, alpha isoform (calcineurin B, type I) (*PPP3R1*, NM\_000945.3), and prokineticin receptor 1 (*PKR1*, NM\_138964.2) were sequenced from leukocyte DNA of an affected member of each family. Novel sequence variations and non-synonymous published allelic variants were confirmed by repeat PCR and sequencing. DNA from each member of that particular pedigree was then sequenced for that region.

Alterations in DNA and protein sequences are described according to HUGO nomenclature guidelines (<http://www.genomic.unimelb.edu.au/mdi/mutnomen/>). Nucleotide and amino acid positions were calculated from the start of transcription or translation, respectively. Gene accession numbers were obtained from NCBI RefSeq files (<http://www.ncbi.nlm.nih.gov/RefSeq/>). Parathyroid expression data were obtained from the EnsMart database (<http://www.ensembl.org/Multi/martview>).

Protein coding regions and splice sites were amplified using primers designed from intronic DNA sequence adjacent to intron-exon boundaries or exonic DNA when internal primers were required for larger exons. Primer sequences are available on request. PCR reactions for individual exons were carried out in 50  $\mu$ l reaction volumes containing 50 ng DNA, 10 pmol each primer, 5  $\mu$ l GeneAmp 10 $\times$  PCR Buffer II (Applied Biosystems, Foster City, CA), 3  $\mu$ l 25 mM MgCl<sub>2</sub> Solution (Applied Biosystems), 500 mU AmpliTaq Gold DNA polymerase (Applied Biosystems), and 250 nM dNTPs. Thermal cycling was carried out in a PC-960G Gradient Thermal Cycler (Corbett Research, Sydney, Australia). Thermocycling conditions consisted of an initial 10 min denaturation step at 95°C followed by 35 cycles of 94°C for 60 s, annealing for 30 s and 72°C for 90 s, with a final extension step at 72°C for 7 min. Annealing temperatures were individualised for each primer pair and are available on request. PCR products were visualised by ethidium bromide staining on 0.8% agarose gel and then purified using an UltraClean PCR Clean-up DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

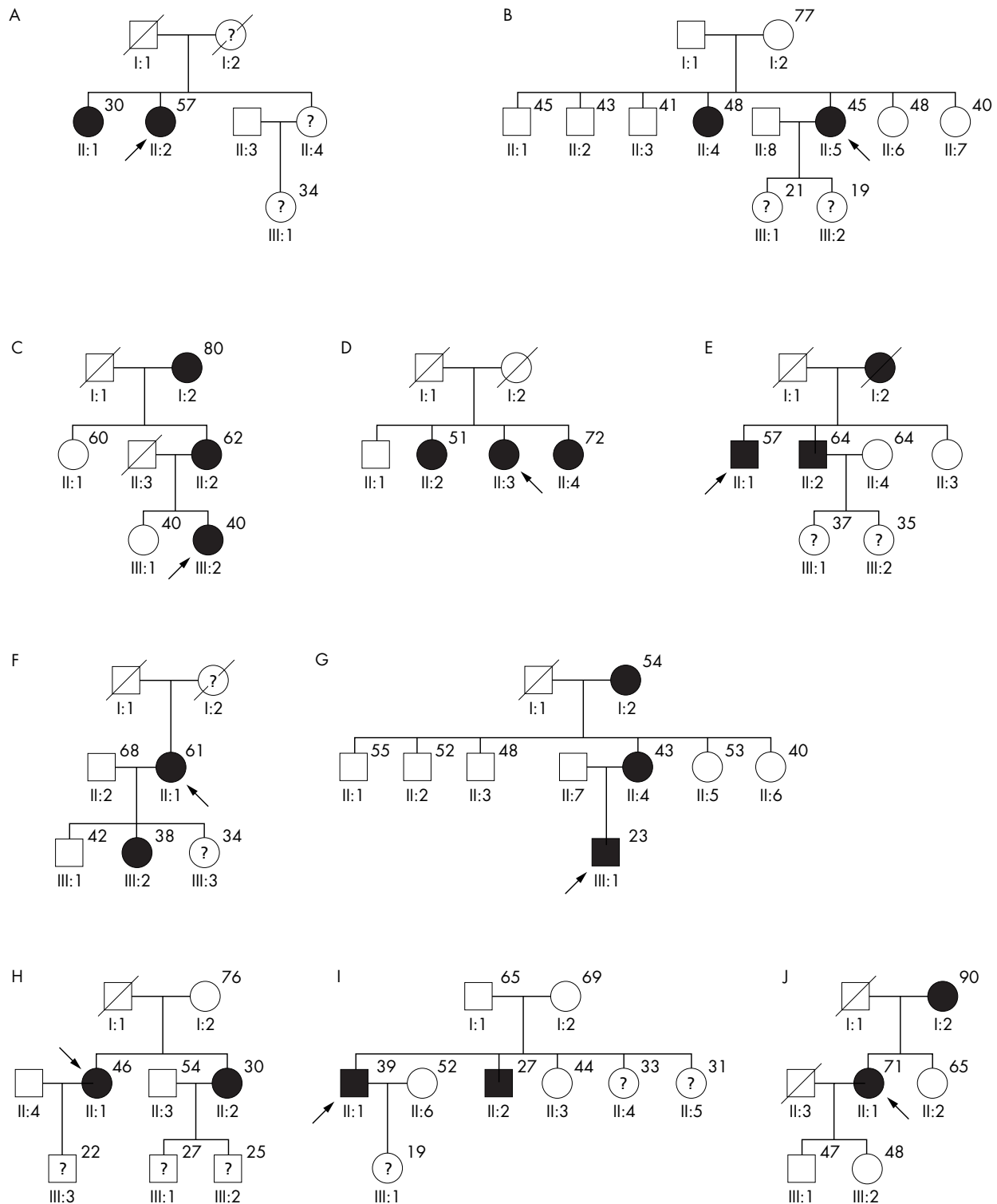
Sequencing reactions employed the same primers used for PCR. Reactions were carried out in 12  $\mu$ l volumes containing 4  $\mu$ l purified PCR product, 1  $\mu$ l BigDye Terminator Version 3.1 (ABI Prism), 3  $\mu$ l BigDye Terminator Version 3.1 sequencing buffer, and 1.6 pmol primer. Thermocycling involved 35 cycles of 95°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The DNA product was analysed on an ABI 3730xl automated sequencer at the AGRF, Brisbane Division.

## RESULTS

### Genome-wide screen

SLINK analysis produced an average maximum total LOD score of 4.27, with 95% and 79% power to detect suggestive (LOD>1.86) and significant (LOD>3.3) linkage (according to accepted criteria),<sup>17</sup> respectively. Therefore, it was concluded that the sample had sufficient power to detect the causative locus in a genome-wide linkage screen.

Two point linkage analysis of genome-wide screen data revealed two genetic loci with LOD scores suggestive of linkage. These were at marker D1S2841 (LOD = 2.1 at a recombination fraction ( $\theta$ ) of 0) and three adjacent markers



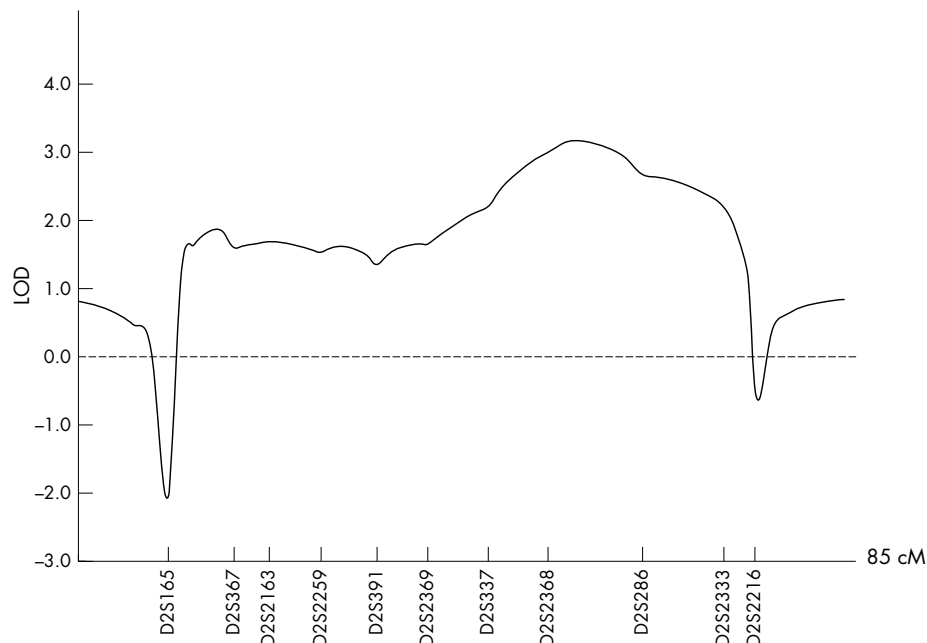
**Figure 1** Pedigrees of families with FIHP. Affected, unaffected, and individuals of unknown phenotype are denoted by black, white, and ? symbols, respectively. Numbers above and to the right of symbols indicate the age in years at diagnosis for affected individuals and ascertainment for others. Individuals without ages noted were not ascertained. Probands are indicated by arrows.

on chromosome 2: D2S2333 (LOD = 2.59 at  $\theta = 0$ ), D2S286 (LOD = 2.36 at  $\theta = 0$ ), and D2S2368 (LOD = 1.91 at  $\theta = 0$ ).

Multipoint analysis did not statistically confirm linkage at D1S2841 (total LOD = 0.42, het LOD = 1.22 with  $\alpha = 0.60$ ). On chromosome 2, however, multipoint analysis gave a maximum total LOD score of 2.68 (and maximum het

LOD = 2.68 with  $\alpha = 1$ ) between D2S2368 and D2S286 with a 77.6 cM (59.7 Mb) interval with LOD >1.0 between D2S165 and D2S2216 (fig 2).

Multipoint analysis also identified an area of suggestive linkage on chromosome 11. A small region around D11S4151 with a total LOD of 2.02 (and het LOD = 2.02 with  $\alpha = 1$ ) was



**Figure 2** GeneHunter postscript graph of total LOD scores for the region of genetic linkage to FIHP on chromosome 2.

observed. The two point LOD score for this marker was 0.10 at  $\theta = 0$ , 0.40 at  $\theta = 0.1$ , 0.43 at  $\theta = 0.2$ , 0.30 at  $\theta = 0.3$ , 0.10 at  $\theta = 0.4$ , and 0.00 at  $\theta = 0.5$ .

None of the regions of suggestive linkage, from either two point or multipoint analysis, was in the vicinity of any genes currently associated with FIHP (*MEN1*, *CASR*, and *HRPT2*).

There were no other regions in which het LOD scores exceeded 1.59, except where already mentioned.

Given that the region of suggestive linkage on chromosome 2 was identified by both analytical methods, had the highest LOD score, was located across several consecutive markers, and was, on examination of reconstructed haplotype diagrams, the only region in which the same, "affected", haplotype was shared by affected members within each pedigree, it was concluded that this region was most likely to contain a gene for FIHP in these pedigrees.

### Fine mapping

The three families that had not been included in the genome-wide screen were then genotyped at markers D2S165, D2S367, D2S2259, D2S391, D2S337, D2S2368, D2S286, D2S2333, D2S2216, and D2S160. All 10 families were also genotyped at D2S2163 and D2S2369 in that round. The resultant maximum multipoint LOD achieved was 3.16 with the region of linkage unchanged (fig 2).

Examination of haplotype diagrams for affected individuals only revealed two intervals of critical linkage between D2S2259 and D2S337, and D2S2368 and D2S2216. The interval between D2S2259 and D2S337 only had a multipoint LOD of 2.22 because of sharing of the affected haplotype by seven out of 17 unaffected siblings or descendants of affected individuals. The other interval corresponded to the peak LOD.

Nine additional markers between D2S2259 and D2S337 (D2S2294, D2S2298, D2S2291, D2S2240, D2S2227, D2S2739, D2S2153, D2S378, D2S2183) and nine in the region between D2S2368 and D2S2216 (D2S358, D2S327, D2S443, D2S1389, D2S2977, D2S99, D2S1374, D2S2110, D2S2109) were therefore genotyped in affected and unaffected individuals from the 10 pedigrees. Figure 3 shows the multipoint LOD result across the region. The interval between D2S2259 and D2S337 increased its LOD close to D2S2259 (LOD 1.83) but decreased

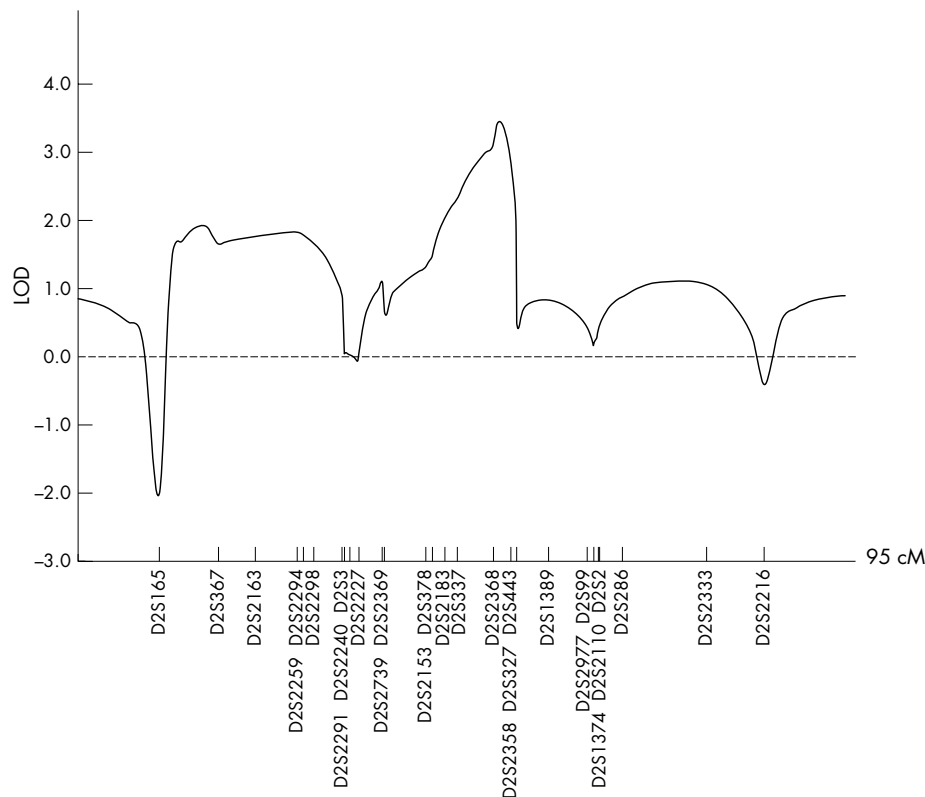
it elsewhere. Haplotype examination of this region revealed a narrower interval of linkage with affected individuals only between D2S2291 and D2S2739.

In the second fine mapped region, a maximum LOD of 3.43 was achieved adjacent to D2S2368. Recombination events observed in reconstructed haplotype diagrams defined the area of linkage to between D2S2368 and D2S358 (fig 4). However linkage was not observed in pedigree B, with individuals II:4 and II:5 not sharing any allele of either marker D2S2368 or D2S358. Both affected members of this pedigree have had mild primary hyperparathyroidism for several years and have declined surgical treatment.

The region of highest LOD score (between D2S2368 and D2S358) corresponds to a physical map position on chromosome 2 between bases 67,125,363 and 68,809,166, according to the May 2004 human reference sequence, based on NCBI Build 35, produced by the International Human Genome Sequencing Consortium. This region is currently thought to contain 10 known and 26 predicted genes, according to the UCSC Genome Browser database. A hierarchy of candidate genes was collated based on the expression and function of known genes. The two most likely candidates, *PPP3R1* (NM\_000945.3) and *PKRI* (NM\_138964.2), were sequenced.

*PPP3R1*, involved in the regulation of PTH gene expression,<sup>18</sup> is composed of six protein coding exons. These, with splice junctions, were sequenced using DNA from an affected member of each pedigree. Six were found to be heterozygous for a known synonymous allelic variant NM\_000945.3:c.99C>T<sup>19</sup> in exon 3. Four individuals were homozygous for thymidine at that site. No other sequence variations were observed in this gene.

*PKRI*, with a role in endocrine gland angiogenesis,<sup>20</sup> is composed of two translated exons which contain one synonymous allelic variant, NM\_138964.2:c.942T>C, and a non-synonymous variant, NM\_138964.2:c.118A>G. Affected individuals from four families were heterozygous 942T>C and the remainder were thymidine homozygotes. Affected individuals II:2 and II:1 of pedigree H were heterozygous 118A>G and homozygous A, respectively. Their unaffected mother (I:2) was a 118A>G heterozygote.



**Figure 3** GeneHunter postscript graph of total LOD scores in the region between D2S165 and D2S2216 after fine mapping.

A novel sequence variation, NM\_138964.2:c.591C>T, was observed in *PKRI* in pedigree C. Synonymous for Ser197, it did not co-segregate with the disease state, being present in individuals III:2 (affected) and III:1 (unaffected) but not in any other individual in that pedigree.

## DISCUSSION

This genome-wide screen has identified a 1.7 Mb interval of significant genetic linkage for FIHP on chromosome 2p13.3–14. The data have also indicated a region of suggestive linkage in close proximity, on 2p16.3–21.

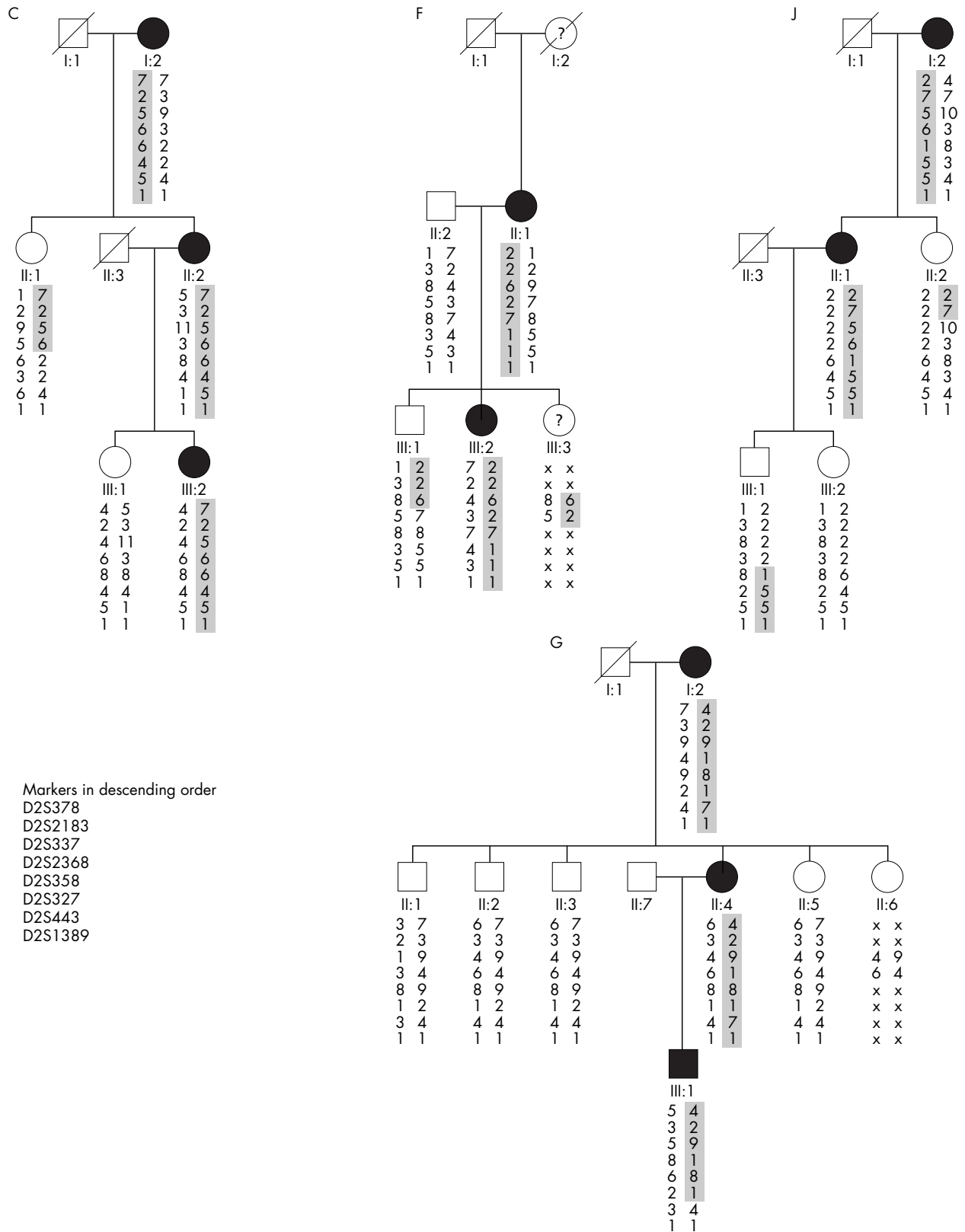
For the interval at 2p13.3–14, the “affected” haplotype is shared by affected members in nine of the 10 pedigrees, consistent with FIHP being a highly penetrant condition. FIHP is known to be genetically heterogeneous, so apparent absence of linkage in one pedigree is neither surprising nor a deterrent to further investigation of the region to identify a gene. Given the range of clinical and histopathological findings in the pedigrees studied<sup>4</sup> and lack of histological information about the unlinked pedigree B, there is no clinicopathological characteristic which distinguishes this family from the other pedigrees.

The region of suggestive linkage between D2S2291 and D2S2739 is physically placed on chromosome 2 from nucleotides 45735915 to 49533802, at 2p16.3–21, according to the May 2004 human reference sequence (NCBI Build 35), of the International Human Genome Sequencing Consortium. All affected individuals in the 10 pedigrees share the “affected” haplotype for that pedigree, but for significant linkage to FIHP to be present, a disease penetrance of only about 60% would have to apply. Current knowledge of related conditions<sup>11–13</sup> and examination of the available pedigree diagrams argue against the penetrance of the FIHP trait being as low as 60%. However, it is

theoretically possible and, if true, the incidence of FIHP is likely to be much higher than assumed, with affected families escaping identification due to the presence of unaffected carriers in one or more generations.

Based on what is known of the disease and its other genetic bases, it seems much more likely that more than one gene for FIHP remains to be identified than that the penetrance of the condition is around 60%. Therefore, the 1.7 Mb interval of linkage between D2S2368 and D2S358 is more likely to contain a gene for FIHP. Sequencing of the two most likely candidate genes in this region, *PPP3R1* and *PKRI*, however, did not reveal any likely causative sequence variations. The synonymous NM\_138964.2:c.591C>T sequence variation observed in *PKRI* in two siblings in pedigree C was probably inherited on their paternal allele.

The two candidates sequenced appeared the most likely, derived from published functional and expression data, but information on functional activity of many of the other genes in the region is not yet available. Those with demonstrated parathyroid expression include *CID* (encoding a DNA binding and apoptosis inducing protein), *LOC 56902*, *C2orf32*, and *C2orf13*. The latter three encode proteins of unknown function. Recent gene expression profiling of sporadic parathyroid adenomata has demonstrated upregulation of two genes, annexin A4 (*ANXA4*) and EGF-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*), compared with parathyroid tissue from age and sex-matched controls. These genes are found at 2p16 and 2p13, respectively,<sup>21</sup> which lie outside the regions of both significant and suggestive linkage. However, it is possible that a pathogenic mutation in a regulatory region of one of these genes, located remotely from the protein coding sequence, could be located within the region of linkage demonstrated in these pedigrees. Gene expression profiling of more diverse, including familial, forms



**Figure 4** Pedigree diagrams showing haplotypes of families with key recombinations in the region of highest LOD score. The disease associated haplotype is shaded. Critical recombination events occurred in individuals II.1 of pedigree C and II.1 of pedigree J. Unavailable allele data are indicated by "x".

of parathyroid neoplasia has also not identified possible positional candidate genes for FIHP in the regions identified in this study.<sup>22</sup>

The interval of suggestive linkage, between D2S2291 and D2S2739, also contains possible candidate genes for FIHP. Most notable is the gene for protein kinase C, epsilon

(*PRKCE*), which is involved in calcium-sensing receptor signalling in parathyroid cells.<sup>23</sup> The *PRKCE* knock-out mouse, however, has not had hypercalcaemia described. To investigate this region, sequencing of candidate genes according to a hierarchy could be undertaken. Using an "affecteds only" approach, ascertainment and genotyping of additional families may narrow the region of linkage. The problem of low disease penetrance, however, would remain.

In summary, a locus for FIHP has been identified on chromosome 2p13.3–14. Two candidate genes in this region have been sequenced without demonstration of likely causative sequence variations. This is a major step towards the eventual precise identification of a gene for FIHP.

The central role of the parathyroid glands in calcium homeostasis makes identification of this gene highly desirable. Analysis of its function under both normal and pathological conditions will improve understanding, and probably management, of primary and secondary parathyroid disease states and enhance general understanding of calcium metabolism.

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## ELECTRONIC-DATABASE INFORMATION



The following URLs have been mentioned in this article: the UCSC Human (*Homo sapiens*) Genome Browser Gateway, <http://www.genome.ucsc.edu/cgi-bin/hgGateway?org=human;MAP-O-MAT.v1.1>, <http://compgen.rutgers.edu/mapomat>; David Duffy's QIMR Homepage, <http://www.qimr.edu.au/davidD/davidd.html>; HGVS Nomenclature for the description of sequence variations, <http://www.genomic.unimelb.edu.au/mdi/mutnomen>; NCBI RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq/>; and Ensembl Multi MartView, <http://www.ensembl.org/Multi/martview>.

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