

1 **An InDel in Phospholipase-C-B-1 is linked with euthyroid multinodular goiter**

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**Abstract**

Euthyroid multinodular goiter (MNG) is common but little is known about the genetic variation conferring predisposition. Previously we reported a family with MNG of adolescent onset in which some family members developed papillary thyroid carcinomas (PTC). We conducted a genome-wide linkage analysis and next generation sequencing to identify genetic variants that may confer disease predisposition. A multipoint nonparametric LOD score of 3.01 was obtained covering 19 cM on chromosome 20p. Haplotype analysis reduced the region of interest to 10 cM; analysis of copy number variation identified an intronic InDel (~1000 bp) in the *PLCBI* gene in all 8 affected family members and carriers (an unaffected person who has inherited the genetic trait); this InDel is present in ~1% of ‘healthy’ Caucasians. Next generation sequencing of the region identified no additional disease-associated variant, suggesting a possible role of the InDel. Since *PLCBI* contributes to thyrocyte growth regulation, we investigated the InDel in relevant Caucasian cohorts. It was detected in 0/70 PTC but 4/81 unrelated subjects with MNG [3 F, age at thyroidectomy 27-59 years, no family history of MNG/PTC]. The InDel frequency is significantly higher in MNG subjects compared with controls;  $X^2 = 5.076$ ,  $p = 0.024$ . *PLCBI* transcript levels were significantly higher in thyroids with the InDel than without ( $p < 0.02$ ).

The intronic *PLCBI* InDel is the first variant found in familial multiple papilloid adenomata-type MNG and in a subset of patients with sporadic MNG. It may function through over-expression and increased PLC activity has been reported in thyroid neoplasms. The potential role of the deletion as a biomarker to identify MNG patients more likely to progress to PTC merits exploration.

## 49 **Introduction**

50 Euthyroid multinodular goiter (MNG) is common and affects at least 4% of the population, although the  
51 prevalence varies with ethnicity and the detection method employed (1). Furthermore, nodular goiter is far  
52 more prevalent in iodine deficient regions (2). Although solitary nodules are considered a risk for thyroid  
53 cancer (3) the situation for MNG is more controversial (4); the reported increase in the incidence of some  
54 thyroid cancers (5) may, in part, be due to increased use of diagnostic tools (6). *BRAF* mutations causing  
55 constitutive activation are the most frequent driver of papillary thyroid cancer (PTC) (7). Several genetic  
56 variations lead to sporadic thyroid cancers including, among others, *RET* chromosomal re-arrangements  
57 (8), translocations between chromosome 2 and 3 generating a PPAR $\gamma$ -PAX8 fusion protein (9), mutations  
58 in *RAS* genes (10) and poly-alanine tract length variation in *FOXEI* (11, 12).

59 Familial non-medullary thyroid cancers account for about 5% of thyroid cancers and have a younger age  
60 of onset than sporadic disease. They are associated with 4 susceptibility loci (13-16) on chromosomes  
61 19p13.2, 2q21, 1q21 and 10q23 (*PTEN*). There is some overlap with familial goiter in which 8 predisposing  
62 loci have been identified (12, 17-20) on chromosomes Xp22, 3q26, 2q, 3p, 7q, 8p 14q13.3 and 14q32, the  
63 last two including the *NKX2.1* (21) and the RNase *DICER1* genes respectively (22). A role for the  
64 predisposing loci on chromosomes 2q.35, 5q.24, 8p.12 and 14q.13 has been confirmed in Chinese families  
65 (23). Genes implicated in familial goiter and cancer generally differ from those in sporadic disease, with  
66 the exception of *NKX2.1* (21) and *FOXEI* (24).

67 Previously, we reported a family (25) exhibiting a type of euthyroid MNG with papillary adenomas of  
68 adolescent onset affecting 8 individuals in 4 generations to date. MNG is known to have progressed to PTC  
69 in 2 of the 8 affected family members. We applied microsatellite analysis to exclude loci described above  
70 on chromosomes 14q, Xp, 3q 9p, 2q and 1q. Since one family member had co-existing breast cancer and  
71 another co-existing kidney disease we investigated genes co-expressed in these tissues and the thyroid, *NIS*  
72 and *PAX8* respectively. Sanger sequencing revealed no abnormality in either gene. Subsequently, the *PTEN*  
73 gene has been fully sequenced in the family member with breast cancer and no mutations were detected.

74 The aim of this study was to apply genome-wide linkage analysis (GWLA) and next generation  
75 sequencing to identify the gene variant(s) responsible for the observed phenotype in this family. We then  
76 aimed to assess the frequency of any variant(s) detected in other relevant cohorts.

77

## 78 **Subjects and Methods**

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### 80 *Genome-Wide Linkage Analysis (GWLA)*

81 We undertook a GWLA of the family described in (25) and summarized in figure 1.

82 All patient samples were obtained with informed consent and Local Research Ethics Committee (LREC)  
83 approval. Genomic DNA was extracted from whole blood from 18 family members (those labelled in the  
84 tree) of whom 8 were affected (7 females, 1 male), according to the manufacturer's instruction (Qiagen)  
85 and quantified using a Nanodrop. Samples (250 ng) were processed following the manufacturer's protocol  
86 and the DNA integrity monitored by agarose gel electrophoresis before being hybridized at 48°C for 18  
87 hours to Affymetrix Genechip™ Human Mapping 10K 2.0 Arrays. The chips were scanned using an  
88 Affymetrix GeneChip scanner 3000; data were acquired using GCOS and analyzed using GTYPE software  
89 respectively.

90 Two quality control steps were performed; the first eliminated SNPs showing 'no call' in more than 4  
91 individuals. The second step would have eliminated data from any individual with >10% 'no calls', but this  
92 did not apply and the data of all 18 family members were retained. Graphical Representation of  
93 Relationships (GRR) software was used to determine how many alleles are shared [identity by state (IBS)]  
94 at each locus. Mendelian errors were tested using PedCheck software. PLINK, was used to merge family  
95 data (founders) with HapMap to investigate ethnicity. Multidimensional scaling (MDS) was performed on  
96 the family merged with HapMap data from 60 European individuals (CEU), 90 Chinese (CHB) & Japanese  
97 (JPT), & 60 Yoruba (YRI). The family were closest to the European cluster (data not shown) thus allele  
98 frequencies were based on CEU HapMap data. Using MERLIN software, the primary analysis was multi-  
99 point non-parametric and the secondary analysis multipoint parametric dominant mode assuming 90%

100 penetrance in females, 50% in males and age of onset later than 12 years (based on clinical information  
101 summarized in figure 1). Single point analyses were also used to support the findings of multipoint analysis.  
102 Since data are derived from a single large family, there is considerable allele sharing and hence the Kong  
103 and Cox exponential (--exp) model was used (for non-parametric analysis) (26).

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#### 105 *Haplotype Analysis*

106 MERLIN software (--best) was also used to perform a haplotype analysis in the region of maximum LOD  
107 score on chromosome 20. The haplotype was also confirmed manually.

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#### 109 *Copy Number Variation Analysis (CNV)*

110 Genomic DNA for CNV analysis of the index patient was quantified and prepared for hybridization to  
111 Illumina Human 660W-Quad BeadChips according to the manufacturer's instructions. Data were analyzed  
112 using PennCNV (27) software; CNVs were required to be 1 kb long and cover at least 10 consecutive  
113 markers (SNP or cnvi) to be considered positive. We focused on the region with a high LOD score identified  
114 in the GWLA.

115

#### 116 *Next Generation Sequencing (NGS)*

117 Primer pools for preparation of DNA libraries were designed using Ampliseq 3.0.1 software  
118 (<https://ampliseq.com/>) according to the manufacturer's protocol. A total of 429 primers were designed  
119 generating 100-300 bp amplicons. The primer pools (details in supplemental table 2) covered the exome  
120 sequences (all coding regions, intron/exon boundaries, proximal promoters and 3' untranslated regions) of  
121 a region spanning from chr20: 8113337 to 11907302. Approximately 10 ng of the genomic DNAs of interest  
122 were amplified according to the manufacturer's instructions. The amplified samples were partially digested  
123 by FuPa reagent (Life Technologies) and ligated with barcode/adaptor mix. DNA libraries were then  
124 purified using Agencourt AMPure XP beads (Beckman Coulter), quantified by qPCR and adjusted to a final  
125 concentration of 100 pM, combined and prepared for Emulsion PCR with Ion OneTouch 2 (Life

126 Technologies). Following enrichment, the ion sphere particles were loaded onto an Ion PI Chip V2 and  
127 sequenced by Ion Torrent Proton sequencer. Sequencing data were analyzed by Ion Torrent Suite software  
128 (4.4.2), using the plug-in variant caller (v 4.2.10) and configuration with generic Personal Genome Machine  
129 (PGM) germ line settings and high stringency analysis mode.

130 NGS was performed on 98 individuals, all 18 family members plus 80 unrelated subjects with MNG (please  
131 see below).

132 Other variants identified in the family using NGS were interrogated in the SHIP cohort (Study of Health in  
133 Pomerania) (28). Relevant genotyping data were available from 986 individuals who were either unaffected  
134 or presented with diffuse goiter (as defined in (29)) and/or MNG (nodules identified by ultrasound). Figure  
135 2 details the filtering steps and evaluations undertaken to assess whether detected variants might be linked  
136 with disease.

137

### 138 *Defining deletion frequency*

139 Primers within and flanking the deleted region were designed using Primer 3 software (supplemental table  
140 2) for PCR amplification of genomic DNA from all family members and 105 unrelated euthyroid  
141 individuals from the UK. PCR amplicons were analyzed by agarose gel electrophoresis and PEG  
142 precipitated for Sanger sequencing using Big Dye Terminator Cycle Sequencing Ready Reaction (ABI  
143 Prism, PE Biosystems) and analysis on an ABI 3100 Genetic Analyser.

144 Tissues from patients recruited in Australia (snap frozen and stored in liquid nitrogen) were also studied  
145 and consisted of 70 PTC and 81 MNG patients. [Ethics approval from the Northern Sydney Area Health  
146 Service Human Research Ethics Committee]. To avoid population stratification, only subjects with self-  
147 reported white European ancestry were included; patient data and tissues were collected between 1992 and  
148 2012 at the Kolling Institute of Medical Research. Genomic DNA for genotyping was obtained from thyroid  
149 tissue using Qiagen kits and analyzed by PCR and Sanger sequencing as described above; these samples  
150 also underwent NGS.

151

152 *High Throughput Screening of PLCBI InDel, analysis of additional cohorts.*

153 We developed a qPCR based genotyping tool using primers within and flanking the *PLCBI* InDel as  
154 described above (Supplementary table 2). The genotyping tool was used to screen 200 breast cancer  
155 patients. Initial optimization experiments revealed that greatest specificity was obtained using primers  
156 flanking the InDel. The qPCR obtained a difference of approximately 10 Ct for samples with and without  
157 the InDel. The qPCR was performed with approximately 100 ng Genomic DNA Input, 1x SyBR green  
158 master qPCR mix (Invitrogen) and 100 nM of each primer in a 25 µl reaction. QPCR conditions included  
159 an initial hold step at 50°C for 2 minutes, then 95°C for 2 minutes followed by 40 cycles of 95°C for 15  
160 seconds and 60°C for 30 seconds then a hold step at 95°C for 1 minute, 55°C for 30 seconds and 95°C for  
161 30 minutes. Samples found to harbor the InDel by qPCR were confirmed by Sanger sequencing.

162

163 *Transcript measurements of PLCBI isoforms*

164 Thyroid tissue was obtained from 3 affected family members heterozygous for the InDel and five subjects  
165 undergoing thyroidectomy for autoimmune thyroid disease expressing two normal *PLCBI* alleles (all  
166 confirmed by genotyping). Thyroid RNA was extracted, reverse transcribed using standard protocols and  
167 qPCR (SYBR Green incorporation measured on a Stratagene MX 3000) was used to measure transcript  
168 levels and evaluate proportions of *PLCBI-a* and *PLCBI-b* isoforms (primers in supplemental table 2, wild  
169 type amplicon identity confirmed by Sanger sequencing). Comparison with standard curves for transcript  
170 levels of isoform 1a and 1b permitted calculations of absolute values for each sample. Transcripts for a  
171 housekeeping gene (*APRT*) were also measured and values were expressed relative to this (transcripts/1000  
172 *APRT*). In a single qPCR experiment, all measurements were made in duplicate; the standard curve was  
173 also run in each reaction. Transcript levels of the various *PLCBI* isoforms were compared between deletion  
174 affected and non-affected thyroids using the Mann Whitney U test and differences where  $p < 0.05$  taken to  
175 be significant.

176

177 **Results**

178

179 *Genome wide linkage, haplotype & copy number variation analyses*

180 We obtained a multipoint nonparametric LOD score of 3.01 over 19.5 cM on chromosome 20p (figure 3  
181 and supplementary figure 1). In secondary analysis, the same region gave a multipoint dominant LOD score  
182 of 2.16, based on a disease model with 0.01 allele frequency, 50% penetrance for males and 90% for  
183 females, both age >12. LOD scores on the remaining 21 autosomes and X chromosome were all below 1  
184 (figure3). Single-point analyses supported the multipoint data for both nonparametric and model-based  
185 linkage on all chromosomes (supplementary table1).

186 Haplotype analysis was employed to identify a possible disease locus and reduced the region of interest to  
187 8.73 cM (3.7 Mbp), which includes 10 genes (supplemental figure 2 and 3). The haplotype was not found  
188 in 503 individuals from the 1000 genome European dataset, although one individual missed only the last  
189 marker suggesting a shorter version of the haplotype (red highlight in supplementary figure 3a).

190 Analysis of copy number variation in an affected individual revealed a deletion of ~900 bp located in the  
191 3<sup>rd</sup> intron in one copy of *phospholipase-C B1 (PLCB1)* in the region of interest (supplementary figure 4;  
192 the log R ratio mean was -0.451, over 14 markers, with at least one marker below -1.00).

193

194 *Defining the deletion frequency in the family and selected cohorts*

195 The length of the deletion was confirmed to be 1077 bp by standard PCR and Sanger sequencing, using  
196 primers flanking and within the deletion, to reveal one copy of full-length and one deleted allele in all  
197 affected and obligate carrier II-3 but only the full-length product in family members free of any sign of  
198 MNG. The sequence of the allele bearing the deletion corresponds to that immediately upstream and  
199 downstream of the deleted region but with an additional 'ATAA' inserted at the junction, hence it is an  
200 InDel.

201 Standard PCR was applied to genotype a selected cohort of 105 Caucasians in whom thyroid function  
202 testing was clinically indicated because of general fatigue. A woman in her forties, with no history of

203 thyroid disease, was heterozygous for the InDel. Further *in silico* analyses, using the database for genomic  
204 variants (30) identified a report which detected the InDel (variation 67651, LRR -0.645) in 2 of 180  
205 Caucasians but none in more than 450 people of other ethnicities (31). Combining our genotyping data with  
206 that of Conrad *et al.* (31) reveals 3 in 285 Caucasians harboring the InDel, suggesting that it is relatively  
207 rare (~1%).

208 Subsequently, genomic DNA was extracted from thyroid tissue from 70 patients undergoing surgery for  
209 non-familial PTC and an additional 81 operated for non-familial MNG. We used PCR analysis to test for  
210 the InDel, as described above. The InDel was not detected in any of the PTC patients but 4 of the 81 MNG  
211 were heterozygous for the InDel and sequencing revealed the same ATAA insertion at the junction.  
212 Comparison of the frequency of the InDel in the general population with that in MNG gives a  $X^2$  value of  
213 5.076 (1 degree of freedom),  $p= 0.024$  (two-tailed). The 4 MNG patients (3 women, 1 man) are unrelated  
214 and with no apparent family history of MNG or PTC at the time of their surgery. The age at thyroidectomy  
215 was between 27 to 59 years and the pathology is variously described as ‘oncocytic neoplasm with variable  
216 patterns of growth’ to ‘cystic degeneration with calcification’. We also investigated whether the *PLCBI*  
217 InDel might be implicated in breast cancer using the qPCR-based screening protocol. Prevalence in this  
218 cohort was similar to that of the general population, i.e. 1%, since just 2 breast cancer patients harbored the  
219 *PLCBI* InDel.

220

#### 221 *Next Generation Sequencing of the Chr20 high LOD score region*

222 The Proton Sequencer generated 9.9 Gbp of data, achieving 98% accurately mapped sequences with >88%  
223 of the percentage of target bases covered by at least 0.2 times the average base read depth.

224 A total of 181 sequence variants between Chr20 8113405 and 11907285 were identified in the family with  
225 the minor allele being on the disease risk haplotype in 12 of these. Given the rarity of PTC and the expected  
226 high penetrance, we expect a pathogenic variant to have a very low population frequency. After referring  
227 to the UCSC genome browser, only 1 of the 12 variants was found to have a minor allele frequency <1%;  
228 its presence in affected family members was confirmed by Sanger sequencing. The variant is at Chr20

229 10036484 (rs56234782) with T (98.8%) or C (1.2%) in the 3' UTR of the *ANKRD5* gene. To investigate  
230 whether it is implicated in goiter and/or thyroid nodule formation, we investigated its frequency in the SHIP  
231 cohort. However, even though the minor allele was more prevalent in the entire cohort, the prevalence in  
232 the affected population (goiters 1.9% and nodules 2.54%) was lower than in the unaffected populations  
233 (2.79% and 2.85% respectively), thereby excluding a role for it in MNG.

234 The MNG cohort was also submitted to NGS analysis. This identified more than 300 different sequence  
235 variants across the 80 patients, however, all were also present in the 1000 genomes cohort at a population  
236 frequency >1%. We therefore considered it unlikely that any of these variants are pathologically relevant  
237 to MNG, thereby confirming the relevance of the InDel.

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#### 239 *Transcript measurements of PLCB1 & effect of knock-down on thyroid growth*

240 Having confirmed that the InDel may contribute to the pathogenesis for MNG (perhaps in combination with  
241 other factors), we investigated how it might promote thyrocyte proliferation. The InDel is in the large 3<sup>rd</sup>  
242 intron of *PLCB1*, the phosphoinositide-specific enzyme which generates IP3 and DAG leading to PKC  
243 activation and also links signaling between the MAPK cascade and G protein coupled receptors (32).  
244 *PLCB1* is present in several isoforms including *PLCB1-a* and *PLCB1-b*, with the latter having a  
245 predominantly nuclear location (33). To test the hypothesis that the InDel causes preferential transcription  
246 of certain *PLCB1* isoforms, RNA was extracted from thyroids from the original family and from subjects  
247 undergoing thyroidectomy for benign disease. In all cases genomic DNA from the donor thyroid was tested  
248 for the *PLCB1* deletion.

249 QPCR analysis of InDel-affected thyroids did not indicate altered expression of the major *PLCB1* isoforms  
250 a and b (sequenced to confirm they were wild type, data not shown). However, qPCR measurements  
251 indicated significantly higher *PLCB1* transcript levels ( $p < 0.02$ ) in thyroids from family members with the  
252 InDel, compared with those from benign thyroid disease who do not harbor the variant (figure 5). Lack of  
253 thyroid tissue precluded analyzing *PLCB1* protein levels.

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255

256 **Discussion**

257 Our GWLA led to the identification of an InDel in the family with a type of MNG, located in the large third  
258 intron of *PLCBI*, a gene encoding an enzyme with a central role in several signaling cascades involved in  
259 regulating thyrocyte growth. Subsequent NGS in the family failed to identify any other disease-linked  
260 variant, thus supporting a role for the *PLCBI* InDel in the pathogenesis of MNG in this family.

261 The InDel comprises the loss of 1077 bp with an ATAA inserted at the junction in all affected family  
262 members and the 4 unrelated patients with MNG. We suggest that this may indicate a ‘cut and paste’ event  
263 indicating transposon activity. Interestingly, a 11-kb transposon cluster has been identified immediately  
264 upstream of the 3.7 Mbp section on chr 20 displaying a non-parametric LOD score of 3.01 in the current  
265 study (34). Of note the LOD score of 3.01, whilst at the lower limit to be considered significant, is higher  
266 than the maximum estimated for a kindred having 8 affected individuals (35).

267 We detected the same InDel in 1 subject of a selected cohort of 105 people in whom measuring thyroid  
268 function was clinically indicated. We also consulted the database of genomic variants and found several  
269 reports of relevance. Conrad et al. found the deletion in 2 of 180 Caucasians but insufficient detail is  
270 provided to know whether it is a simple CNV or the same InDel identified in our studies. Combining our  
271 genotyping data with that of Conrad et al. reveals that 3 in 285 Caucasians harbor the deletion, suggesting  
272 that it is rare (31). Several other authors did not observe this deletion, but aware of the difficulty in detecting  
273 small CNVs, we did not include these in our calculation. In addition, 200 patients with breast cancer have  
274 been screened for the InDel with only two harboring this deletion. Hence, the prevalence was similar to the  
275 general population suggesting that there is no connection of the InDel with breast cancer.

276 We then considered how the deletion or novel *PLCBI* InDel might exert its effects. The region was  
277 explored using the Encyclopedia of DNA elements (ENCODE) (although compiled without inclusion of  
278 thyroid tissue or cell lines) (36), which revealed the existence of a binding site for the estrogen receptor  
279 alpha (ER $\alpha$ ) within the deletion. This is of potential importance since all thyroid diseases are more prevalent  
280 in women than men (1). The incidence of thyroid disorders increases in the years immediately following

281 puberty and *in vitro* studies have demonstrated that estrogen can promote thyrocyte proliferation (37) by  
282 several mechanisms. The *PLCBI* InDel is located in an intron; while many functional transcription factor  
283 binding sites are found in promoters, a systematic search for ER $\alpha$  binding sites in the human genome  
284 identified >1000 with >95% of them residing in introns and not promoters (38).

285 We also conducted experiments to determine whether the deletion alters the ratio of *PLCBI-a* and *PLCBI-*  
286 *b*, which are generated by alternative splicing. Differences in their C terminal sequence mean that only  
287 *PLCBI-a* has a nuclear export signal. We found no alteration in the ratio of *PLCBI-a* and *b* isoforms but  
288 in all cases transcript levels for *PLCBI* were higher in thyroids from people heterozygous for the InDel  
289 than in thyroids with two full-length copies. This suggests that the InDel may contribute to MNG  
290 development through overexpression of *PLCBI*. Furthermore, total PLC enzyme activity is elevated in  
291 thyroid neoplasms (39) but unfortunately PLC inhibitors lack the specificity required to identify which  
292 isoform is responsible. Increased *PLCBI* expression has also been reported in small cell lung carcinoma  
293 (40) and expression of *PLCB2* is substantially increased in breast cancer and is used as a prognostic marker  
294 (40).

295 As mentioned above, PLC enzymes activate PKC and genes implicated in this signal pathway are  
296 upregulated in euthyroid MNG (41). They also link signaling via Gq (which can also be activated via the  
297 thyrotropin receptor) to the MAPK cascade and in the thyroid disruption of this pathway, by thyrocyte-  
298 targeted Cre/Lox P knock-down of the Gq $\alpha$  subunit, produces mice which are resistant to goiter formation  
299 when fed a goitrogenic diet (42). However, when we performed western blots with protein extracts of  
300 thyroid tissue from family members with the *PLCBI* InDel we were surprised to observe that pMAPK  
301 levels were substantially lower than in thyroid tissue from patients with autoimmune thyroid disease or  
302 MNG without the *PLCBI* InDel (Supplementary Figure 5).

303 In conclusion, the *PLCBI* InDel identified in this family with MNG also occurs in a proportion of sporadic  
304 MNG, and may provide a biomarker to identify MNG patients more likely to progress to PTC. The *PLCBI*  
305 InDel appears to predispose to goiter formation, possibly by increasing *PLCBI* transcription with  
306 subsequent downstream effects.

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308 **Supplemental Data**

309 The supplemental data comprises 5 figures and 2 tables;

310 Supplemental Figure 1; LOD scores of all Chromosomes

311 Supplementary Figure 2; Genes in high LOD score region chromosome 20

312 Supplemental Figure 3; Haplotype Frequency in 1000 genomes European dataset

313 Supplementary Figure 4; Copy number variation in high LOD score region chromosome 20

314 Supplementary Figure 5; Densitometry ratios for pERK/total ERK

315 Supplemental Table 1; Single point LOD scores all chromosomes

316 Supplemental Table 2; Primers used for NGS and to define deletion frequency

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318

319 **Web Resources**

320 The March 2006 human reference sequence (NCBI Build 36.1) produced by the International Human

321 Genome Sequencing Consortium, was used as a reference genome (UCSC Genome Browser;[http://genome-](http://genome-euro.ucsc.edu/cgi-bin/hgGateway?hgsid=192302910&clade=mammal&org=Human&db=hg18)

322 [euro.ucsc.edu/cgi-bin/hgGateway?hgsid=192302910&clade=mammal&org=Human&db=hg18](http://genome-euro.ucsc.edu/cgi-bin/hgGateway?hgsid=192302910&clade=mammal&org=Human&db=hg18)).

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332

333 **Declaration of interest**

334 There is no conflict of interest that could be perceived as prejudicing the impartiality of the research  
335 reported.

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