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Title

New disease gene location and high genetic heterogeneity in idiopathic scoliosis

Running title

Gene location in idiopathic scoliosis

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1 **Abstract**

2 Idiopathic scoliosis is a spine disorder of unknown origin with 1.5-3% prevalence in the
3 general population. Besides the large multifactorial form sample of idiopathic scoliosis, there
4 is a good evidence for the existence of a monogenic subgroup in which the disease is inherited
5 in a dominant manner. However, results from literature suggest a strong heterogeneity in the
6 locations of the mutated genes. Using a high resolution genome-wide scan, we performed
7 linkage analyses in three large multigenerational idiopathic scoliosis families compatible with
8 dominant inheritance including 9 to 12 affected members or obligate carriers. In two of these
9 families, our results suggested intra-familial genetic heterogeneity whereas, in the other, we
10 observed a perfect marker disease co-segregation in two regions at 3q12.1 and 5q13.3. We
11 can state that one of these two locations is a novel idiopathic scoliosis disease gene locus,
12 since the probability of having this perfect co-segregation twice by chance in the genome is
13 very low ($p=0.001$). Lastly, in all three families studied, linkage to the previously mapped
14 dominant idiopathic scoliosis loci on chromosomes 19p13.3, 17p11.2, 9q34, 17q25 and 18q is
15 unlikely, confirming that there is a high genetic heterogeneity within the subgroup of
16 dominant forms of idiopathic scoliosis.

17

18 **Introduction**

19 Idiopathic scoliosis (IS) is a spine deformity of unknown cause commonly defined by a
20 rotation of vertebral bodies leading to a structurally fixed lateral curvature of the spine with a
21 Cobb's angle of at least 10°. Idiopathic scoliosis affects up to 3% of the population ¹, girls
22 being more commonly affected than boys ^{2 3}. Adolescent idiopathic scoliosis (AIS, MIM
23 181800) occurs in otherwise healthy adolescents and accounts for approximately 85% of IS
24 cases, other cases being infantile or juvenile (15%). About 3 per 1,000 children present with
25 severe AIS forms over 30° which may require braces or surgical treatment and may have
26 severe functional and aesthetic consequences.

27 Aside from idiopathic forms, scoliosis may also occur secondary to other hereditary disorders
28 including defects in extracellular matrix components (e.g. Marfan syndrome, Ehlers-Danlos
29 syndromes), neuromuscular disorders (e.g. Friedreich ataxia, muscular dystrophies),
30 osteochondrodystrophies (e.g. osteogenesis imperfecta, neurofibromatosis), vertebral
31 segmentation defects or other malformation syndromes including CHARGE syndrome and
32 Smith-Magenis (del17p11.2) syndrome.

33 Several population studies established that up to 40% of IS are familial ⁴ and that there is a
34 high concordance rate of this disease in monozygotic twins ⁵. Many studies concluded that the
35 most likely mode of inheritance was multifactorial, postulating that predisposing alleles were
36 required together with environmental factors for phenotypic expression ⁶. Although the exact
37 mode of inheritance remains unknown in many IS cases, there is now evidence for a
38 monogenic subgroup. A segregation analysis using a model with age and gender effects
39 established a significant contribution of a major causal gene transmitted according to an
40 autosomal dominant inheritance with sex-dependent incomplete penetrances ⁷. A small
41 number of model-dependent linkage analyses using an autosomal dominant model in large
42 multigenerational IS families have been performed successfully to date. Indeed such large

43 multiplex IS families compatible with dominant inheritance are very rare. A genome-wide
44 scan of seven unrelated multiplex families of Chinese descent with AIS concluded that there
45 was a linkage to chromosome 19p13.3 (IS1, MIM 181800) ⁸. This finding was replicated in
46 several IS multiplex families in Alden et al's study ⁹. A similar study in an Italian family
47 mapped a second locus to 17p11.2 (IS2, MIM 607354) ¹⁰. Another study reported on the
48 mapping of two loci for IS on chromosome 9q31.2-q34.2 (IS4, MIM 612238) and 17q25.3-
49 qtel (IS5, MIM 612 239), the latter by combining information obtained from two unrelated IS
50 families ¹¹. Recently, a locus for IS and pectus excavatum was found in a single large family
51 on chromosome 18q ¹².

52 In the present study, we performed model-dependent linkage analyses in three large IS
53 families compatible with autosomal dominant inheritance of the disease trait with a high
54 number of informative meioses. We compared our results to published linkage data.

55

56 **Families and Methods**

57 *Patient and Family Inclusion*

58 We selected large multiplex IS families and we genotyped the largest families, i.e. those
59 including the highest number of affected individuals or obligate carriers. A positive diagnosis
60 for IS was reached by combining clinical examination of the spine, including the forward
61 bending test (Adams test), and the Cobb's angle measurement on radiographic evaluation.
62 Both a Cobb's angle above 15° (in order to minimize the risk of phenocopies) and vertebral
63 rotation were required for the positive diagnosis of IS. Patients harbouring a spine curvature
64 but no rotational component were assigned an "unknown status", as were those with any other
65 associated anomaly of the spine or, more generally, with atypical IS. Patients with normal
66 clinical examination and strictly normal spine on radiograph were considered "unaffected".
67 All individuals underwent clinical examination by at least one clinical geneticist (PE) and one

68 orthopaedist (BB and/or JCB). Each radiograph was carefully and often repeatedly checked,
69 by the clinical geneticist and one or both orthopaedists.

70 Pedigrees of the three selected IS families are shown in the figure. All families are three-
71 generation pedigrees suggestive of the transmission of an autosomal dominant mutation with
72 incomplete penetrance. Clinical data on patients belonging to the families studied are
73 provided in table 1.

74

75 *DNA sample collection*

76 We selected for genotyping 57 individuals including 27 affected patients or obligate carriers
77 belonging to the three families presented here. Blood samples were collected from each
78 participant (IS patients and their first-degree affected or unaffected relatives) after providing
79 them with an information sheet describing the research study and obtaining their informed
80 written consent. This research study was approved by the local Ethical Committee and was
81 promoted by the Hospices Civils de Lyon, France. Genomic DNA was extracted from blood
82 samples according to standard procedures. EBV-transformation of lymphocytes was
83 performed in at least one affected individual per family. After the cell-lines were established,
84 transformed lymphocytes were stored frozen in liquid nitrogen. Normal standard
85 chromosomal studies on blood lymphocytes were obtained from at least one affected
86 individual in each family ruling out gross chromosomal abnormalities.

87

88 *Genotyping*

89 Considering the large number of possible IS candidate genes with regard to their function and
90 the wide genetic heterogeneity of IS, we decided to favour genome-wide approaches rather
91 than candidate loci mapping strategies. We genotyped the three large multiplex IS families
92 using a high density 1,000 microsatellite DNA marker set, with an average spacing of 3.7 cM
93 between each marker, where genetic locations are based on the deCODE map¹³.

94

95 *Statistical analyses*

96 The classical method used for mapping a disease gene under monogenic transmission is the
97 Morton Lod Score analysis (1955). A lod score value of 3.00 (for autosomal loci) means that
98 the hypothesis of linkage between the disease trait locus and the marker locus is 1,000 times
99 more likely than the hypothesis of no linkage. This method is very powerful when the disease
100 is due to a mutation of the same gene in all families with full penetrance for mutation carrier.
101 In the present situation, it is realistic to assume that the disease is due to segregation of a
102 dominant mutation in the families selected for this study. However, the results from literature
103 suggest a strong heterogeneity in the mutated genes. In addition, penetrance values may differ
104 according to the genes and mutations involved. Clerget-Darpoux et al ¹⁴ showed that a
105 misspecification of the penetrance values results in a lod score decrease and hence in a loss of
106 the power to detect linkage.

107 To overcome the interfamilial genetic heterogeneity, we performed a lod score analysis
108 separately in each family. Analyses were performed assuming the segregation of a dominant
109 mutation, with and without the possibility of phenocopies. In linkage analysis, marker
110 segregation was considered only in affected individuals. The status of other family members,
111 including unaffected individuals, was assumed to be unknown. In such an analysis, the results
112 do not depend on the absolute penetrance values, but only on the relative ones (probability to
113 be affected for a non mutation carrier versus this probability for a mutation carrier).

114 In a first step, we measured in each family the lod score value that could be reached at the
115 disease gene locus in the situation of no phenocopy and full informativity. This theoretical
116 maximum lod score value $Z_{i\max}$ can be simply inferred from the number T_i of disease allele
117 transmissions to affected individuals in each family. If we denote M_i the number of meioses

118 between a common ancestor and all affecteds, n_i the number of common ancestors and $T_i =$
119 $M_i - n_i$, in each family F_i :

120 $Z_{i\max} = \log_{10} [L(\theta=0)/L(\theta=0.5)]^{T_i} = - T_i * \log_{10} (1/2)$ where $L(\theta=0)$ is the likelihood of H1
121 (hypothesis of linkage at $\theta=0$, i.e. no recombination event between the marker and the disease
122 locus) and $L(\theta=0.5)$ is the likelihood of H0 (hypothesis of no linkage).

123 In a second step, we estimated the probabilities under H0 to reach the maximum lod score on
124 each arm of autosomes 0, 1 or more than once. This maximum occurs according to a Poisson
125 distribution with a parameter λ equal to the probability of reaching $Z_{i\max}$ if hypothesis H0 is
126 true $= (0.5)^{T_i}$. On the whole genome (44 chromosome arms), this parameter λ is equal to
127 $44 * (0.5)^{T_i}$.

128 In a third step, we computed maximum Morton lod scores using Merlin software ¹⁵ separately
129 in each of the selected families.

130 In a final step, we tested in each of the three families studied the compatibility with linkage to
131 the five loci previously considered to be IS loci (19p13.3, 17p11.2, 9q34, 17q25, 18q), using
132 both the genetic model without phenocopy described here and a more flexible model allowing
133 for various rates of phenocopies (0.01, 0.03, 0.10 and 0.020).

134

135 **Results**

136 Table 2 gives the values M_i , n_i , T_i and the expected maximum lod score $Z_{i\max}$ in each family
137 F_i . It also gives the probabilities to reach it 0, 1 or more than once under the H0 hypothesis in
138 whole genome analysis. Expected maximum lod scores are 1.51, 3.01 and 2.71 in families F1,
139 F2 and F3 respectively. The probability of never reaching $Z_{i\max}$ under H0, and thus only once
140 (under H1), in whole genome analysis, is 25% for F1, 96% for F2, and 92% for F3.

141 Table 3 gives, for each family F1, F2 and F3, the maximum lod score (max Z) and marker
142 informativity at this location. In families F1 and F3, the maximum lod scores obtained after

143 whole genome scan were significantly lower than the expected Z_{max} previously estimated.
144 Affected individuals never shared a same transmitted allele in any chromosome arm.
145 Consequently, the hypothesis that the disease is due to the transmission of a dominant
146 mutation has to be reconsidered in these families. The variable severity in these two families
147 suggests a possible intra familial genetic heterogeneity. In family F1, the severity of IS in
148 affected individuals is highly variable since, in several patients of this family, Cobb's angles
149 are between 16° and 24° and over 50° in four patients. Similarly, patient III1 of family F3 is
150 affected with a severe form of IS (Cobb's angle of 65°), whereas angles for the five other
151 patients are between 15° and 24° . When patient III1 of family F3 is removed from the linkage
152 study, the lod score reaches the theoretical maximum value of 2.11 at the D15S979 locus.
153 In family F2, the lod score Z_{max} of 3.01 is reached both on chromosome 3 (3q12.1) and on
154 chromosome 5 (5q13.3). As noted in table 2, the probability of having twice obtained this
155 value randomly (under H_0) is very small ($p=0.001$). It means that there is strong evidence that
156 a disease gene lies in one of these two regions. The lengths of chromosome intervals shared
157 by all patients at the two possible disease gene locations are 7cM and 12cM respectively
158 (table 3). The boundaries of these intervals,]D3S3690-D3S3045[and]D5S2851-D5S1397[
159 respectively, were deduced from recombination events in affected individuals as observed on
160 haplotypes. To test whether the study of unaffected members of family F2 may help in the
161 discrimination between these two locations, we performed a mod score analysis¹⁶ with all
162 genotyped family members (affected and non affected) using the software Genehunter-
163 Modscore 2.0.1¹⁷. The results from four unaffected individuals in this family, II7, II8, III6,
164 III7 (see the figure) proved to be informative. The mod scores obtained at chromosome 3 and
165 5 loci respectively were identical and did not allow us to discriminate between the two
166 possible locations.

167 Lastly, we considered lod scores obtained in families F1, F2 and F3 on the 5 regions claimed
168 to be idiopathic scoliosis loci. All lod scores were negative when assuming no phenocopy and
169 a maximum value of 0.00 was reached when the model allowed for phenocopies.

170

171 **Discussion**

172 Using a set of 1000 polymorphic markers (decode set), we performed a genome-wide linkage
173 analysis under a dominant mode of transmission in three large multiplex IS families. Several
174 pitfalls such as inter-familial genetic heterogeneity, possible phenocopies or unknown
175 penetrance for mutation carrier were taken into account. First, lod score analyses were
176 computed for each family independently. Second, in order to decrease the risk of
177 phenocopies, individuals with a Cobb's angle of at least 15°, instead of the widely accepted
178 threshold of 10°, were considered as affected. Indeed, mild scoliosis or even mild curvature
179 and/or rotation of the spine are likely to be caused by a combination of environmental and
180 minor gene effects rather than by a major gene effect ⁷. In addition, previous reports and our
181 own observations showed that there are important variations in Cobb's angle measurements of
182 up to 5° among different physicians ^{18,19}.

183 This study shows the mapping of a new locus for dominant IS in a large family (F2) either on
184 5q13-q14 or on 3q11-q13. Indeed, in this family, the observed Zmax reached the expected
185 Zmax value of 3.01, on both genomic locations. Since the probability of obtaining a Zmax
186 value in the genome twice by chance is very low (p=0.001), we can conclude that there is
187 strong evidence that a novel IS disease gene lies in one of these two locations. This IS gene
188 still remains to be identified. Among the genes in the two chromosomal regions, many are
189 involved in cellular signalling, some are coding for hypothetical/uncharacterized proteins yet
190 to be discovered, and others were cloned but their function is not yet fully documented.
191 Mutation screening using either exome sequencing or targeted high throughput sequencing,

192 gene expression studies, and *in vivo* functional analysis of candidate genes within these loci
193 need to be performed.

194 In this study, we also provide further evidence for a high heterogeneity among monogenic
195 forms of IS. In each of the three families studied, the lod scores are either negative or
196 inconclusive, depending on the phenocopy rate assumption, at the five previously suggested
197 locations for IS genes, namely 19p13.3, 17p11.2, 9q34, 17q25 and 18q. Such high genetic
198 heterogeneity favours the view that computing IS families independently, when performing
199 linkage analyses based on a monogenic model hypothesis, is critical to obtaining accurate
200 results.

201 We also show that the selection of multiplex IS families diagnosed with strict clinical criteria
202 does not warranty that the apparently dominant inheritance is simply explained by the
203 segregation of a unique gene mutation. We have to consider the difficulty of selecting
204 families with a high number of informative meioses without introducing intra-familial
205 heterogeneity or phenocopies, to be able to detect linkage at a family level. It seems that the
206 criteria of a minimum angle of 15° is not sufficient to ensure intra-familial genetic
207 homogeneity, and we hypothesize that clinical variability between family members also has to
208 be minimized. Indeed, in both families F1 and F3, the Z_{\max} values obtained were far beyond
209 the values expected under the assumption of a single causative mutation segregating in all
210 affected members. In family F1, IS patients have a wide range of Cobb's angle values
211 extending from 16° to 130° , and in family F3, one patient is affected with a severe form of IS
212 (Cobb's angle of 65°), whereas angles for the five other patients are between 15° and 24° .
213 When the patient with the Cobb's angle of 65° from family F3 is removed from the analysis,
214 we observe a perfect co-segregation of the disease and the D15S979 marker. Therefore, in
215 such studies, we suggest paying careful attention to the clinical aspects of scoliosis, possibly
216 including Cobb's angle values, even within single families. Such stratification by the severity

217 of the phenotype or by other discrete clinical aspects of scoliosis may minimize the risk of
218 intra-familial heterogeneity. The results provided in our study, supporting a high degree of
219 inter- and intra-familial genetic heterogeneity in IS, raise questions regarding several
220 previously reported mapping studies based on linkage data obtained by pooling together
221 independent IS families. It should be noted that *a posteriori* selection of families compatible
222 with linkage to a specific locus may lead to false primary linkage and/or fine mapping. Thus,
223 in genetically heterogeneous disorders in which a different mutated gene in each family may
224 be dealt with, pooling linkage data obtained from unrelated families should be considered
225 with caution. Accurate linkage mapping studies are required to allow the subsequent
226 identification of dominant IS genes. To date, no dominant IS gene has been identified, and we
227 hope that the present study will prove to be an important step toward this goal. Also, although
228 dominant IS genes, yet to be identified, may account for a restricted number of IS cases, they
229 may provide insights into the genetic bases of multifactorial IS, which remain largely
230 unknown²⁰. Future genetic and functional analyses of candidate genes located within these
231 defined regions will hopefully reveal gene defects accounting for IS, which will allow, in
232 turn, the identification of secondary molecular factors that may point towards yet
233 unrecognized IS pathophysiological pathways.

234

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240 Lyon 1 and the Association GEN'HOM.

241

242 **Competing interests:** none

243 **Web resources**

244 Online Mendelian (OMIM), <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>

245 National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>

246 deCODE Genetics, <http://www.decode.com/>

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Figure Titles and Legends

Title to Figure. Simplified pedigrees for families 1-3.

Figure Legend: Simplified pedigrees are drawn for families 1-3. All pedigrees are compatible with autosomal dominant inheritance of the disease trait. Cobb's angles are indicated. Antero-posterior spine radiographs are shown for affected individuals. Question marks indicate uncertain status. ND: status not determined. Several unaffected individuals, who have been genotyped, are not included since they were not useful in linkage analysis. In family 2, four unaffected individuals are included since they contributed to the mod score analysis.

Titles to tables

Table 1. Clinical data for patients included in this study

Table 2. Informativity studies performed in each family. Only affected individuals have been taken into account.

Table 3. Maximum lod score values obtained in each family using only affected individuals.

Table 1

| Family | Patient | Age at diagnosis (years old) | Cobb's angle on radiograph (age: years old) | Cobb's angle | Apical vertebrae | Therapy | |
|-----------|-----------|------------------------------|---|---|---|----------------|------|
| F1 | IV-2 | 13 | 76° (65) | right thoracic | D9 | physiotherapy | |
| | IV-3 | adolescence | 55° ^a (61) | right thoraco-lumbar | D12 | spinal surgery | |
| | IV-4 | fortuitous | 18° (50) | right lumbar | L2 | none | |
| | IV-6 | 12 | 35°/28°(64) | right thoracic/left lumbar ^b | D8-D9/L2-L3 | bracing | |
| | IV-7 | 6 | 90°/93° (20) | right thoracic/left lumbar ^b | D7/L2 | unknown | |
| | IV-8 | 9 | 21° ^a (26) | right thoracic/left lumbar ^b | D8/L3 | spinal surgery | |
| | V-1 | 15 | 16° (15) | left lumbar | L3 | none | |
| F2 | II-1 | fortuitous | 27° (73) | right thoraco-lumbar | D12-L1 | none | |
| | II-3 | fortuitous | 19° (74) | right lumbar | L1 | none | |
| | II-4 | fortuitous | 23° (71) | right lumbar | L2 | none | |
| | II-6 | adolescence | 41° (67) | right thoraco-lumbar | L1 | none | |
| | III-3 | adolescence | 15° (49) | right thoraco-lumbar | L1 | bracing | |
| | III-4 | fortuitous | 29° (46) | right thoraco-lumbar | D12 | none | |
| | III-8 | fortuitous | 15° (42) | right thoraco-lumbar | L1 | none | |
| | IV-1 | adolescence | 29°/29° (20) | right thoracic/left lumbar ^b | D8/L2 | physiotherapy | |
| | IV-2 | fortuitous | 15° (16) | right thoracic | D7 | none | |
| | IV-3 | fortuitous | 14°/16° (19) | right thoracic/left lumbar ^b | D8/L1 | none | |
| | IV-4 | 12 | 18° (15) | left thoraco-lumbar | L1 | physiotherapy | |
| | F3 | II-3 | fortuitous | 14°/16°(58) | right thoracic/left lumbar ^b | D8/L1 | none |
| | | II-8 | fortuitous | 24°(66) | right thoracic | D8 | none |
| III-1 | | adolescence | 65°(18) | right thoracic-left lumbar | D8 | spinal surgery | |
| III-2 | | fortuitous | 15°(32) | right lumbar | L2 | none | |
| IV-1 | | 9 | 22°(15) | right thoracic | D8 | physiotherapy | |
| IV-2 | | 10 | 20° (15) | right thoraco-lumbar | L1 | bracing | |

a Cobb's angle after arthrodesis

b Double major curve

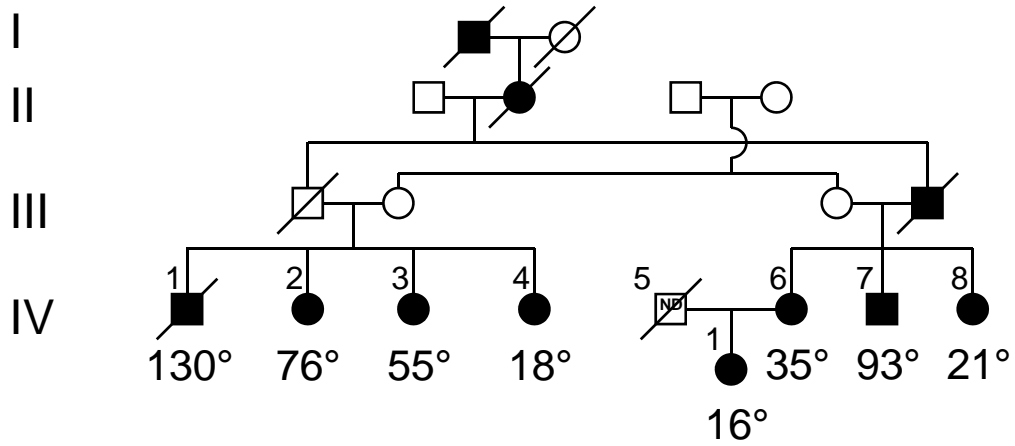
Table 2

| Family | Number of meioses between a common ancestor and all affected individuals | Number of common ancestors | Number of disease allele transmissions to affected individuals | Expected Maximum lod score | Probabilities to reach $Z_{i_{max}}$ under H_0 , 0, 1 or more than once in whole genome analysis | | |
|--------|--|----------------------------|--|---------------------------------------|--|-------|-------|
| F_i | M_i | n_i | $T_i = M_i - n_i$ | $Z_{i_{max}} = -T_i * \log_{10}(0.5)$ | 0 | 1 | >1 |
| F1 | 9 | 4 | 5 | 1.51 | 0.253 | 0.346 | 0.401 |
| F2 | 12 | 2 | 10 | 3.01 | 0.958 | 0.041 | 0.001 |
| F3 | 10 | 1 | 9 | 2.71 | 0.918 | 0.079 | 0.003 |

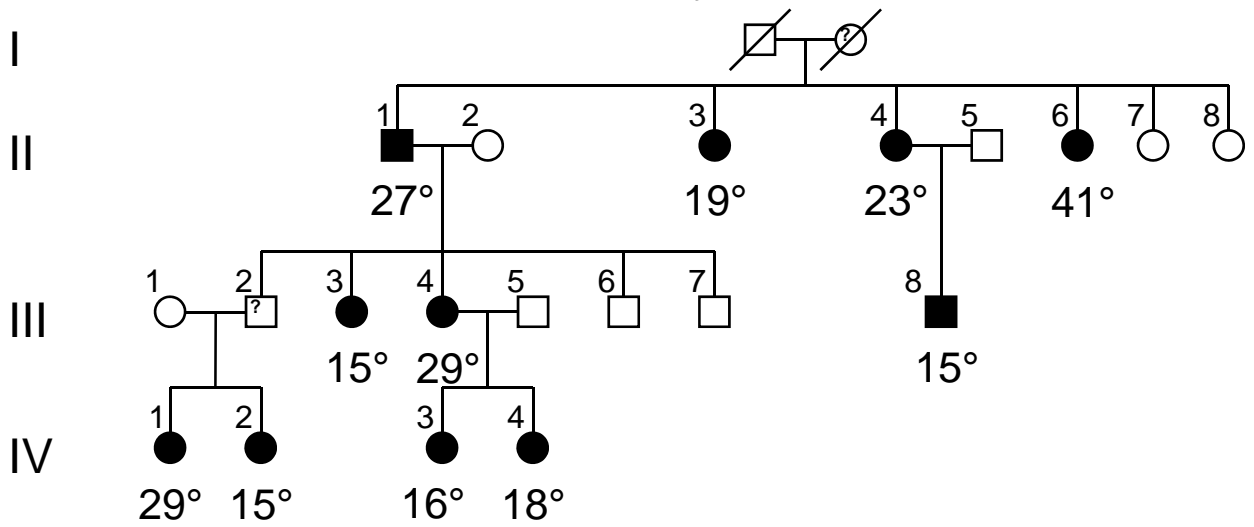
Table 3

| family | Expected Z _{max} | Observed Z _{max} | informativity | Localisation (interval length) | Chromosomal location |
|--------|------------------------------|------------------------------|---------------|-------------------------------------|-------------------------|
| F1 | 1.51 | 0.85 | 0.79 | D14S274 | 14q23.1 |
| F2 | 3.01 | 3.00 | 0.99 | D5S2003 (D5S2851-D5S1397, 12 cM) | 5q13.3 |
| | 3.01 | 3.01 | 0.99 | D3S2462 (D3S3690-D3S3045, 7 cM) | 3q12.1 |
| F3 | 2.71 | 1.23 | 0.87 | D15S979 | 15q25.1 |

Family 1



Family 2



Family 3

