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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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Abstract

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

Introduction

Idiopathic scoliosis (IS) is a spine deformity of unknown cause commonly defined by a rotation of vertebral bodies leading to a structurally fixed lateral curvature of the spine with a Cobb's angle of at least 10°. Idiopathic scoliosis affects up to 3% of the population¹, girls being more commonly affected than boys^{2 3}. Adolescent idiopathic scoliosis (AIS, MIM 181800) occurs in otherwise healthy adolescents and accounts for approximately 85% of IS cases, other cases being infantile or juvenile (15%). About 3 per 1,000 children present with severe AIS forms over 30° which may require braces or surgical treatment and may have severe functional and aesthetic consequences.

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

Several population studies established that up to 40% of IS are familial⁴ and that there is a high concordance rate of this disease in monozygotic twins⁵. Many studies concluded that the most likely mode of inheritance was multifactorial, postulating that predisposing alleles were required together with environmental factors for phenotypic expression⁶. Although the exact mode of inheritance remains unknown in many IS cases, there is now evidence for a monogenic subgroup. A segregation analysis using a model with age and gender effects established a significant contribution of a major causal gene transmitted according to an autosomal dominant inheritance with sex-dependent incomplete penetrances⁷. A small number of model-dependent linkage analyses using an autosomal dominant model in large 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.

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

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

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

DNA sample collection

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

Genotyping

Considering the large number of possible IS candidate genes with regard to their function and the wide genetic heterogeneity of IS, we decided to favour genome-wide approaches rather than candidate loci mapping strategies. We genotyped the three large multiplex IS families using a high density 1,000 microsatellite DNA marker set, with an average spacing of 3.7 cM 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_{imax} 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 $Z_{\text{imax}} = \log_{10} [L(\theta=0)/L(\theta=0.5)]^{T_i} = -T_i \cdot \log_{10} (1/2)$ 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_{imax} if hypothesis H0 is
126 true $= (0.5)^{T_i}$. On the whole genome (44 chromosome arms), this parameter λ is equal to
127 $44 \cdot (0.5)^{T_i}$.

128 In a third step, we computed maximum Morton lod scores using Merlin software¹⁵ 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_{imax} 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_{imax} 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.

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

Discussion

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

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

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

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

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

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

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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

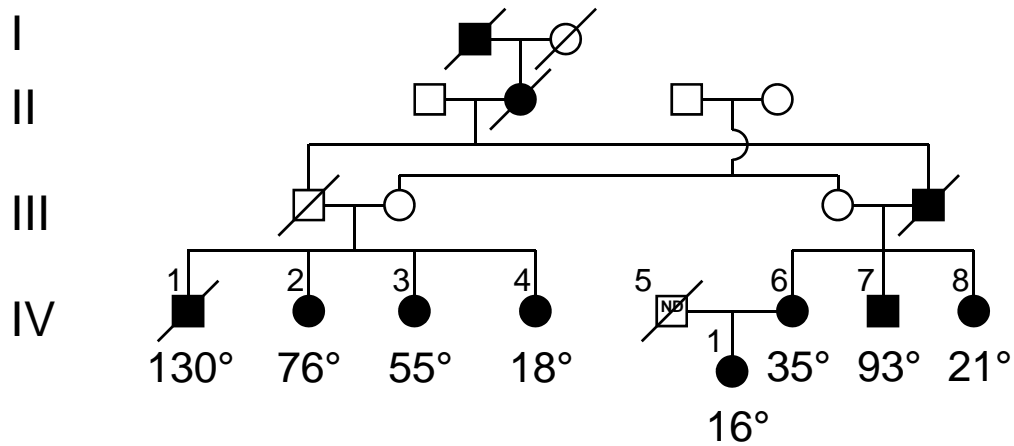
Table 2

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_{imax} under H_0 , 0, 1 or more than once in whole genome analysis		
F_i	M_i	n_i	$T_i = M_i - n_i$	$Z_{\text{imax}} = -T_i \cdot \log_{10}(0.5)$	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

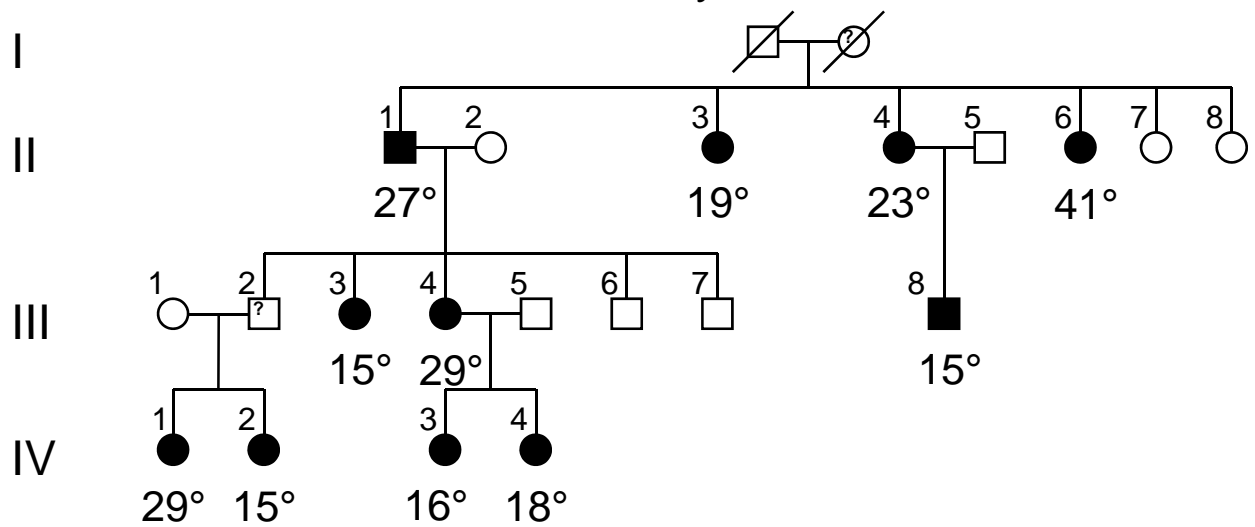
Table 3

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

