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Estrogen receptor polymorphism, estrogen content and idiopathic scoliosis in human: A possible genetic linkage☆

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ABSTRACT

Idiopathic scoliosis (IS) is a largely diffused disease in human population but its pathogenesis is still unknown. There is a relationship between scoliotic phenotype and the patient age, since in the early stage the pathology shows a ratio of 50% between male and female teenagers. During puberty the sex ratio is 8.4/1 (female/male), suggesting a sex-conditioned manifestation of the disease. Genetic inheritance of idiopathic scoliosis is still unclear although some authors claim for its X-linked dominant inheritance. There is large agreement in considering the IS as a sex-conditioned disease, in terms of steroid content and their receptor activity, although no evidence has been found yet.

The blood content of 17β-estradiol in teenagers with IS shows lower levels than teenagers of the same age without IS. Also testosterone and progesterone content are lower in IS girls with respect to the control girls. Furthermore, we extracted DNA from white blood cells of IS patients and their relatives until the third generation in order to examine estrogen receptor α polymorphisms, considering this tool a plausible molecular marker for IS prognosis. In this respect, we identified four polymorphisms in the exons encoding for the steroid binding domain and two other in the trans-activation domain. Our results show a clear relationship with clinical manifestation of IS.

1. Introduction

Scoliosis refers to lateral curvatures of the spine. Scoliotic curvatures are due to congenital abnormalities or neuromuscular, myopathic, mesenchymal, metabolic, nutritional and endocrine disorders, and traumatic causes. The structural changes in the vertebrae responsible for scoliosis are principally due to the development of a wedge-like deformity of the vertebral body.

The idiopathic scoliosis (IS) of unknown origin is the largest group and accounts for 80–90% of cases. In this form of scoliosis the deformity consists of lateral deviation of the spine with rotation of the vertebrae; it is classified according to both age at onset and location of the structural curve. The direction of the convexity varies with age of onset and location [1,2]. Similar curves have been noted in identical twins [3]. The most deforming curves originate early in the life.

Many studies indicate a significantly increased incidence of idiopathic scoliosis in close relatives, indicating a dominant form of inheritance [4,5]. On the basis of radiographic examination of entire families, the idiopathic scoliosis was inherited as a sex-linked dominant trait, although the characteristic had variable expressivity and limited penetrance [6].

Five recent studies have reported possible relationship between marker loci and IS [7–11]. Wise et al. reported evidence for linkage on chromosome 6p, distal 10q, and 18q in a single extended family with IS in an affected-only analysis [8]. Salehi et al. studied a single three-generation Italian family with IS and reported evidence of linkage to chromosome 17p11.2 [9]. Chan et al. reported linkage to a 5.2 cM region on chromosome 19p13.3 in a group of 7 Chinese candidates and noted a second candidate region on chromosome 2 [10]. In an earlier analysis of the families evidence of linkage was reported with markers on chromosome Xq23-26 in a subset of the families most likely to be X-linked dominant [11].

Thus, as a consequence of the above studies, the inheritance and the chromosome/gene related to the IS appear to be not yet understood.

It is usually accepted, by the scientific community, that the IS phenotype is mainly present in girls than in boys and its recruitment starts during puberty. This let to hypothesize the IS as a sex-related disease.

In this respect we have undertaken the present study in order to investigate among the endocrine status of girls showing the IS phenotype either in terms of sex steroid content or in terms of

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Table 1
Clinical parameters of the IS teenagers and no IS teenagers.

	Age	Menarca	Cobb angle	Risser value
No IS girls (control group) ($n = 104$)	14.8 ± 2.5	11.6 ± 1.1	No	1.45 ± 1.18
IS girls ($n = 174$) (47% unaffected sisters)	14.9 ± 3.2	11.7 ± 1.1	24.13 ± 10.77	1.54 ± 1.48

estrogen receptors polymorphisms, in the view of a clear relationship between estrogen, estrogen receptor α and bone metabolism [12–16].

2. Materials and methods

2.1. Blood samples

Teenagers (174) ranging between 13 and 19 years with either clinical or radiographic diagnosis of IS and teenagers of the same age ($n = 104$) without IS (control group) have been studied (Table 1). Both groups of teenagers were not under steroid or other pharmacological treatment. All teenagers were subjected to genetic counselling with their parents. After the counselling and with their written informed consent, blood samples from teenagers were collected at 7, 14 and 21 days of the menstrual cycle. We also collect blood samples from their relatives. Teenagers' samples were divided in two aliquots for both DNA extraction and steroids determination. Blood samples from their relatives were used for DNA extraction.

2.2. Chemicals

All chemicals and glassware were molecular biology grade. Primers for exons amplification and assay kits for steroids were purchased from Sigma (St. Louis, MO).

2.3. DNA extraction

Blood samples were obtained from all participants and genomic DNA was extracted with standard purification protocols [17,18].

2.4. Determination of blood 17β-estradiol, progesterone and testosterone content

Plasma concentrations of 17β-estradiol, progesterone and testosterone were measured with commercially available kit assay (IBL, Hamburg, Germany), using classical RIA [19].

2.5. Primers and estrogen receptor α (ERα) exons amplification

Primers were designed (Table 2) from sequences present in GenBank (AL356311.6, BC128573.1, BE084831.1, AL078582.13) and Human Genome Browser. PCR was performed accordingly to the manufacturer of PCR kit Roche (Roche Diagnostics, Italy). Products of amplification reactions were purified with standard methods and used for sequencing. Amplification products were sequenced from both ends.

Table 2
Exons, primers sequences, chromosome position and expected length of the amplified product for the exons 5–8 of the human estrogen receptor α gene.

Exon	Primer sequence	Position	Length (bp)
5	(F) 5′ GGGCATAAGGCAAGTAATTTA 3′	203700	599
	(R) 5′ GAATGAACTATGATCGTAAAGA 3′	204298	
6	(F) 5′ GTTACATTATTTGAAGGCTATG 3′	253003	697
	(R) 5′ AAACGTGTGTCAGTAGTTTCT 3′	253697	
7	(F) 5′ CATGGACAATTCATGATGGTG 3′	286509	741
	(R) 5′ CCAAGCGTAAGTATCGCTTC 3′	287249	
8	(F) 5′ TGGGAAGTGACGTGAGAGAT 3′	290703	1009
	(R) 5′ CATAGACTGAGTTCAGCTGT 3′	291711	

2.6. Exons sequencing

The product of PCR amplification was sequenced from both strands according to the conditions out-lined by the Big Dye Terminator Kit (PerkinElmer/Applied Biosystem) and runs on ABI 377 Sequencing System (PerkinElmer/Applied Biosystem).

2.7. RFLP of sequenced exons

Sequenced exons were digested using appropriate restriction enzymes in order to generate restriction fragments. The exon 5 was digested with XbaI (New England Biolabs), the exon 6 was digested with Pst1 (New England Biolabs), the exon 7 with StuI (New England Biolabs), and the exon 8 with MseI (New England Biolabs). The restriction fragments were separated onto agarose gel (1.8%) and the electrophoresis results were photographed with appropriate apparatus.

2.8. Statistics

Significance of differences was evaluated by using Duncan's test (at $p < 0.05$ and $p < 0.01$) for multigroup comparisons.

3. Results

3.1. Steroid content

Blood samples of all teenagers (IS girls and no IS girls) were collected during the menstrual cycle. Here are reported the results obtained during the preovulatory phase, being this the phase of maximum production of steroids.

The results of the analysis of 17β-estradiol content in IS teenagers and control teenagers (no IS and same range of age) are shown in Fig. 1. In IS girls the 17β-estradiol content results significantly ($p < 0.01$) lower than that found in control girls.

The same picture has been observed for progesterone content (Fig. 2) where the values in IS teenagers are significantly lower

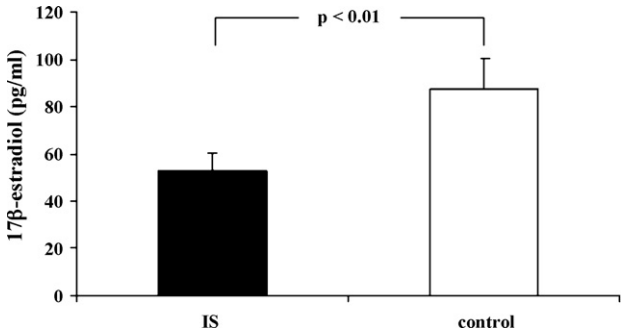

Fig. 1. 17β-estradiol content in blood samples from teenagers with IS phenotype (IS) and in teenagers not showing IS (control). Each blood sample was collected during the ovulatory phase. In IS teenagers the 17β-estradiol content was significantly ($p < 0.01$) lower than no IS teenagers (control).

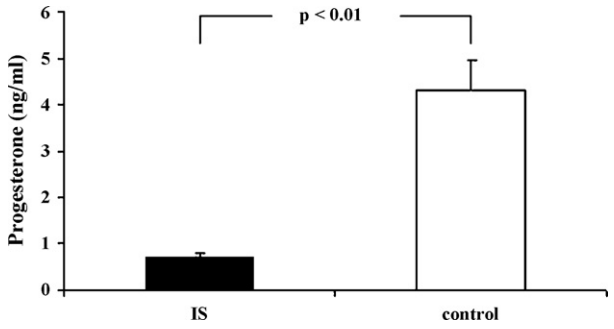

Fig. 2. Progesterone content in blood samples from teenagers with IS phenotype (IS) and in teenagers not showing IS (control). Each blood sample was collected during the ovulatory phase. In IS teenagers the progesterone content was significantly ($p < 0.01$) lower than no IS teenagers (control).

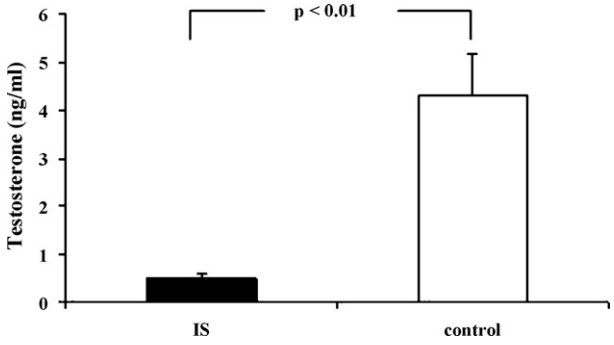

Fig. 3. Testosterone content in blood samples from teenagers with IS phenotype (IS) and in teenagers not showing IS (control). Each blood sample was collected during the ovulatory phase. In IS teenagers the testosterone content was significantly ($p < 0.01$) lower than no IS teenagers (control).

($p < 0.01$) than no IS teenagers. Data shown refer to blood content of progesterone during the luteal phase of menstrual cycle.

Among androgen (testosterone), the IS teenagers once again show blood levels significantly ($p < 0.01$) lower than no IS teenagers (Fig. 3).

3.2. ERα polymorphism

The analyses of the sequences of amplified exons, show 4 polymorphisms in the exons 5–8. The base mutations cause the loss of restriction sequences for the following enzymes: XbaI (exon 5), Pst1 (exon 6), StuI (exon 7) and MseI (exon 8). The restriction fragments generated by the above mutation are shown in Figs. 4–7. We never found more than one mutation/sample.

4. Discussion

Clinical reports of the familial occurrence of idiopathic scoliosis have appeared in the literature since the early 1930s. Population studies have documented the increased incidence of scoliosis within families compared to that in the general population, suggesting that scoliosis may be a hereditary disorder [20–22]. Twin studies have consistently shown that monozygotic twins are more concordant for the idiopathic scoliosis than are di-zygotic twins [23,24]. Despite the evidence that the etiology of this disorder may have a major genetic component, the mode of inheritance in still unresolved.

As a consequence of the above studies, the inheritance and the chromosome/gene related to the IS appear to be controversial and not yet understood, although the hypothesis of a multi-factorial inheritance should be seriously considered.

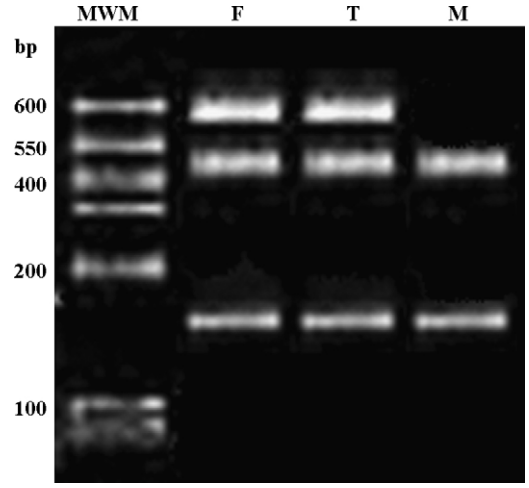

Fig. 4. Analysis of FRLP generated by digestion with XbaI enzyme of amplified products of the exon 5 belonging form ERα gene. F = father; T = teenager; M = mother.

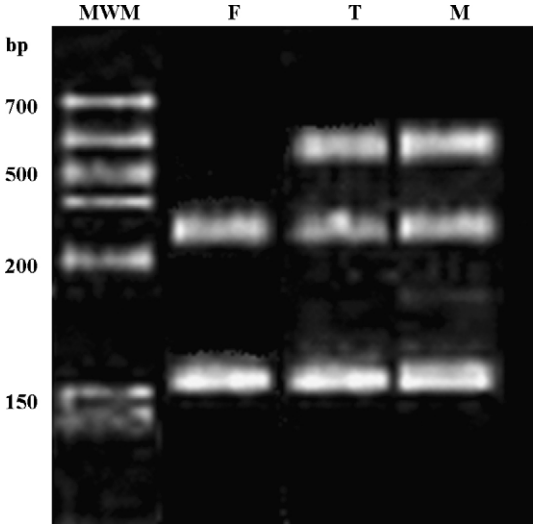

Fig. 5. Analysis of FRLP generated by digestion with Pst1 enzyme of amplified products of the exon 6 belonging form ERα gene. F = father; T = teenager; M = mother.

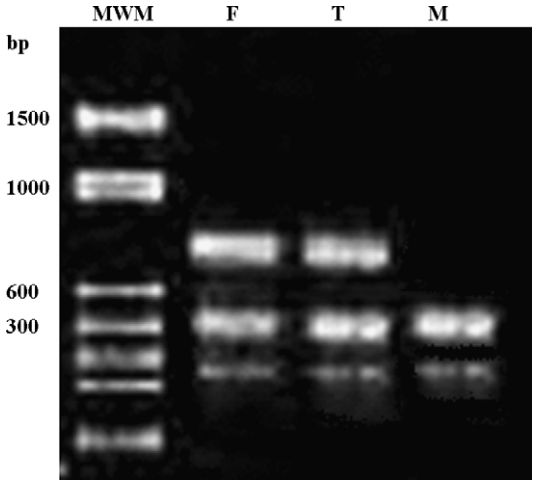

Fig. 6. Analysis of FRLP generated by digestion with StuI enzyme of amplified products of the exon 7 belonging form ERα gene. F = father; T = teenager; M = mother.

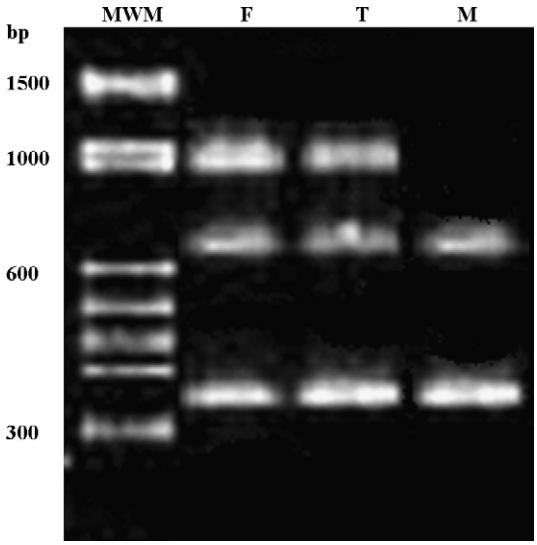

Fig. 7. Analysis of FRLP generated by digestion with MseI enzyme of amplified products of the exon 8 belonging form ERα gene. F = father; T = teenager; M = mother.

A part the above consideration, IS must be considered a bone disease whose recruitment starts during puberty and is mainly present in girls than in boys. This let to hypothesize IS as a sex-related disease.

The structural changes that occur in IS, concerns directly the osseous part of spine, its ligamentous and capsular apparatus as well as muscular system. In this respect, bone development, occurring during puberty in human is a critical period for a well development of bone in general and spine in particular. On the other hand, the role of steroid hormones in the development and maintenance of bone is a well-established phenomenon. Traditionally the point of view is that estrogens and androgens were the main sex steroids influencing bone maturation and maintenance in women and men, respectively. Studies reported that serum 17β-estradiol well correlated with bone mass density at various sites although also T has some effects [14–16,25–31]. It has been shown that serum 17β-estradiol is important in the increase in bone mass during the puberty [32]. This claims for a central role of estrogen with respect to skeletal metabolism. Our result, focused on the endocrine status of teenagers affected by IS, well correlate with international reports on the role of steroid on bone development during the puberty. The low levels of 17β-estradiol, testosterone and progesterone, have been found only in girls affected by IS, while normal values have been found in the control group. This may be due either to low level of steroid production by follicular cells or to low levels of the enzymes involved in steroid conversion. In fact, in patients with aromatase deficiency 17β-estradiol levels lesser than 20 pg/ml are required to complete bone maturation and mineralization [33–36]. However, by our study, it seems that the lower endocrine status, of girls affected by IS, with respect to no IS girls, is probably due to a reduced production of steroids than to a reduced conversion. This finding let us to hypothesize that in teenagers, affected by IS, a reduced levels of steroidogenesis occur.

The cortical bone size is, at least partly, due to sex steroid exposure during sexual maturation. In mice, it has been demonstrated that androgen receptor (AR) activation results in cortical radial bone expansion [37], since male AR−/− mice have reduced cortical bone development. This also claims, for a role of T in bone development. In addition, male ERα−/− but not ERβ−/− mice show reduced cortical radial bone growth during sexual maturation. These results indicate that ERα but not ERβ activation is also important for a normal cortical radial bone growth [12]. Similarly as seen for cortical bone, both AR and ERα but not ERβ activation regulates trabecular bone mass in mice [13,36,37]. Studies on the effect of ER activation on trabecular bone have been performed in order to better understand which ER is mainly involved in bone development. In these studies, orchidectomized wild type (WT) and ER-inactivated mice were treated with the non-aromatizable androgen dihydrotestosterone (DHT), 17β-estradiol, or vehicle. Both ERα and AR but not ERβ activation preserved the amount of trabecular bone. ERα activation resulted both in a preserved thickness and number of trabeculae. ERα is the principal ER for the regulation of both trabecular and cortical bone in female mice [38–42].

Thus, the activation of ERα, may have a central role in the bone homeostasis. In the present study we have found polymorphic sequences in the exons 5–8 of the ERα. It is noteworthy that the exons 5,6 and 7 encode for the steroid binding domain of ERα and each point mutation has the effect to change the amino-acid sequence of the domain itself. In the exon 5 the Leu^{379} is substituted by Glu. Thus a non-polar amino-acid is substituted by a basic one. In the exon 6, the Glu^{441} is substituted by Gln. Thus a basic amino-acid is substituted by a polar one. In the exon 7 the Leu^{495} is substituted by Met with not relevant changes in amino-acid characteristics. To our knowledge, no report of such mutations has been reported in the international literature, despite numerous mutation of the ERα has been found or experimentally induced [43]. Furthermore, these point mutations, are not present in the control girls, and in the IS girls we never found more than one mutation.

It is conceivable that the above mutation could have an effect on the reduction of the affinity of the ligand pocket with steroid, that in turn could modify the Kd of steroid/receptor binding. On the other hand, the reduced levels of steroids in teenagers with IS are probably *per se* the mastermind in the regulation of the homeostasis of estrogen/ERα function. However, it is also conceivable that both the reduced stroidogenesis and ERα mutations, could be implied in IS phenotype.

At present we cannot exclude other factors than the above can influence the estrogen effects on bone metabolism. However, the teenagers affected by IS, show in all cases lower levels of steroids when compared to teenagers that do not have the IS phenotype.

In conclusion we postulate that there is a linkage between IS phenotype and endocrine status of teenagers affect by the disease, and that the IS show a clear cut sex-dependent manifestation.

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