

# **Molecular biological investigation of adolescence idiopathic scoliosis**

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Ph.D. Thesis

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## INTRODUCTION

Recently the medical studies focus on revealing etiology of major common complex diseases. Complex diseases are believed to be caused by a several number of genes, usually interacting with various environmental factors include cardiovascular diseases, diabetes, obesity and some psychiatric disorders. Evaluation of genetic background of common complex disease is currently a hot area in human medical research thanks for the recent development of the Human Genome and HapMap Projects. The association between genetic polymorphism and disease phenotype based on population case-control study could reveal predisposing genetic factors of complex disorders.

Adolescence idiopathic scoliosis, a multifactorial disease represents a three-dimensional deformity of spine with lateral curvature in which the causes are unknown. Many studies are conducted to uncover contributing factors of AIS, however, pathomechanism underlying AIS have not been identified yet. Adolescent idiopathic scoliosis occurs at the peripubertal period (between 10 and 16 years of age) supposing the role in AIS etiology the changes in endocrine, metabolic and biomechanical alterations during growth spurt. It is widely accepted that scoliosis has a sex-conditioned manifestation, girls have a higher risk for development of curve progression the ratio of girls to boys is 3.4/1 with curves greater than 30 degrees. In the last 40 years many different research area were involved in hunting for etiology factors of spine deformity like connective tissue abnormalities, asymmetries in the central nervous system, hormonal variation and genetics.

According to the most accepted model for inheritance of AIS the genetic factors can be divided into predisposing factors, initiating factors and contributing factors in accordance with their role in the pathogenesis of AIS. Moreover, the current view is that there are no major genes causing AIS, but combinations of sequence variants of possible predisposition genes may have synergic effects on determining the deformity. Therefore, it is important to identify such sequence variants and combined genotype pattern of these susceptibility genes to get a better understanding of AIS pathogenesis.

The major objectives of the work presented in my dissertation were the investigation of the genetic background of AIS and the identification of novel molecular alterations that are associated with this disease.

## **AIMS OF THE STUDY**

The major objectives of the work presented in my dissertation were the investigation of the genetic background of idiopathic scoliosis and the identification of novel molecular alterations that are associated with this disease. For these studies I used two different molecular biological approaches.

**A1.** In the first approach I proposed to investigate the gene expression pattern of the paravertebral muscles responsible for the stability of the spinal column. By using the DNA microarray technology in transcriptome profiling experiments I compared:

- I. The gene expression pattern of the paravertebral muscles in healthy control individuals and AIS patients.**
- II. The gene expression pattern of the paravertebral muscles in the concave and the convex side of the spinal column deformity.**

My aim was to collect those genes that might be good candidate genes for AIS according to the following criteria:

1. They are significantly accumulated in a functionally relevant Gene Ontology group.
2. They are accumulated in a functionally relevant metabolic or signal transduction pathway.
3. They are located in a chromosome region has been previously reported to be involved in AIS.
4. According to the scientific publications reliable hypothesis could be built regarding its involvement in AIS pathomechanism.

**A2.** Quantitative real-time polymerase chain reaction (QRT-PCR) would be used for validation of microarray findings in several genes.

**B1.** In the second approach the identified candidate genes were investigated in case-control association studies of polymorphism in their promoter regions.

**B2.** The genotyping data were subjected to different statistical evaluation processes suitable for the identification of the genetic interactions.

## **Materials and Methods**

### Experimental samples

Tissue samples were collected in the Department of Orthopedics, Medical School University of Pécs from patients subjected preliminary clinical investigations. The diagnosis of AIS was accepted only in the lack of any neurological, metabolic or muscle disorder. The RNA and DNA samples were originated from muscle biopsies taken from *bilateral musculus latissimus dorsi* during correction surgery interventions. Patients were informed very accurately, and their samples were used in my investigation only with their explicit consent.

### Methods

1. Purification DNA and RNA from different tissues.
2. Testing the quality of RNA samples by Northern agarose gel electrophoresis.
3. Reverse transcription of the RNA samples.
4. Microarray analyses.
5. Statistical analysis of microarray results.
6. Annotation analysis of gene set selected in microarray experiments.
7. Quantitative real-time polymerase chain reaction (QRT-PCR).
8. Restriction length polymorphism of polymerase chain reaction fragments (PCR-RFLP).
9. Statistical evaluation of genotyping data.

## RESULTS

### A.1.

For revealing the gene expression differences in AIS disease 95 total RNA samples have been prepared from muscle biopsies of AIS patients subjected to correction surgery interventions. In collaboration with the Functional Genomics Group of the BRC **two microarray analyses have been performed**. In the first experimental layout the gene expression levels in healthy and AIS individuals were compared. In the second the gene expression patterns of muscle derived from the concave and the convex sides of the spinal column deformity were investigated. As a result of these two microarray experiments I **obtained lists containing 187 and 63 genes** showing significant gene expression differences. To select genes for further genetic investigations, the elements of the gene lists have been evaluated regarding to the following four criteria:

1. The members of the gene lists has been classified according to their Gene Ontology annotation, and the significantly overrepresented GO categories that functionally might be involved in the AIS etiology have been identified. Genes from the **lipid metabolism (GO:0006629) and morphogenesis (GO:0007275) categories were enriched** in gene set showing expression difference in the concave and convex side of the spinal cord deformity. These GO categories contained 18 out of the total 63 genes of this list.
2. The simultaneous accumulation of genes with significantly altered expression in particular metabolic or signal transduction

networks might help to reveal molecular or cell physiological processes involved in the appearance of a disease phenotype. In our microarray investigations the **adipocyte signaling cascade** (*KEGG\_Pathway: hsa04920*) proved to be overrepresented in the gene list, indicating the potential role of lipid metabolism in AIS disorder.

3. I have identified the **chromosomal localization of genes** showing significant expression difference in microarray experiments, and selected 8 of them have been previously reported in family based linkage analysis as could be involved in AIS.
4. Performing very exhaustive data mining in different biomedical literature and gene annotation databases like Pubmed, GenCards or OMIM, I tried to find connections between the known functions of the selected genes and the different AIS pathogenesis models suggested up till now by the scientific community working in this field. Two of the identified genes might be very interesting in this respect. **Dystonin** has well characterized role in affecting the muscle strength, while **tensin** has an important function in the regeneration process of the wounded muscles.

## A.2.

Microarray experiment can be a good starting point for functional genomics studies since it can draw the attention to new genes that previously haven't been connected to the investigated phenomena. However, it is always necessary in the next step to validate the microarray results by an independent method, to check if the observed differences in

gene expression levels indeed exist. In bulk majority of the currently applied experimental strategies this independent method is the quantitative real time polymerase chain reaction (QRT-PCR). **Out of the 13 genes that were selected for QRT-PCR validation the leptin(LEP) gene was the only one** where the gene expression tendencies detected in the microarray investigations could be confirmed. In QRT-PCR assay the abundance of leptin mRNA was 2.5 times higher in the convex side of the spinal in AIS patients. These findings inspired me to make further studies concerning the involvement of leptin gene in AIS pathogenesis.

### **B.1.**

The involvement of leptin in the development of AIS was recently described in a publication where the authors demonstrated that the serum level of leptin in young AIS females is lower than in the appropriate healthy control population. In a case-control association study I have investigated the association of a promoter SNP in the leptin gene with the manifestation of the AIS phenotype. In the same assay I have also analyzed another SNP from IL6 gene promoter region that has been reported to be associated with AIS. By using the PCR-RFLP method for the concurrent detection of **LEP G-2548A and IL6 G-174C polymorphisms I have determined the genotypes in 126 AIS and 197 healthy unrelated individuals.** Both investigated populations were in Hardy-Weinberg equilibrium at these loci. No significant differences were found for allele and genotype frequencies of the polymorphisms of LEP G-2548A and IL6 G-174C between cases and controls. However, the determination of the odds ratio of the different genotypes indicates that the carrying of the LEP-2548A allele might be a susceptibility factor for

AIS. In my studies the odds ratio of LEP-2548 AA vs. LEP-2548 GG was OR=1.513 (CI:0.894-2.562), which is generally accepted as an indicator for susceptibility alleles in the case of complex diseases. The odds ratio value was further increased (LEP-2548 AA vs. LEP-2548 GG: OR=2.02(CI:0.824-4.961)) when the female subpopulations was subjected suggesting that in females LEP-2548A allele might have a greater impact on the manifestation of AIS than in males. In the other hand I didn't find any association between the alleles determined by these SNPs and the extent (Cobb<sup>o</sup>) of the spinal curve. In my studies I couldn't confirm the association of some particular IL6 G-174C alleles with AIS phenotype as it has been described by other investigators recently. The lack of reproducibility of findings seen in association studies is a well known phenomenon in the complex disease research.

## **B.2.**

Since in complex diseases the genetic factors involved act in mutual manner, I have investigated if there is any epistatic interaction between the alleles of leptin and IL6 in respect of the manifestation of AIS. I used two different approaches for revealing the gene-gene interactions. MDR analysis is suitable for detection of epistatic interactions in such situations when the sample size is relatively small for the investigation in addition this method gives reliable results when there is no difference between the distribution of the alleles in the healthy and the affected group. As a result of this study the genotype combinations had increased risk of the appearance of AIS could be emerged. The other statistical process based on the maximum likelihood estimation can give a quantitative answer

how a particular allelic combination increases the chance of AIS manifestation compared to the other allelic patterns. By performing these studies I **have demonstrated that the IL6 CC-LEP AA allele combination has the strongest susceptibility effect**, which is 4.667 times higher to that of appear in the presence of IL6 GG-LEP GG alleles. This relative odds ratio is even higher in the case of female target population (OR= 6.667 (CI:0.597-74.506) indicating a higher genetic risk for idiopathic scoliosis. I couldn't detect any statistically significant association between the different genotype combinations and the extent of the spinal curve. These findings suggest that the LEP AA-IL6 CC genotype combination is rather involved in the onset than in the progression of AIS disease.

### SUMMARY

In the studies described in my Ph.D. dissertation I used a novel approaches, namely microarray analyses in AIS research for the investigation of the gene expression pattern of the paravertebral muscles. I could identify several genes where the altered gene expression indicates their potential role in AIS pathogenesis. An independent method (QRT-PCR) was successfully used to collect supporting evidence regarding the detected gene expression difference in the case of leptin. Further genotyping studies and statistical investigations lead to the identification of a gene-gene interaction resulting in increased susceptibility for AIS. I could demonstrate that the simultaneous occurrence of allelic variants determined by single nucleotide polymorphisms in the regulatory promoter regions of two adipokine genes (IL6 and leptin) might be a susceptibility factor in AIS etiology.

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