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RESEARCH ARTICLE

ASSOCIATION STUDY DESIGN OF COMPLEX DISEASES: IDIOPATHIC SCOLIOSIS

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ABSTRACT

The current concept of idiopathic scoliosis (IS) consists of a multifactorial disease involving genetic and non-genetic factors in the occurrence and progression of curvature. The candidate gene association study begins with selection of a putative candidate gene based on hypotheses, including biological systems involved in the development of deformity and assumptions based on results of clinical observations. The aim of a genome wide association study (GWAS) is to detect significant associations in a population between common diseases and common genetic variants. The results from previous association studies based on hypotheses and from whole genome scan suggest involvement of polymorphic variants of different candidate genes with different impact on the etiopathogenesis of IS in different population groups. The identification of molecular markers with diagnostic and prognostic value could be useful in clinical practice for early diagnosis of scoliosis among relatives and for more accurate prognosis of the risk of rapid progression of the deformity among affected individuals. That will permit prophylaxis and early treatment with less invasive procedures.

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INTRODUCTION

The current concept of idiopathic scoliosis (IS) consists of a multifactorial disease involving genetic and non-genetic factors in the occurrence and progression of curvature. According to the common disease – common variant hypothesis (CDCV hypothesis), there is an accumulation of common genetic variants creating a predisposition that is triggered under the influence of non-genetic factors. During the period from 1992 to 2016 a number of molecular genetic studies on the etiopathogenesis of IS were conducted. Most of them were case-control studies investigating the potential association between a particular clinical phenotype and common polymorphisms in one or few candidate genes.

METHODS

Candidate gene association studies

The candidate gene association study begins with selection of a putative candidate gene based on hypotheses, including biological systems involved in the development of deformity and assumptions based on results of clinical observations (1). This is followed by assessment and selection of polymorphisms as tag Single Nucleotide Polymorphisms (SNPs) and/or functional polymorphisms affecting gene regulation or the protein product (2, 3). Finally, the gene variant is verified for association with a disease (trait) by observing its occurrence in random test subjects (cases) having the disease and in selected control subjects which do not; and is then evaluated for its association with disease prognosis and diagnosis and its future potential as a biomarker (1). This makes the knowledge derived from candidate gene studies valuable and clinically relevant as a potential disease diagnostic tool and for personalised medicine initiatives in future treatments of genetic disorders (4).

Single nucleotide polymorphisms

SNPs are classified according to their location in the gene locus, which also most times dictates the functional downstream effects of the polymorphic allele (5). SNPs in the coding region which leads to a change in the translated amino acids and thus in the encoded protein are categorised as non-synonymous SNPs (nsSNPs). While the functional role of nsSNPs is relatively straight forward, SNPs located in regulatory and intronic regions have recently gained importance upon recognition of their potential to deregulate transcriptional efficiency, gene expression and splicing (6-9).

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Table 1. Candidate gene association studies

Candidate-genes	Number of positive associations References)	Number of negative associations (References)
AANAT (SNAT)	0	2 (21, 32)
ACAN	0	2 (58, 59)
ACE	0	1 (36)
ACTN3	0	1 (36)
AMPD1	0	1 (34)
ASMT (HIOMT)	0	1 (32)
BMP4	0	2 (15, 18)
CALM1	2 (27, 28)	0
COL1A1	0	1 (60)
COL1A2	0	2 (60, 61)
COL2A1	0	1 (60)
CYP17	0	1 (62)
DPP9	0	1 (63)
ELN	0	1 (61)
ESR1	5 (27, 37, 38, 39, 40)	3 (41, 42, 43)
ESR2	1 (45)	2 (42, 46)
FBN1	0	1 (61)
GHR	0	2 (64, 65)
GPER (GPR30)	1 (48)	1 (29)
GPR50	0	1 (30)
IL-6	3 (11, 12, 13)	1 (15)
IGF-1	2 (25, 73)	3 (31, 34, 65)
LAPTM4B	1 (73)	0
LCT	0	1 (70)
LEP	0	2 (15, 18)
LEPR	1 (26)	0
LOX1, 2, 3, 4, 5	0	1 (66)
MATN1	3 (22, 23, 51)	2 (31, 70)
MMP3	1 (11)	3 (12, 15, 16)
MTNR1A	0	3 (32, 67, 68)
MTNR1B	1 (20)	6 (15, 30, 31, 32, 33, 69)
NTF3	1 (19)	1 (29)
RANK	0	1 (179)
RANKL	0	1 (179)
TGFB1	1 (14)	0
TIMP2	1 (24)	1 (29)
TNFRSF11B (OPG)	1 (72)	0
TPH1	1 (21)	2 (31, 32)
VDR	1 (71)	3 (34, 62, 70)

Table 2. Genome wide association studies

Population (Reference)	N, cases/controls	Phenotype,Cobb	Gene, Locus	SNP	P-value
Caucasian	Initial study:				
(52)	419 trios	AIS	CHL1	rs1400180	7.91×10^{-8}
	Replication study I: 375/444	> 10°	(3p26.3)	rs10510181	2.58×10^{-8}
	Replication study II: 187/222				
Caucasian	Total: 137/2126	AIS	IL17RC (3p25.3)	rs708567 (S111L)	1.18×10^{-9}
(78)		> 10°			
			Between NEK7 and ATP6V1G3		
			(9 chr)	rs10758121	2.83×10^{-8}
Caucasian	Total: 1000/1000	AIS			
(79)		> 10°	Between TLX1 and LBX1	rs11190878	2.45×10^{-11}
			(10 chr)	rs7893223	1.53×10^{-7}
			Near PRICKLE 1 (12 chr)	rs7138732	3.87×10^{-8}
				rs11181576	2.59×10^{-7}
Caucasian	Total: 906/1480	AIS	LBX1 (10q24.31)	rs11190870	5.43×10^{-9}
(77)		> 15°		rs11190878	4.18×10^{-9}
Asian	Initial study: 1050/1474	AIS			
(53)	Replication study: 326/9823	> 15°	LBX1 (10q24.31)	rs11190870	1.24×10^{-19}
	Japanese study: 1819/25 939				Japanese:
Asian	Chinese study:	AIS	GPR126 (6q24.1)	rs6570507	2.25×10^{-10}
(57)	N/A	> 15°			Chinese:
					1.27×10^{-14}
	Initial study: 554/1474		Near SOX9 and KCNJ2 (17q24.3)	rs12946942	Japanese:
Asian	Replication study I: 268/9823	AIS			4.00×10^{-8}
(74)	Replication study II: 571/326	> 40°			Chinese:
					6.43×10^{-12}
Asian	Total:	AIS	BNC2 (9p22.2)	rs10738445	2.46×10^{-13}
(75)	2109/11 140	> 15°			
			Near AJAP1 (1p36.32)	rs241215	2.95×10^{-9}
Asian	Total:	AIS	Between PAX3 and	rs13398147	7.59×10^{-13}
(76)	4317/6016	> 20°	EPHA4 (2q36.1)		
			Near BCL-2 (18q21.33)	rs4940576	2.22×10^{-12}
			LBX1AS1 (10q24.32)	rs678741	9.68×10^{-37}

SNPs within the regulatory elements of the gene can disrupt gene expression by altering transcription factor binding sites, influencing the strength of enhancers and promoters, making these SNPs of prime importance to be considered for candidate gene association studies (10). Until now, in Caucasian population molecular genetic studies found single associations with the promoter SNPs of *IL-6* (11, 12, 13), *MMP-3* (11), *TGF-b* (14) and double associations with genotype combinations of *IL-6-Lep*, *MMP-3-BMP4* and *MMP-3-MTNR1B* (15) and *IL-6-MMP-3* (12). In Asian populations these data differ due to the fact that *IL- 6* (-174G/C) is not polymorphic (16), and *TGF-b* was associated only with the progression of the deformity (17). Results for *MMP-3* were not confirmed in a large Chinese cohort (16). There are no single associations between functional polymorphisms of *Lep* and *BMP4* and idiopathic scoliosis in the studied population samples (15, 18). In Chinese population statistically significant associations with the promoter SNPs of *NTF3* (19), *MTNR1B* (20), *TPH1* (21), *MATN1* (22, 23), *TIMP2* (24), *IGF-1* (25), *LEPR* (26), *CALM1* (27, 28) were found. Most of these associations were not confirmed in Japanese (29, 30, 31) and Caucasian population (15, 32, 33, 34). The functional *ACTN3* and *ACE* polymorphisms are studied only within a large Brazilian family (35). The first association study in Caucasian population did not establish a correlation with the deformity (36). It needs association studies type of case-control study of the role of the common functional polymorphisms of *ACE* and *ACTN3* in etiopathogenesis of IS. Case-control studies in European, Japanese and Chinese populations also established associations with common polymorphisms with poorly explored functional effect e. g. restriction polymorphisms in the *ESR1* gene (27, 37, 38, 39, 40) or lack of associations with the same SNPs (41, 42, 43). The interpretation of these positive results is hampered by the fact that there is no direct evidence of the impact of the restriction polymorphisms on the levels and activity of the gene product. However, there is evidence that these polymorphisms affect gene transcription (44). In a Chinese study, a common variant of *ESR2* was associated with susceptibility and progression of IS (45), as the association was not confirmed in a larger Japanese study (42) and one European study (46).

Rare polymorphisms

Inability to explain the phenotypic variability points to epigenetic factors, rare highly penetrant alleles, intergenic or non-allelic interactions, changes at different levels of regulation of gene expression and interaction gene - environment. Rare variants can occur as point mutations, or as gene deletions/duplications (47). Three rare tag SNPs in the *GPR30* gene showed association with the progression of IS, but not with the etiology of IS in Chinese (48). In Japanese patients there was no association between these three polymorphisms and curve severity (29). A recent study in Caucasian population established an association between rare variants of *FBN1* and *FBN2* and progression of IS, using a technique called exome sequencing. Subsequently the team confirmed the findings in a Chinese population sample (49). A recent study in Caucasian population involving linkage analysis in combination with exome sequencing identified a rare missense substitution of gene *POC5*, which co-segregates with IS in a large family with multiple affected members. Subsequently replacement was found in other families and another 3 cases. Another substitution was coupled with deformity in one family; a rare third substitution was identified in 5 cases of IS. In zebrafish model organisms the increased expression of mRNA for each of the three functional *POC5* variants leads to spinal deformity without affecting other skeletal structures (50).

Microsatellite markers

In a population of 81 trios Italian researchers found that one intergenic microsatellite polymorphism (short tandem repeat) of an untranslated region of the *MATN1* gene is more frequent in index patients with IS compared to other alleles (51).

Genome-wide association studies

The aim of a genome wide association study (GWAS) is to detect significant associations in a population between common diseases and common genetic variants. In particular, a GWAS is designed to examine millions of SNPs in the genome, using commercially available chips to survey the genotypes of thousands of individuals. Variant SNP alleles that are differentially associated with a disease cohort compared to controls are thought to denote susceptibility regions that contain genetic correlates to the disease (i.e. genes or genetic deletions/duplications). Any positive association should be confirmed in a different population using a larger sample size (47).

Single nucleotide polymorphisms

First, Sharma *et al*. (2011) generated a list of the top 100 significantly IS-associated SNPs from a genomic survey in Caucasian population. The results suggested that *CHL1*, a member of the L1 gene family of neural cell adhesion molecules and a neural recognition molecule that may be involved in signal transduction pathways, might be associated with the susceptibility of IS. The authors then surveyed variants significantly associated with other genes involved in the axon guidance pathway: *DSCAM* and *CNTNAP2* (52). Next, Takahashi *et al*. (2011) demonstrated an association in an East Asian population between IS and common variants near *LBX1*, a transcription factor required for the development of inhibitory interneurons in the dorsal horn of the spinal cord as well as migration and further development of hypaxial muscle precursor cells. They suggested the relevance of somatosensory pathways in the disease etiology (53). For the three associated SNPs, authors of a replication study using a Chinese Han population observed the same direction of effect - increased risk in IS population (54-56). To identify additional IS susceptibility loci, Kou *et al*. (2013) extended the previous GWAS in Japanese population. The most significant SNP identified was located in an intron of the *GPR126* gene. *GPR126* is involved in the process of myelination and in the growth and development of the spine during childhood. Functional analysis by the group showed that the knockdown of *gpr126* in zebrafish embryos caused delayed ossification of the developing spine. Further, the authors replicated the association and the same effect size in Han Chinese and

European-ancestry populations (57). Taken together, the recent GWAS evidence for the association of three genes (*CHL1, LBX1* and *GPR126*) with IS (52, 53, 57), barely accounts for 1% the observed phenotypic variance (47). The results from the candidate gene association studies and genome wide association studies are summarized on Table 1 and Table 2, respectively.

Discussion

Candidate gene based association studies are the predominant type of studies on IS genetics. They are inexpensive and quick to perform and are focused on the selection of individual candidate genes that have been in some way related to the disease previously and thus come with prior knowledge about gene function. The main weakness of these studies is that significant associations rarely found confirmation in replication studies. False positive or false negative findings could be the reason for non-replication (1). First, the observed differences in the results could be explained with different selection criteria for the samples (pre-analytical stage). Over 90% of research is on the most common form of IS – adolescent and on scoliosis in females. A smaller number of studies recruit the participants among patients with infantile, juvenile and adolescent idiopathic scoliosis, separate the cases in subgroups according to age, gender, Cobb angle, curve pattern and familial history and investigate the associations in the general sample and in the different subgroups. In some studies detailed information is not described. There is a need for studies of early IS - infantile and juvenile, as well as scoliosis in males. It is possible the participation of different genetic variants in the etiopathogenesis of early and late onset scoliosis, progressive and non-progressive forms, sporadic and familial cases, males and females etc. At the same time there are many studies of the relationship between susceptibility to IS and various candidate genes, while studies on the relationship between progression and candidate genes with potential modifying effect is not so much. Additionally, the design of the association studies is very different in terms of clinical criteria for assessment of progression (indications for surgical treatment, an increase in the Cobb angle per month and per year, annual spinal growth etc.) and patient selection (minimal Cobb angle, age of onset) and controls (age, origin - random sampling, subpopulations, samples from biobanks). So far, case-control studies of individual candidate genes are the predominant type of studies on IS genetics. Case-only studies will be also needed to compare the genotype and allele distribution between cases with different curve pattern or other clinical characteristics and to investigate a possible association between candidate genes and bracing.

Second, the various results could be explained with technical errors (analytical stage). Most candidate gene studies use identical technical approaches as amplification-restriction (PCR-RFLP) protocol, real-time PCR or direct sequencing. One of the reasons for identifying a number of false positive findings could involve systemic genotyping errors and lack of statistical power due to smaller samples. Conversely, the negative results obtained in small cohorts do not exclude the potential involvement of the candidate gene with minor effect in the etiopathogenesis of the disease. False negative findings can be attributed to under evaluation of gene-gene interactions and gene environment interactions (1). Third, the observed differences in the results could be explained with differences in the preferred statistical methods with or without corrections (post-analytical stage). Pearson's chi-squared test (χ^2) or Fisher's exact test are usually used. Suitable adjustments to compute the statistical power of the study are needed. Additionally, the multiple comparisons issue due to accounting for the same SNP in various tests can lead to false discovery rates. This can be addressed by computing Bonferroni adjustments of the significance criterion (alpha) according to the number of examined genes or SNPs. Finally, the genotype and allele frequencies could be different in the different population and even ethnical groups. In recent years, the identification of new candidate genes is the goal of GWAS and some other approaches as exome sequencing.

There are some important limitations to consider with GWAS. First, to avoid false significant associations by testing approximately 500 000 SNPs in several thousand individuals, a threshold of p value less than 10^{-7} or 10^{-8} must be used. At the same time, however, this decreases the power to detect SNPs of minor effect independently, which may have strong associations when considering the interplay of genes and environmental factors. Second, the effectiveness of a study is subject to multiple factors. When evaluating the results of a GWAS, it is important to consider the sample sizes, the odds ratios, the allele frequencies, the threshold of significance, and the performance of the commercial microarrays in a population (47).

The results from previous association studies based on hypotheses and from whole genome scan suggest involvement of polymorphic variants of different candidate genes with different impact on the etiopathogenesis of IS in different population groups.

Conclusion

In conclusion, the studies on idiopathic scoliosis have indicated substantial genetic heterogeneity in the etiology of the disease. There are predisposition genes that usually have low penetrance and are associated with a moderate increase of the risk of developing the disease. In addition to predisposition to the development of idiopathic scoliosis, genetic factors could also influence the severity of the disease. The concept of disease-modifier genes as an element of genetic heterogeneity has been widely accepted and reported. Additionally, there are genetic variants that are independent predisposing and/or modifying factors, and there are genetic markers of minor modifying effect. The identification of molecular markers with diagnostic and prognostic value could be useful in clinical practice for early diagnosis of scoliosis among relatives and for more accurate prognosis of the risk of rapid progression of the deformity among affected individuals. That will permit prophylaxis and early treatment with less invasive procedures.

Conflict of Interests

The authors declare no conflict of interest regarding the publication of this paper.

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