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Does the association of *TIMP2* gene polymorphisms with curve initiation or progression of thoracic idiopathic scoliosis in Caucasian population exist?

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Abstract

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Study Design Genetic Association Case-Control Study (Level III)

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Objective. To determine whether one promoter and eight intragenic DNA single nucleotide polymorphisms of the metalloproteinase 2 (*TIMP2*) gene are associated with idiopathic scoliosis (IS) initiation or progression in a homogenous group based on curve pattern, clinically relevant curve progression criteria and joint hypermobility.

Summary of Background Data. Idiopathic scoliosis is a spinal deformity with strong genetic susceptibility. Predicting curve progression reveals important for clinical practice. Genetic association study of the rs8179090 of the tissue inhibitor of metalloproteinase 2 gene previously reported on the association with IS progression in Han Chinese, not confirmed in Japanese population. There is no reports regarding intragenic polymorphisms (rs9916809, rs2277700, rs2377005, rs11658743, rs11077401, rs2376999, rs8068674, rs4789934 and rs8179090) associations in idiopathic scoliosis patients.

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Methods. We recruited 100 girls with right thoracic IS, Cobb angle ≥20° and 100 healthy females. Scoliosis patients were subdivided accordingly: (1) peak annual progression rate, into progression (>12°/year) *vs.* non-progression (<12°/year) group, (2) curve severity into Cobb angle >50° *vs.* Cobb <50°, (3) joint mobility into hypermobile (Beighton≥5) *vs.* not hypermobile (Beighton<5) and (4) curve pattern into right thoracic (Lenke 1/Ponseti IV) *vs.* double major curve (Lenke 3/Ponseti III). Allele-specific PCR based on fluorescence resonance energy transfer was applied to evaluate the associations of the *TIMP2* polymorphisms.

Results. Distribution of genotype and alleles frequency in only one out of nine analyzed polymorphisms (rs11658743) differed in case-control study. Four of the polymorphisms (rs2277700, rs11077401, rs2376999, rs4789934 showed non-equal distributions either in genotype or/and allele distributions in the patients of different progression rate. The rs11077401 was additionally related to curve severity patients distinction, and the rs8179090 differed patients with joint mobility level. Two polymorphisms eighter differed statistically in curve patterns subgrouping (rs8068674 and rs8179090) or slight tendency toward significance was shown in recessive model of alleles distribution (rs9916809 and rs8179090). Remaining two polymorphisms showed no association with clinical and radiographic results obtaining for the patients.

Conclusions. The influence of the G allele of the rs8179090 on the clinical course of scoliosis has not been confirmed. Four polymorphisms (rs11077401, rs2376999, rs2277700, rs4789934) have been identified, associated with a higher risk of progressive form of IS. Further association studies based on suggested clinical criteria for progression would be necessary to identify SNPs associated with the curve progression.

Keywords

idiopathic scoliosis; tissue inhibitor of metalloproteinase 2 gene (*TIMP2)*; curve progression; genetic association study; Cobb angle; joint hypermobility; Beighton score

Mini Abstract

A prospective genetic association study of promoter and intragenic SNPs of *TIMP2* gene was conducted in 100 patients and 100 controls in homogenous group based on curve pattern and clinically important curve progression criteria and joint mobility.

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Introduction

Adolescent idiopathic (AIS) scoliosis is a common disease with an overall prevalence of 0.47–5.2 % [1]. It is the most common spine pathology occurring in children and attended by orthopedic surgeon [2,3]. It is a tridimensional spine deformity with a lateral curvature greater than 10 degrees with vertebrae rotation [3,4] and sagittal plane imbalance [5]. Progression of the deformity occurs in 10%-15% of cases [6–8] and is most common in females during pubertal growth spurt [1,8–11].

Although wide research was conducted in the field, etiopathogenesis of IS remains poorly understood [9,12–14]. Genetic factors are supposed to be crucial in idiopathic scoliosis etiology [15]. However, genetic factors might vary from population to population and may be reflected in prevalence differences. These assumption are supported by the fact, that the prevalence of scoliosis differs among races, and curve severity is associated with race [16–19].

Many different genes seem to play role both in curve initiation and progression. Genome-wide linkage analyses have identified plausible regions associated with scoliosis in almost half of chromosomes with one candidate gene identified, encoding chromodomain-helicase-DNA-binding protein 7. Recently, genetic association studies indicated several IS predisposition genes. Single nucleotide polymorphisms (SNPs) associated with IS have been described within genes encoding both estrogen receptors (*ESR1* and *ESR2*), matrilin 1 (*MATN1*), melatonin receptor 1B (*MTNR1B*), tryptophan hydroxylase 1 (*TPH1*), ladybird homeobox 1 (*LBX1*), close homolog of L1 (*CHL1*) and many more [20]. Furthermore, SNPs associated with the curve severity of IS have been described in case of *ESR1, ESR2, MATN1,* and in insulin-like growth factor 1 (*IGF1*). Many of those studies were replicated in different populations with conflicting results [1,21,22].

Clinical observations made in mid-twentieth century revealed that the lateral curvature developing in the thoracic spine is usually associated with thoracic hypokyphosis. The concept of described as relative anterior spine overgrowth (RASO) was further reinforced with magnetic resonance imaging studies [23]. It was postulated that this process of faster anterior column endochondral ossification leads to rotational spine instability and relative tethering of neural structures as one of the causes of deformity [24,25]. It was demonstrated that normal endochondral ossification is dependent on extracellular matrix degradation and remodeling that is mainly regulated by matrix metalloproteinases and their tissue inhibitors (TIMPs). In 2012 Jiang *et al.* identified that promoter polymorphism of *TIMP2* (rs8179090; -418G/C) may be associated with scoliosis progression in Chinese population [26]. This result was not replicated in Japanese population as stated by Ogura *et al.* in 2013 [27].

Other polymorphisms in *TIMP2* gene were described in the term of e.g. susceptibility of osteoarthritis in the elderly population (rs4789934) [28], myopia (rs2376999) [29] or breast cancer patients survival (rs9916809 and 2376999) [30]. Conflicting results regarding rs8179090 and IS susceptibility or progression could be race/ethnicity-dependent, as mentioned above, and never have been evaluated in Caucasian population. Other polymorphisms, we considered in this study, were not examined in relation with scoliosis.

Genetic data referring *TIMP2* single nucleotide alternations in this study were analyzed in patients divided into separate groups according to: (1) peak annual progression rate (APR) (2) curve severity (3) joint mobility (as its role in IS was suggested in the literature [31,32] and may be related to TIMP2 dependent extracellular matrix remodeling) and (4) curve pattern. An association study was performed in each group to investigate the possible relationship between *TIMP2* gene polymorphisms and IS patients clinical and radiological picture.

Materials and Methods

The study has been approved by University of Medical Sciences Poznan Institutional Review Board and written informed consent was obtained from all study participants (approval No 562/11).

Study participants

We recruited 100 girls with IS and 100 healthy girls. For the study group full history, clinical examination and radiological examination was obtained. IS diagnosis was ascertained by two experienced orthopedic surgeons. Patients with congenital scoliosis, neuromuscular

disorders, skeletal dysplasia, and syndromic scoliosis were excluded. Only patients with clinically relevant right-sided thoracic curve of Cobb angle greater than 20° were included (Lenke type 1 and 3). For the control group scoliosis was excluded by medical history analysis and standard clinical examination with scoliometer. Any congenital abnormalities, disorders affecting bone growth and metabolism, hereditary diseases affecting musculoskeletal system were also exclusion criteria.

Association Design Study Server calculated study design power of 87%, based on expected odds ratio (OR) of 3.0 and disease prevalence of 0.03. Genotype analyses were performed using allele-specific PCR based on fluorescence resonance energy transfer (FRET) hybridization probes designed with ProbeDesign software (Roche).

Genetic analyses

Genomic DNA was isolated using AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., Union City, CA, USA) accordingly manufacturers' protocol.

In FRET, both anchor and sensor probes were labeled with either fluorescein or LC® Red-640 dye as applicable to the orientation of the SNP detection. The regions of interest were amplified by the use of forward and reverse primer pairs, complementary to the flanking regions of target DNA. Both probes were designed to be localized on the same DNA strand to anneal on the target sequence in a head-to-tail arrangement. The SNP specific sensor probe with the 3'-end-conjugated-fluorescein dye was designed two nucleotide apart from the anchor probe (with 5'-end-conjugated LC® RED-640 dye) to facilitate the energy transfer between the two fluorescent dyes in proximity.

As described before [33], in short, during the melting phase, and fluorescein excitation energy transfer referred to as FRET occurs if both fluorescent dyes are in approximate distance. This absorbed excitation energy is transferred from the anchor to the sensor probe and the emitted by LC® Red-640 fluorescence energy is detected. Gradual temperature-dependent fluorescence intensity decrease as the probes melt. The sensor probe with a perfect match to the target SNP melts at a higher temperature contrary to the mismatch that melts at a lower temperature (ca. 5 Celsius degrees lower). Therefore, the difference in the melting temperature remains as a basis to differentiate the genotypes. All PCR reactions followed by melting curve analysis were performed using the LightCycler® 2.0 instrument.

Two macros modules of the LightCycler® 2.0 dedicated software 4.1 – melting curve and genotyping were used for the analyses. PCR amplifications were carried out in a 20 μL total volume containing: 50 ng of template DNA, 1x LC® HybProbe® Master Mix, specific primer pair and both probes in a defined concentration (Supplementary Table S1). Thermal cycling parameters are presented in supplementary Table S2. The primer pair and hybridization probes are shown in supplementary Table S3.

Statistical analyzes

Purpose of the entire region of the *TIMP2* gene analyses was to implement the SNP tagging method. To determine the tagSNP set, the Association Design Study Server (http://design.cs.ucla.edu./index.cgi) was used, which uses the tagging method described by de Bakker *et al.* [34] and simultaneously calculates the statistical power of the projected study. Input data has been entered to the server: Population: CEU (Caucasian), chromosome 17, positions from 74355657 to 74438067 (length = 82410). MAF for SNPs: 0.01, total number of SNPs in the region: 79, significance level for the region: 0.05, expected relative risk (odds ratio): 3, disease incidence: 0.03, number of cases and controls: 100 and 100, total number of causative SNPs: 75.

Clinical and radiological results differences among the subgroups were compared by using the Mann-Whitney U test. The association between the SNP and initiation or progression of IS and the Hardy-Weinberg equilibrium of the genotypes were examined by χ^2 test. A level of the $P < 0.05$ and confidence interval 95% were considered to be statistically significant. The values for interval scale were showed as mean±standard deviation Analyses were performed using R statistical software [35]. The D' and LOD score (logarithm (base 10) of odds), were generated with Haploview software [36].

Results

There was no significant differences between patients and controls in age as well as first menarche onset ($p \geq 0.05$). Patients in case-only analyzes were divided into subgroups. First grouping was accordingly annual progression rate and followed-up until skeletal maturity or surgical endpoint. APR of 12 degrees per year was set as cut-off point for inclusion into progression group. Second subdivision covered maximum Cobb angle (MCA) to assess the curve severity. Patients with MCA of 50° and more were included into „severe" group. Patients were also analyzed according to the presence of joint hypermobility, and the last one was related to the thoracic curve. There was no significant differences between patients and controls in age as well as first menarche onset ($p \geq 0.05$) except age of the patients with lower and higher progression of the scoliosis. These groups were included in case-only analysis. Patients designation is shown in Table 1.

Table 1. Clinical and radiographic results

	Whole group		Progression						MCA						Beighton						Thoracic curve					
			yes		no				≤ 50°		> 50°				≤ 3		≥ 4				Lenke 1 /Ponseti IV		Lenke 3 /Ponseti III			
	N=100 Mean [SD]		N=53 Mean [SD]		N=47 Mean [SD]		p-value		N=63 Mean [SD]		N=37 Mean [SD]		p-value		N=28 Mean [SD]		N=72 Mean [SD]		p-value		N=41 Mean [SD]		N=59 Mean [SD]		p-value	
Age (years)	12.7	[1.5]	13.2	[1.5]	12.1	[1.4]	.0001		13.0	[1.5]	12.1	[1.5]	.0032		12.7	[1.6]	12.7	[1.5]	.4577		13	[1.7]	12.5	[1.4]	.0590	
Follow-up time (years)	2.9	[1.8]	2.5	[1.8]	3.2	[1.7]	.0139		2.7	[1.8]	3.2	[1.7]	.0549		2.8	[1.6]	2.9	[1.8]	.3449		2.7	[2.]	2.9	[1.6]	.1598	
Initial thoracic ATR (°)	10.5	[3.8]	9.3	[3.7]	11.9	[5.2]	.0003		9.6	[3.8]	12.1	[5.5]	.0012		10.1	[4.6]	10.7	[4.5]	.2607		10.8	[5.1]	10.3	[4.2]	.3287	
Final thoracic ATR (°)	12.4	[5.1]	9.5	[3]	15.7	[5.5]	<.0001		9.8	[3.0]	16.9	[5.6]	<.0001		12.4	[5.1]	12.4	[5.4]	.4783		13.3	[5.8]	11.7	[4.8]	.1069	
Joint hypermobility[1]	2	[0-9]	2	[0-7]	2	[0-9]	.0538		2	[0-7]	0	[0-9]	.0573		5	[4-9]	1	[0-3]	<.0001		2	[0-9]	2	[0-9]	.4986	
Initial Cobb (°)	31.3	[12.2]	27.7	[7.5]	35.4	[15]	.0010		26.7	[7.1]	39.2	[14.9]	<.0001		29.0	[9.9[	32.2	[13.0]	.1677		31.7	[14]	31	[10.9]	.4154	
MCA (°)	45.1	[21.2]	30.8	[10]	61.2	[19]	<.0001		31.4	[8.7]	68.5	[14.7]	<.0001		44.1	[18.3]	45.5	[22.4]	.4862		44.1	[21.2]	45.8	[21.4]	.2935	
Peak APR (°/year)	9.1	[7.9]	2.9	[3.1]	16.1	[5.3]	<.0001		4.7	[5.2]	16.7	[5.6]	<.0001		9.8	[8.9]	8.9	[7.5]	.3225		2.4	[1.2]	2.4	[1]	.2733	
Age at menarche (years)	12.8	[1.4]	12.6	[1.3]	13	[1.4]	.0674		12.6	[1.3]	13	[1.4]	.0674		12.8	[1.3]	12.7	[1.4]	.3649		13	[1.6]	12.6	[1.1]	.2875	

Legend: [1] – Median [MIN-MAX], ATR – Angle of Trunk Rotation, MCA – Maximum Cobb Angle, APR – Annual Progression Rate, SD – standard deviation

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For the genetic analyses, the Association Design Study Server has designed a list of 8 tagSNP. In empirical power simulation, 500 panels of 10,000 exceeded the χ^2 threshold of 7.21, so this threshold was used as a positive result. 8,828 panels of 10,000 exceeded the χ^2 threshold, thus the estimated statistical power is 87.3% with an approximate 95% confidence interval of ± 1.31%.

Case-Control Study

In the most cases of analyzed polymorphism, minor allele frequencies were similar to those obtained from 1000G database especially for Estonian population (closest geographical location to our patients). Only two of them differ (rs11077401 and rs8179090) (Table 2).

Table 2. Allele association of *TIMP2*, with minor allele frequencies observed in this study and in databases of 1000genomes and Estonian population, case-control study.

#	Name	Position	Association allele	ObsHET	PredHET	MAF	1000G	EST	Alleles
1	rs9916809	78860177	C	.286	.306	.188	.185	n/a	C:A
2	rs2277700	78870629	A	.295	.298	.182	.335	.165	A:G
3	rs2377005	78874211	C	.060	.139	.075	.121	.061	T:C
4	rs11658743	78877679	C	.010	.226	.130	.211	.149	C:T
5	rs11077401	78892915	G	.253	.351	.227	.044	n/a	G:T
6	rs2376999	78894782	T	.190	.219	.125	.356	.125	T:C
7	rs8068674	78911363	C	.431	.493	.439	.369	.430	C:T
8	rs4789934	78915454	T	.110	.113	.060	.051	.059	T:C
9	rs8179090	78925807	G	.315	.453	.347	.058	.009	C:G

Legend: MAF – minor allele frequency, ObsHET – observed heterozyosity, PredHET – predicted heterozyosity, 1000G – 1000 Genomes Project MAF, EST – Estonian population MAF

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In four of nine analyzed alleles, the deviation from Hardy-Weinberg equilibrium was significant (Table 3), but the allele frequencies were similar to those observed in Estonians. Additionally, the LOD score, generated with Haploview software indicate, that the SNP markers linkage is less likely and the genetic alternations of the *TIMP2* gene are inherited separately and a strong evidence of recombination exist, the highest r^2 value was 0.544 (Figure 1).

Table 3. Genotype and allele frequencies case-control study

SNP	Genotype /Allele	controls N	controls (%)	cases N	cases (%)	HWE	X^2 p-value	Model	OR [CI]	model
rs9916809	A	39	20	36	18		.7620	———	0.926 [0.562-1.525]	*C vs A*
	C¹	161	81	162	82					
	AA	7	7	2	2		.0853	R	3,73 [0,76-18,39]	AA *vs* AC+CC
	AC	25	25	32	32	.47	.7685	I	1,28	
	CC¹	68	68	65	65		.6899	D	1,13 [0,63-2,03]	CC *vs* AC+AA
rs2277700	**A¹**	162	81	165	83		.7252	———	1.095 [0.661-1.813]	A *vs* G
	G	38	19	35	18					
	AA¹	65	65	69	69		.7203	D	0,85 [0,47-1,53]	AA *vs* AG+GG
	AG	32	32	27	27	1	.7262	I	1,05	
	GG	3	3	4	4		.7203	R	0,76 [0,17-3,48]	GG *vs* AG+AA
rs2377005	**C¹**	14	7	16	8		.7466	———	1.131 [0.537-2.383]	C *vs* T
	T	186	93	184	92					
	CC¹	4	4	5	5		.7561	R	0,81 [0,21-3,1]	CC *vs* TC+TT
	TC	6	6	6	6	**.0002**	.7963	I	1,1	
	TT	90	90	89	89		.8551	D	1,09 [0,44-2,69]	TT *vs* TC+CC
rs11658743	T	38	19	14	7		**.0003**	———	0.314 [0.164-0.600]	C *vs* T
	C¹	162	81	186	93					
	TT	18	18	7	7		**.0163**	R	2,98 [1,19-7,49]	TT *vs* TC+CC
	TC	2	2	0	0	**<.0001**	**.0092**	I	1,76	
	CC¹	80	80	93	93		**.0061**	D	0,3 [0,12-0,73]	CC *vs* TC+TT
rs11077401	T	50	26	40	20		.2942	———	0.780 [0.490-1.242]	G *vs* T
	G¹	146	74	160	80					
	TT	9	9	11	11		.5517	R	0,76 [0,3-1,89]	TT *vs* GT+GG
	TG	32	32	18	18	.1828	.3549	I	1,11	
	GG¹	57	57	71	71		.0919	D	0,61 [0,34-1,09]	GG *vs* GT+TT
rs2376999	**T¹**	172	86	178	89		.4096	———	1.281 [0.710-2.310]	T *vs* C
	C	28	14	22	11					
	TT¹	75	75	81	81		.3571	D	0,73 [0,38-1,42]	TT *vs* TC+CC
	TC	22	22	16	16	.3985	.4368	I	1,19	
	CC	3	3	3	3		.9804	R	1,02 [0,2-5,18]	CC *vs* TC+TT
rs8068674	T	93	47	80	41		.2244	———	0.782 [0.525-1.163]	C *vs* T
	C¹	105	53	116	59					
	TT	27	27	17	17		.0874	R	1,81 [0,91-3,59]	TT *vs* TC+CC
	TC	39	39	46	46	**.0434**	.2506	I	1,27	
	CC¹	33	33	35	35		.7647	D	0,91 [0,51-1,64]	CC *vs* TC+TT
rs4789934	**T¹**	186	93	190	95		.5025	———	1.321 [0.585-2.983]	C *vs* T
	C	14	7	10	5					
	TT¹	87	87	90	90		.6265	D	0,81 [0,34-1,9]	TT *vs* TC+CC
	TC	12	12	10	10	.3864	.5065	I	1,66	
	CC	1	1	0	0		.3113	R	3,09 [0,12-76,76]	CC *vs* TC+TT
rs8179090	**G¹**	64	32	75	38		.2481	———	1.275 [0.844-1.926]	G *vs* C
	C	136	68	125	63					
	GG	17	17	21	21		.3222	R	1,32 [0,76-2,31]	GG *vs* GC+CC
	GC	30	30	33	33	**.0025**	.312	I	1,2	
	CC¹	53	53	46	46		.4709	D	0,77 [0,38-1,57]	CC *vs* GC+GG

Legend: A/G – allele genotype; ¹ – risk allele/genotype; MCA – maximum Cobb angle; OR – odds ratio; CI – confidence intervals; HWE – Hardy-Weinberg equilibrium p-value; OR – odds ratio; [CI] – confidence interval, models: R – recessive, I – independent, D – dominant.

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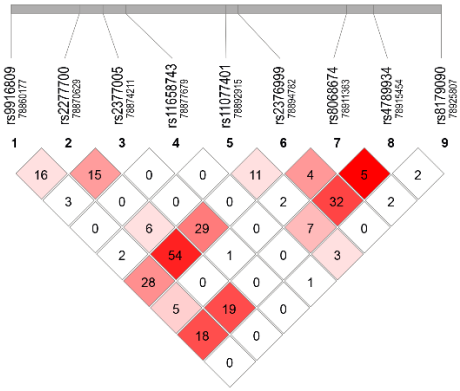

Figure 1. **Standard D'/LOD display obtained from Haploview software.** The values in diamonds shows how many of the SNPs in the dataset have been successfully tagged by the set of chosen tests. D'<1 and LOD<2 are indicated in white. D'<1 and LOD≥2 are indicated in shades of pink/red. D'=1 and LOD ≥2 as bright red. There is no D'=1 and LOD≥2. There is a strong evidence of recombination and the highest r^2 value is 0.544.

In the case-control study, no statistically significant difference were noted neither in allele nor genotype distribution of between patients and controls for all analyzed polymorphisms except rs11658743. In this SNP the differences were statistically significant for genotypes (p<0.0003) as well as all recessive, independent and dominant models (p=0.163; OR [95%CI]=2.98[1.19-7.49], p=0.0092; OR=1.76; and p=0.061; OR=0.3[0.12-0.73], respectively). Two of the analyzed polymorphisms showed a slight tendency toward significance in recessive model (p<0.9). Frequencies of alleles and genotype are presented in Table 3.

Case-Only Study

Genotype and Allele Association with Scoliosis Progression and MCA Outcome

Accordingly annual progression rate followed-up until skeletal maturity or surgical endpoint, slightly more girls (N=53) showed annual progression rate of 12 degrees or more per year and were assigned to progression group. With lower APR values in no-progression group were qualified 47 girls. Both groups differ significantly in age (p=0.0001), and the patients more progressive were older (13.2±1.5 *vs.* 12.1±1.4). The patients differ in all analyzed clinical and radiographic results (p<0.05) except joint hypermobility and age at menarche (p>0.05). Mean MCA±SD for cases group was 45.1°±21.2°. For the progression *vs.* non-progression group, the mean MCA was 61.2°±19° *vs.* 30.8°±10.0° (p<0.0001) and mean peak APR was 16.1°±5.3°/year *vs.* 2.9°±3.1°/year (p<0.0001) respectively. Taking under consideration the Maximum Cobb Angle, similarly to described above sub-groups, the patients with MCA≤50° were more numerous (63 *vs.* 37 cases of MCA>50º group). They differ in all clinical and radiographic result (p<0.05) except follow-up time, joint hypermobility and menarche onset (p>0.05). For the MCA≤50º *vs.* MCA>50º, the mean MCA was 68.5°±14.7° *vs.* 31.4°±8.7° (p<0.0001) and mean peak APR was 4.7°±5.2°/year *vs.* 16.7°±5.6°/year (p<0.0001), respectively (Table 1).

The frequency of genotype and allele distribution in patients with different progression rate were altered in four of analyzed polymorphisms, i.e. rs2277700, rs11077401, rs2376999, and rs4789934. In the remaining five, we did not observed any dissimilarities neither in genotype nor in allele distributions. In case of rs2277700 the genotype A in both groups was more often present, but in the group designed as progressive, G genotype was more often present than A (p<0.01; OR=0.34 [0.16-0.74]). The dominant and independent models differ statistically significant (p<0.01; OR=3.74 [1.42-8.52] and p<0.01, OR=2.59, respectively). Genotype and alleles frequencies obtained for rs11077401 significant changes in their distributions. The recessive genotype T was more frequent present in progressive patients (p<0.01; OR=0.13 CI [0.05-0.31]. The dissimilarities between both groups were shown in alleles' distribution too. Statistically significant differences were observed in all three models: recessive (p=0.01; OR=0.17 [0.03-0.81]), independent (p<0.01; OR=3.47) and dominant (p<0.01; OR=10.01 [3.39-29.62]). What is more, this polymorphisms distribution differs between MCA≤50º and MCA>50º subgroups. The T genotype was more common in MCA>50º group (p<0.01; OR=0.22 [0.11-0.48]. Recessive allele distribution model was almost significant (p=0.05; OR=0.29 [0.08-1.07], and we observed significance differences in remaining models – independent and dominant (p<0.01; OR=2.46 and p<0.01; OR=5.59 [2.2-14.23], respectively (Table 1).

In case of rs2376999 heterogeneous genotype distribution between both progression groups were present (p=0.04; OR=0.37 [0.15-0.99] as well as allele distributions in dominant model (p=0.04; OR=3 [1.03-8.67]. The independent model was almost significant (p=0.05; OR=2.06). Analyzing progression rate, distributions of genotypes as well as alleles in dominant and independent models differ statistically in rs4789934 (p=.048; OR=0.21 [0.04-1]; p=0.03; OR=5.23 [1.05-26.02] and p=0.03; OR=5.23, respectively). The T genotype and the TT allele were more frequent in both groups (Table 1).

A slight tendency toward significance in genotype distributions was shown in case of rs8179090 between MCA≤50º and MCA>50º subgroups (p=0.07).

Association of Genotype and Allele with Joint Hypermobility

Accordingly sub-groups of joint mobility less girls (N=28) showed values ≤3 *vs.* ≥4 accordingly Beighton scale. However, the difference was not statistically significant. No significant differences were observed in clinical and radiographic results (p>0.05), except those of joint mobility on which the division was based (Table 1).

No statistically significant difference were noted in allele and genotype distributions were observed except of rs8179090 between groups. The genotype distribution in rs8179090 was not equal, and the percent of G genotype was higher in the group designated as Beighton ≥4 (p<0.01; OR=0.39 [0.21-0.74]. We observed differences in allele distributions in dominant and independent model (p=0.01 in both; OR=3.55 [1.34-9.38] and OR=2.06, respectively). A slight tendency toward significance in genotype distributions was observed in recessive model (p=0.09, OR=0.39 [0.21-0.74]. We observed another tendency toward significance in genotype distribution of rs2277700 (p=0.08; OR=0.51 [0.24-1.1]. Frequencies of alleles and genotype are presented in Table 4.

Genotype Distributions and Thoracic Curve

The right thoracic (Lenke 1/Ponseti IV) patients (N=41) *vs.* double major curve (Lenke 3/Ponseti III) (N=59) do not differ in any of obtained clinical and radiographic results (p>0.05) (Table 1). Considering genotype and allele distributions, similarly to differences observed in subgroups divided accordingly Beighton scale, the groups vary in genotype distribution of rs8179090 between patients with different thoracic curve (p=0.04; OR=0.54 [0.3-1]). In both, recessive and independent models, a slight tendencies toward significance were observed (p=0.07; OR=0.37 [0.13-1.12] and p=0.08; OR=1.67, respectively). In two cases of rs9916809 and rs4789934 only tendencies toward significance in recessive models were observed (p=0.08; OR=7.73 [0.36-16.5] and p=0.06; OR=0.37 [0.13-1.12], respectively). Additionally, in case of rs8068674, the differences in genotype distribution were significant (p=0.04; OR=0.88 [0.49-1.57]). Frequencies of allele and genotypes are presented in Table 4.

4cm

Table 4. Genotype and allele frequencies – case only

Progression

A/G	No N	No %	Yes N	Yes %	HWE	X^2 p-val	OR [CI]	model
rs9916809								
A¹	22	21	14	15		*.31*	0.69 [0.33-1.43]	A *vs* C
C	84	79	78	85				
AA¹	2	4				*.18*	4.52 [0.21-96.49]	AA *vs* AC+CC
AC	18	34	14	30	*1*	*.29*	2.22	
CC	33	62	32	68		*.45*	0.72 [0.31-1.67]	CC *vs* AC+AA
rs2277700								
A¹	95	90	70	74		***<.01***	0.34 [0.16-0.74]	A *vs* G
G	11	10	24	26				
AA¹	43	81	26	55		***<.01***	3.47 [1.42-8.52]	AA *vs* AG+GG
AG	9	17	18	38	*.44*	***<.01***	2.59	
GG	1	2	3	6		*.25*	0.28 [0.03-2.81]	AA *vs* AG+GG
rs2377005								
C	6	6	10	11		*.19*	0.5 [0.18-1.44]	T *vs* C
T¹	100	94	84	89				
CC	2	4	3	6		*.55*	0.58 [0.09-3.6]	CC *vs* TC+TT
TC	2	4	4	9	***.004***	*.31*	1.43	
TT¹	49	92	40	85		*.24*	2.14 [0.59-7.85]	TT *vs* TC+CC
rs11658743								
T	6	6	8	9		*.43*	0.65 [0.22-1.93]	C *vs* T
C¹	100	94	86	91				
TT	3	6	4	9		*.58*	0.65 [0.14-3.04]	TT *vs* TC+CC
TC	0	0	0	0	***<.01***	*.58*	1.25	
CC¹	50	94	43	91		*.58*	1.55 [0.33-7.32]	CC *vs* TC+TT
rs11077401								
T	7	7	33	35		***<.01***	0.13 [0.05-0.31]	G *vs* T
G¹	99	93	61	65				
TT	2	4	9	19		***.01***	0.17 [0.03-0.81]	TT *vs* TG+GG
TG	3	6	15	32	***.01***	***<.01***	3.47	
GG¹	48	91	23	49		***<.01***	10.01 [3.39-29.62]	GG *vs* TG+TT
rs2376999								
T¹	99	93	79	84		***.04***	0.37 [0.15-0.99]	T *vs* C
C	7	7	15	16				
TT¹	47	89	34	72		***.04***	3 [1.03-8.67]	TT *vs* TC+CC
TC	5	9	11	23	*.19*	*.05*	2.06	
CC	1	2	2	4		*.49*	0.43 [0.04-4.93]	CC *vs* TC +TT
rs8068674								
T¹	44	42	36	39		*.65*	0.88 [0.49-1.55]	T *vs* C
C	60	58	56	61				
TT¹	8	15	9	19		*.59*	0.75 [0.26-2.13]	TT *vs* TC+CC
TC	28	53	18	38	*.58*	*.66*	1.09	
CC	16	30	19	40		*.28*	0.63 [0.28-1.45]	CC *vs* TC+TT
rs4789934								
T¹	104	98	86	91		***.048***	0.21 [0.04-1]	T *vs* C
C	2	2	8	9				
TT¹	51	96	39	83		***.03***	**5.23** [1.05-26.02]	TT *vs* TC+CC
TC	2	4	8	17	*1*	***.03***	**5.23**	
CC	0	0	0	0		*1*	0.89 [0.02-45.62]	CC *vs* TC+TT
rs8179090								
G¹	35	33	40	43		*.17*	1.50 [0.85-2.67]	G *vs* C
C	71	67	54	57				
GG¹	8	15	13	28		*.12*	0.47 [0.17-1.25]	GG *vs* CG+CC
GC	19	36	14	30	*.21*	*.22*	1.41	
CC	26	49	20	43		*.51*	1.3 [0.59-2.87]	CC *vs* CG+GG

MCA

A/G	≤ 50° N	≤ 50° %	> 50° N	> 50° %	HWE	X^2 p-val	OR [CI]	model
rs9916809								
A¹	27	21	9	13		*.12*	0.52 [0.23-1.19]	A *vs* C
C	99	79	63	88				
AA¹	2	3				*.28*	2.97 [0.14-63.53]	AA *vs* AC+CC
AC	23	37	9	24	*.72*	*.10*	2.88	
CC	38	60	27	73		*.14*	0.51 [0.2-1.26]	CC *vs* AC+AA
rs2277700								
A¹	107	85	58	78		*.24*	0.61 [0.31-1.35]	A *vs* G
G	19	15	16	22				
AA¹	46	73	23	62		*.26*	1.65 [0.69-3.92]	AA *vs* AG+GG
AG	15	24	12	32	*.62*	*.25*	1.48	
GG	2	3	2	5		*.58*	0.57 [0.08-4.26]	AA *vs* AG+GG
rs2377005								
C	8	6	8	11		*.26*	0.56 [0.2-1.56]	T *vs* C
T¹	118	94	66	89				
CC	3	5	2	5		*.89*	0.88 [0.14-5.49]	CC *vs* TC+TT
TC	2	3	4	11	***<.01***	*.37*	1.26	
TT¹	58	92	31	84		*.20*	2.25 [0.63-7.95]	TT *vs* TC+CC
rs11658743								
T	8	6	6	8		*.64*	0.77 [0.26-2.31]	C *vs* T
C¹	118	94	68	92				
TT	4	6	3	8		*.74*	0.77 [0.16-3.64]	TT *vs* TC+CC
TC		0	0	0	***<.01***	*.74*	1.14	
CC¹	59	94	34	92		*.74*	1.3[0.28-6.16]	CC *vs* TC+TT
rs11077401								
T	7	7	33	35		***<.01***	0.22 [0.11-0.48]	G *vs* T
G¹	99	93	61	65				
TT	4	6	7	19		*.05*	0.29 [0.08-1.07]	TT *vs* TG+GG
TG	6	10	12	32	***<.01***	***<.01***	2.46	
GG¹	53	84	18	49		***<.01***	5.59 [2.2-14.23]	GG *vs* TG+TT
rs2376999								
T¹	113	90	65	88		*.69*	0.83 [0.34-2.05]	T *vs* C
C	13	10	9	12				
TT¹	52	83	29	78		*.61*	1.3 [0.47-3.61]	TT *vs* TC+CC
TC	9	14	7	19	*.11*	*.71*	1.11	
CC	2	3	1	3		*.89*	1.18 [0.1-13.48]	CC *vs* TC +TT
rs8068674								
T	46	37	34	47		*.16*	0.66 [0.37-1.19]	C *vs* T
C¹	78	63	38	53				
TT	8	13	9	24		*.42*	1.44 [0.6-3.44]	TT *vs* TC+CC
TC	30	48	16	43	*1*	*.17*	1.53	
CC¹	24	38	11	30		*.13*	0.44 [0.15-1.28]	CC *vs* TC+TT
rs4789934								
T	119	94	71	96		*.75*	0.72 [0.18-2.87]	T *vs* C
C¹	7	6	3	4				
TT	56	89	34	92		*.63*	0.59 [0.01-30.38]	TT *vs* TC+CC
TC	7	11	3	8	*1*	*.63*	1.07	
CC¹	0	0	0	0		*.63*	0.71 [0.17-2.92]	CC *vs* TC+TT
rs8179090								
G	49	39	26	35		*.07*	1.17 [0.65-2.13]	C *vs* G
C¹	77	61	48	65				
GG	13	21	8	22		*.91*	0.94 [0.35-2.54]	GG *vs* CG+CC
GC	23	37	10	27	*.07*	*.65*	1.1	
CC¹	27	43	19	51		*.41*	0.71 [0.31-1.61]	CC *vs* CG + GG

Beighton

A/G	≤ 3 N	≤ 3 %	≥ 4 N	≥ 4 %	HWE	X^2 p-val	OR [CI]	model
rs9916809								
A¹	7	13	29	20		*.19*	0.56 [0.23-1.36]	A *vs* C
C	49	88	113	80				
AA¹			2	3		*.37*	2.05 [0.1-44.05]	AA *vs* AC+CC
AC	7	25	25	35	*.72*	*.17*	2.62	
CC	21	75	44	61		*.22*	0.54 [0.2-1.45]	CC *vs* AC+AA
rs2277700								
A¹	42	75	123	85		*.08*	0.51 [0.24-1.1]	A *vs* G
G	14	25	21	15				
AA¹	16	57	53	74	*.63*	*.11*	2.09 [0.84-5.22]	AA *vs* AG+GG
AG	10	36	17	24		*.09*	1.86	
GG	2	7	2	3		*.32*	0.37 [0.05-2.78]	AA *vs* AG+GG
rs2377005								
C¹	5	9	11	8		*.77*	0.84 [0.28-2.55]	C *vs* T
T	51	91	133	92				
CC¹	1	4	4	6		*.68*	1.59 [0.17-14.86]	CC *vs* TC+TT
TC	3	11	3	4	***<.01***	*.81*	1.04	
TT	24	86	65	90		*.51*	1.55 [0.42-5.76]	TT *vs* TC+CC
rs11658743								
T¹	2	4	12	8		*.36*	2.46 [0.53-11.37]	T *vs* C
C	54	96	132	92				
TT¹	1	4	6	8		*.40*	2.46 [0.28-21.36]	TT *vs* TC+CC
TC	0	0		0	***<.01***	*.40*	1.57	
CC	27	96	66	92		*.40*	0.41 [0.05-3.55]	CC *vs* TC+TT
rs11077401								
T	15	27	25	17		*.13*	0.57 [0.28-1.19]	G *vs* T
G¹	41	73	119	83				
TT	5	18	6	8		*.17*	0.42 [0.12-1.5]	TT *vs* TG+GG
TG	5	18	13	18	***.01***	*.21*	1.52	
GG¹	18	64	53	74		*.36*	1.55 [0.61-3.94]	GG *vs* TG+TT
rs2376999								
T¹	48	86	130	90		*.35*	0.64 [0.26-1.64]	T *vs* C
C	8	14	14	10				
TT¹	21	75	60	83		*.34*	1.67 [0.58-4.79]	TT *vs* TC+CC
TC	6	21	10	14	*.12*	*.39*	1.37	
CC	1	4	2	3		*.83*	0.77 [0.07-8.86]	
rs8068674								
T	24	43	56	40		*.71*	0.84 [0.47-1.67]	C *vs* T
C¹	32	57	84	60				
TT	5	18	12	17		*.93*	0.95 [0.3-3.01]	TT *vs* TC+CC
TC	14	50	32	44	*.8*	*.72*	1.11	
CC¹	9	32	26	36		*.64*	1.25 [0.49-3.16]	CC *vs* TC+TT
rs4789934								
T¹	52	93	138	96		*.48*	0.57 [0.15-2.08]	T *vs* C
C	4	7	6	4				
TT¹	24	86	66	92		*.37*	1.83 [0.48-7.06]	TT *vs* TC+CC
TC	4	14	6	8	*1*	*.37*	1.83	
CC	0	0		0		*1*	0.39 [0.01-20.29]	CC *vs* TC+TT
rs8179090								
G	45	31	30	54		***<.01***	0.39 [0.21-0.74]	C *vs* G
C¹	99	69	26	46				
GG	12	17	9	32		*.09*	0.42 [0.15-1.16]	GG *vs* CG+CC
GC	21	29	12	43	***.01***	***.01***	2.06	
CC¹	39	54	7	25		***.01***	3.55 [1.34-9.38]	CC *vs* CG+GG

Thoracic curve

A/G	Lenke 1 /Ponseti IV N	Lenke 1 /Ponseti IV %	Lenke 3 /Ponseti III N	Lenke 3 /Ponseti III %	HWE	X^2 p-val	OR [CI]	model
rs9916809								
A¹	18	15	18	23		*.19*	0.62 [0.3-1.28]	A *vs* C
C	100	85	62	78				
AA¹	0	0	2	5		*.08*	7.73 [0.36-16.53]	AA *vs* AC+CC
AC	18	31	14	34	*1*	*.17*	2.67	
CC	41	69	24	59		*.33*	0.66 [0.28-1.53]	CC *vs* AC+AA
rs2277700								
A¹	95	81	70	85		*.37*	0.71 [0.33-1.52]	A *vs* G
G	23	19	12	15				
AA¹	40	68	29	71		*.75*	1.15 [0.48-2.73]	AA *vs* AG+GG
AG	15	25	12	29	*.57*	*.39*	2.49	
GG	4	7				*.09*	0.15 [0.01-2.84]	AA *vs* AG+GG
rs2377005								
C¹	7	6	9	11		*.2*	0.51 [0.18-1.43]	C *vs* T
T	111	94	73	89				
CC¹	2	3	3	7		*.38*	2.25 [0.36-14.1]	CC *vs* TC+TT
TC	3	5	3	7	***<.01***	*.31*	1.52	
TT	54	92	35	85		*.33*	0.54 [0.15-1.91]	TT *vs* TC+CC
rs11658743								
T¹	6	5	8	10		*.20*	0.5 [0.17-1.49]	T *vs* C
C	112	95	74	90				
TT¹	3	5	4	10		*.37*	2.02 [0.43-9.54]	TT *vs* TC+CC
TC	0	0		0	***<.01***	*.37*	1.42	
CC	56	95	37	90		*.37*	0.5 [0.11-2.34]	CC *vs* TC+TT
rs11077401								
T¹	21	18	19	23		*.35*	0.72 [0.36-1.44]	T *vs* G
G	97	82	63	77				
TT¹	6	10	5	12		*.75*	1.23 [0.35-4.33]	TT *vs* TG+GG
TG	9	15	9	22	***.02***	*.44*	1.21	
GG	44	75	27	66		*.34*	0.66 [0.28-1.57]	GG *vs* TG+TT
rs2376999								
T	107	91	71	87		*.36*	0.66 [0.27-1.61]	C *vs* T
C¹	11	9	11	13				
TT	50	85	31	76		*.25*	0.56 [0.2-1.53]	TT *vs* TC+CC
TC	7	12	9	22	*.54*	*.40*	1.27	
CC¹	2	3	1	2		*.78*	0.71 [0.06-8.13]	CC *vs* TC +TT
rs8068674								
T	48	42	32	39		***.04***	0.88 [0.49-1.57]	C *vs* T
C¹	66	58	50	61				
TT	9	15	8	20		*.63*	1.29 [0.45-3.7]	TT *vs* TC+CC
TC	30	51	16	39	*.32*	*.67*	1.08	
CC¹	18	31	17	41		*.31*	1.54 [0.67-3.54]	CC *vs* TC+TT
rs4789934								
T¹	110	93	80	98		*.20*	0.34 [0.07-1.66]	T *vs* C
C	8	7	2	2				
TT¹	51	86	39	95		*.15*	3.06 [0.62-15.22]	TT *vs* TC+CC
TC	8	14	2	5	*1*	*.15*	3.06	
CC	0	0	0	0		*.06*	0.54 [0.3-1]	CC *vs* TC+TT
rs8179090								
G	51	43	24	29		***.04***	0.54 [0.3-1]	C *vs* G
C¹	67	57	58	71				
GG	16	27	5	12		*.07*	0.37 [0.13-1.12]	GG *vs* CG+CC
GC	19	32	14	34	*.27*	*.08*	1.67	
CC¹	24	41	22	54		*.20*	1.69 [0.76-3.77]	CC *vs* CG+GG

Legend: A/G – allele genotype; ¹ – risk allele/genotype; MCA – maximum Cobb angle; OR – odds ratio; CI – confidence intervals; HWE – Hardy-Weinberg equilibrium p-value

5p

2cm

2cm

arial
narrow
5.5p

4cm

Additionally, the backward stepwise regression analysis was conducted and three polymorphisms were significant and remained in the model: rs9916809 (p=0.018, R^2 (accumulated)=0.057; b*=0.25), rs4789934 (p=0.014, R^2 (accumulated)=0.116; b*=-0.37) and rs2376999 (p=0.041, R^2 (accumulated)=0.116; b*=0.25). Multiple R=0.39, F(3.93)=5.69, R^2=0.15503292, p=0.0013.

Discussion

Although many theories have been suggested, etiopathogenesis of idiopathic scoliosis remains unsolved; however, the role of genetic factors in initiation and progression of the disease is widely discussed [14,37,38]. The search for genetic risk factors is important from clinical standpoint as we lack reliable diagnostic markers to predict IS presence, curve progression or joint mobility in the disease development. Such a prediction factor could lead to effective non-surgical treatment as well as aid early surgical intervention decision-making process [39].

Of all polymorphisms studied, only in the case of rs11658743, a statistically significant difference in the frequency of alleles and distribution of genotypes in case-control study was found. The T allele was much more common in women from the control group (OR=3.18, p = 0.00027), demonstrating a lower risk of scoliosis in this group. Also, the distribution of genotypes confirms this relationship, yielding OR=2.98 with p=0.0163 for the recessive inheritance model. On the one hand, this result, however, should be considered carefully because of the deviation from the Hardy-Weinberg Equilibrium (F=0.93, p<0.0001). On the other hand, it was comparable with previously published genotype distributions included in the HapMap database. Smaller deviations from Hardy-Weinberg's equilibrium were also observed for rs8179090 and rs2377005, but there is currently no good data on the distribution of genotypes of these two polymorphisms in the Caucasian population [40]. Back to the data obtained for rs11658743, we did not observed any differences in genotype and allele distributions in case-only study nor progression rate, MCA, Beighton nor in thoracic curve sub-group assigned patients.

In different papers, the most widely discussed is the rs8179090 (-418G/C) polymorphism, localized upstream coding region, in promoter sequence of *TIMP2* gene [41,42]. Its C allele was confirmed to be associated with lower transcription activity leading to low *TIMP2* gene expression due to changing consensus sequence affinity to Sp1 transcription factor binding site [42,43]. Alternation of the DNA sequence has to be improved to be associated with pathogenesis of many diseases such as chronic obstructive pulmonary disease [44], aortic aneurysms [45], neoplasms [43] and many more, including related to different types of muscles [46–50].

As described before in Jiang *et al.* study [26], which was consistent with Ogura *et al.* work [27] also we did not find association of rs8179090 in case-control study. However, we could not confirm the relationship of this polymorphism with Cobb angle curvature. Differences in ethnicity may cause the discrepancy. However, is more likely due to selective, clinically relevant criteria for patient inclusion and definition of scoliosis progression applied in the studies. We included only clinically significant scoliosis patients with Cobb angle of 20 degrees or greater and defined progression as a time relative value of APR. This approach was rarely used in genetic research of scoliosis up to date [51], but yields more reliable results from clinician perspective. It is also a likely cause of deviation from HWE equilibrium noted in case-control part of the study.

It is important to note that thorough clinical examination of patients allowed us to identify an association of rs8179090 with lower incidence of joint hypermobility in scoliotic patients (OR=2.22, p=0.01, independent model and OR=3.55; 95%CI=[1.34-9.38], p=0.01, recessive model). The frequencies in allele distributions differ also significant (C *vs.* G p<0.01, OR=0.39 [0.21-0.74]. Differences in progression and non-progression groups regarding joint hypermobility were almost significant (p=0.053), and we found that in case of thoracic curve subgrouping, the distribution of genotype was not equal therefore the role of *TIMP2* gene in pathogenesis of IS needs further research.

Genetic markers in which the causative allele can be located, changing the sequence of the protein or modifying the gene expression. The remaining polymorphisms, selected for this

study, are located within the introns of the *TIMP2* gene and do not affect the protein structure. They could indirectly affect the level of transcription by altering the structure of DNA-binding site of regulatory proteins [52,53] or the effect on mRNA stability [54] or lead to DNA-histone binding strength alternations, but such relationships have not yet been described in the literature for any of the designated SNPs.

We found in different papers regarding the role of rs2376999, rs4789934, and rs9916809 and in relation to myopia [29], osteoarthritis [28] and breast cancer susceptibility and survival [30]. The first of these was not statistically significant in the disease development [29] but the rs4789934 and showed rs9916809 showed significance with susceptibility of osteoarthritis and ovarian cancer [28,30].

Analysis of the prevalence of alleles divided into patients with low and high progression rates showed remarkably significant difference for the rs11077401 polymorphism T allele ($p < 0.00001$) and less significant for the rs2376999 polymorphism C allele ($p = 0.034$), G rs2277700 allele ($p = 0.004$) and the C 4749934 allele ($p = 0.04$).

Dependencies have also been demonstrated in the analysis of the distribution of genotypes. The highest relative risk of the occurrence of progressive scoliosis was observed in patients with TT and TC genotype rs11077401 polymorphism (independent odds ratio OR=3.465, p=0.00003). In the dominant inheritance model the relative risk was 10.017 ($p<0.00001$). Patients with this genotype also showed more than twice (independent model, p=0.0006) and five times (dominant model, $p = 0.00016$) higher risk of curvature of Cobb angle above 50 degrees. The risk independent for rs2376999 was 2.057, and it was almost significant (p=0.052), obtaining statistical significance with the recessive model of inheritance (OR=2.995, p=0.037). For the rs2277700 polymorphism, the independent risk was 2.585 (p=0.006) and increased to 3.473 (p=0.005) assuming a recessive inheritance model). The distribution of genotypes was also significantly different for rs4789934 and was associated with a more than 5-fold increase in the risk of progressive form both independently and assuming a dominant inheritance model (OR = 5.231, $p = 0.027$). For this polymorphism, there was no difference in distribution of genotypes when cut-off progression point was lower ($p = 0.29$).

Three of analyzed polymorphisms (rs9916809, rs2377005, and rs11659743) showed no association with any of the analyzed clinical and radiographic results. However, the rs9916809 showed weak tendency to be significant in case of allele recessive model (AA *vs.* AC+CC) and thoracic curve (p=0.08).

Our results suggest a significant effect of the *TIMP2* gene on the development of progressive curves, and the presence of at least one of the genotypes mentioned above in 83% of cases was associated with a progression rate of more than 12 degrees a year. It should also be noted that two analyzed polymorphisms (rs2376999 and rs11077401) burdened with higher risk are located in the area of the *CEP295NL*/*KIAA1731* gene. This gene is called a nested gene and most likely deal of regulatory function in the *TIMP2* gene expression process, encoding the differential display clone 8 (DDC 8) protein. The highest level of expression was observed in the testes during spermatogenesis, indicating possible differences in regulatory processes of extracellular matrix rebuilding, dependent on *TIMP2* in relation to gender [55].

There were no differences in the frequency of alleles or genotype distributions for any of the tagSNP region of the *TIMP2* gene being tested. The result confirms the assumption that right thoracic distortion and double-thoracic right-sided lumbar with a thoracic predominance constitute a homogeneous group regarding genetic basis. There were no differences in the frequency of alleles tagSNP region of the TIMP-2 gene being tested, but only genotypes distributions in two polymorphisms.

Despite many different theories on the causes of lateral curvature of the spine in children, researches have not yet been able to explain the mechanisms responsible for its formation and progression clearly. Within each of the current theories, the role of genetic factors as predisposing or modulating the course of this disease is accepted [14,38]. The screening for genetic risk factors that are the subject of the presented study is extremely important from the clinician's point of view because we currently do not have suitably sensitive and specific markers to predict the progression of the curvature in IS initial period. Detection of the patient's genetic risk factor allows better planning

of both the intensity of conservative treatment and may support decisions about surgical treatment [39].

The obtained results, despite the lack of confirmation of the role of the promoter polymorphism observed in the Eastern populations [26,27], indicate the importance of the *TIMP2* gene in the etiopathogenesis of idiopathic scoliosis, not described in the literature so far. The indicated odds ratios are higher than the majority observed so far in the association studies of candidate genes. The explanation for this may be a very homogeneous group of patients regarding the clinical form of scoliosis and the use of clinically relevant criteria for determining the progressive form.

The methodology adopted in the work does not allow for accurate identification of the causative alleles or proposing a mechanism of how the *TIMP2* gene influences the formation and progression of the curvature, but narrows the search area and determines the direction of research in the future.

Conclusions

The incidence of polymorphisms in the *TIMP2* gene was consistent with that reported so far in the literature for the Caucasian population however, the influence of the G allele of the rs8179090 on the clinical course of scoliosis suggested in the Chinese population has not been confirmed. Four polymorphisms (rs11077401, rs2376999, rs2277700, rs4789934) have been identified, associated with a significantly higher risk of scoliosis in a progressive form, suggesting a large role of the TIMP-2 gene in the etiopathogenesis of this disease. The associations of the rs8179090 of *TIMP2* gene with IS occurrence or progression are not definite. Further association studies based on suggested clinical criteria for progression would be necessary to identify SNPs associated with the curve progression.

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Conflicts of Interest and Source of Funding

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Supplementary Table S1. Reaction mix reagent concentrations used for FRET analysis

Reagent	Initial concentration	Final concentration
Water	-	-
$MgCl_2$	25mM (1mM[1])	3mM
Forward primer	5mM	400/600nM[2]
Reverse primer	5mM	400/600nM[2]
Donor probe	1mM	200nM
Acceptor probe	1mM	400nM
LC® HybProbe® Master Mix	10x	1x
DNA template	Variable	400ng

Legend: [1]in LC® HybProbe® Master Mix Mg^{2+} concentration is at 1mM
[2]higher concentration for primer promoting synthesis of the probe-priming strand

Supplementary Table S2. Thermal cycling parameters of FRET method

Cycle(s)	Step	rs9916809	rs2277700	rs2377005	rs11658743	rs11077401	rs2376999	rs8068674	rs4789934	rs8179090	Ramp rate
1	**Initial denaturation**	95°C, 10 min									20°C/s
45	**Amplification**										20°C/s
	Denaturation	95°C, 10 s									
	Primer annealing	60°C, 5 s									
	Elongation	72°C									
	Fluorescence detection/acquisition	11s	10s	9s	10s	7s	8s	9s	7s	7s	
1	**Melting curve analysis**										20°C/s
	Denaturation	95°C, 25 s									
	Annealing	50°C, 2 min									
	Denaturation with continuous fluorescence acquisition	50°C–80°C									0.1°C/s
1	**Cooling**	40°C									20°C/s

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Supplementary Table S3. Probes and primers sequences used for polymorphisms evaluation

	Forward primer	Reverse primer	Probe 1	Probe 2
rs9916809	GGCAGGAGAATCACTTGAAC	CTTCTCAAGGAATTGCCCAT	GTCTTC**[C]**AAACAAAAAAAACAAAAACAAAAACAAA-Fluorescein	LC Red 640-AAACCTCATCCCTCCTGCTACAACAG-P
rs2277700	GCCAATTTCCTGGGAAATTTGA	GCAGGTCTCGTGCATAG	GAAAGGGTTTCTCTGCCCTGCATGTC-Fluorescein	LC Red 640-CAGCATCC**[C]**GCACTCGC-P
rs2377005	CCCAGCCACCAGTGAGTA	TCCTCCTCCTTCCACGA	ACAGAAAC**[C]**GCCTTGGTCACAA-Fluorescein	LC Red 640-GACTCTGGGCTTCTGGCTTGGG-P
rs11658743	GCAGAATCCAAAGGAATCAACA	AGGAGAATTGCTTGAACCC	CGGCCTTTATTACAGTCTGCTGAATTCTGTATGTT-Fluorescein	LC Red 640-TTTGGAAATT**[C]**CTGGAGCTGCTTTTC-P
rs11077401	GTAGTGCCGAGACTGACA	CTTCCTCCTTTCCTTTACGA	LC Red 640-CCTC**[C]**ATCCCTACCTGAATCCTAACC-P	TGTTTCCAAGGGCTGCCCTTTGTG-Fluorescein
rs2376999	GCAAATATTTCCTTCCAGTCCGTA	TAGGTGTATATTCATGACCTTGAGT	TGTTCAGCTTATCCGTTTCTT**[C]**GTTTATTGG-Fluorescein	LC Red 640-GTGCTTTTGGTGTTGTATCTAAGAAATCTTTGCCT-P
rs8068674	TCTTCCTTAAGGGCTCCTTG	TGAGCTGAGATCACGCCA	CAA**[C]**ATTCTAGAAGGGTCTAGGACAAACT-Fluorescein	LC Red 640-CTAACTATTGAGCCCCTCCAGTGCTTTTGTT-P
rs4789934	CAGAGTGAGACTCTGTCTCAAA	CCATCCGTCACCTGTAG	TGCTGATTCAGACTGATA**[C]**TGGCTTG-Fluorescein	LC Red 640-GATGAATGGTCTAGGCTGTGGAGCTGG- P
rs8179090	TCCTGTCAGTTTCTCAATAGGC	TCGACTCTGGAGGGCTAC	CGA**[C]**GCTGGGCTCGAAGG–Fluorescein	LC® Red 640–CCCCGGGGTGGCGGG–P

Legend: [C] – perfect match/mismatch base, probe linked with fluorescein is the donor probe