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Etiopathogenesis of adolescent idiopathic scoliosis: Expression of melatonin receptors 1A/1B, calmodulin and estrogen receptor 2 in deep paravertebral muscles revisited

JOSEF ZAMECNIK[1*], LENKA KRSKOVA[1*], JAROMIR HACEK[1], IVANA STETKAROVA[2] and MARTIN KRBEC[3]

[1]Department of Pathology and Molecular Medicine, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, 15006 Prague; [2]Department of Neurology, Charles University; [3]Department of Orthopedics and Traumatology, 3rd Faculty of Medicine, Charles University in Prague and University Hospital Královské Vinohrady, 10034 Prague, Czech Republic

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Abstract. The pathogenesis of adolescent idiopathic scoliosis (AIS), including the associated local changes in deep paravertebral muscles, is poorly understood. The asymmetric expression of several molecules involved in the melatonin signaling pathway, including melatonin receptors 1A/1B (MTNR1A/MTNR1B), estrogen receptor 2 (ESR2) and calmodulin (CALM1), has previously been suggested to be associated with AIS. However, this hypothesis is based on single studies in which the data were obtained by different methodological approaches. Therefore, to evaluate the symmetry of the mRNA expression levels of these molecules, 18 patients with AIS and 10 non-scoliotic controls were enrolled in the present study. Muscle biopsy samples from deep paraspinal muscles (from the convexity and concavity of the scoliotic curve in patients with AIS, or from the left and right sides in controls) were obtained during spinal surgery. For each sample, the relative mRNA expression levels of MTNR1A, MTNR1B, CALM1 and ESR2 were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and were quantified according to the quantification cycle method. The results indicated that the mRNA expression levels of none of the investigated molecules were significantly different between samples obtained from the convex and concave side of the scoliotic curve in patients with AIS. In addition, no difference in expression was detected between the patients with AIS and the controls. With regards to MTNR1A and MTNR1B, their expression was very weak in paravertebral muscles, and in the majority of cases their expression could not be detected by repeated RT-qPCR analysis. Therefore, these data do not support the previously suggested role of the asymmetric expression of molecules involved in the melatonin signaling pathway in deep paravertebral muscles in the pathogenesis of AIS.

Correspondence to: Professor Josef Zamecnik, Department of Pathology and Molecular Medicine, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, V Uvalu 84, 15006 Prague, Czech Republic
E-mail: josef.zamecnik@lfmotol.cuni.cz

[*]Contributed equally

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Introduction

Adolescent idiopathic scoliosis (AIS) is characterized by deformity of the spine, which develops without a known cause, predominantly in previously healthy individuals during the adolescent growth spurt (1,2). It affects between 2 and 4% of the global population, and is more prevalent in girls (gender ratio, 8:1) (3-5). Since there is currently no early preventive treatment available, and a significant proportion of patients with AIS require intensive brace therapy or invasive surgical correction (2,6,7), improved understanding regarding the etiopathogenesis of AIS is required. Several suggestions have been proposed regarding the etiology of AIS, including neuromuscular, genetic, mechanical, growth-related or developmental hypotheses; however, at present, no single key factor has been identified (8,9).

The potential role of local changes to deep paravertebral muscles in the development of AIS has been the subject of several analyses. Numerous histochemical studies have reported significant differences in fiber size and the proportion of muscle fiber types between the convex and concave sides of the scoliotic curve (10-12). Therefore, muscle imbalance and asymmetry of paravertebral muscles in patients with AIS may be considered to serve an important role in the development of spinal deformity (10-13).

The asymmetric expression of several molecules in bilateral paravertebral muscles has also been suggested to be involved in the pathogenesis of AIS. Among the various molecular hypotheses, melatonin deficiency (14,15) and dysfunctional melatonin signaling (16,17) have received

major attention. The expression of melatonin receptors 1A/1B (MTNR1A/MTNR1B; also referred as MT1 and MT2 receptors) in paravertebral muscles was previously investigated by Qiu *et al* (18), and MTNR1B expression was shown to be asymmetric. At the protein level, Acaroglu *et al* (19) demonstrated an asymmetric distribution of calmodulin (CALM1) in the paravertebral muscles of patients with AIS. This finding is of particular interest, since CALM1 not only regulates the contractile properties of muscle fibers by regulating calcium transport through the cellular membranes (20), but also acts as a neurotransmitter in the regulation of melatonin secretion (21). Crosstalk between estrogens and the melatonin signaling pathway has previously been reported (22), and the existence of an anomaly specific to the estrogen system has been suggested in patients with scoliosis (23). In addition, the expression levels of estrogen receptor 2 (ESR2) have previously been investigated in the paravertebral muscles of patients with AIS; the results indicated that ESR2 exhibited asymmetrical expression, albeit not unidirectional (24).

Although the hypothesis that asymmetric molecular alterations to the melatonin signaling pathway in paravertebral muscles are associated with AIS appears promising, it is based on single studies in which data were obtained by different methodological approaches. In order to verify and provide more evidence for this hypothesis, the present study examined the relative mRNA expression levels of MTNR1A, MTNR1B, ESR2 and CALM1 in deep paravertebral muscles from patients with AIS using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Materials and methods

Patient characteristics. The present study recruited 18 patients with AIS and 10 control subjects in University Hospital Kralovske Vinohrady (Prague, Czech Republic) between March 2012 and April 2015. The AIS group consisted of 15 female and 3 male patients that were undergoing posterior instrumentation and fusion for treatment of AIS. The mean age was 17.2±5.5 years (range, 11-31 years), and all patients had relatively severe spinal curves, with a mean Cobb angle measurement of 50.5°±8.7° (range, 31°-70°). All but one subject had thoracic convexity to the right. The control group comprised 6 female and 4 male patients without scoliosis (including 4 cases of thoracolumbar trauma with burst fractures, 4 disc herniations and 2 cases of degenerative stenosis) undergoing posterior surgery. The mean age was 32.7±11.9 years (range, 12-55 years). Written informed consent was obtained from each patient/guardian prior to the enrollment. The present study was approved by the ethics committee of the 3rd Faculty of Medicine, Charles University and University Hospital Kralovske Vinohrady.

Muscle biopsy. Muscle biopsies (size, ~10x5x5 mm) were obtained during surgery. The biopsies were taken bilaterally from the multifidus muscle at the apex of the scoliotic curve between the 9th and 12th thoracic vertebral levels in patients with AIS, and bilaterally from the same region in individuals of the control group.

Muscle tissues were snap-frozen in isopentane (2-methylbutane; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and were maintained in liquid nitrogen. From each tissue block, 3 μm cryosections were examined by routine hematoxylin-eosin staining to ensure the quality and the relevance of the harvested tissue. Muscle samples were subsequently stored at -80°C.

RNA extraction and preparation of cDNA by RT. Total RNA was isolated from cryosections of the samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA was prepared by RT from 10 μl RNA in a 20 μl reaction volume. The reaction mixture consisted of 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiotreitol; 0.5 mM each dNTP; 12.5 mM random hexamers and 50 units MMLV Reverse Transcriptase (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT included an incubation period at 37°C for 60 min.

RT-qPCR. RT-qPCR analyses were performed using LightCycler® 480 Instrument II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). RT-qPCR analysis of the housekeeping gene β2-microglobulin was conducted using a hydrolyzation probe in order to evaluate the amount and amplificability of cDNA. The primers were designed as reported by Bijwaard *et al* (25) and are presented in Table I.

Relative mRNA expression levels of CALM1 (id. Hs 003000085_s1), ESR2 (id. Hs 01100353_m1), MTNR1A (id. Hs 00195567_m1) and MTNR1B (id. Hs 001737947_m1) were evaluated by qPCR using TaqMan Master Mix II, and primers and probes contained in TaqMan assays (cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, including the thermocycling conditions.

For confirmation of the observed low or absent expression levels of melatonin receptors, the results of the TaqMan assays were re-analyzed using another set of primers and a TaqMan probe for MTNR1A (26) using TaqMan Master Mix II, and a set of primers with SYBR Green detection [Go Taq® qPCR Master Mix (Promega Corporation, Madison, WI, USA)] for MTNR1B (27) (Table I). The program for confirmatory assay for MTNR1B consisted of initial denaturation at 95°C for 3 min, followed by 55 cycles at 95°C for 20 sec, and 60°C for 60 sec (single fluorescence measurement) followed by melting curves analysis at 95°C for 15 sec and 65°C for 10 sec and 97°C (continuous fluorescence measurement; ramping rate, 0.1) Four archive samples of invasive ductal breast carcinoma were obtained from the archives of the Department of Pathology and Molecular Medicine, University Hospital Motol (Prague, Czech Republic) with known expression of both melatonin receptors (28) were co-analyzed by both methods, in order to ensure functionality of the TaqMan assays and/or primers and probes used in the present study.

All analyses were performed in duplicate and the mean values were obtained for further analysis. Representative amplification curves obtained for the genes of interest are presented in Fig. 1.

Evaluation of RT-qPCR results. The mRNA expression levels of CALM1, ESR2, MTNR1A and MTNR1B were calculated

Table I. Primers and probes used in the present study, including those used for confirmatory analysis of melatonin receptor gene expression (Metabion International AG, Planegg, Germany).

Gene	Sequences
β2-microglobulin	F: 5'TGACTTTGTCACAGCCCAAGATA3' R: 5'AATCCAAATGCGGCATCTTC3' Probe: 5'TGATGCTGCTTACATGTCTCGATCCCA3'
Confirmatory analysis	
MTNR1A	F: 5'-CGGTGTATCGGAACAAGAAGCT R: 5'-AGGTCTGCCACCGCTAAGC TaqMan probe: 5'-6-Fam-TCACCACAAAGATGTTTCCT GCGTTCCT-Tamra-3'
MTNR1B	F: 5'-CGGAACGCAGGTAATTTGTT R: 5'-TAATGGCGATGGCAGTGATA

MTNR1A, melatonin receptor 1A; MTNR1B, melatonin receptor 1B; F, forward; R, reverse.

Table II. Results of the reverse transcription-quantitative polymerase chain reaction analysis of the relative mRNA expression levels (ΔCq) of CALM1 and ESR2 in the subgroups of patients with AIS and control individuals.

Group	n	ΔCq CALM1	ΔCq ESR2
AIS	36	3.69±0.70	13.39±1.32
Convex side	18	3.79±0.53	12.93±1.42
Concave side	18	3.60±0.82	13.86±1.01
Control	20	3.51±0.70	12.48±1.18
Left side	10	3.50±0.73	12.39±1.35
Right side	10	3.52±0.66	12.57±0.98

AIS, adolescent idiopathic scoliosis; CALM1, calmodulin; ESR2, estrogen receptor 2.

according to the ΔCq relative quantification method (29,30), which is based on the expression levels of a target gene versus the reference housekeeping gene. Briefly, fluorescence was measured continually during the melting curve cycle and a melting curve analysis was performed. Data were analyzed using LightCycler® 480 Software, version 1.5 (Roche Diagnostics Deutschland GmbH). Results were obtained as quantification cycle (Cq) values. The software determines a threshold based on the baseline fluorescent signal, and the data point that meets the threshold is considered the Cq value, which is inversely proportional to the starting number of template copies. The average Cq value for β2-microglobulin was subtracted from the average Cq value of the gene of interest to yield the ΔCq value.

Statistical analysis. Results are presented as the mean ± standard deviation. Differences between the numeric variables of two groups were evaluated using the Mann-Whitney U test. Correlations between numeric variables were assessed according to the Spearman's rank correlation analysis. Statistical analysis was considered using JMP IN 5.1 software (SAS Institute, Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA expression of CALM1 and ESR2. The results of the RT-qPCR analysis of the mRNA expression levels of CALM1 and ESR2 in the specific subgroups are summarized in Table II. For mRNA expression of both CALM1 and ESR2 no statistically significant differences were observed when comparing the AIS and control groups (P=0.09 and 0.47, respectively), as well as when comparing the concave and convex samples from the patients with AIS AIS (P=0.53 and 0.75, respectively). In addition, no statistically significant correlation was observed between the value of the Cobb angle and the mRNA expression levels of the studied genes (data not shown).

mRNA expression of MTNR1A and MTNR1B. In general, for MTNR1A and MTNR1B, the observed mRNA expression levels were very weak or absent in patients with AIS, as well as in control individuals.

MTNR1A mRNA expression near the detection limit was observed in 9/18 samples from the concave side of the curve in patients with AIS (mean Cq value, 37.06±1.43) and in 11/18 convex scoliosis samples (mean Cq value, 37.18±1.19). Similarly in the control group, MTNR1A mRNA expression was weakly detectable in a small proportion of samples (3/10 from the left and 2/10 from the right side; mean Cq values, 38.55±0.17 and 39.77±0.55, respectively). The remaining samples were completely negative for MTNR1A mRNA expression. The results of the mRNA expression analysis of MTNR1B were completely negative in both AIS and control groups, as determined using TaqMan assay and have not been presented.

The results of the present study were confirmed by repeating the analysis using different sets of primers and probes. The confirmatory analysis for MNTR1A revealed

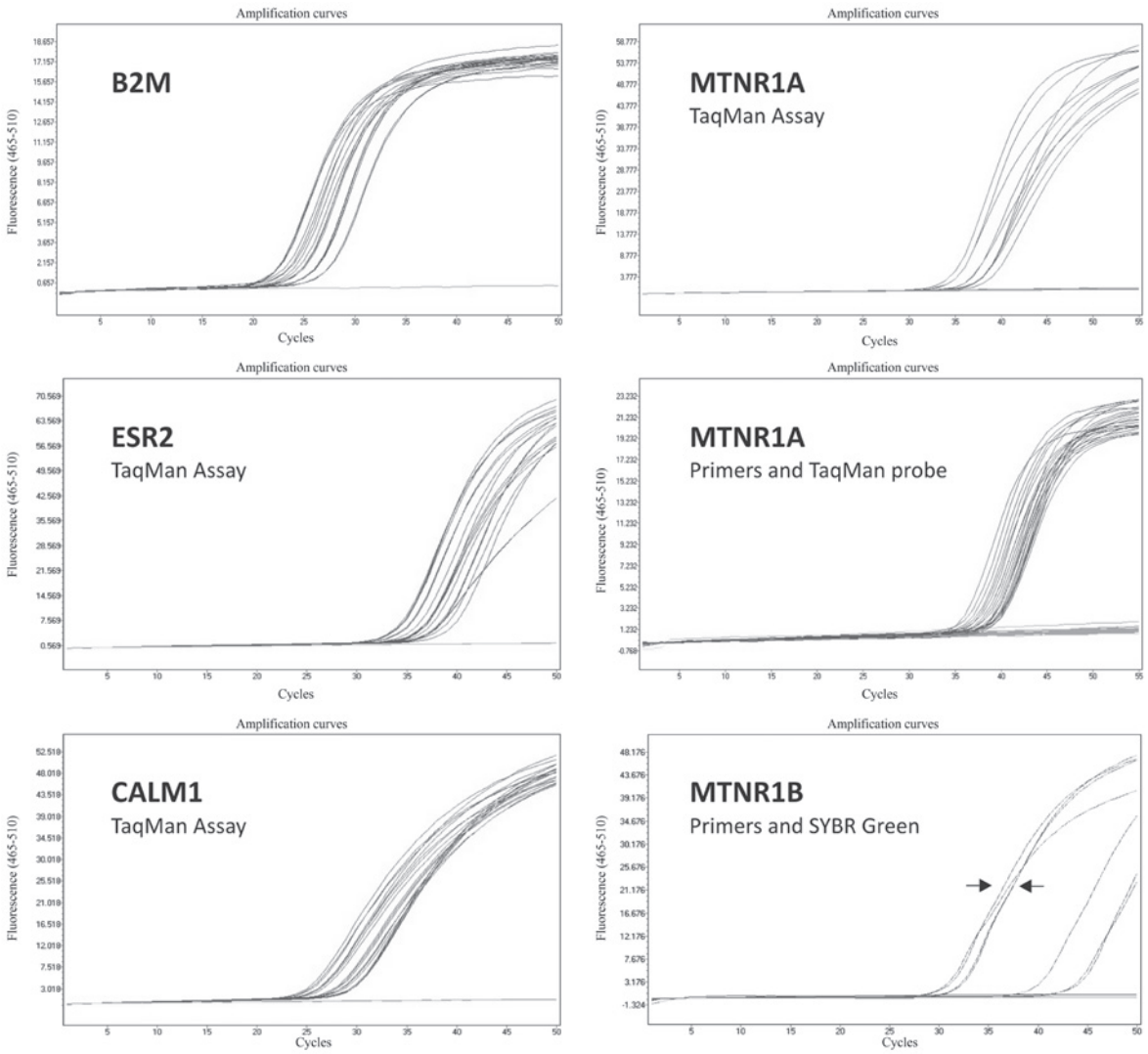

Figure 1. Representative RT-qPCR amplification plots in semi-logarithmic view for B2M housekeeping gene, and for ESR2, CALM1 and MTNR1A using TaqMan assays. Furthermore, the results of the confirmatory analysis of MTNR1A expression using primers and a TaqMan probe, and of MTNR1B expression using primers and SYBR Green are presented. Arrows represent cases of invasive ductal breast carcinoma with known MTNR1B mRNA expression (Cq values around the 30th cycle, compared with the studied samples where Cq values are around the 40th cycle or are completely negative). X-axes indicate RT-qPCR cycle number, Y-axes indicate fluorescence intensity. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; B2M, β2-microglobulin; ESR2, estrogen receptor 2; CALM1, calmodulin; MTNR1A, melatonin receptor 1A; MTNR1B, melatonin receptor 1B.

similar results as the TaqMan assay. MNTR1A mRNA expression near the detection limit was observed in 10/18 AIS samples from the convex and concave sides (mean Cq values, 38.10±4.50 and 37.87±2.37, respectively). Furthermore, MNTR1A mRNA expression near the detection limit was observed in 1/10 control samples from the left side and in 2/10 control samples from the right side (mean Cq values, 38.32 and 37.31±3.00, respectively).

Using MTNR1B primers and SYBR Green, MTNR1B mRNA expression was detected in 10/18 concave and convex AIS samples (mean Cq values, 46.13±3.28 and 45.21±4.56, respectively). In the control group, mRNA expression of MTNR1B was detected in 4/10 left-sided and 2/10 right-sided samples (mean Cq values, 45.49±2.78 and 47.54±2.45, respectively). The remaining samples were completely negative for MTNR1B expression.

The present study used two independent sensitive detection techniques to determine the mRNA expression levels of MTNR1A and MTNR1B. The results indicate that MTNR1A and MTNR1B expression was absent in a marked proportion of AIS and control samples. In the remaining samples, expression was near the detection limit, with Cq values ranging between 37 and 47; however, the majority of these results should also be interpreted as negative, since Cq values >40 approach the sensitivity limits of the RT-qPCR system (31,32).

No statistically significant differences were revealed when comparing the mRNA expression levels of MTNR1A and MTNR1B between the studied subgroups. In addition, no correlation was observed between the expression levels of melatonin receptors and the value of the Cobb angle (data not shown).

Discussion

The hypothesis that melatonin deficiency and dysfunctional melatonin signaling have roles in the etiopathogenesis of AIS

remains controversial. Early experiments, which revealed the development of scoliosis after pinealectomy in chickens (33,34), and the prevention of experimentally-induced scoliosis development following intramuscular implantation of the pineal body into pinealectomised chickens (33), led to the hypothesis that defects in the neurohormonal system of the pineal body may serve a major role in the development of AIS. Recently, however, major concerns have been raised regarding the scientific validity and limitations of the melatonin-deficient experimental animal models used for studying the etiopathogenesis of AIS in humans (35). Furthermore, the hypothesis that melatonin deficiency is a causative factor in the etiology of AIS has not been supported by data from several studies in human patients with AIS, as reviewed by Girardo *et al* (36).

The involvement of melatonin receptors, particularly MTNR1B, in the development of AIS has been suggested by a few reports published by a single center, which have reported that the protein and mRNA expression levels of MTNR1B are significantly reduced in cultured osteoblasts from girls with AIS compared with controls (37,38). Furthermore, functional abnormality of the melatonin signaling pathway, resulting in an abnormal proliferative and differentiative response of cultured growth plate chondrocytes (GPCs) to melatonin, alongside significantly reduced MTNR1B mRNA expression, has been detected in AIS GPCs compared with in controls (39). However, at present, these associations have not been replicated and validated. Another group investigated the paravertebral muscles in patients with AIS, and demonstrated that the mRNA expression levels of MTNR1B were higher on the concave side of the scoliotic curve compared with on the convex side, as determined by endpoint PCR; however, MTNR1A mRNA expression exhibited no significant difference (18). The present study did not observe significant differences in MTNR1A mRNA expression, nor in MTNR1B expression, as determined using RT-qPCR. Notably, RT-qPCR is a more precise method for quantitative gene expression analysis, which displays a dynamic range compared with quantification by endpoint PCR (40). Furthermore, in a marked proportion of patients with AIS and control individuals, the expression of MTNR1B was undetectable, even when employing two different approaches, and the remaining positive samples had Cq values that reached the sensitivity limits of the RT-qPCR system.

The pathogenic role of melatonin receptors in AIS has been further questioned, since no significant association has been reported between mutations in any known melatonin-related receptors and AIS (41). Although an MTNR1B gene polymorphism has been revealed to be associated with AIS in Chinese patients (42), it was not confirmed in the Japanese population (43) nor by a recently published meta-analysis (44).

Among other molecules involved in the melatonin signaling pathway, the role of estrogen receptors and CALM1 have attracted the attention of several investigators. Previous experimental studies have demonstrated that although selective estrogen receptor modulators, and CALM1 antagonists, do not prevent the occurrence of scoliotic deformities, they can decrease the rate of progression of the deformity in experimental animals (45-47). Conversely, replication studies and a meta-analysis did not confirm the previously suggested association between estrogen receptor gene polymorphisms and AIS predisposition or curve severity in humans (48-51). In the paravertebral muscles of patients with AIS, the expression of ESR2 was investigated in a previous study (24). The observed asymmetry of ESR2 expression, although not unidirectional, could not be supported by the present results. The present study observed no significant difference in the expression of ESR2 between the convex and concave sides of the scoliotic curve, or between patients with AIS and non-scoliotic controls. Similar results were observed with regards to the mRNA expression levels of CALM1 in paravertebral muscles. Therefore, the present study provided no support for the results of two previous studies, which reported the asymmetric distribution of CALM1 (at the protein level) in the paravertebral muscles of patients with AIS; one study reported that its expression was higher at the convex side and lower at the concave side (19), whereas the other study indicated the opposite (52).

In conclusion, the present study does not support the hypothesis that the expression of molecules involved in the melatonin signaling pathway (MTNR1A, MTNR1B, ESR2 and CALM1) in the paravertebral muscles is associated with development of AIS.

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References

1. Lonstein JE: Adolescent idiopathic scoliosis. Lancet 344: 1407-1412, 1994.
2. Altaf F, Gibson A, Dannawi Z and Noordeen H: Adolescent idiopathic scoliosis. BMJ 346: f2508, 2013.
3. Weinstein SL: Adolescent idiopathic scoliosis: Prevalence and natural history. Instr Course Lect 38: 115-128, 1989.
4. Brooks HL, Azen SP, Gerberg E, Brooks R and Chan L: Scoliosis: A prospective epidemiological study. J Bone Joint Surg Am 57: 968-972, 1975.
5. Rogala EJ, Drummond DS and Gurr J: Scoliosis: Incidence and natural history. A prospective epidemiological study. J Bone Joint Surg Am 60: 173-176, 1978.
6. Weinstein SL and Ponseti IV: Curve progression in idiopathic scoliosis. J Bone Joint Surg Am 65: 447-455, 1983.
7. Weinstein SL, Dolan LA, Wright JG and Dobbs MB: Effects of bracing in adolescents with idiopathic scoliosis. N Engl J Med 369: 1512-1521, 2013.
8. Dayer R, Haumont T, Belaieff W and Lascombes P: Idiopathic scoliosis: Etiological concepts and hypotheses. J Child Orthop 7: 11-16, 2013.
9. de Séze M and Cugy E: Pathogenesis of idiopathic scoliosis: A review. Ann Phys Rehabil Med 55: 128-138, 2012 (In English, French).
10. Meier MP, Klein MP, Krebs D, Grob D and Müntener M: Fiber transformations in multifidus muscle of young patients with idiopathic scoliosis. Spine (Phila Pa 1976) 22: 2357-2364, 1997.
11. Mannion AF, Meier M, Grob D and Müntener M: Paraspinal muscle fibre type alterations associated with scoliosis: An old problem revisited with new evidence. Eur Spine J 7: 289-293, 1998.
12. Green RJ: Histochemistry and ultrastructure of the paraspinal muscles in idiopathic scoliosis and in control subjects. Med Lab Sci 38: 197-216, 1981.
13. Ford DM, Bagnall KM, McFadden KD, Greenhill BJ and Raso VJ: Paraspinal muscle imbalance in adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 9: 373-376, 1984.
14. Sadat-Ali M, al-Habdan I and al-Othman A: Adolescent idiopathic scoliosis. Is low melatonin a cause? Joint Bone Spine 67: 62-64, 2000.

15. Machida M, Dubousset J, Yamada T and Kimura J: Serum melatonin levels in adolescent idiopathic scoliosis prediction and prevention for curve progression-a prospective study. J Pineal Res 46: 344-348, 2009.
16. Azeddine B, Letellier K, Wang da S, Moldovan F and Moreau A: Molecular determinants of melatonin signaling dysfunction in adolescent idiopathic scoliosis. Clin Orthop Relat Res 462: 45-52, 2007.
17. Moreau A, Wang DS, Forget S, Azeddine B, Angeloni D, Fraschini F, Labelle H, Poitras B, Rivard CH and Grimard G: Melatonin signaling dysfunction in adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 29: 1772-1781, 2004.
18. Qiu Y, Wu L, Wang B, Yu Y and Zhu Z: Asymmetric expression of melatonin receptor mRNA in bilateral paravertebral muscles in adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 32: 667-672, 2007.
19. Acaroglu E, Akel I, Alanay A, Yazici M and Marcucio R: Comparison of the melatonin and calmodulin in paravertebral muscle and platelets of patients with or without adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 34: E659-E663, 2009.
20. Cheung WY: Calmodulin plays a pivotal role in cellular regulation. Science 207: 19-27, 1980.
21. Xia Z and Storm DR: Calmodulin-regulated adenylyl cyclases and neuromodulation. Curr Opin Neurobiol 7: 391-396, 1997.
22. Letellier K, Azeddine B, Parent S, Labelle H, Rompré PH, Moreau A and Moldovan F: Estrogen cross-talk with the melatonin signaling pathway in human osteoblasts derived from adolescent idiopathic scoliosis patients. J Pineal Res 45: 383-393, 2008.
23. Marty-Poumarat C, Scattin L, Marpeau M, Garreau de Loubresse C and Aegerter P: Natural history of progressive adult scoliosis. Spine (Phila Pa 1976) 32: 1227-1234; discussion 1235, 2007.
24. Rusin B, Kotwicki T, Głodek A, Andrusiewicz M, Urbaniak P and Kotwicka M: Estrogen receptor 2 expression in back muscles of girls with idiopathic scoliosis-relation to radiological parameters. Stud Health Technol Inform 176: 59-62, 2012.
25. Bijwaard KE, Aguilera NS, Monczak Y, Trudel M, Taubenberger JK and Lichy JH: Quantitative real-time reverse transcription-PCR assay for cyclin D1 expression: Utility in the diagnosis of mantle cell lymphoma. Clin Chem 47: 195-201, 2001.
26. Toma CD, Svoboda M, Arrich F, Ekmekcioglu C, Assadian O and Thalhammer T: Expression of the melatonin receptor (MT) 1 in benign and malignant human bone tumors. J Pineal Res 43: 206-213, 2007.
27. Adi N, Mash DC, Ali Y, Singer C, Shehadeh L and Papapetropoulos S: Melatonin MT1 and MT2 receptor expression in Parkinson's disease. Med Sci Monit 16: BR61-BR67, 2010.
28. Lai L, Yuan L, Cheng Q, Dong C, Mao L and Hill SM: Alteration of the MT1 melatonin receptor gene and its expression in primary human breast tumors and breast cancer cell lines. Breast Cancer Res Treat 118: 293-305, 2009.
29. Morse DL, Carroll D, Weberg L, Borgstrom MC, Ranger-Moore J and Gillies RJ: Determining suitable internal standards for mRNA quantification of increasing cancer progression in human breast cells by real-time reverse transcriptase polymerase chain reaction. Anal Biochem 342: 69-77, 2005.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
31. Burns MJ and Valdivia H: Modelling the limit of detection in real-time quantitative PCR. Eur Food Res Technol 226: 1513-1524, 2008.
32. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, *et al*: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.
33. Machida M, Dubousset J, Imamura Y, Iwaya T, Yamada T and Kimura J: An experimental study in chickens for the pathogenesis of idiopathic scoliosis. Spine (Phila Pa 1976) 18: 1609-1615, 1993.
34. Dubousset J, Queneau P and Thillard M: Experimental scoliosis induced by pineal and diencephalic lesions in young chickens: Its relation with clinical findings. Orthop Trans 7: 7-12, 1983.
35. Man GC, Wang WW, Yim AP, Wong JH, Ng TB, Lam TP, Lee SK, Ng BK, Wang CC, Qiu Y and Cheng CY: A review of pinealectomy-induced melatonin-deficient animal models for the study of etiopathogenesis of adolescent idiopathic scoliosis. Int J Mol Sci 15: 16484-16499, 2014.
36. Girardo M, Bettini N, Dema E and Cervellati S: The role of melatonin in the pathogenesis of adolescent idiopathic scoliosis (AIS). Eur Spine J 20 (Suppl 1): S68-S74, 2011.
37. Man GC, Wong JH, Wang WW, Sun GQ, Yeung BH, Ng TB, Lee SK, Ng BK, Qiu Y and Cheng JC: Abnormal melatonin receptor 1B expression in osteoblasts from girls with adolescent idiopathic scoliosis. J Pineal Res 50: 395-402, 2011.
38. Yim AP, Yeung HY, Sun G, Lee KM, Ng TB, Lam TP, Ng BK, Qiu Y, Moreau A and Cheng JC: Abnormal skeletal growth in adolescent idiopathic scoliosis is associated with abnormal quantitative expression of melatonin receptor, MT2. Int J Mol Sci 14: 6345-6358, 2013.
39. Wang WW, Man GC, Wong JH, Ng TB, Lee KM, Ng BK, Yeung HY, Qiu Y and Cheng JC: Abnormal response of the proliferation and differentiation of growth plate chondrocytes to melatonin in adolescent idiopathic scoliosis. Int J Mol Sci 15: 17100-17114, 2014.
40. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ and Reed MW: Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Anal Biochem 285: 194-204, 2000.
41. Shyy W, Wang K, Gurnett CA, Dobbs MB, Miller NH, Wise C, Sheffield VC and Morcuende JA: Evaluation of GPR50, hMel-1B and ROR-alpha melatonin-related receptors and the etiology of adolescent idiopathic scoliosis. J Pediatr Orthop 30: 539-543, 2010.
42. Qiu XS, Tang NL, Yeung HY, Lee KM, Hung VW, Ng BK, Ma SL, Kwok RH, Qin L, Qiu Y and Cheng JC: Melatonin receptor 1B (MTNR1B) gene polymorphism is associated with the occurrence of adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 32: 1748-1753, 2007.
43. Takahashi Y, Matsumoto M, Karasugi T, Watanabe K, Chiba K, Kawakami N, Tsuji T, Uno K, Suzuki T, Ito M, *et al*: Lack of association between adolescent idiopathic scoliosis and previously reported single nucleotide polymorphisms in MATN1, MTNR1B, TPH1 and IGF1 in a Japanese population. J Orthop Res 29: 1055-1058, 2011.
44. Yang M, Wei X, Yang W, Li Y, Ni H, Zhao Y, Chen Z, Bai Y and Li M: The polymorphisms of melatonin receptor 1B gene (MTNR1B) (rs4753426 and rs10830963) and susceptibility to adolescent idiopathic scoliosis: A meta-analysis. J Orthop Sci 20: 593-600, 2015.
45. Demirkiran G, Dede O, Yalcin N, Akel I, Marcucio R and Acaroglu E: Selective estrogen receptor modulation prevents scoliotic curve progression: Radiologic and histomorphometric study on a bipedal C57Bl6 mice model. Eur Spine J 23: 455-462, 2014.
46. Akel I, Kocak O, Bozkurt G, Alanay A, Marcucio R and Acaroglu E: The effect of calmodulin antagonists on experimental scoliosis: A pinealectomized chicken model. Spine (Phila Pa 1976) 34: 533-538, 2009.
47. Akel I, Demirkiran G, Alanay A, Karahan S, Marcucio R and Acaroglu E: The effect of calmodulin antagonists on scoliosis: Bipedal C57BL/6 mice model. Eur Spine J 18: 499-505, 2009.
48. Yang M, Li C and Li M: The estrogen receptor α gene (XbaI, PvuII) polymorphisms and susceptibility to idiopathic scoliosis: A meta-analysis. J Orthop Sci 19: 713-721, 2014.
49. Takahashi Y, Matsumoto M, Karasugi T, Watanabe K, Chiba K, Kawakami N, Tsuji T, Uno K, Suzuki T, Ito M, *et al*: Replication study of the association between adolescent idiopathic scoliosis and two estrogen receptor genes. J Orthop Res 29: 834-837, 2011.
50. Ogura Y, Takahashi Y, Kou I, Nakajima M, Kono K, Kawakami N, Uno K, Ito M, Minami S, Yanagida H, *et al*: A replication study for association of 5 single nucleotide polymorphisms with curve progression of adolescent idiopathic scoliosis in Japanese patients. Spine (Phila Pa 1976) 38: 571-575, 2013.
51. Ogura Y, Takahashi Y, Kou I, Nakajima M, Kono K, Kawakami N, Uno K, Ito M, Minami S, Yanagida H, *et al*: A replication study for association of 53 single nucleotide polymorphisms in a scoliosis prognostic test with progression of adolescent idiopathic scoliosis in Japanese. Spine (Phila Pa 1976) 38: 1375-1379, 2013.
52. Zhao Y and Qiu GX: Expression of calmodulin and nNOS in the paraspinal muscles in idiopathic scoliosis. Zhonghua Yi Xue Za Zhi 84: 1358-1361, 2004 (In Chinese).