Mol Genet Genomics (2017) 292:789–794
DOI 10.1007/s00438-017-1315-3

ORIGINAL ARTICLE

Genetic variant of BNC2 gene is functionally associated with adolescent idiopathic scoliosis in Chinese population

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Received: 20 January 2017 / Accepted: 22 March 2017 / Published online: 25 March 2017

Abstract Adolescent idiopathic scoliosis (AIS) is a structural curvature of the spine that was estimated to affect millions of children worldwide. Recent study shows that the functional variant rs10738445 could add to the risk of AIS through the regulation of BNC2 gene. This study aims to investigate whether the rs10738445 of BNC2 gene is a functional susceptible locus for AIS in the Chinese population and to further clarify the association of the BNC2 expression with the curve severity. SNP rs10738445 was genotyped in 1952 patients and 2492 controls, and further replicated in 693 patients and 254 controls. We found that patients have a significantly higher frequency of CC than the controls (21.9 vs. 17.7%, $p=0.004$ for stage 1; 12.6 vs. 7.9%, $p=0.03$ for stage 2). Allele C can significantly add to the risk of AIS with an OR of 1.14–1.24. AIS patients were found to have significantly higher BNC2 expression than the controls. The BNC2 expression was significantly correlated with the curve severity ($r=0.316$, $p=0.02$). In conclusion, our study suggests a functional role of BNC2 in the development and progression of the spinal deformity in AIS.

Keywords Adolescent idiopathic scoliosis · BNC2 · Susceptibility · Polymorphism · Functional variant

Communicated by S. Hohmann.

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Introduction

Adolescent idiopathic scoliosis (AIS) is a structural curvature of the spine that was estimated to affect millions of children worldwide (Weinstein 1989). To date, numerous studies have been performed to investigate the pathogenesis of AIS but with no consensus reached on its etiology (Ahn et al. 2002; Parent et al. 2005). Genetic factors were believed to contribute to AIS as indicated by the familial aggregation of the patients (Miller et al. 1996; Morcuende et al. 2003). However, the inconclusive findings of earlier genome-wide linkage studies suggested the genetically heterogeneous of AIS (Gurnett et al. 2009; Raggio et al. 2009). Through candidate gene association analysis, many susceptible genes of AIS were reported, including ERα, MTNR1B, MATN1, and TPH1 (Chen et al. 2009; Qiu et al. 2006; Wang et al. 2008; Wu et al. 2006). It is noteworthy that few of these genes can be successfully replicated in different populations. It is apparent that the relatively small sample size and the inherent drawbacks of selecting candidate genes have weakened the reliability of these studies.

Recently, the genome-wide data were utilized to explore the genetic background of AIS. Buchan et al. performed the first whole-genome exome sequencing (WES) in a cohort of 91 severe AIS cases and 373 normal controls (Buchan et al. 2014). Through the rare variant burden analysis, FBN1 and FBN2 were identified as the susceptible gene

associated with AIS (Buchan et al. 2014). In addition to the WES, several genome-wide association studies (GWASs) of AIS were performed in the White (Sharma et al. 2011), the Japanese (Kou et al. 2013; Ogura et al. 2015; Takahashi et al. 2011), and the Chinese (Zhu et al. 2015) Han populations, respectively, which yielded much more reliable results than the traditional association study. The first GWAS performed in the Caucasian population showed that the CHL1 gene is strongly associated with AIS (Sharma et al. 2011). Subsequently, LBX1 gene, GPR126 gene, and BNC2 gene were identified as the susceptible genes of AIS in the Japanese population (Kou et al. 2013; Ogura et al. 2015; Takahashi et al. 2011). Based on the meta-analysis of the GWAS data in the Caucasian and the Japanese population, Sharma et al. found that PAX1 gene was associated with the development of AIS (Sharma et al. 2015). Recently, three new genes including PAX3, BCL2, and AJAP1 were reported to be associated with AIS in the Chinese population (Zhu et al. 2015). In the era of genome-wide big data, the genetic etiology of AIS is being deciphered with intriguing findings.

Although GWAS has great capability to reveal novel susceptible genes, variants identified by GWAS are commonly located in non-coding regions, thus making it difficult to determine the functional role in the etiology of AIS. To date, a limited number of susceptible genes were proven functionally implicated in the development of AIS through the animal model and in-vitro cellular experiments. Ogura et al. (2015) observed that the functional variant rs10738445 could add to the risk of AIS through the regulation of BNC2 gene. To our knowledge, there is a paucity of knowledge concerning the functional role of this variant in the Chinese AIS population. Therefore, the primary purpose of this study was to investigate whether the rs10738445 of BNC2 gene is a susceptible locus for AIS in Chinese population, and to further clarify its role in the regulation of BNC2 expression in AIS patients.

Methods

Subjects

The current case–control study was composed of two stages. Under the approval of the ethics committees of the local institution, female AIS patients who visited our Joint Scoliosis Center in Nanjing and in Hong Kong between June 2007 and October 2015 were reviewed for the eligibility to be included in this study. The healthy participants were recruited during the physical examinations prior to college admission. All the control subjects were verified through Adam's Forward Bend Test by an experienced orthopedic surgeon (X.L.). Overall, there were 2645 patients and 2746 controls included in our study, all from Chinese Han population. The stage 1 comprised 1952 patients and 2492 controls collected in Nanjing of Jiangsu Province, and the stage 2 comprised 693 patients and 254 controls collected in Hong Kong. Informed consent was obtained from all the participants and from the guardians of the adolescents. Baseline characteristics of the patients including initial age and curve magnitude were recorded at their first visit to the center.

Sample collection

Blood sample was collected with the informed consent obtained from the participants or their parents. Genomic DNA was subsequently extracted using the commercial kit (QIAGEN) according to the standard protocol. Bilateral facet joint was collected from 34 patients during the surgical intervention. Besides, 12 age-matched congenital scoliosis (CS) patients undergoing posterior spinal correction surgery in our clinics also gave their informed consent for the collection of facet joint that was used as the control. All the samples were collected at the convex and concave side of the apex. All above-mentioned tissue samples were collected in Nanjing of Jiangsu Province. The total RNA was extracted subsequently from the facet joint with a commercial kit (CWBio. Co. Ltd). Specifically, before extraction, ceramic pestle and mortars were baked under 200 °C for 6 h to destroy RNase. Liquid nitrogen was used to prechill ceramic pestle and mortars immediately before transferring the bone into mortar. The facet joint was grinded into uniform fine powder. Liquid nitrogen was introduced to maintain low temperature during grinding. Reverse transcription of two micrograms of the total RNA was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA).

Genotyping of the target locus

SNP rs10738445 of the BNC2 gene was genotyped using TaqMan SNP Genotyping Assay as reported in previous studies. The results of genotyping assay were interpreted by ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Twenty percent of the samples were randomly selected to validate the reliability of the genotyping results. The overall call rate was 99.8%.

Tissue expression of the BNC2 gene in AIS patients and controls

Real-time PCR was carried out using ABI 7900HT mentioned above. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene for the normalization of mRNA expression. The specific primers

are as follows: forward 5′-TCAGCGTCCTGAAGCAAGA-3′, reverse 5′-AACCGCAGAAACTGCTGAAG-3′ for the BNC2 gene, and forward 5′-GAGTCAACGGATTTGGTCGT-3′, reverse 5′-TTGATTTTGGAGGGATCTCG-3′ for the GAPDH. All amplifications were performed in triplicate.

Statistical analysis

The Hardy–Weinberg equilibrium (HWE) test was performed for both patients and controls. The Chi-square test was used to evaluate the differences of genotype and allele frequency between AIS patients and healthy controls. Odds ratio (OR) was calculated with allele C used as reference. The Student t test was used to compare the difference of BNC2 expression between the patients and the controls, and between the concave and the convex side. One-way ANOVA test was used to compare the BNC2 expression and the curve severity among different genotypes of rs10738445. The Pearson correlation analysis was carried out to investigate the relationship between the BNC2 expression and the curve severity. All the statistical analyses were performed with the SPSS software (version 17.0, Chicago, IL). Statistical significance was set at $p < 0.05$.

Results

Baseline characteristics of the subjects

For the 5391 subjects included in the genotyping analysis, the mean age was 12.5 ± 2.1 years for the patients and 16.9 ± 2.8 years for the controls, respectively. For the 46 subjects included in the tissue expression analysis, the mean age was 14.3 ± 1.2 years for the AIS patients and 14.5 ± 1.1 years for the CS patients, respectively. The mean curve magnitude of AIS patients undergoing surgical intervention was 56.2 ± 14.3 degrees, which showed no significant difference with that of the CS patients (58.3 ± 10.2 degrees, $p = 0.72$).

Association of the rs10738445 with the development of AIS

The SNP rs10738445 was successfully genotyped for all subjects. As shown in Table 1, HWE test showed no remarkable difference regarding the genotype frequency in the patients and the controls ($p > 0.05$). The genotype and allele frequencies of rs10738445 were significantly different between the patients and the controls in both stage 1 and stage 2. Patients were found to have a significantly higher frequency of CC than the controls (21.9 vs. 17.7%, $p = 0.004$ for stage 1; 12.6 vs. 7.9%, $p = 0.03$ for stage 2). Besides, the frequency of allele C was found to be remarkably higher in the patients than the controls (45.6 vs. 42.3%, OR 1.14, $p = 0.002$ for stage 1; 37.5 vs. 32.5%, OR 1.24, $p = 0.04$ for stage 2).

Tissue expression of the BNC2 in AIS and the controls

Table 2 summarized the expression level of the BNC2 in the patients and in the controls, respectively. AIS patients were found to have significantly higher expression of the BNC2 as compared with the controls. There was no

Table 2 Comparison of BNC2 expression between patients and controls

Expression of BNC2	Patients ($n = 34$)	Controls ($n = 12$)	p
Convex side	0.00062 ± 0.00026	0.00039 ± 0.00021	0.008
Concave side	0.00067 ± 0.00031	0.00043 ± 0.00025	0.021
Mean value	0.00064 ± 0.00029	0.00041 ± 0.00023	0.017

Table 1 Distribution of the genotype and allele frequency of rs10738445 in patients and controls

	Genotype			p	Allele		p	Odds ratio (95% CI [a])	p[a]
	CC	CT	TT		C	T			
Stage 1				0.004			0.002	1.14 (1.05–1.25)	
Patients ($n = 1952$)	428	926	598		1782	2122			0.06
Controls ($n = 2492$)	442	1225	825		2109	2875			0.73
Stage 2				0.03			0.04	1.24 (1.01–1.54)	
Patients ($n = 693$)	87	345	261		519	867			0.10
Controls ($n = 254$)	20	125	109		165	343			0.06

95% CI indicates 95% confidential interval

[a] Indicates p value of HWE test

Table 3 Comparison of BNC2 expression among patients with different genotypes

Genotype	BNC2 expression	
	Convex side	Concave side
CC ($n=8$)	0.00082 ± 0.00027[a]	0.00087 ± 0.00032[a]
CT ($n=15$)	0.00062 ± 0.00021	0.00071 ± 0.00035
TT ($n=11$)	0.00049 ± 0.00017	0.00043 ± 0.00024

[a] $p<0.05$ as calculated by One-way ANOVA test

Table 4 Comparison of curve severity among patients with different genotypes

Genotype	Curve severity (degrees)	p[a]
CC ($n=515$)	41.3 ± 13.5	<0.001
CT ($n=1271$)	38.1 ± 12.9	
TT ($n=859$)	35.4 ± 14.1	

[a] Calculated by One-way ANOVA test

significant difference between the expression of the BNC2 in the concave side and in the convex side of two groups (0.00062 ± 0.00026 vs. 0.00067 ± 0.00031, $p=0.47$ for AIS; 0.00039 ± 0.00021 vs. 0.00043 ± 0.00025, $p=0.67$ for CS).

Relationship between the genotype of rs10738445 and the BNC2 expression

Results of the comparison of the BNC2 expression among patients with different genotypes are shown in Table 3. The mean value of BNC2 expression was 0.00083 ± 0.00029 for genotype CC, 0.00066 ± 0.00028 for genotype CT, and 0.00045 ± 0.00021 for genotype TT. Patients with genotype CC were found to have a remarkably higher BNC2 expression than those with genotype TT in both convex and concave sides ($p=0.03$ for convex side; $p=0.02$ for concave side).

Association of BNC2 with the curve severity of AIS

The BNC2 expression was significantly correlated with the curve severity ($r=0.316$, $p=0.02$). As shown in Table 4, patients with genotype CC had remarkably larger Cobb angle than those with genotype TT (41.3 ± 13.5 vs. 35.4 ± 14.1 degrees, $p<0.001$).

Discussion

For genetic research of complex traits, replication study is critical to verify the association of previously reported susceptible loci. On the basis of a large cohort of patients and controls, we confirmed that the association of rs10738445 in BNC2 with AIS was successfully replicated in the Chinese Han population. In our study, subjects were recruited from two geographically distant clinic centers. We observed remarkably different minor allele frequency (MAF) between the controls recruited in our center (MAF = 0.423) and those recruited in Hong Kong (MAF = 0.325). Apparently, subjects in the two stages were composed of both Northern Han population and Southern Han population, which we believed could strengthen the validity of our findings. Through the two-stage genotyping experiment, we observed that allele C of rs10738445 can add to the risk of AIS with an OR of 1.14–1.24. This finding was consistent with that of the previous study, which reported that allele C was the risky allele of AIS in Japanese population (Ogura et al. 2015).

As a functional variant of the BNC2 gene, rs10738445 may bind to the transcription factor YY1 and thus regulate the BNC2 expression, which has been well described by Ogura et al. (2015) through a series of in-vitro experiments. It is noteworthy that the role of rs10738445 in the regulation of BNC2 expression remains obscure in patients with AIS. For the first time, we analyzed the tissue expression of BNC2 gene in patients with AIS. Compared with the control group, AIS patients were found to have a remarkably elevated expression of BNC2 in the bone tissue. Moreover, patients with genotype CC were confirmed to have a significantly elevated expression of BNC2 than those with TT. These findings were consistent with the results of genotyping analysis of rs10738445 that patients have obviously higher frequency of genotype CC than normal controls. Comparably, Ogura et al. (2015) reported that the risk allele C could resulted in a promoted enhancer activity as shown by the luciferase assay. Herein, we can conclude that rs10738445 plays an important role in the regulation of BNC2 expression in AIS patients.

No studies have focused on the relationship between the expression of BNC2 and the curve severity. In the current study, we observed that the expression of BNC was remarkably correlated with the curve severity. Patients with genotype CC were found to have more severe curve magnitude. Interestingly, Ogura et al. (2015) reported that abnormal expression of BNC2 can lead to scoliosis in zebrafish, the severity of which was observed to be dose-independent. BNC2 is a zinc finger protein concentrated in the nuclear speckles, possibly playing a role in nuclear processing of mRNA (Vanhoutteghem et al. 2011). As the knowledge concerning the function of BNC2 is still limited, further

study is warranted to investigate the underlying mechanism of BNC in the development and the progression of AIS.

Several limitations in the present study should be mentioned. First, this study included a relatively small sample size of the controls. It should be noted that we have strictly matched the cases and the controls in terms of age, and such inclusion criteria could add to the reliability of the expression analysis. Second, all the tissue samples were obtained from patients with severe curvature who underwent surgical intervention. Herein, the relationship between BNC2 expression and the curve severity reported in the present study should be interpreted cautiously. Further studies are required to clarify whether lower expression of BNC2 exists in AIS with mild scoliosis.

To conclude, our large-scale case–control study validated that the functional variant rs10738445 of BNC2 is associated with the development of AIS in the Chinese population. Remarkably higher BNC2 expression was observed in the bone tissue of AIS. Moreover, the elevated expression of BNC2 was associated with more severe curvature of AIS. These findings suggest a functional role of BNC2 in the development and progression of the spinal deformity in AIS.

Acknowledgements This work was supported by the Natural Science Foundation of China (No. 81501849, No. 81661168013, No. 81171672, & No. 81501932), Natural Science Foundation of Jiangsu Province (BK20150099), and Nanjing Medical Science and technology development Foundation (Grant No. YKK15060).

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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