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Analysis of the association between *CDH2* gene polymorphism and osteoarthritis risk

Guanglei Zhao#, Jingsheng Shi#, Jun Xia*

> Objective: to define the cadherin 2 (CDH2) gene polymorphism in Chinese osteoarthritis and control populations and to explore the correlation between *CDH2* gene polymorphism and the risk of osteoarthritis.

Method: a total of 476 patients with osteoarthritis were collected and 380 control subjects were included in the study. Clinical data such as gender, age and functional score were collected. The blood and tissue samples were collected and genotyped by PCR. Data analysis was performed using SPSS 19.0, Hapioview 4.2 and SNPstats softwares.

Results: the association of rs11083271 and osteoarthritis was initially validated in this study population (P = 0.016, OR = 1.43 (1.07-1.93)]. The risk of OA was significantly higher in heterozygous T/C than in homozygous T/T and C/C in rs11083271. By adjusting the age, according to gender stratification analysis, the heterozygous T/C genotype in rs11083271 significantly increased the risk of OA incidence in males [p = 0.011, 3.40 (1.55-7.43)]. The remaining rs sites were not significantly associated with OA. Notably, the association of rs11564299 with OA, regardless of genotyping, gene frequency and RNA expression levels in the study population, was not confirmed.

Conclusion: in this study, we have analyzed the association between *CDH2* gene polymorphism and OA in Chinese population. We found that rs11083271 heterozygous T/C genotype significantly increases the risk of OA and the severity of the disease. By contrast, the rs11564299 locus and OA have no significant correlation in the Chinese population. The role of rs11083271 in the regulation of CDH2 expression levels and the mechanisms by which it impacts OA remain to be further studied. <

Key words: CDH2, correlation, gene polymorphism, osteoarthritis, rs11083271 locus.

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Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease affected daily activities and quality of life of the elderly population in serious harm. Due to the rapid aging of China population in this century, the cumulative incidence of osteoarthritis that has increased linearly over the last two decades, results already in a huge social and family economic burden. It is an urgent problem to reduce the incidence of osteoarthritis. OA is a complex disease caused by congenital genetic factors and acquired environmental factors. The literature suggests that genetic factors account for at least 50% of the causative factors. [1] Exploring the genetic pathogenesis of osteoarthritis can help to find targets for new drugs and therapeutic methods and could provide direction for better prevention and treatment of osteoarthritis. [2] Gene loci polymorphism has been shown to be one of the genetic factors of OA susceptibility [1-5]. Polymorphisms of multiple loci have been found to be related to the development of OA. OA susceptibility genes are ethnically-related and many OA susceptibility gene loci have not been confirmed yet in different ethnic groups. For example, OA susceptibility in the European population is not necessarily verified in the Asian population [1,6-8]. Several meta-analysis studies which increased in sample size have confirmed that genes such as growth and differentiation factor 5 (GDF5) [8], cartilage oligomeric matrix protein (COMP) [9], chondroadherin-like (CHADL) [9], aldehyde dehy-

drogenase 1 family member A2 (ALDH1A2)[1], iodothyronine deiodinase 2 (DIO2) [10] are related to OA. Furthermore, there are many susceptible genes loci that need further validation.

The N-cadherin protein is encoded by the *CDH2* gene that regulates the intercellular adhesion of functional proteins [11]. It has been shown to co-express with cadherin 11 in mouse synovial cells and it plays an important role in the regulation of tumor invasiveness [12]. A literature review shows that the down-regulation of N-cadherin may lead to the decrease of intercellular adhesion between osteosarcoma cells [13]. Therefore, the down-regulation of N-cadherin may increase tumor cell invasiveness. By contrast, overexpression of N-cadherin in stromal cells and pancreatic tumor cells can also increase the invasiveness of tumor cells [14]. Anke Ruedel [15] et al who analyzed the genotype of 312 OA patients and 259 healthy controls found that N-cadherin may be involved in the development of OA. They found that the SNP rs11564299 (A/G) is an important susceptible locus for OA and that the minimum frequency of gene G in the healthy group is significantly higher than that in OA group ($P<0.05$). The expression level of *CDH2* in A/G heterozygotes was significantly higher than that in A/A homozygotes. The expression of N-cadherin may lead to the decrease of cell migration ability. Thus, the authors of this study suggested that *CDH2* may be a susceptible gene of OA, and the SNP rs11564299 may affect the expression of *CDH2* level by a single nucleotide change. However, the studies have been limited to the Caucasian population so far and not validated with mongoloid or negroid ethnic groups. Thus, in the present study, we have investigated the association between *CDH2* gene polymorphism and OA susceptibility in the Chinese population.

Materials and Methods

Study populations

Case group

The patients diagnosed with knee osteoarthritis according to the American Association of rheumatism osteoarthritis diagnostic criteria [16] at the Huashan Hospital Affiliated to Fudan University from April 2013 to May 2016 were included in the study. Exclusion criteria were as follows: 1) rheumatoid arthritis, ankylosing spondylitis, infectious arthritis and other systemic or autoimmune diseases; 2) previous history of trauma knee arthritis; 3) alcohol abuse more than 6 months, smoking more than 12 months. A total of 476 OA patients were enrolled in the case group

Control group

The patients with diagnosed non-OA disease, no OA clinical and imaging signs from April 2013 to May 2016 at the Huashan Hospital Affiliated to Fudan University while meeting the exclusion criteria of the case group. A total of 380 subjects were enrolled in the control group. All informed study patients or family members have written consent and a study agreement approved by the Ethics Committee of Huashan Hospital. There was no genetic relationship between the subjects. The study protocol was approved by the Ethics Committee of Huashan Hospital Affiliated to Fudan University (No).

Selection of research sites

The *CDH2* gene is located on chromosome 18 (18q12.1) and encodes a N-cadherin protein consisting of 906 amino acids. In order to verify whether the SNP polymorphism of CDH2 increased the susceptibility of OA, three SNPs reported in the literature and a high frequency locus were studied. Rs11083271 is located at the distal end of the promoter of the *CDH2* gene. Rs11083255 and rs11564299 are located at the proximal end of the promoter. The minimum gene frequency of rs41274322 is C = 0.046 in the global population. However, rs41274322 is a high frequency site in Chinese population and the MAF is 0.08. Thus, the high frequency site was included in our study

DNA extraction

Blood samples included in the study were stored in a 2 mL EDTA anticoagulant and stored in a -80 ° freezer within 1h after collection. All samples DNA were extracted using the QIAamp DNA Blood Mini Kit kit, and DNA extraction was performed according to the manufacturer's instructions for use. The extracted DNA samples were tested for DNA concentration and purity by spectrophotometer. Only DNA samples showing an OD260/OD280 ratio in the range of 1.6-2.0 were included in the experiment.

Genotyping

All samples were genotyped by KASP (Kompetitive Allele Specific PCR). The results were read on a Pherastar microplate reader (LGC genomics, England). Primers are indicated in *Table 1*.

KASP reactions were done following the manufacturer's protocol (Zhongyu, Beijing using 50 ng of DNA in a 5 µl reaction volume. Reactions were carried-out in a 96-Well Light Cycler 480 II (Roche, CA) at the following conditions: 94 °C for 15 min, then 10 cycles of 94 °C for 20 s, 61 °C for 1 min, followed by 26 cycles of 94 °C for 20 s and 1 min at 55 °C. Following the PCR, fluorescence was detected using a single quantification cycle of 37 °C for 1 s after cooling at 37 °C for 1 min.

RNA expression levels comparison

Synovial specimens were obtained from patients undergoing total knee arthroplasty and arthroscopic surgery. The synovial tissues were immediately stored in RNAlater (Sigma, USA) for 4 h and kept at -80 °C All synovial tissues RNA were extracted by the Rneasy MiNi Kit (Qiagen, USA). Once the concentration was measured by NanoDrop2000 and the OD260 / OD280 ratio was in the range of 1.8-2.0, the RNA was stored. RNA samples were then used to generate cDNA templates using the

ID	Primer_AlleleFAM	Primer_AlleleHEX	Primer_Common
rs11083255	ATCATTTAAGAATCTGATGATGAACCTTC	GATCATTTAAGAATCTGATGATGAACCTTT	CAGTTATGTCAAGAAATTAGTAGCTGGTTT
rs11564299	AATAATAAATAATAGGCCTCTGATTACAAGA	ATAATAAATAATAGGCCTCTGATTACAAGG	CTCCAACAGCATGATTTTAGACTAGACTA
rs11083271	ATTTGATTGACTTAATCCACTAAAAGAACG	AATATTTGATTGACTTAATCCACTAAAA-GAACA	GCAATTCTTTTGCTCCACAAACATCTACTT
rs41274322	GCCACTTGCCACTTTTCCTGC	GCCACTTGCCACTTTTCCTGG	CAAGTTCCTGATATATGCCCAAGACAAA

Table 1. *Primers used for genotyping.*

	OA group	Control group	P value
Total	476	380	
Age	64.24 ±5.32	46.48±8.58	<0.01
Gender			0.12
Male	85	73	
Femal	391	307	
BMI	29.4±3.4	27.6±3.2	0.015
K-L grade			
0-1		380	
2	95		
3	148		
4	235		

Table 2. *Characteristics of the studied Chinese population.*

PrimeScript™ RT reagent Kit with gDNA Eraser reverse transcription kit (Takara, Japan) according to the instructions. Real-time PCR primers were designed and Real-time PCR was performed using the Taqman method.

Measurement and classification of clinical indicators

All patients were enrolled through a unified clinical method and classification. All subjects included were subjected to an ipsilateral X-ray examination. X-ray were read by two different high-grade orthopedic surgeons and imaging studies were based on the Kellgren-Lawrence grading [17]. At the same time, the functional scores of all patients were reviewed using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score [18].

Statistics and analysis

The Spss 19.0 software was used for data analysis. The chi-square test was used to compare gender differences and to analyze the association between the *CDH2* genotyping and the K-L classification. Differences in age, BMI and mRNA expression of different genotypes were compared with the Student's t test. The Spearman correlation test was used to analyze the association between WOMAC knee function score and different genotypes. The sensitivity of the selected loci linkage disequilibrium (LD) was determined by using the Hapioview 4.2 and SNPstats softwares.

Results

Characteristics of study population

A total of 856 subjects were included in this study, including 476 cases (55.61%) in the case group and 380 cases (44.39%) in the control group. The average age (62.99 ± 5.32 vs 58.87 ± 8.58, p<0.01) was significantly different. There was no significant difference in gender composition (χ^2 =0.2572, p = 0.12). The case group was divided into four grades according to the K-L classification. Specific demographic characteristics are shown in *Table 2*.

Hardy-Weinberg equilibrium (HWE)

The multiple logistic regression model adjusted for age, sex and BMI for HWE analysis was applied. The results of the analysis of the selected four sites are shown in *Table 2*. All sites detected were in line with HWE (rs41274322, p = 1; rs11083271, p = 0.91; rs41274322, P = 1; rs11083255, p = 1) *(Table 3)*.

Polymorphism of the *CDH2* gene locus and correlation analysis with the OA group

There was no significant difference in allele frequency of all the four SNPs between the OA group and the control group. The genotyping of rs11564299 showed a A/A frequency of 96.8% and of 3.2% for A/G in the OA group. In the control group, the frequency of A/A was 96% and of A/G 4%. There was no significant difference between the two groups when the genotyping of rs11564299 was performed [OR = 1.27 (0.61-2.68), p = 0.52]. In addition, the rs11083255 genotyping showed that the control group and the OA group are homozygous (T/T).

	Location		Allele	MAF		HWE
SNP	chromosome	Gene	A/B	Case	Control	P
rs11083271	24180057	5' Intergenic	C/T	0.38	0.38	0.91
rs11083255	24013162	Promoter	T/C	0	0	1
rs11564299	24014026	Promoter	A/G	0.02	0.02	1
rs41274322	28013749	Intergenic	G/C	0.06	0.04	1

Table 3. *SNP in the four studied loci.*

After adjusting the sex, age and BMI, the statistical comparison was carried out using different models. The results are shown in *Table 4.1.* Rs11083271, in the Over-dominant model analysis of heterozygous T/C and homozygous (C/C and T/T) genotypes, may increase the risk of incidence of OA [p = 0.016, OR =1.43 (1.07-1.93)]. According to gender stratification analysis, by adjusting the age, the T/C genotype significantly increased the risk of male patients with OA [p = 0.011, 3.40 (1.55-7.43)]. The rs11564299 locus was not significantly different between the two groups. Similarly, no significant difference was observed when a gender stratification analysis was applied. The other sites were not significantly correlated with the incidence of OA *(Table 4-2)*.

The Hapioview software was used for the four sites analysis of linkage disequilibrium. A linkage disequilibrium was found between rs11564299 and rs11083271 and rs41274322. *(Figure 1)*. Also, there was no correlation between the haploidy of different gene loci and OA *(Table 5)*.

Correlation between the severity of osteoarthritis (K-L), the functional score (WOMAC) and the polymorphism at the *CDH2* gene locus

We then analyzed the association between OA and SNP loci in the case group and in the control group. Patients in the OA group were divided into mild group (II) and severe group (III-IV) according to the K-L grading criteria. After adjusting for sex and age, we found that patients with severe OA and rs11083271 heterozygosity (T/C) were significantly more likely to have a severe OA ($\chi 2$ =107.00, P<0.01). There was no significant association between other genotypes and OA severity. Similarly, we also found that, in heterozygous (T/C) patients, the WOMAC score was significantly lower than the homozygote carriers (T/T + C/C)

Verification of differential expression of *CDH2* gene in different genotypes

In order to confirm the differences between rs11564299 and rs11083271 in OA and the control group, RNA levels in the synovial tissue were evaluated. Eight patients with the rs11564299 A/A genotype and 8 with the A/G genotype were selected. For the site rs11083271, we defined the T/C genotype as a positive SNP and the non-T/C genotype as negative SNP. Therefore, we selected 6 (T/C)/6 (C/C + T/T) samples for analyzing the CDH2 RNA level. There was no significant difference in the rs11564299 genotype between the groups, but there was a significant difference when the rs11083271 genotype was analyzed. T/C was significantly higher than the C/C+T /T genotypes *(Figure 2)*.

Discussion

The role of genetic factors in the pathogenesis of osteoarthritis has been paid more and more attention. A large number of studies have focused on the possible association between gene polymorphism and osteoarthritis. The *CDH2* gene was found to be associated with OA in 2014 by Anke Ruedel et al[15]. Up to now, there is no report to further document the association of the *CDH2* gene with OA. The association of *CDH2* gene polymorphism with OA in patients has been evaluated for the first time in the present study. We found no significant correlation between the rs11083271 allele frequency and OA in the Chinese population. However, we also found that the rs11083271 T/C genotype significantly increases the risk of the occurrence of OA. By the Over-dominant model analysis, the rs11083271 T/C genotype carriers have an increased risk of OA by 43% as compared with the C/C and T/T genotype carriers. In contrast, the recessive model analysis showed that the rs11083271 T/T genotype may have a protective effect for OA and may reduce the risk of OA. However, the statistical results did not show any significant differences. By stratifying the samples according to gender, we found that T/C genotype carriers in males were more prone to OA, but also an average increase of 3.40 times OA risk for C/C and T/T genotype carriers. T/C heterozygous genotypes may play an important role in the mechanism of OA, especially in male patients. However, the incidence of female OA patients is significantly higher than in male patients. [19] Accordingly, OA male patients in the present study accounted for only 17.8% of the total patients. The male sample size is too small to conclude about the impact of genotype differences, and the follow-up study should expand the number of male OA patients to verify the T/C genotype gender differences. Ruedel [15] et al detected six SNP loci in a set of case-control cohorts in which rs11083271, rs11564299 and rs11083255 were found to be associated with susceptibility to osteoarthritis. Of note, rs11564299 was identified as correlating with the knee OA risk. However, the association of the two SNPs, rs11564299 and rs11083255, with OA has not been demonstrated in the present study. The results of the genotyping of the rs11083255 locus in the Chinese population by other

rs41274322	Model	Genotype	OA group	Control group	OR (95% CI)	P-value*
		G/G	376 (89.1%)	335 (91.8%)	1.00	
	Codominant	G/C	43 (10.2%)	30 (8.2%)	0.78 (0.48-1.27)	0.086
		C/C	3 (0.7%)	0 (0%)	0.00 (0.00-NA)	
	Dominant	G/G	376 (89.1%)	335 (91.8%)	1.00	0.19
		G/C-C/C	46 (10.9%)	30 (8.2%)	0.73 (0.45-1.18)	
	Recessive	G/G-G/C	419 (99.3%)	365 (100%)	1.00	0.049
		C/C	3 (0.7%)	0 (0%)	0.00 (0.00-NA)	
	Overdominant	G/G-C/C	379 (89.8%)	335 (91.8%)	1.00	0.33
		G/C	43 (10.2%)	30 (8.2%)	0.79 (0.48-1.28)	
		G	795	700	2.3621*	0.124
		C	49	30		
	Log-additive	---	---	---	0.70 (0.44-1.10)	0.12
rs11083271	Codominant	C/C	159 (42.5%)	137 (38.8%)	1.00	0.033
		T/C	144 (38.5%)	167 (47.3%)	1.35 (0.98-1.85)	
		T/T	71 (19%)	49 (13.9%)	0.80 (0.52-1.23)	
	Dominant	C/C	159 (42.5%)	137 (38.8%)	1.00	0.31
		T/C-T/T	215 (57.5%)	216 (61.2%)	1.17 (0.87-1.57)	
	Recessive	C/C-T/C	303 (81%)	304 (86.1%)	1.00	0.062
		T/T	71 (19%)	49 (13.9%)	0.69 (0.46-1.02)	
	Overdominant	C/C-T/T	230 (61.5%)	186 (52.7%)	1.00	0.016
		T/C	144 (38.5%)	167 (47.3%)	1.43 (1.07-1.93)	
		T	462	441	0.0756*	0.7834
		C	286	265		
	Log-additive	---	---	---	0.97 (0.79-1.19)	0.79
rs11564299		A/A	418 (96.8%)	354 (95.9%)	1.00	0.52
		G/A	14 (3.2%)	15 (4.1%)	1.27 (0.61-2.68)	
		A	850 723		0.3804	0.5374
		G	14	15		

Table 4-1. *Analysis of the correlation between genotyping and gene frequency with OA.*

groups of research vary widely. No significant difference was found between the control group and the case group about the rs11564299 alleles, even when using various models of analysis.

We have also attempted to correlate the K-L grade and knee function score with the genotyping of the OA patients. The patients exhibiting a rs11083271 T/C genotype could be divided into two groups according to K-L grade. The severity of OA in patients with the T/T genotype was significantly lower as compared with patients harboring the T/C and C/C genotypes. By contrast, the severity of OA in patients with a rs11083271 T/C genotype was significantly higher than in patients harboring the other two genotypes. This

rs11083271		OA group	Control group		P-value
				OR (95% CI)	0.65
Female	C/C	127	116	1.00	
	T/C	126	127	1.10 (0.78-1.57)	
	T/T	55	42	0.84 (0.52-1.34)	
				OR (95% CI)	0.011*
Male	C/C	32	21	1.00	
	T/C	18	40	3.40 (1.55-7.43)	
	T/T	16	7	0.67 (0.23-1.89)	

Table 4-2. *Stratified analysis of rs11083271 locus gender.*

indicates that some SNPs in the rs11083271 locus not only increase the risk of OA, but also leads to more severe clinical consequences. The remaining sites, rs11564299, rs41274322, rs11083255, did not exhibit a significant correlation with OA severity and function scores in our study. So far, only one report [15] has described a correlation between rs11083271 and OA in Caucasians. This has not been reported in the Chinese population.

In this study, we found that there was a difference in the level of RNA expression in the rs11083271 genotypes (T/C; C/C+T/T) between OA and control groups. This difference of expression was not verified with the rs11564299 genotypes. Differences in *CDH2* expression were detected in knee synovial samples with different genotypes of sites rs11083271 (6/6) and rs11564299 (8/8). We found that the *CDH2* expression in patients with a T/C genotype was significantly higher than in patients with other genotypes (C/C+T/T). The was no significant differences *in CDH2* expression in patients with the different genotypes of rs11564299. This result again suggests that the different genotypes of rs11083271 affect the expression of *CDH2* gene and increase the risk of OA. CDH2 is an important molecule for cell adhesion. Ruedel et al. [16] found that CDH2 expression in heterozygote A/G carriers is significantly higher than that in A/A carriers. In addition, they found that the transcription factor hnRNPK can be bound by CDH2n [16] and suggested that the site rs11564299 genotype G will change the expression level of CDH2 and, hence, modulate its binding to hnRNPK.

CDH2 is thought to regulate the ability of cell migration and invasion. CDH2 overexpression may increase the ability of migration and invasion of synovial cells, and thus destroy cartilage, induce and exacerbate osteoarthritis [12, 21-23]. However, the present study did not detect an association of rs11564299 with OA when exploring the CDH2 gene polymorphism in our large cohort. Surprisingly, we found a correlation between rs11083271 and OA. The results confirmed that the heterozygous rs11083271 T/C genotype could increase the *CDH2* gene expression levels, increasing the risk of OA. Finally, in our study, the rs11564299 locus susceptibility of OA was not confirmed, and the

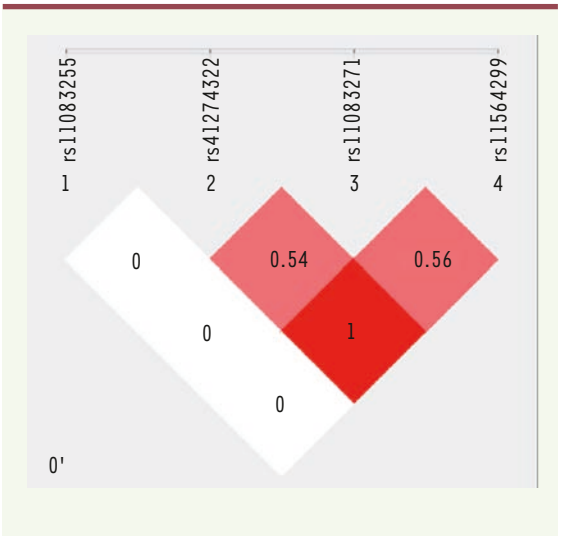

Figure 1. *Linkage disequilibrium analysis.*

main reason may be related to the ethnic differences of the two populations studied [24-25]. Although Ruedel [15] findings are comprehensive, they have not been validated in Chinese populations, suggesting the genetic complexity underlying the induction and severity of osteoarthritis.

Conclusion

Our results show that the *CHD2* gene polymorphism is correlated with OA. However, we demonstrate herein that there is no significant correlation between rs1564299 and OA in Chinese population based on genotyping correlation analysis, analysis of RNA level and clinical analysis. Rs11083271 heterozygous T/C genotype significantly increased the risk of OA and the

rs11083255	rs41274322	rs11083271	rs11564299	Freq	OR (95% CI)	P-value*
T	G	C	A	0.5817	1.00	1.00
T	G	T	A	0.3499	0.98 (0.78 - 1.22)	0.85
T	C	C	A	0.0321	0.71 (0.35 - 1.44)	0.34
T	C	T	A	0.0182	0.68 (0.24 - 1.95)	0.47
T	G	T	G	0.0111	1.00 (0.34 - 2.94)	1

Table 5. *Correlation analysis of CDH2 haploidy with OA.*

grading / function score		Genotype			χ2		R	
K/L	Total	CC(%)	T/C(%)	TT(%)		P value		P value
2	95	54	8	33	113.00*	0	0.531	0
3	122	55	42	25				
4	157	50	94	13				
WOMAC	374	76.1±14.5	65.6±15.6	73.4±13.5	7.9274*	0.001		

Table 6. *Correlation analysis of K-L grading / function score with the rs11083271 genotype.*

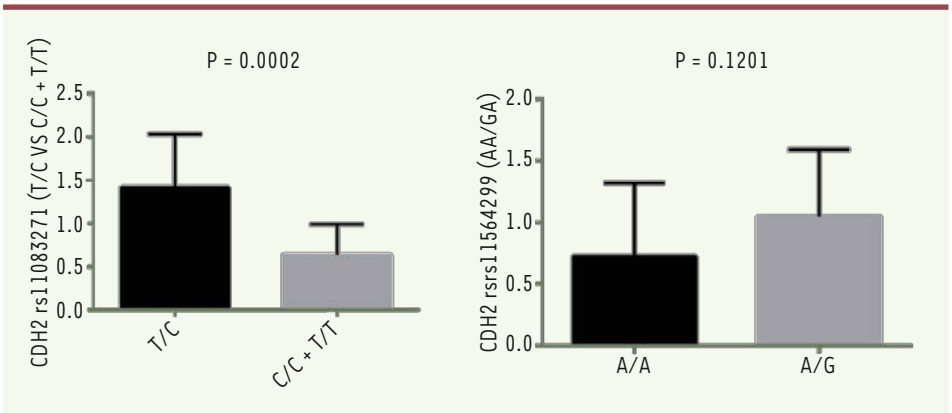

Figure 2. *Differential expression of different genotypes CDH2.*

severity of the disease, especially for male patients. Its involvement in the regulation of CDH2 expression and the specific mechanism of the occurrence of OA remain to be further studied. ◊

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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