ORIGINAL RESEARCH

Association of CLTA-4 Gene Polymorphisms with Diabetes Mellitus: A Study Based on the Han Population of Northern China

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Objective: This study aimed to investigate the association of CLTA-4 gene polymorphism with Type 1 and 2 diabetes mellitus (DM) (T1DM and T2DM) in the Han population of northern China.

Methods: A total of 234 Chinese Han DM patients and 187 non-diabetic subjects were included in the study. Two gene polymorphisms of the CLTA-4, including CTLA-4+49A/G (rs231775) and CTLA4-318C/T (rs5742909), were studied using polymerase chain reaction-restriction endonuclease fragment length polymorphism (PCR-RFLP) analysis. Chi-square analysis and Student's *t*-test were performed to determine the distribution of the gene polymorphism and alleles at the locus of CTLA4-318C/T and CTLA4+49A/G among T1DM and T2DM patients with or without diabetic ketosis (DK), diabetic nephropathy (DN) or autoantibodies (Ab).

Results: Our results indicated that the distribution frequencies of genotypes and alleles at the CTLA-4+49A/G and CTLA4-318C/T loci of T1DM patients were not significantly different from those of the controls. However, the distribution frequencies of genotypes and alleles at the CTLA-4+49A/G loci of T2DM patients were significantly different from the controls (P=0.024 for genotypes and P=0.004 for A and G alleles). Besides, the A and G alleles at the CTLA4-318C/T loci of T2DM DK+ patients showed significantly different distribution frequencies compared to those of the T2DM DK patients (P=0.001).

Conclusion: Our data suggest that the gene polymorphisms of CTLA-4, including CTLA-4+49A/G and CTLA4-318C/T, are important predictors of DM. CTLA-4 may be a susceptibility gene for T2DM. Patients with T2DM carrying the T allele at the CTLA4-318 C/T locus are more predisposed to diabetic ketosis.

Keywords: diabetes mellitus, gene polymorphism, CTLA-4, diabetic ketosis, diabetic nephropathy, autoantibodies

Introduction

Diabetes mellitus (DM) is a highly prevalent disease characteristic of elevated glucose level as a result of dysregulated metabolism. There are currently 285 million people with diabetes worldwide, and nearly 500 million people is expected to develop diabetes by 2030.[1] The chronic vascular complications of diabetes pose a great threat to patients' lives and impose a heavy economic burden. The World Health Organization estimated that the economic loss due to diabetes in China has amounted to $557.7 billion between 2005 and 2015.[2]

The etiology of diabetes is yet to be fully understood. It is now recognized that diabetes is not a disease of a single cause, but a syndrome of multiple causes, among which are genetic, autoimmune and environmental factors.[3,4] There are two major subtypes of diabetes mellitus: type 1 and type 2 diabetes. Type 1 diabetes results from cell-mediated autoimmune destruction of β-cells, causing decreased insulin production.[5] Type 2 diabetes is characteristic of progressive loss of islet function and insulin resistance in peripheral tissues, eventually requiring insulin therapy. Therefore, immunological factors are extremely important in the pathogenesis of both type 1 and 2 diabetes.[6]

The capability of some pancreatic β-cells to secrete insulin is lost by the time diabetes develops symptoms, therefore prediction and prevention of diabetes are very important.[5] It is helpful to know the genetic profile of diabetes to prevent it.

Understanding the genetic underpinnings of DM is critical to develop effective treatments of the disease.[7] A recent study suggested that CTLA-4, a costimulatory molecule that activates T cells, might be one of the candidate biomarkers related to DM susceptibility.[8] *CTLA-4* consists of four exons and three introns and is located on the long arm of chromosome 2q33. Similar to CD28, CTLA-4 also belongs to the immunoglobulin superfamily, but has a higher affinity to B7 molecule expressed on the antigen-presenting cells (APCs) than CD28, making CTLA-4 a potent suppressor of T-cells.[9]

CTLA-4 has multiple polymorphic loci. The exon 1+49 A/G polymorphism is the sole polymorphism that alters the major amino acid sequence of CTLA4.[10] An in vitro study showed that exon +49 A/G mutations in CTLA-4 caused abnormal glycosylation of the endoplasmic reticulum (ER), resulting in decreased cell surface expression.[11] In contrast, the CTLA-4 promoter −318 C/T polymorphism is associated with higher promoter activity, which increases CTLA-4 expression and thus decreases T-cell activity, and this polymorphism could be protective against the pathogenesis of autoimmune diseases.[12]

In this study, we applied PCR-RFLP method[13] to detect the gene polymorphisms of promoter exon −318C/T and +49A/G of CTLA-4, and demonstrated the correlation between the gene polymorphisms of −318C/T and +49A/G of CTLA-4 and type 1 and 2 diabetes in Han population in northern China, which provides a new molecular epidemiological basis for the genetic study of diabetes and for predicting the development and genotyping of DM. Our data suggest that the CTLA-4 polymorphisms are important predictors of DM and CTLA-4 may be a susceptibility gene for type 2 diabetes. Patients with type 2 diabetes carrying the T allele at the CTLA4-318 C/T locus are more predisposed to diabetic ketosis. Our study provides a new valuable genetic diagnostic tool for stratifying patients with type 2 diabetes.

Materials and Methods

Study Subjects

In total, 234 Chinese Han diabetic patients who were hospitalized from November 2008 to August 2010 were enrolled in the study. The participants included 13 cases of type 1 diabetes, 221 cases of type 2 diabetes, 26 cases of type 2 diabetes with diabetic ketosis, 53 cases of diabetic nephropathy, 26 patients with positive antibodies to any of DAD65, IAA and IA-2 antibodies, and 107 patients with negative antibodies. In total, 187 non-diabetic subjects who underwent health checkups at a medical examination center were enrolled as controls. Diagnosis of diabetes was made referring to the 1999 WHO diagnostic criteria for diabetes and the Chinese population diagnostic criteria for diabetes of our diabetes association in October 1999. The study was approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University. 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.

All subjects were not related to each other and had no family history of hereditary immune diseases or other autoimmune diseases. The subjects were sex- and age-matched.

PCR-RFLP

Peripheral venous blood of 2 mL was collected and stored in −20 °C until further use. Genomic DNA extraction was performed using the TIANGEN DNA extraction kit in accordance with manufacturer's recommendations. PCR was performed using the following primers: CTLA4+49A/G (rs231775): forward, 5-GCTCTACTTCCT-GAAGACCT-3; reverse, 5-AGTCTCACT-CACCTTTGCAG-3. CTLA4-318C/T (rs5742909): forward, 5-AAATGAATTGGACTGGATGGT-3; reverse, 5-TTACGAGAAAGGAAGCCGTG-3. The annealing temperature for rs231775 and rs5742909 were 58°C and 56°C, respectively, and the product lengths were 162 and 247 bp, respectively. The following conditions were used for PCR: Pre-denaturation: 95°C for 5 minutes; Denaturation: 95°C for 30 seconds; Annealing: 30 seconds; Extension: 72°C for 30 seconds; Repeat for 30 cycles; Extension: 72°C for 5 minutes.

PCR products underwent electrophoresis using 1% agarose gel and the target bands were purified using the AXYGEN kit. The representative gel images for CTLA-4-318C/T and CTLA-4+49A/G detection are shown in Supplementary Figures S1 and S2. The restriction endonucleases, reaction conditions and fragment lengths of each SNP are shown in Table 1. PCR products containing AG and CT were selected for recovery using the AXYGEN kit and sent to TaKaRa for sequencing. The results of sequencing were compared with those of enzymatic determination to confirm the reliability of

Table 1 Conditions for PCR-RFLP

Reference SNP ID		Restrict Enzyme	Temperature	Fragments
rs231775	CTLA4+49A/G	BbvI	37°C	AA:162bp AG:162bp+88bp+74bp GG:88bp+74bp
rs5742909	CTLA4-318C/T	MseI	37°C	CC:226bp+21bp CT:226bp+130bp+96bp+21bp TT:130bp+96bp+21bp

the enzymatic results. Representative sequencing results of the CTLA-4+49A/G and CTLA-4-318C/T loci are shown in Supplementary Figures S3 and S4.

Statistical Analysis

SPSS16.0 software package was used for data analysis. The comparison of alleles and genotypes was performed by chi-square and Student's *t*-tests. The genetic equilibrium coincidence was analyzed using the Hardy–Weinberg equilibrium method. Differences with P<0.05 were determined to be statistically significant.

Results
Study Design and Participants Characteristics

In this study, we enrolled a total of 234 Chinese Han DM patients and 187 non-diabetic healthy subjects. The differences in terms of age and gender between the diabetic group and the control group were not statistically significant and were comparable. The clinical characteristics of the two groups are shown in Table 2. The Hardy–Weinberg equilibrium test was performed on the genotype frequencies of the two loci in the control group, and the equilibrium test was met at both loci (P>0.05), indicating that the sampling was representative of the population (Table 3).

Table 2 Participants' Clinical Characteristics

	Gender		Age (Years)	Fasting Blood Glucose (mM)	C Peptide (ng/mL)
	Male	Female			
DM	132(56.4%)	102(43.6%)	56.9±13.2		
T1DM	8(61.5%)	5(38.5%)	43.8±15.3	14.76±5.33	0.59±0.51
T2DM	124(56.1%)	97(43.9%)	57.6±12.6	10.38±4.35	2.29±1.03
Control group	116(62%)	71(38%)	48.6±19.3	4.87±2.35	0.71±0.34

Abbreviations: T1DM, type 1 diabetes; T2DM, type 2 diabetes.

Table 3 Hardy–Weinberg Equilibrium Test for CTLA-4+49A/G and −318C/T Gene Polymorphisms

	Genotype	Observed	Expected	χ^2	P
CTLA4+49A/G	AA	34	29.213	0.784	0.134
	AG	75	84.574	1.083	
	GG	66	61.212	0.374	
CTLA4-318C/T	CC	139	139.674	0.003	0.638
	CT	40	38.651	0.047	
	TT	2	2.674	0.169	

Genotypes and Allele Frequency Distribution of CTLA-4+49A/G and −318C/T Loci in DM Patients Compared to Control Subjects

The frequency distributions of CTLA-4+49A/G genotype and A and G alleles were not significantly different between T1DM patients and control subjects (P>0.05, Table 4). However, the frequency distribution of CTLA4+49A/G genotype and alleles between T2DM patients and normal controls were statistically significant (P<0.05, OR=1.487, 95% confidence interval 1.116–1.981 for genotypes; and P<0.05, OR=1.55, 95% confidence interval 1.15–2.091 for alleles) (Table 5).

Our analysis also indicated that at the CTLA4-318C/T locus, no significant differences were seen for the genotype and allele frequency distribution of T1DM or T2DM patients compared to healthy controls (Tables 4 and 5).

Table 4 Distribution of the CTLA-4 Polymorphisms in Patients with Type 1 Diabetes and Controls

		Genotype			Allele	
		AA	**AG**	**GG**	**A**	**G**
CTLA-4+49A/G	T1DM	2(16.7%)	3(25%)	7(58.3%)	7(29.2%)	17(70.8%)
	Control	34 (19.4%)	75(42.9%)	66(37.7%)	143(41%)	207(59%)
	χ^2	2.112			1.278	
	P	0.348			0.258	
CTLA4-318C/T		**CC**	**CT/TT**		**C**	**T**
	T1DM	12 (92.3%)	1 (7.7%)		0 (0%)	25(96.2%)
	Control	139(76.8%)	40(22.1%)		2(1.1%)	318(87.8%)
	χ^2	2.185			0.923	
	P	0.335			0.337	

Table 5 Distribution of the CTLA-4 Polymorphisms in Patients with Type 2 Diabetes and Controls

		Genotype			Allele	
		AA	**AG**	**GG**	**A**	**G**
CTLA-4+49A/G	T2DM	51 (29.1%)	79(45.1%)	45(25.7%)	181(52%)	169(48%)
	Control	34 (19.4%)	75(42.9%)	66(37.7%)	143(41%)	207(59%)
	χ^2	7.477			8.297	
	P	0.024			0.004	
CTLA4-318C/T		**CC**	**CT/TT**		**C**	**T**
	T2DM	155(79.1%)	40(20.4%)		1(0.5%)	350(89.3%)
	Control	139(76.8%)	40(22.1%)		2(1.1%)	318(87.8%)
	χ^2	0.614			0.386	
	P	0.736			0.534	

CTLA-4 Polymorphisms in T2DM Patients with or without Diabetic Ketosis

Table 6 summarizes the frequency distribution of CTLA-4-318C/T and +49A/G genotypes and alleles of T2DM patients with (DK+) or without (DK-) diabetic ketosis. Our results indicated that CTLA-4+49A/G and −318C/T genotypes both showed no significant differences in terms of genotype distribution frequency between the DK+ and DK- groups (P>0.05). CTLA-4+49A/G also showed no significance in the A and G allele distribution (P>0.05). However, in terms of CTLA4-318C/T, C and T allele frequencies were significantly different between the two groups, and T allele frequencies were higher in DK+ patients than in DK- patients in T2DM (P<0.05, OR=0.334, 95% confidence interval 0.166–0.671).

CTLA-4 Polymorphisms in T2DM Patients with or without Diabetic Nephropathy

Table 7 summarizes the frequency distribution of CTLA-4-318C/T and +49A/G genotypes and alleles of T2DM patients with (DN+) or without (DN-) diabetic nephropathy. It turned out that CTLA-4+49A/G and −318C/T genotypes both showed no significant differences in terms of genotype and allele distribution frequency between the DN+ and DN- groups (P>0.05).

Table 6 Distribution of the CTLA-4 Polymorphisms in Type 2 Diabetes Patients with DK+ and DK

		Genotype			Allele	
		AA	**AG**	**GG**	**A**	**G**
CTLA-4+49A/G	T2DM DK+	4 (23.5%)	9 (52.9%)	4(23.5%)	17 (50%)	17(50%)
	T2DM DK -	47 (29.7%)	70 (44.3%)	41(25.9%)	164(51.9%)	152(48.1%)
	χ^2	0.494			0.044	
	P	0.781			0.833	
CTLA4-318C/T		CC	CT/TT		C	T
	T2DM DK+	16(69.6%)	7(30.4%)		39(73.6%)	14(26.4%)
	T2DM DK-	137(79.2)	36(20.8%)		309(89.3%)	37(10.7%)
	χ^2	1.098			10.19	
	P	0.295			0.001	

Table 7 Distribution of the CTLA-4 Polymorphisms in Type 2 Diabetes Patients with DN+ and DN

		Genotype			Allele	
		AA	**AG**	**GG**	**A**	**G**
CTLA-4+49A/G	T2DM DN+	15 (35.7%)	20 (47.6%)	7(16.7%)	50(59.5%)	34(40.5%)
	T2DM DN-	36 (27.1%)	59 (44.4%)	38(28.6%)	131(49.2%)	135(50.8%
	χ^2	2.653			2.7	
	P	0.265			0.1	
CTLA4-318C/T		CC	CT/TT		C	T
	T2DM DN+	37(80.4%)	9(19.6%)		0(0%)	83(90.2%)
	T2DM DN-	116(77.3%)	33(22%)		1(0.7%)	265(88.3%)
	$\chi 2$	0.677			0.251	
	P	0.713			0.617	

Table 8 Distribution of the CTLA-4 Polymorphisms in Type 2 Diabetes Patients with Ab+ and Ab

		Genotype			Allele	
		AA	**AG**	**GG**	**A**	**G**
CTLA-4+49A/G	T2DM Ab+	3(21.4%)	8(57.1%)	3(21.4%)	14(50%)	14 (50%)
	T2DM Ab-	21(21.6%)	45(46.4%)	31(32%)	87(44.8%)	107(55.2%)
	χ^2	0.769			0.262	
	P	0.681			0.609	
CTLA4-318C/T		CC		CT/TT	C	T
	T2DM Ab+	13(76.5%)		4(23.5%)	29(85.3%)	5(14.7%)
	T2DM Ab-	73(76%)		23(24%)	169(88%)	23(12%)
	χ^2	0.001			0.19	
	P	0.969			0.663	

Association of Antibody Positivity Rate with CTLA-4 Polymorphisms in T2DM Patients

Table 8 summarizes the frequency distribution of CTLA-4-318C/T and +49A/G and genotypes and alleles of T2DM patients with (Ab+) or without (Ab-) antibody positivity. Our analysis suggested that CTLA-4+49A/G and −318C/T genotypes both showed no significant differences in terms of SNPs distribution frequency between the Ab+ and Ab- groups (P>0.05).

Discussion

Recent studies have found that there are many important risk loci that are closely associated with the development of diabetes, among which CTLA-4 has been identified as a susceptibility gene for T1DM.[8,9] Studies correlating CTLA-4 polymorphisms with T1DM have been carried out among different populations in several countries. It has been shown that the occurrence of T1DM in Korean, Portuguese, Chilean, and Azerbaijani people is not significantly associated with CTLA-4+49 A/G gene polymorphism;[14–16] whereas in European, Chinese, African, Iranian, Croatian, Polish, Finnish, Belgian, Estonian, Tunisian, and Egyptian people there is a significant association, where the GG genotype of the CTLA-4+49 locus is a risk factor for T1DM.[17,18] In contrast, there are contradictory studies in Japan, with some studies showing that CTLA-4 exon 1+49 GG genotype is closely associated with type 1 diabetes and others showing no association between the two.[19] The present study showed that the AA genotype of the CTLA-4 exon 1+49 locus was strongly associated with the development of T2DM, but not with T1DM, which is inconsistent with the previous findings. This discrepancy may stem from the relatively small patient number and hence suboptimal representation of T1DM patient population. However, it was found that DM patients with the G allele at the CTLA-4+49 locus were more likely to develop T1DM,[18] while those with the A allele were more likely to develop T2DM.[20] No association was found between the CTLA-4+49A/G polymorphism and DK and DN in the study.

The CTLA-4-318C/T gene polymorphism and T1DM have been relatively rarely studied. In our study, no correlation was found between CTLA-4-318C/T gene polymorphism and T1DM. The CTLA-4-318C/T gene polymorphism was not found to correlate with diabetic nephropathy in T2DM neither in the study. However, T2DM patients with a T allele at CTLA-4 promoter −318 were found to be predisposed to DK. This could potentially provide a new predictive basis for ketosis-prone diabetes (KPD).

Approximately 10% of the patients with T2DM have autoantibodies in their body fluids at the time of diagnosis, particularly GAD65,[21] and these patients are insulin resistant, with gradually decreasing islet function as the disease progresses and eventually requiring insulin therapy. The presence of islet-specific antibodies in latent autoimmune

diabetes in adults (LADA) is similar to that of T1DM, but its symptoms are similar to those of T2DM.[22] Some studies have shown that the GG or AG type of CTLA-4+49 is a susceptibility gene for LADA and can be used to diagnose and differentiate LADA from T2DM.[23] No relevant comparison was made in this study because the number of LADA patients was too small, which is a limitation of our study. Similarly, our study included a relatively small number of patients with T1DM, which could limit the rigor of comparison among patients with different genotypes. Further, more comprehensive analysis of the CTLA-4 polymorphism in patients with different diseases using Hardy–Weinberg equilibrium test and multivariate logistic regression analysis could likely enhance our understanding of the pathogenetic role of the CTLA-4 polymorphism. At last, there has also been no study on the frequencies of the polymorphisms among Chinese Han population, therefore the representation of the study sample pool to the general population should also be verified in the future once relevant data become available.

Conclusion

Using PCR-RFLP analysis, we showed that CTLA4 gene polymorphism is closely linked to the pathogenesis of diabetes. The frequency distribution of CTLA4+49A/G genotype and alleles between T2DM patients and normal controls were statistically significant. T2DM patients carrying the T allele at the CTLA4-318 C/T locus are more predisposed to DK. In terms of CTLA4-318C/T, C and T allele frequencies were significantly different between the two groups, and T allele frequencies were higher in DK+ patients than in DK- patients in T2DM. Further studies are warranted to investigate how genetic and environmental factors affect the pathogenesis of DM to provide a basis for early diagnosis, genotyping and treatment of DM.

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Disclosure

The authors declare that they have no competing interests.

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