



BASIC SCIENCE ARTICLE

LCK rs10914542-G allele associates with type 1 diabetes in children via T cell hyporesponsiveness

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BACKGROUND: Abnormal lymphocyte-specific protein tyrosine kinase (LCK)-related T cell hyporesponsiveness was discovered in type 1 diabetes (T1D). This study aims to investigate the potential associations between LCK single-nucleotide polymorphisms (SNPs) and the susceptibility of T1D.

METHODS: DNAs were extracted from blood samples of 589 T1D patients and 596 healthy controls to genotype seven SNPs of the LCK gene using PCR and Sanger sequencing. Associations of these SNPs with the susceptibility of T1D were determined by χ^2 test. LCKs were knocked out in peripheral blood mononuclear cells (PBMCs) using CRISPR-Cas9 to investigate the role of LCK SNP in T-lymphocyte activation in T1D.

RESULTS: SNP rs10914542 but not the other six SNPs of the LCK gene was significantly associated with (C vs. G, odds ratio (OR) = 0.581, 95% confidence interval (CI) = 0.470–0.718, P value = $4.13E - 7$) the susceptibility of T1D. Peripheral T-lymphocyte activation in response to T cell receptor (TCR)/CD3 stimulation is significantly lower in the rs10914542-G-allele group than in the C-allele group. In vitro experiments revealed that rs10914542 G allele impaired the TCR/CD3-mediated T-cell activation in PBMCs.

CONCLUSIONS: This study reveals that the G allele of SNP rs10914542 of LCK impairs the TCR/CD3-mediated T-cell activation and increases the risk of T1D.

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INTRODUCTION

Type 1 diabetes (T1D), also known as insulin-dependent diabetes, is a chronic disease caused by the lack of insulin. T1D is the common type of diabetes in children and teens, affecting ~1 in every 400–600 children,¹ which is why it was called juvenile diabetes. Different factors, including genetics and some viruses, have been found to contribute to many complex diseases, as well as T1D.^{2,3} Single-nucleotide polymorphisms (SNPs) of many genes, such as PTPN22⁴ and PTPN2,⁵ have been found to be associated with the susceptibility of T1D.

Lymphocyte-specific protein tyrosine kinase (LCK) encodes a kinase that plays an essential role in the choice and maturation of developing T cells in the thymus and in the function of mature T cells. Autoreactive T cell is one of the causes of T1D via specific autoimmune destruction of the insulin-producing islet cells.⁶ Abnormal LCK expression positively correlated with the defect of T-lymphocyte activation induced by T cell receptor (TCR)/CD3 in T1D patients.⁷ Moreover, gene polymorphisms of PTPN22, an interacting protein of LCK, have been found to be associated with the risk of T1D.⁴ It leads to the hypothesis that gene polymorphisms of LCK may also associate with the susceptibility of T1D. However, a previous study found no association between gene polymorphisms and protein levels of LCK in T1D patients.⁸ They also indicated that their study was not statistically powerful enough to detect weak or moderate genetic associations in a complex disease like T1D. Hulme et al.⁹ genotyped 13 SNPs of LCK and found no associations with T1D in British population. Although the selection of SNPs in their study was according to the frequency in British population, there are many

high-frequency SNPs in other populations that were not involved in this study.

Therefore, we selected seven high-frequency SNPs (minor allele frequency (MAF) > 0.05) of LCK in Chinese population. To investigate whether these SNPs are associated with the susceptibility of T1D, we collected blood samples from 589 T1D patients and 596 healthy people to genotype seven SNPs (rs1202592, rs10914542, rs670025, rs145088108, rs695161, rs1042546, and rs11576042) of LCK. Thereafter, we analyzed the potential associations between these SNPs and the susceptibility of T1D by using χ^2 test. SNP rs10914542 but not the other six SNPs of the LCK gene showed significant association with (C vs. G, odds ratio (OR) = 0.581, 95% confidence interval (CI) = 0.470–0.718, P value = $4.13E - 7$) the susceptibility of T1D. It suggested a potential contribution of gene polymorphism of LCK to the pathogenesis of T1D.

MATERIALS AND METHODS

Participants

T1D patients and healthy participants enrolled in this study were collected in Nantong Municipal Maternal and Child Health Hospital between January 1, 2015 and October 31, 2018. This study was approved by the ethics committee of Nantong Municipal Maternal and Child Health Hospital and followed the principle of Declaration of Helsinki. We obtained written consents from all participants or their parents after a full rationalization of the study. T1D patients were diagnosed by a specialist according to the Clinical Practice Guidelines of T1D. All participants were of Chinese population.

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Genotyping of SNPs

A QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used to collect DNA from blood samples of all participants. DNA sequences having selected SNPs were amplified by polymerase chain reactions (PCRs). The PCR reaction system consists of 2 µl of DNA, 2 µl of primers, 0.25 µl of *Taq* enzyme (Takara, Japan), 4 µl of dNTPs, and 5 µl of PCR buffer. The PCR products were sequenced by using an ABI-PRISM 3730 genetic analyzer (Sequenom Inc.). Seven SNPs of the LCK gene with minor allele frequencies (MAFs) >0.05 in Chinese population from the dbSNP database (www.ncbi.nlm.nih.gov/SNP) were selected for this study.

Cell-proliferation assays

Ficoll-Hypaque density centrifugation was used to collect PBMCs from 30 ml of heparinized peripheral venous blood. PBMCs were cultured in RPMI-1640 (add 4 mM glutamine and 10% fetal bovine serum) overnight. Nonadherent PBMCs were collected for further study. PBMCs were seeded on wells with 10 µg/ml anti-CD3 or phosphate-buffered saline at a density of 6 × 10⁵ cells/ml. Then, PBMCs were pulsed for 8 h after 3 days with 1 µCi of [³H]thymidine

to harvest with glass fiber filters. A Matrix Cell Counter (Packard, Zurich, Switzerland) was used to determine the radioactivity.

Cell transfection

The CRISPR-Cas9 vectors were obtained from Addgene (Cambridge, USA). To knock out LCK in PBMCs, the LCK-CRISPR-Cas9 system was designed according to previously established procedures¹⁰ and by using the tools supplied by Dr. Zhang Feng. PBMCs were seeded in 24-well plates at a density of 6 × 10⁵ cells/well and incubated overnight. Transfection of the LCK-CRISPR-Cas9 vectors, LCK-rs10914542-C-allele vectors, LCK-rs10914542-G-allele vectors, and control vectors (Life Technologies, Beijing, China) was conducted using a Lipofectamine 2000 transfection reagent (Invitrogen, Beijing, China) with 1 µg/ml DNA plasmid, respectively.

Statistical analysis

All statistical analysis was conducted on an R platform. Hardy-Weinberg equilibrium (HWE) test was conducted in both the T1D and healthy population. The associations between SNPs of LCK and T1D were determined by comparing genotype and allele frequencies between T1D and healthy people using the χ^2 test. The OR with a 95% CI was used to determine the relative risks of alleles. *D'* and *R*² were used to determine the linkage disequilibrium (LD) between SNPs. A *P* value < 0.05 was considered significant.

Table 1. Characteristics of participants

	Patients (n = 589)	Control (n = 596)
Age (years)	8.32 ± 3.97	8.82 ± 4.18
Male	283	301
Female	306	295
Population	Han	Han
Duration of diabetes (years)	2.9 (1–5)	NA
HbA1c (%)	9.35 (8.21–10.76)	NA

HbA1c hemoglobin A1c, *NA* not available

RESULTS

rs10914542 associates with the susceptibility of T1D

We obtained blood samples from 589 T1D patients and 596 healthy children participants from the Chinese population (Table 1). The age for T1D patients is 8.32 ± 3.97 (mean ± SD), while that for healthy participants is 8.82 ± 4.18 (mean ± SD). The sex ratio is close to 1 in both T1D patients and healthy participants (Table 1). All participants are from Chinese Han population.

Table 2. Comparison of allele and genotype frequencies of SNPs between patients and controls

Group	Allele frequency		<i>P</i> value	OR	95% CI	Genotype frequency			<i>P</i> value	HWE
rs1042546	A	G				AA	AG	GG		
Control	1091	101				498	95	3		0.499349
rs10914542	C	G				CC	CG	GG		
Control	1020	172				425	170	1		0.000154
rs11576042	T	C				TT	TC	CC		
Control	1077	115				490	97	9		0.104714
rs12025920	T	C				TT	TC	CC		
Control	1106	86				514	78	4		0.582822
rs145088108	G	A				GG	GA	AA		
Control	1128	64				534	60	2		0.82023
rs670025	T	A				TT	TA	AA		
Control	626	566				157	312	127		0.22557
rs695161	A	G				AA	AG	GG		
Control	721	471				228	265	103		0.088273

SNP single-nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval, *HWE* Hardy-Weinberg equilibrium

For T1D patients, their average duration of diabetes was 2.9 years and their average hemoglobin A1c levels were 9.35% (Table 1). Then, we selected seven SNPs of LCK with an MAF above 0.05 in Chinese population and genotyped them in those collected blood samples from both T1D and healthy participants. Among these SNPs, rs145088108 is a missense SNP, rs1042546 and rs11576042 locate at the 3'-untranslated region, and rs670025, rs695161, rs10914542, and rs12025920 locate at the intron region. rs670025 is the SNP with the highest MAF in both T1D and healthy populations, and rs145088108 is the one with the lowest MAF. The genotypes of these SNPs were in HWE (P value > 0.0001) in all healthy participants. All other SNPs, except for rs10914542, were in HWE in the T1D population (Table 2). rs10914542 showed significant differences (P value > 0.05) in both allele frequencies and genotypes between T1D and healthy populations (Table 2). The C-allele frequency of rs1129740 was lower in T1D patients than in healthy participants (C-G OR = 0.581, 95% CI = 0.470–0.718). The other six SNPs did not show significant differences either in frequency of allele or frequency of genotype between T1D and healthy populations.

LD of the selected SNPs

The LD analysis indicated that there was no strong LD ($D' > 0.8$) among selected SNPs in either T1D or healthy population (Table 3). Only rs12025920 showed medium LD ($0.8 > D' > 0.5$) with rs670025 and rs695161 in T1D population, and with rs10914542 in healthy population (Fig. 1). The other SNP pairs only showed weak LD ($D' < 0.5$) (Table 3). Therefore, no LD block analysis was conducted either in T1D or healthy population.

The G alleles of rs10914542 impaired the TCR/CD3-mediated T-cell activation

Although the association between rs10914542 and T1D was discovered in this study, the molecular mechanisms underlying

this phenomenon are still unknown. Since LCK is essential for T-lymphocyte activation,¹¹ we investigate TCR/CD3-mediated proliferative response in PBMCs from 589 T1D patients and 596 healthy children participants. The results showed a lower proliferative response of PBMCs from T1D patients than healthy controls (Fig. 2), which indicated a reduction of T cell responsiveness. Moreover, T cell hyporesponsiveness was also observed in rs10914542 GG and CG genotype carriers (Fig. 3).

To investigate how rs10914542 functions in the pathogenesis of T1D, we knocked out the wild-type LCK of PBMCs using CRISPR-Cas9. Then, these LCK-knockout PBMCs were transfected

Table 3. The linkage disequilibrium analysis of the SNPs

SNP1	SNP2	Patients			Controls		
		D'	LOD	R^2	D'	LOD	R^2
rs12025920	rs10914542	0.086	0.55	0.002	0.524	4.61	0.017
rs12025920	rs670025	0.613	9.5	0.035	0.288	15.7	0.06
rs12025920	rs145088108	0.022	0.07	0	0.359	3.45	0.013
rs12025920	rs695161	0.536	9.14	0.036	0.437	5.95	0.023
rs12025920	rs1042546	0.068	0.88	0.004	0.38	3.26	0.012
rs12025920	rs11576042	0.076	0.93	0.004	0.458	10.32	0.039
rs10914542	rs670025	0.366	10.59	0.041	0.277	1.68	0.007
rs10914542	rs145088108	0.213	1.86	0.008	0.078	0.8	0.004
rs10914542	rs695161	0.309	9.82	0.039	0.032	0.13	0.001
rs10914542	rs1042546	0.21	3.59	0.016	0.107	1.65	0.008
rs10914542	rs11576042	0.24	5.51	0.024	0.251	4.26	0.021
rs670025	rs145088108	0.371	1.98	0.008	0.313	3.49	0.014
rs670025	rs695161	0.318	19.6	0.076	0.238	2.34	0.009
rs670025	rs1042546	0.384	4.19	0.016	0.308	2.84	0.011
rs670025	rs11576042	0.334	3.72	0.014	0.471	14.55	0.057
rs145088108	rs695161	0.478	4.28	0.017	0.036	0.26	0.001
rs145088108	rs1042546	0.121	1.48	0.007	0.098	1.68	0.008
rs145088108	rs11576042	0.226	0.09	0	0.19	4.2	0.02
rs695161	rs1042546	0.445	7.31	0.029	0.048	0.39	0.002
rs695161	rs11576042	0.23	2.3	0.009	0.157	3.39	0.016
rs1042546	rs11576042	0.049	0.47	0.002	0.171	2.89	0.013

SNP single-nucleotide polymorphism, LOD limit of detection

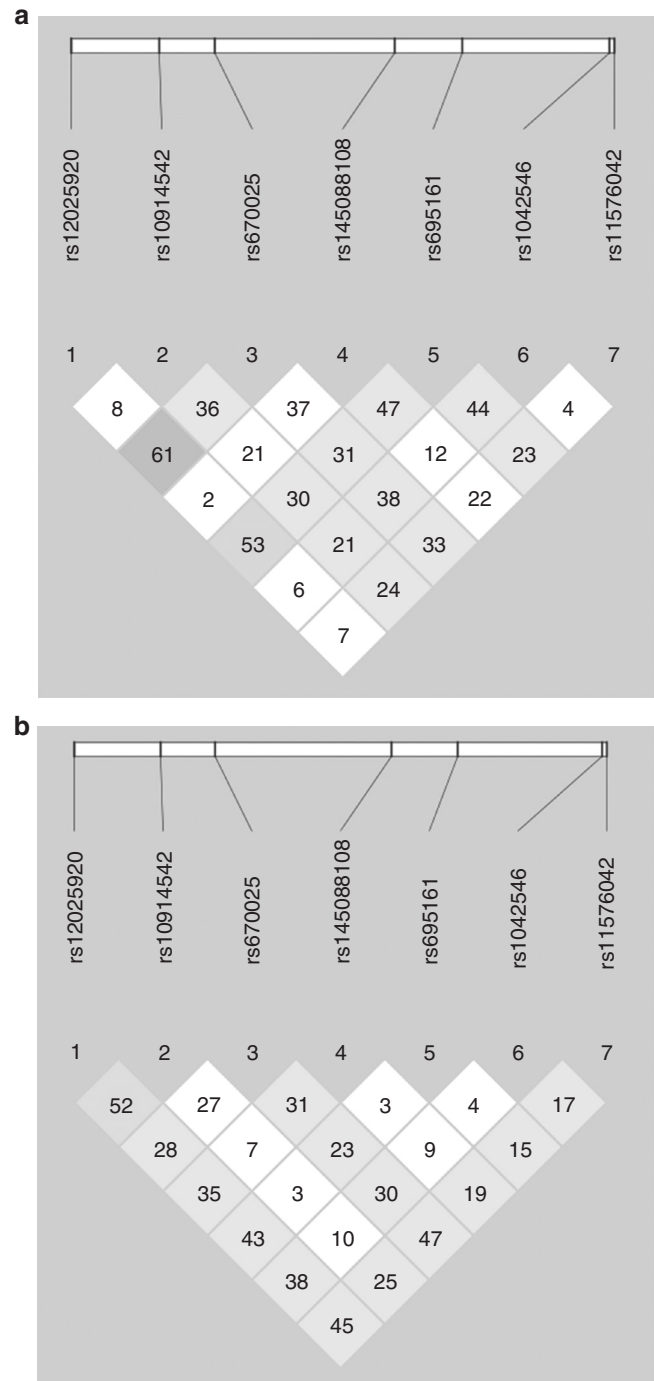


Fig. 1 The linkage disequilibrium analysis of selected single-nucleotide polymorphisms (SNPs) in type 1 diabetes (T1D) (a) and healthy (b) population

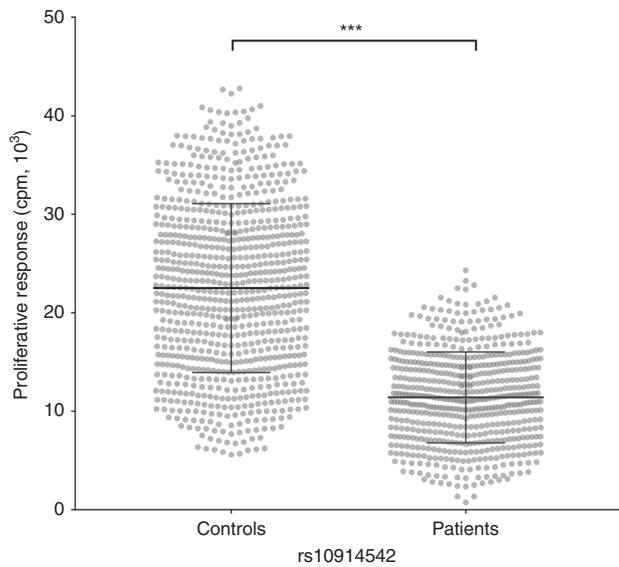


Fig. 2 T cell receptor (TCR)/CD3-mediated proliferative response in peripheral blood mononuclear cells (PBMCs) from 589 T1D patients and 596 healthy controls. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001

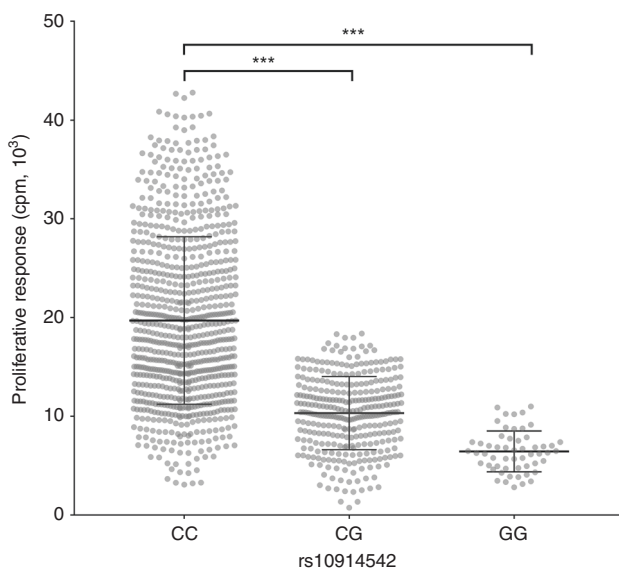


Fig. 3 T cell receptor (TCR)/CD3-mediated proliferative response in peripheral blood mononuclear cells (PBMCs) from rs10914542 CC, CG, and GG genotype carriers. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001

with rs10914542-C-allele LCK and rs10914542-G-allele LCK, respectively. The results revealed that the G allele of SNP rs10914542 of LCK impairs the TCR/CD3-mediated T-cell activation (Fig. 4).

DISCUSSION

T1D is a chronic disease caused by the lack of insulin.¹² Therefore, it is also known as insulin-dependent diabetes. T1D was also called juvenile diabetes. It is the common type of diabetes in children and teens. There is one T1D patient in every 400–600 children.¹ Genetic factors, such as SNPs of many genes, such as PTPN22⁴ and PTPN2,⁵ have been found to be associated with the susceptibility of T1D.

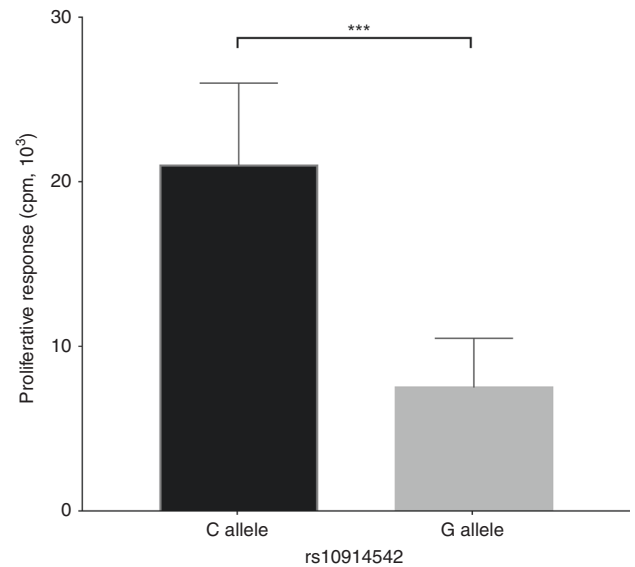


Fig. 4 T cell receptor (TCR)/CD3-mediated proliferative response in peripheral blood mononuclear cells (PBMCs) transfected with rs10914542-C-allele LCK and rs10914542-G-allele LCK, respectively. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001

LCK encodes a kinase that is important in developing T cells in the thymus and in the function of mature T cells, which may be involved in T1D.⁶ Moreover, SNPs of PTPN22, an interacting protein of LCK, have been found to be associated with the risk of T1D.⁴ It leads to the hypothesis that gene polymorphisms of LCK may also associate with the susceptibility of T1D.

In this study, we selected seven high-frequency SNPs (rs1202592, rs10914542, rs670025, rs145088108, rs695161, rs1042546, and rs11576042) (MAF > 0.05) of LCK and genotyped them in blood samples from 589 T1D patients and 596 healthy people. We found that SNP rs10914542, but not the other six SNPs of the LCK gene, showed significant association with (C vs. G, OR = 0.581, 95% CI = 0.470–0.718, *P* value = 4.13E – 7) the susceptibility of T1D. Further in vitro experiments revealed that rs10914542 G allele impaired the TCR/CD3-mediated T-cell activation. It suggested a potential contribution of rs10914542 G allele of LCK to the pathogenesis of T1D via T-cell hyporesponsiveness. Since SNPs of PTPN22, an interacting protein of LCK, also associate with the risk of T1D, it implied a potential joint function between LCK and PTPN22 in T1D, which is common in complex diseases.^{3,13,14} It will promote the discovery of a new combination therapy for T1D.^{15,16}

The results are different from what Hulme et al.⁹ reported on T1D in the British population.⁹ They genotyped 13 SNPs of LCK and found no associations with T1D in the British population. The choice of SNPs in their study was according to the frequency in the British population. So, the SNPs they selected are different from what we used in this study. SNP rs10914542 locates in the intron region of LCK. Since Nervi et al.⁸ found no association between gene polymorphisms and protein levels of LCK in T1D patients, rs10914542 may change the function of LCK by affecting the alternative splicing of messenger RNA.^{17–19}

Generally, this study reveals that the G allele of SNP rs10914542 of LCK impairs the TCR/CD3-mediated T-cell activation and increases the risk of T1D. This is the first study to report an association between LCK SNP and the risk of T1D. The results of this study give us a clue on discovering the role of LCK in the pathogenesis of T1D and will deepen our understanding of the molecular mechanisms underlying T1D.

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ADDITIONAL INFORMATION

Competing interests: The authors declare that they have no competing interests.

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