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Original Article

Potentials of long non-coding RNAs to differentiate latent autoimmune diabetes in adults from type 2 diabetes

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Abstract: Latent autoimmune diabetes in adults (LADA), which characteristically overlaps with type 2 diabetes (T2D), is often misdiagnosed. In this study, we aim to access potentials of lncRNAs to differentiate LADA from T2D. We analyzed 156 participants with T2D, 101 with LADA, and 100 normal glucose tolerance (NGT). Expression levels of lncRNAs (n342533, n335556, and n336109) were detected in the three groups. Receiver operating coefficient curve was used to evaluate the potentials of n342533, n335556 and n336109 to differentiate LADA from T2D. Expression level of n342533 in T2D group was higher than that in LADA and NGT groups. n342533 had the potential discriminatory ability in T2D versus LADA group (AUC_{roc} = 0.758, P < 0.001). Expression level of n342533 was stratified in three tertiles, and 62.8% patients in T2D group were in the high tertile. Patients with LADA were mainly in the low and middle tertiles. The expression level of n342533 was correlated to fasting C peptide, triglycerides, HDL-C, HOMA2-IR, and visceral adiposity index values. We concluded that the expression level of lncRNA n342533 was declined in LADA, and had the potential to differentiate LADA from T2D.

Keywords: Latent autoimmune diabetes in adults, type 2 diabetes, long non-coding RNAs, insulin resistance

Introduction

Latent autoimmune diabetes in grown-ups (LADA) is characterized by an over age of 30 years old, positive islet autoimmune antibody and without need of insulin treatment in the first 6 months after diagnosis [1]. Approximately 4%-14% patients who were initially diagnosed as T2D meet the criteria of LADA [2]. LADA is a highly heterogeneous disease. It is reflected by various clinical phenotypes (e.g., widespread insulin resistance and insulin deficiency), and high implications for treatment [3]. In clinical practice, it is usually difficult to distinguish LADA from T2D. Owing to misdiagnosis, the majority of patients with LADA were not appropriately treated.

Long non-coding RNAs (lncRNAs) generally refer to non-encoding RNA transcripts over 200 bp [4]. Our previous study found that lncRNAs are expressed aberrantly in Han Chinese T2D cases, and revealed that n342533, n335556, and n336109 were three most upregulated lncRNAs in T2D [5]. However, we do not know their expression levels in LADA. We suppose that these lncRNAs may help differentiate between LADA and T2D.

In this study, we aim to compare the expression levels of n342533, n335556, and n336109 among T2D, LADA and healthy control, and evaluate their potentials to distinguishing LADA from T2D.

Materials and methods

Patients

257 patients of diabetes mellitus were analyzed, including T2D (n = 156) and LADA (n = 101), from January 2015 to December 2018 in the First Affiliated Hospital of Shihezi (China). The protocol here complied with Helsinki's dec-

laration; it was authorized by the First Affiliated Hospital's ethics committee (School of Medicine, Shihezi University). All participants have signed an informed consent. The diagnosis of T2D met standard criteria [6]. LADA was defined as follows [1]: 1) over 30 years of age, 2) positive glutamic acid decarboxylase (GAD) antibodies, 3) without need of insulin treatment in the first six months after diagnosis. The NGT group (n = 100) comprised healthy volunteers without diabetes mellitus of communities in Shihezi, China. 75-g oral glucose tolerance test for normoglycemia was performed for the control group (2-hour plasma glucose < 7.8 mmol/L, 3.9 mmol/L < fasting plasma glucose < 6.1 mmol/L). Clinicopathological and demographic information (e.g., previous diseases, ethnicity and age) was acquired.

Participants were overall Han Chinese. Individuals with acute complications related to diabetes at the time of enrollment, those who received immunosuppression or hormone-containing drugs, and cancer history, pregnancy, breastfeeding, immunodeficiency or infection were excluded.

Anthropometry and examination

Before breakfast, anthropometric and body composition measurements were conducted on all participants. Weight (kg) divided by the square of the height (m) indicates body mass index (BMI). We took blood samples from the cubital vein and gathered them in a vacuum tube with preservatives (heparin sodium). We incubated the tubes at 4°C until they were centrifuged. Besides, with the use of an automated D10 analyzer (Bio-Rad, Hercules, CA, USA), 1 mL of whole fresh blood samples were collected in evacuated sample tubes with ethylene diamine tetra-acetic acid to measure hemoglobin A1c (HbA1c) level. With the use of an autoanalyzer (Hitachi, Tokyo, Japan), we analyzed plasma glucose, overall cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) levels in an enzymatical manner. With electrochemiluminescence (E170, Roche, Basel, Switzerland), C-peptide levels were detected. By immunoblotting (Blot, Shenzhen, China), GAD antibody was tested. Insulin resistance is denoted as HOMA2-IR index, and β-cell function by HOMA2-β which is obtained based on paired values of glucose and C-peptide fasting plasma levels with the use of the Homeostasis Model Assessment 2 released as a computer HOMA Calculation software from University of Oxford (https://www.dtu.ox.ac.uk/homacalculator/). By the published formula [7], the visceral adiposity index (VAI) was calculated as:

$$\text{Male: } VAI = \left[\frac{WC}{39.68 + (1.88 \times BMI)}\right] \times \left(\frac{TG}{1.03}\right) \times \left(\frac{1.31}{HDL}\right)$$

$$\text{Female: } VAI = \left(\frac{WC}{36.58 + (1.89 \times BMI)}\right) \times \left(\frac{TG}{0.81}\right) \times \left(\frac{1.52}{HDL}\right)$$

Following the manufacturer's instructions, we took out total RNA from peripheral blood to achieve purification and isolation, with the PAXgene Blood RNA kit (Qiagen, Dusseldorf, Germany) [8]. In brief, in PAXgene® Blood RNA tubes (Becton Dickinson, Franklin Lakes, NJ, USA), we incubated nearly 2.5 mL of blood from each subject for 2 h at ambient temperature, and then we centrifuged it for 10 min at 3000 g. Subsequently, we incubated cell pellets with 40 μL of proteinase K and a mixture of 300 μL of binding buffer for 10 min at 55 °C while being shaken. The resultant lysate underwent centrifugation for 3 min in a PAXgene® Shredder spin column (Qiagen) at 16300 g. Subsequently, in the PAXgene® RNA spin column, we mixed the supernatant with 350 μL of 100% ethanol, and then the mixture was centrifuged for 1 min at 16300 g. The purified RNA was eluted using an elution buffer, denatured by the incubation at 65°C for 5 min, chilled on iced, and then stored at -20°C until being used in gene expression studies.

We developed primers for quantitative real-time polymerase chain reaction (qRT-PCR) according to the previous study of the authors [5]. Then, they underwent synthesis and purification at Shenggong, Shanghai, China. With the use of a 7500 Fast RT-PCR System (Applied Biosystems) and a cDNA synthesis kit (Qiagen, Dusseldorf, Germany), we performed the reverse transcription reactions on extracted total RNA. With the use of the ABI 7500 Fast, qRT-PCR was conducted. The qRT-PCR cycle was as follows: 95°C for 2 min, 40 cycles at 95°C for 5 s and at 60°C for 30 s, and undergoing a final melting curve analysis at the temperature 60-95°C. With the use of the $2^{-\Delta ct}$ method against β-actin (ACTB) to achieve normalization, qRT-PCR results were quantified. The data refer

Table 1. Demographic and metabolic characteristics of the study population

Variable	NGT (n = 100)	T2D (n = 156)	LADA (n = 101)	*P*-value
Male (%)	57.0	62.2	55.4	0.284
Age (years)	51.8 ± 9.6	53.3 ± 11.7	51.7 ± 10.5	0.456
Systolic blood pressure (mmHg)	129 ± 12	133 ± 16	131 ± 15	0.065
Diastolic blood pressure (mmHg)	76 ± 10	79 ± 13[a]	78 ± 8	0.039
Waist circumference (cm)	86 ± 8	92 ± 8[a]	85 ± 6[b]	< 0.001
Body mass index (kg/m^2)	24.9 ± 2.4	26.9 ± 2.6[a]	25.1 ± 2.1[b]	< 0.001
Fasting glucose level (mmol/L)	4.7 (4.5-5.2)	9.6 (7.6-11.8)[a]	8.4 (7.0-9.9)[a]	< 0.001
Fasting C peptide (nmol/L)	0.63 (0.42-0.94)	1.16 (0.69-1.39)[a]	0.70 (0.46-1.13)[a,b]	< 0.001
HbA1c (%)	5.5 (5.2-5.7)	9.2 (7.7-10.6)[a]	8.1 (7.2–9.4)[a]	< 0.001
HOMA2-IR	1.4 (1.0-2.0)	3.2 (1.8-3.8)[a]	2.0 (1.3-2.8)[a,b]	< 0.001
HOMA2-β	123.0 (103.2-164.3)	56.1 (34.5-79.6)[a]	47.4 (27.5-78.1)[a,b]	< 0.001
Total cholesterol (mmol/L)	4.1 ± 0.7	4.8 ± 1.1[a]	4.4 ± 1.2	0.049
Triglycerides (mmol/L)	1.5 (0.9-2.1)	3.1 (2.0-3.8)[a]	1.7 (1.3-2.6)[b]	< 0.001
HDL-C (mmol/L)	1.3 ± 0.3	0.9 ± 0.3[a]	1.0 ± 0.4[a,b]	< 0.001
LDL-C (mmol/L)	2.3 ± 0.8	3.0 ± 0.9[a]	2.8 ± 0.8[a]	< 0.001
Visceral adiposity index	1.7 (1.1-2.6)	5.3 (3.6-7.2)[a]	2.4 (1.3-3.8)[a,b]	< 0.001

a, Compared with NGT group, *P* <0.05; b, Compared with T2D group, *P* <0.05; Data are reported as means ± standard deviation, number (%) or median (interquartile range). T2D, type 2 diabetes; LADA, latent autoimmune diabetes in adults; NGT, normal glucose tolerance; HbA1c, haemoglobin A1c; HOMA2-IR, homeostasis model assessment 2 insulin resistance index; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

to 3 experiments' means. Primers employed in qRT-PCR are presented as follows:

Target	Primer (5' → 3')
n342533	Forward, TGGCTGTTCTGAAGAGTGTTACTG
	Reverse, GCCAGAGGGTCTGTAGTTCTGAT
n335556	Forward, TTCTTGGAATTGGCCTCCTG
	Reverse, GATCCGTGCTCCGACAAATA
n336109	Forward, CACCCTGGAGCTGGTAGTGAC
	Reverse, TTCAGTGCCGTATGGCTGTG
ACTB	Forward, ACTGGGACGACATGGAGAAA
	Reverse, TAGCACAGCCTGGATAGCAA

Statistical analysis

With the use of SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA), we conducted all analyses, and a *P* value of < 0.05 was considered statistically significant. Data were expressed as median or mean ± SD (interquartile range) as appropriate. With one-way ANOVA or Mann-Whitney U test as appropriate, the comparison between groups was drawn. Categorical variables were reported as numbers (%), and to assess categorical variables distribution, the chi-square test was performed. For examining the association between biochemical parameters and lncRNA expression levels, we employed Spearman's rank correlation coefficient.

Results

Patient demographic and metabolic characteristics

357 subjects were recruited in total, including 156 cases with T2D (97 men and 59 women), 101 patients with LADA (56 men and 45 women), and 100 patients with NGT (57 men and 43 women). **Table 1** lists patients' metabolic and demographic features in the three groups. Diastolic blood pressure, waist circumference, BMI, fasting glucose levels, fasting C-peptide levels, HbA1c, HOMA2-IR, LDL-C, HDL-C, triglycerides, total cholesterol, HOMA-β, and VAI were significantly different (P < 0.05). Other variables, such as gender distribution, age, and systolic blood pressure were comparable (P > 0.05).

Comparison of n336109, n342533, n335556 expression levels in each group

n336109, n335556 and n342533 expression levels were higher in the T2D group, compared with those in the NGT group (P < 0.05) (**Figure 1A-C**). n342533 and n335556 expression levels were higher in the T2D group than those in the LADA group (P < 0.05) (**Figure 1A**, **1B**). Compared with those in the NGT group,

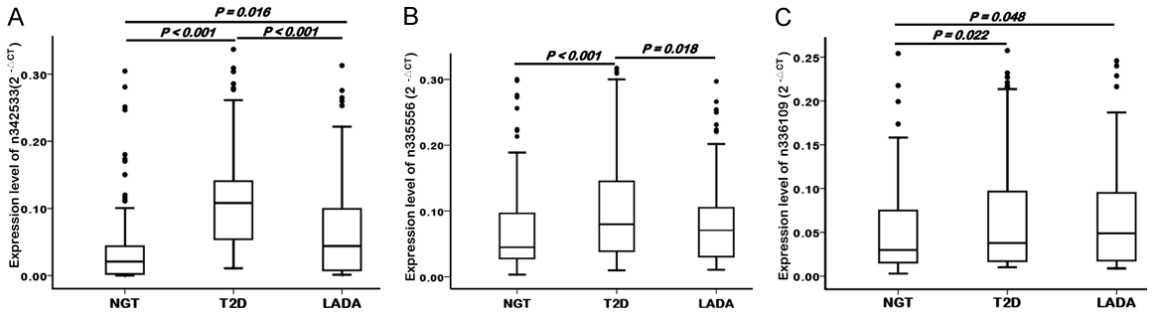

Figure 1. Comparison of n342533, n335556 and n336019 expression levels in T2D, LADA and NGT groups. The expression levels of 342533 (A), n335556 (B) and n336019 (C) in the T2D group was higher than those in LADA and NGT groups.

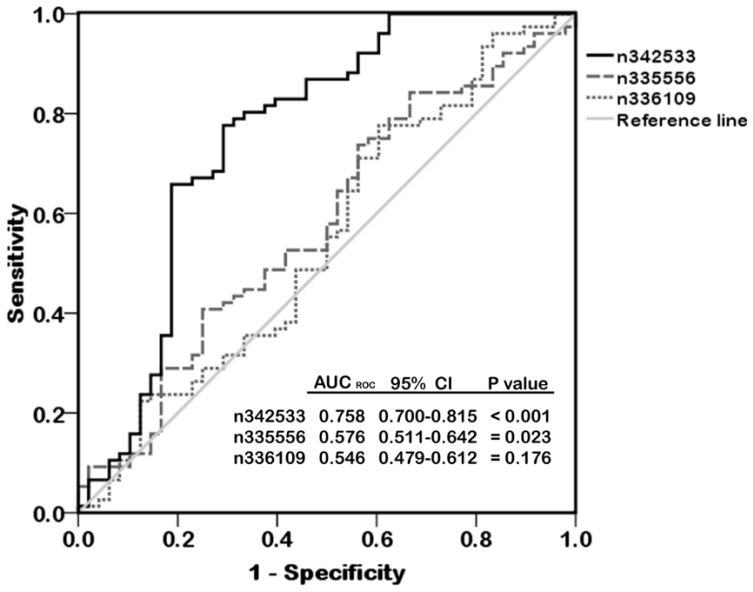

Figure 2. ROC curve showing the relationship between sensitivity (true positive) and one-specificity (true negative) in determining the discriminatory ability of n342533, n335556 and n336019 as predictors for LADA vs. T2D.

n342533 and 336109 expression levels were higher in the LADA group (P < 0.05) (**Figure 1A**).

n342533 helps to discriminate T2D and LADA patients

Diagnostic models were constructed with expression levels of n342533, n335556 and n336109. We noted that n342533 had the best discriminatory ability in the T2D group versus the LADA group (AUC ROC = 0.758; P < 0.001) (**Figure 2**).

T2D, LADA and NGT groups distribute in different tertiles of n342533

We stratified the expression level of n342533 in low, middle, and high tertiles. 62.8% patients in T2D group were in the high tertile. Patients in LADA group were mainly in the middle (42.6%) and low (40.7%) tertiles, while 59.7% in NGT group were distributed in the low tertile. The tertile distribution of n342533 was significantly different in each group (P < 0.05) (**Figure 3**).

Associations between expression of n342533 and metabolic parameters

Results of the univariate analysis for the association between expression level of n342533 and metabolic parameters of patients with diabetes are presented in **Table 2**. The n342533 expression level was proportional to waist circumference, BMI, fasting glucose level, fasting C-peptide level, HOMA2-IR, triglycerides, and visceral adiposity index values (P < 0.05), and correlated with HDL-C in a negative manner. After age, body mass index and waist circumference were adjusted, expression level of n342533 remained proportional to fasting C-peptide level, HOMA2-IR, triglycerides, and visceral adiposity index values, and negatively correlated with HDL-C (P < 0.05).

Discussion

n342533, n335556 and n336109 expression levels in T2D, LADA and NGT patients were compared here. Patients with T2D were proved to have increased expression level of n342533 compared with LADA and NGT. Diagnostic mod-

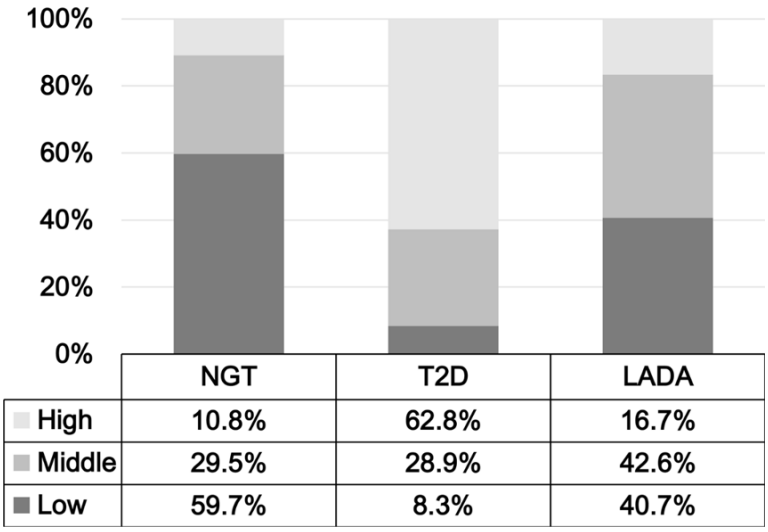

Figure 3. The expression level of n342533 was stratified into low, middle and high tertiles and T2D, LADA and NGT groups distributed in different tertile respectively.

Table 2. Univariate associations between expression of n342533 and metabolic parameters

Metabolic parameters	n342533	
	r	*P* value
Waist circumference	0.275	< 0.001
Body mass index	0.196	< 0.001
Fasting blood glucose	0.149	0.003
Fasting C peptide	0.157	0.002
HOMA2-IR	0.189	< 0.001
Triglycerides	0.328	< 0.001
HDL-C	- 0.191	< 0.001
Visceral adiposity index	0.376	< 0.001
Adjusted age, waist circumference and body mass index		
Fasting C peptide	0.152	0.003
HOMA2-IR	0.163	0.002
Triglycerides	0.286	< 0.001
HDL-C	- 0.153	0.003
Visceral adiposity index	0.332	< 0.001

HOMA2-IR, homeostasis model assessment 2 insulin resistance index; HDL-C, high density lipoprotein cholesterol;

els demonstrated that n342533 had the potent discriminatory ability in the T2D compared to the LADA group. Furthermore, we stratified the expression level of n342533 in low, middle, and high tertiles and observed that T2D, LADA and NGT groups were separately distributed in different tertiles. In addition, we performed univariate analysis to evaluate the association between expression level of n342533 and metabolic parameters and observed that n342533 expression level was proportional to fasting C peptide, triglycerides, HOMA2-IR, and visceral adiposity index values, and negatively correlated with HDL-C. As far as we know, this study is the first to evaluate the discriminatory ability of long lncRNA in T2D and LADA patients.

LADA is a subtype of autoimmune diabetes with slow β-cell destruction. Recent studies suggested that LADA is the most common adult-onset autoimmune diabetes [2, 9]. LADA shares clinical features of both classical T1D and T2D [10]. Although T1D and LADA have similar autoantibodies, it was easier to distinguish between T1D and LADA because of their differences in the age at onset of diabetes and β-cell function. However, LADA cases are more similar to T2D than T1D cases [3, 11]. Early-stage LADA is hard to identify from T2D based on clinical phenotype. Several studies reported that a considerable proportion of patients diagnosed with T2D were initially detected with T1D-associated autoantibodies [2, 9, 12]. In this study, clinical and metabolic features of the LADA group, such as age, blood pressure, glycometabolism, lipid profile, were similar to the T2D group, which was coincident with previous reports [10, 12-14]. A percentage of patients with LADA, especially those with high titer of GADA, would require early insulin therapy [15]. Owing to misdiagnosis, most patients with LADA were initially not appropriately treated. Early diagnosis of patients with LADA may result in more frequent and focused follow-up as well as early administration of insulin therapy [16], which may facilitate improved and early

glycemic control as well as preservation of residual β-cell function [17, 18]. At present, the diagnosis of LADA mainly relies on the circulating islet autoantibodies; however, there is no cut-off titer of these antibodies for distinguishing between T1D and LADA. Novel biomarkers for early detection LADA would greatly improve the care of such patients.

In the recent decade, a growing list of lncRNAs involved in glucose homeostasis and the development of diabetes is emerging [19-22]. In addition, lncRNAs may function as diagnostic markers for clinical applications in the management of diabetes [23]. Our previous study found that lncRNAs are expressed aberrantly in Han Chinese T2D patients, and that n342533, n335556, and n336109 were the top three upregulated lncRNAs in T2D patients [5]. Here, the three lncRNAs in T2D, LADA and NGT individuals were tested, and it was observed that these three lncRNAs' expression levels were different in each group. We hypothesized that these lncRNAs may help differentiate between T2D and LADA. Furthermore, we prepared diagnostic models, which showed that n342533 had good discriminatory ability in the T2D compared to the LADA group. Previous studies reported some novel biomarkers of LADA. Wen et al. [24] reported that glycoprotein phospholipase D1 (GPLD1), which is upregulated in LADA, served as a potential candidate plasma protein to ascertain early-stage LADA and T2D. Esmeralda et al. [25] reported that low-grade inflammatory markers may help differentiate between T2D and LADA; however, their study was mainly conducted in overweight or obese patients and they did not include a normal control group. Here, we showed that n342533 expression level fell in low, middle, and high tertiles and observed that T2D, LADA and NGT were separately distributed in different tertiles. This indicated that n342533 had the potential to distinguish between T2D and LADA. However, because the expression level of n342533 was tested by fluorescent quantitative PCR, which is a relatively quantitative approach, the cut-off value could not be determined at present.

LncRNA n342533 is located in the positive strand of chromosome 21 (start site 39601884, end site 39663669) and consists of 202 nucleotides. Its newest ID is NONHSAT082081 in NONCODE v5.0 (https://www.bioinfo.org/NONCODE2016). It is mainly expressed in the kidneys, thyroid, and white blood cells in humans [26]. At present, there are no studies on the function of n342533. In our study, it was found that n342533 expression level was associated with some glucolipid metabolism index, like HDL-C, triglycerides, fasting C peptide, HOMA2-IR, and visceral adiposity index. It indicates that n342533 may be related to lipid metabolism and glucose regulation, insulin resistance or islet β cell function. Visceral obesity and hypertriglyceridemia is strongly correlated with insulin resistance [27, 28]. It was reported that some lncRNAs promoted insulin sensitivity [29, 30]. Future studies should investigate whether n342533 participates in the pathophysiological process of insulin resistance.

The present study has some limitations. First, the expression level of n342533 was tested by a relatively quantitative approach, so that the cut-off value of LADA versus T2D could not be determined based on the present data. Second, this study was a cross-sectional design, which could not explain the direction of causality, e.g., change of lncRNA n342533 expression and insulin resistance. Third, this study has relatively small sample size, which may result in a reduced statistical power. Therefore, replication and verification of our present results in larger cohorts are necessary in the future.

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Disclosure of conflict of interest

None.

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