Influence of rs1292037 Genetic Variant on miR-21 Gene Expression in Patients with type 1 Diabetes Mellitus

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Abstract

Background

MicroRNAs (miRNAs) are small non-coding RNA molecules that play a pivotal role in the central dogma of molecular biology by regulating gene expression. Alterations in the expression pattern of miRNAs are seen to be linked with several human diseases including autoimmune diseases such as pediatric type 1 diabetes mellitus (T1DM). Single nucleotide polymorphism (SNP) of the miRNAs coding genes can influence pancreatic development and insulin secretion. We contemplated a relation between *miR-21* expression level as well as *miR-21 rs1292037* SNP and pediatric T1DM.

Results

The heterozygous T/C genotype was seen to be more common amongst T1DM patients than amongst controls (OR = 2.74 (1.78-4.27), P<0.0001). The C allele was more frequent in patients than in control subjects (OR = 1.36 (1.03-0.8), P = 0.02). *miR-21* expression was seen to be upregulated in patients compared to the controls by more than twofold (p<0.0001). In the study population, *miR-21* was found to be significantly upregulated when carrying the T/C genotype.

Conclusions

We report that the miR-21 *rs1292037* variant is related to T1DM. Our study also suggests that the *miR-21* expression level is upregulated in T1DM patients compared to the control subjects.

1- Background

Type 1 diabetes mellitus (T1DM) is a multifactorial autoimmune disease with long-lasting and extreme impacts on individuals, their families, and society (1). T1DM occurs with selective loss of pancreatic β-cells, which leads to insulin deficiency (2). T1DM can severely affect multiple organs including the heart, kidney, and nerves (3). It has been well-documented that genetic factors play a pivotal role in the incidence and pathogenicity of T1DM (4). The epidemiological studies on pediatric T1DM have raised concerns due to the increasing rate of its prevalence in the world (5). It is estimated that 0.3% of children are diagnosed with T1DM in the United States, annually (6). It has been well-established that early diagnosis of T1DM can significantly prevent T1DM complications and would increase the effectiveness of T1DM treatments including stem cell transplantation and β cells regeneration strategies (7, 8).

microRNAs (miRNAs) are endogenous, evolutionary conserved single-stranded molecules with about 22 nucleotides; miRNAs act in cell communication via regulating gene expression by base-pairing with different sites of the transcriptome, mainly the 3' untranslated region (3'UTR) of their target mRNAs (9). Studies have illustrated that miRNAs play a significant role in the development and proper function of β cells (10).

Single-nucleotide polymorphisms (SNPs) are the most common and basic form of genetic mutations, which are well-contemplated, and can induce devastating phenotypic outcomes (11). Studies have vastly reported that SNPs in the miRNAs coding genes can affect the binding affinity and binding ability of the miRNAs and even can alter the target mRNA (12). miRNAs-related SNPs are known to be associated with the incidence of several diseases including T1DM (13). Limited studies have investigated the association between miR-21 *rs1292037* SNP and human-related diseases to this date. In a study on cervical cancer patients by Zhang and their colleagues, it is demonstrated that miR-21 *rs1292037* SNP decreases the cumulative survival of patients by almost 30%. In the same study, miR-21 *rs1292037* was seen to increase the chemoresistance in patients (14). However, no relation was detected between this reference SNP and either breast cancer or ischemic stroke (15, 16). Furthermore, alteration in the expression level of miR-21 is reported to be linked with the incidence of T1DM by inhibiting T cells apoptosis, which consequently can induce autoimmunity (17). Considering these data, we speculated that miR-21 *rs1292037* T > C may be associated with the incidence of pediatric T1DM. To evaluate this speculation, we employed molecular genetic methods in a case-control study.

2- Results

Features of the Study Subjects

The study is comprised of 250 T1DM patients and 250 control subjects. The clinical and biochemical characteristics are depicted in Table 1. There were no statically significant differences in age, gender, and body mass unit. Nonetheless, the hemoglobin A1C was significantly higher in T1DM patients compared to the control subjects. The mean age of diagnosis in T1DM patients was observed to be 8.119 ± 3.69. Polyuria, polydipsia, and lethargy conditions were detected in 100%, 96.8%, and 61.2%, respectively. 93.6% of patients reported significant weight loss, and 64.4% of them disclosed abdominal pain.

Table 1
Clinical and biochemical characteristics of the study population.

Variables	Case n (%)	Control n (%)	P-value
Age	10.26 ± 3.57	10.81 ± 3.58	0.11
Gender	114 (45.6%)	116 (46.4%)	0.19
Male	136 (54.4%)	134 (53.6%)	0.07
Female			
BMI[a]	16.67 ± 1.61	17.00 ± 1.60	0.29
Hemoglobin A1C	9.64 ± 1.65	5.13 ± 0.26	< 0.0001
Age of Diagnosis	8.119 ± 3.69		
Family History of Diabetes	24 (9.6%)	3 (1.2%)	< 0.0001
Type 1	137 (54.8%)	15 (6%)	< 0.0001
Type 2	89 (35.6%)	232 (92.8%)	< 0.0001
None			
Polyuria	250 (100%)		
Yes	0 (0%)		
No			
Polydipsia	242 (96.8%)		
Yes	8 (3.2%)		
No			
Weight Loss	234 (93.6%)		
Yes	16 (6.4%)		
No			
Abdominal Pain	161 (64.4%)		
Yes	89 (35.6%)		
No			

[a]BMI= Body mass unit

Variables	Case n (%)	Control n (%)	P-value
Lethargy	153 (61.2%)		
Yes	97 (38.8%)		
No			
[a]BMI= Body mass unit			

miR-21 rs1292037 is Associated with T1DM

Table 2 depicts detected genotypes as well as allele frequencies with their estimated ORs. In a codominant model, the T/C genotype was discovered significantly more frequent amongst T1DM patients (32.4%) than amongst controls (14.8%). In a dominant model, the frequency of the T/C + C/C genotypes was significantly higher in the T1DM subjects (43.6%) when compared to the controls (32%). In a recessive model, the T/C genotype was found to be significantly associated with the incidence of T1DM (OR = 2.74 (1.78–4.27), P < 0.0001). The minor C allele was significantly more prevalent in patients than controls (OR = 1.36 (1.03–0.8), P = 0.02).

Table 2
Genetics of miR-21 SNP in patients and controls

Model	Patients N (%)	Controls N (%)	OR (95% CI)
Codominant genotype			
T/T	141 (56.4%)	170 (68%)	1.00
T/C	81 (32.4%)	37 (14.8%)	2.63 (1.68–4.13)[a]
C/C	28 (11.2%)	43 (17.2%)	0.78 (0.46–1.32)[b]
Dominant genotype			
T/T	141 (56.4%)	170 (68%)	1.00
T/C + C/C	109 (43.6%)	80 (32%)	1.64 (1.14–2.36)[c]
Recessive genotype			
T/T + T/C	222 (88.8%)	207 (82.8%)	1.00
C/C	28 (11.2%)	43 (17.2%)	0.6 (0.36–1.01)[d]
Over-dominant genotype			
T/T + C/C	169 (67.6%)	213 (85.2%)	1.00
T/C	81 (32.4%)	37 (14.8%)	2.74 (1.78–4.27)[a]
Allele			
T	363 (72.6%)	377 (75.4%)	1.00
C	137 (27.4%)	123 (24.6%)	1.36 (1.03–1.8)[e]

[a]p<0.0001, [b]p=0.36, [c]p=0.007, [d]p=0.056, [e]p=0.02. OR = Odds ratio, CI = Confidence interval

miR-21 expression is upregulated in T1DM patients

Quantitative real-time PCR (qRT-PCR) was used to determine the relative expression of the miR-21 gene in the T1DM patient and control groups. The acquired data revealed a significant difference in the *miR-21* expression level between T1DM patients and normal controls. As illustrated in Fig. 1, the mean expression level of *miR-21* in the control subjects and T1DM patients was calculated to be 1 and 2.34, respectively. The results stipulate that the miR-21 gene is significantly increased in T1DM patients compared to the control group by nearly twofold ($p < 0.0001$). In the study population, those who carried the T/C genotype were recognized to have a significantly higher *miR-21* expression (Fig. 2).

3- Discussion

miR-21 (*rs1292037*) SNP was determined in 250 T1DM patients and 250 population-matched controls. We report a significant association between the *miR-21 rs1292037* T/C genotype and the incidence of T1DM. It was also found that *miR-21* is significantly overexpressed in T1DM patients comparing to the control group. Moreover, this study demonstrated that *miR-21* was significantly upregulated in subjects with the T/C genotype.

MiRNAs, as regulators of gene expression, are seen to contribute to several pathological conditions including T1DM (18). SNPs in the miRNAs coding gene are reported to affect the biological function of these non-coding RNAs, which can lead to the incidence of numerous human diseases (19). Multiple studies have investigated the role of miRNAs-relates SNPs in the incidence and prognosis of T1DM. For instance, miR-196a *rs11614913* is reported to be associated with the pathogenesis of T1DM (13). Moreover, studies have shown that SNPs in miRNAs coding genes can determine the development of pancreatic cells and insulin secretion. For instance, Wang et al. have detected a link between *miR-124a rs531564* and *miR-27a rs895819*, and pancreatic development, adipocyte differentiation, and insulin secretion (20). It is observed that miR-21 targets genes that act in cell proliferation and cell apoptosis including *Ras* and *Pdcd4* (21). SNPs in the miRNA coding genes are seen to affect the binding affinity and binding ability of miRNAs, which can consequently affect the expression of the miRNA target genes (9). Although miR-21 *rs1292037* is seen to be correlated with the prognosis of hepatocellular carcinoma and cervical cancer, there is no reported evidence on the association of this reference SNP and T1DM (14, 22).

In addition to the critical role of miR-SNPs in the incidence and prognosis of autoimmune diseases such as T1DM, alteration in the expression level of miRNAs in autoimmune diseases is manifested by several researchers (23). In a research conducted by Mostahfezian et al., it is reported that miR-21 is upregulated in T1DM patients. They postulated that upregulated levels of miR-21 inhibit T cells apoptosis, which leads to autoimmunity (17). Compelling evidence has shown that upregulation of miR-21 can expose β cells to proinflammatory cytokines. This unfortunate phenomenon promotes cell death, which stimulates T1DM (24). Upregulation of miR-21 in T1DM patients is also reported by Fouad and their colleagues. Analyzing the plasma of T1DM patients, they tracked the upregulated levels of miR-21. Moreover, they claimed that in the first 5 years of the disease onset, when is even sooner than the development of microalbuminuria, upregulated levels of this miRNA can be detected (25).

Analyzing miRNAs expression level and/or miRNAs-related SNPs have been proposed as a biomarker in medicine and biotechnology for the early detection of diseases with genetic backgrounds (26, 27). Regarding T1DM, the early detection of this disease not only increases the quality of life in patients but also elevates the possibility of preventing T1DM complications such as heart disease, foot ulcer, retinopathy, and neuropathy (28). More importantly, early detection of T1DM can positively affect the honeymoon period (a transition remission phase after the commencement of insulin treatment that insulin doses can be significantly decreased or even totally withdrawn) (29). In this case, the specialists

will be given more time to take considerable actions (e.g., adjunctive therapy) to prolong the reduction of residual β cell function in T1DM patients (30). Taken together, the results of this study suggest that *miR-21 rs1292037* T/C genotype can be considered as a putative biomarker for early detection of T1DM. Moreover, analyzing *miR-21* expression patterns can be also evaluated for this purpose.

Some limitations should be considered when the acquired data are analyzed. Firstly, this study included 250 T1DM patients and 250 control subjects, and therefore, expanded research with a larger case and control population is recommended. Secondly, this research was limited to the Iranian population, and the authors realize that there might be differences in this SNP in the T1DM characteristics of distinct populations. For this reason, we recommend further investigations into other ethnic/racial groups.

4- Conclusions

In conclusion, after genotyping 250 patients with T1DM and 250 control subjects, we discovered that *miR-21 rs1292037* T/C genotype is significantly linked to the incidence of T1DM. Additionally, we observed that *miR-21* is significantly overexpressed in T1DM patients when compared to control subjects. We also report that the *miR-21* level is seen to be significantly elevated in the study subjects who carried the T/C genotype.

5- Materials And Methods

5.1- Subjects

We designed a case-control study involving 250 randomly-selected patients diagnosed with T1DM and 250 randomly-chosen healthy control subjects, from 17 Shahrivar hospital, Rasht, Iran. The peripheral venous blood from subjects was collected in EDTA-containing tubes. T1DM was diagnosed based on the criteria published by the American Diabetes Association in 2014 (31). Cases with at least 6 months of T1DM with concomitant insulin injection were included and patients with severe complications of T1DM such as nephropathy were excluded. All healthy individuals were interviewed in order to collect information regarding the clinical and biochemical characteristics of the study subjects. Control subjects with a history of autoimmune diseases and/or pathological findings were excluded. The two groups were age-matched: the mean [SD] for cases and controls are 10.26 [3.57] and 10.81 [3.58], respectively (P = 0.112). The study was performed according to the principles of the 1964 Helsinki declaration and its further amendments.

5.2- Methods

Genotyping

The genomic DNA was extracted from peripheral blood samples of all study subjects using Triton X-100. The concentration and purity of the extracted DNA were evaluated by the NanoDrop spectrophotometer

(Thermo Fisher Science, USA). Genotyping of *rs1292037* was achieved using polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP). Forward and reverse primers of miR-21 *rs1292037* were 5'-ACTGTCTGCTTGTTTTGCCTA-3' and 5'- TGAAAGAGATGAACCACGACT-3', respectively. The PCR amplification was performed in a 20 µl reaction volume consisting of 3 µl of the extracted DNA (30 ng/µl), 10 µl of Taq DNA polymerase Master Mix Red (Ampliqon, Denmark), and 1 µl (10 pmol/µl) of each primer (forwards and reverse). Samples were subjected to amplification in an MJ Mini thermocycler (BioRad, USA). The following PCR condition was employed for this purpose: an initial denaturation step at 94°C for 5 min, amplification for 35 rounds at 94°C for 45 sec, 57°C for 45 sec, and 72°C for 45 sec, followed by an elongation step at 72°C for 5 min. The PCR products (540 bp) were then analyzed by 1% agarose gel electrophoresis in Tris-Boric acid-EDTA buffer and stained by RedSafe Nucleic Acid Staining Solution (Boca Scientific, USA). In order to detect allelic variations, the amplicons were digested by the *Tsp*RI restriction enzyme (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. After digestion with *Tsp*RI, the PCR product was cut into 338 bp and 202 bp fragments in the presence of the C allele whereas the T allele remained uncut (540 bp). The enzyme-digested products were then separated on 2% agarose gel and stained by RedSafe.

RNA extraction and quantitative Real-Time PCR

miRNAs were isolated from the peripheral blood using the SanPrep Column microRNA Mini-Prep Kit (Bio Basic, Canada) according to the manufacturer's instruction. RNA integrity was analyzed using 2% agarose gel electrophoresis. RNA purity was then assessed using a NanoDrop spectrophotometer.

The complementary DNA (cDNA) was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with a miR-21 stem-loop primer (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATCAACA-3'), according to the manufacturer's guideline. This stem-loop primer was specifically designed for this study. The process then was followed by reverse transcription- PCR (RT-PCR).

qRT-PCR was performed to evaluate the expression level of miR-21 in T1DM patients and control subjects. Genotyping was performed in a 20 µl reaction mixture consisting of 1 µl of cDNA (2µg/µl), 10 µl of SYBR-Green 2X Mastermix (TB Green Premix Ex Taq II Tli RNase H Plus; Takara, Japan), 1 µl (5pmol/µl) of each specific forward and reverse primers. Amplification was performed using an MJ Mini Thermo Cycler (BioRad, USA). Cycling conditions were 5 min incubation at 95°C, 45 cycles of 95°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec, followed by an extension time of 5 min at 72°C. The *U48* was used as the endogenous control to normalize the expression value of miR-21. The used primers are as follows: *miR-21* (F): 5'-GGTGTAGCTTATCAGACTGATG-3', *miR-21* (R): 5'-AGGGTCCGAGGTATTCGC-3', *U48* (F): 5'-GAGTGATGATGACCCCAGGTAA-3', and *U48* (R): 5'- GTGCAGGGTCCGAGGT-3'. All reactions were performed in triplicate. The fold changes in the expression level were calculated by the $2^{-\Delta\Delta CT}$ method (32).

Statistical Analysis

The chi-squared (χ^2) test was used to evaluate the differences in the genotype and allelic distribution between T1DM patients and healthy controls. The Hardy-Weinberg Equilibrium (HWE) was assessed by a goodness-of-fit χ^2 test. To investigate the strength of association, odds ratios (ORs) with 95% confidence intervals (CIs) were used. P-values less than 0.05 were considered statistically significant. SNP genotyping-related data were analyzed using MedCalc statistical software (version 19.5.3; Belgium). All other data and information were analyzed and studied using Graphpad Prism (version 8.0.2, USA).

List Of Abbriviations

T1DM: Type 1 diabetes mellitus

miRNAs: microRNAs

3'UTR: 3' untranslated region

SNPs: Single-nucleotide polymorphisms

PCR: Polymerase chain reaction

qRT-PCR: Quantitative real-time PCR

ORs: Odds ratios

CIs: Confidence intervals

Declarations

Ethics approval and consent to participate

All subjects, both patients and controls, signed written informed consent after receiving an extensive disclosure of the purpose of the study. The study was approved by the responsible ethical committee of Guilan University of Medical Sciences (Approval ID: IR.GUMS.REC.1397.427).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Competing interest

The authors declare no potential conflict of interest.

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Authors contribution

Reza Bayat contributed to the laboratory work and drafted the manuscript. Zivar Salehi defined the study protocol, drafted the manuscript, and supervised the whole project. Setila Dalili defined the study protocol and drafted the manuscript. Farbod Bahreini provided the final revision of the manuscript, and was in charge of the submission process. All the authors have given the final approval of the version to be published and they take responsibility for appropriate portions of the content.

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Figures

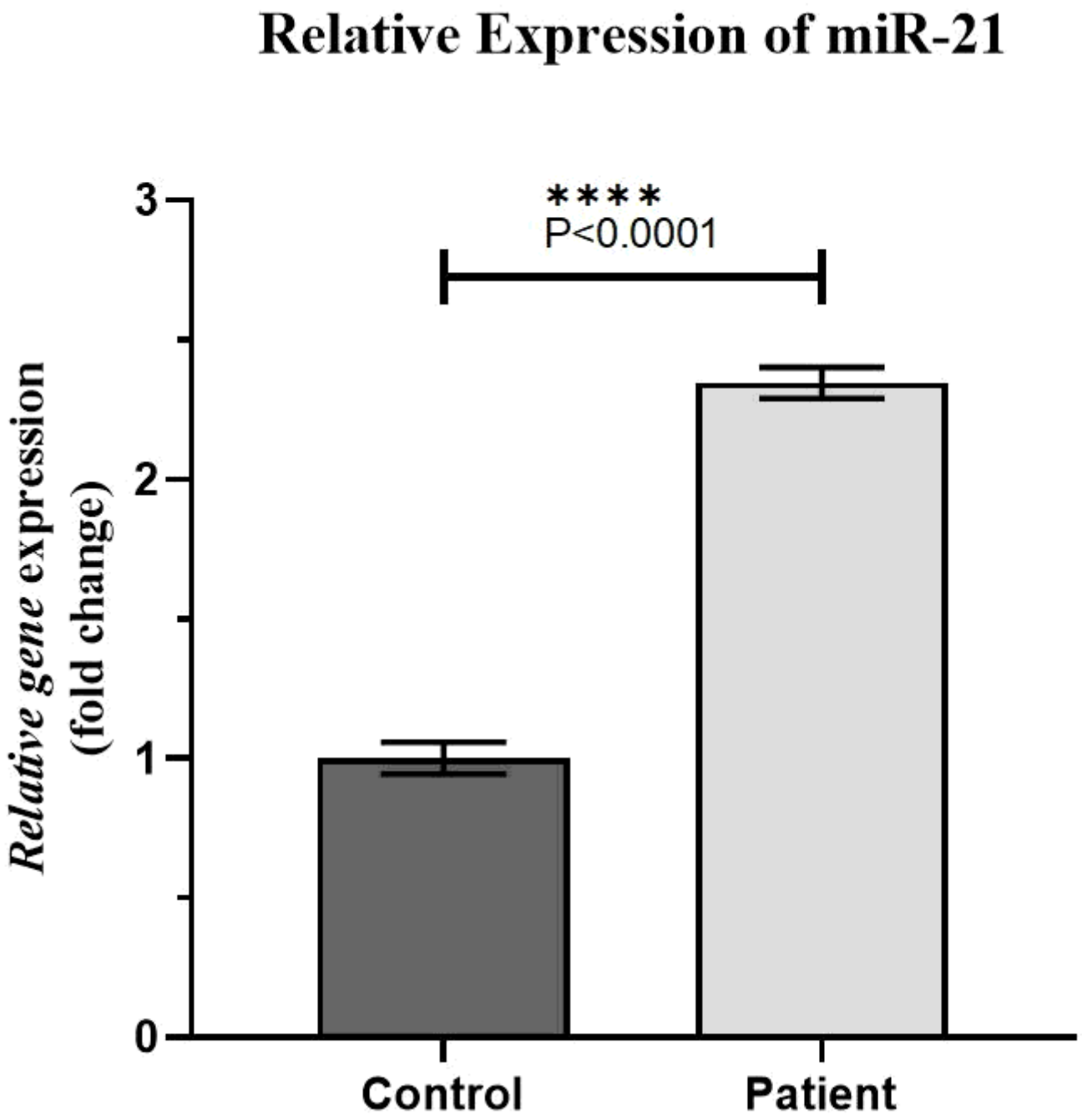

Figure 1

Relative gene expression of miR-21 in control subjects and T1DM patients. miR-21 is significantly upregulated in patients.

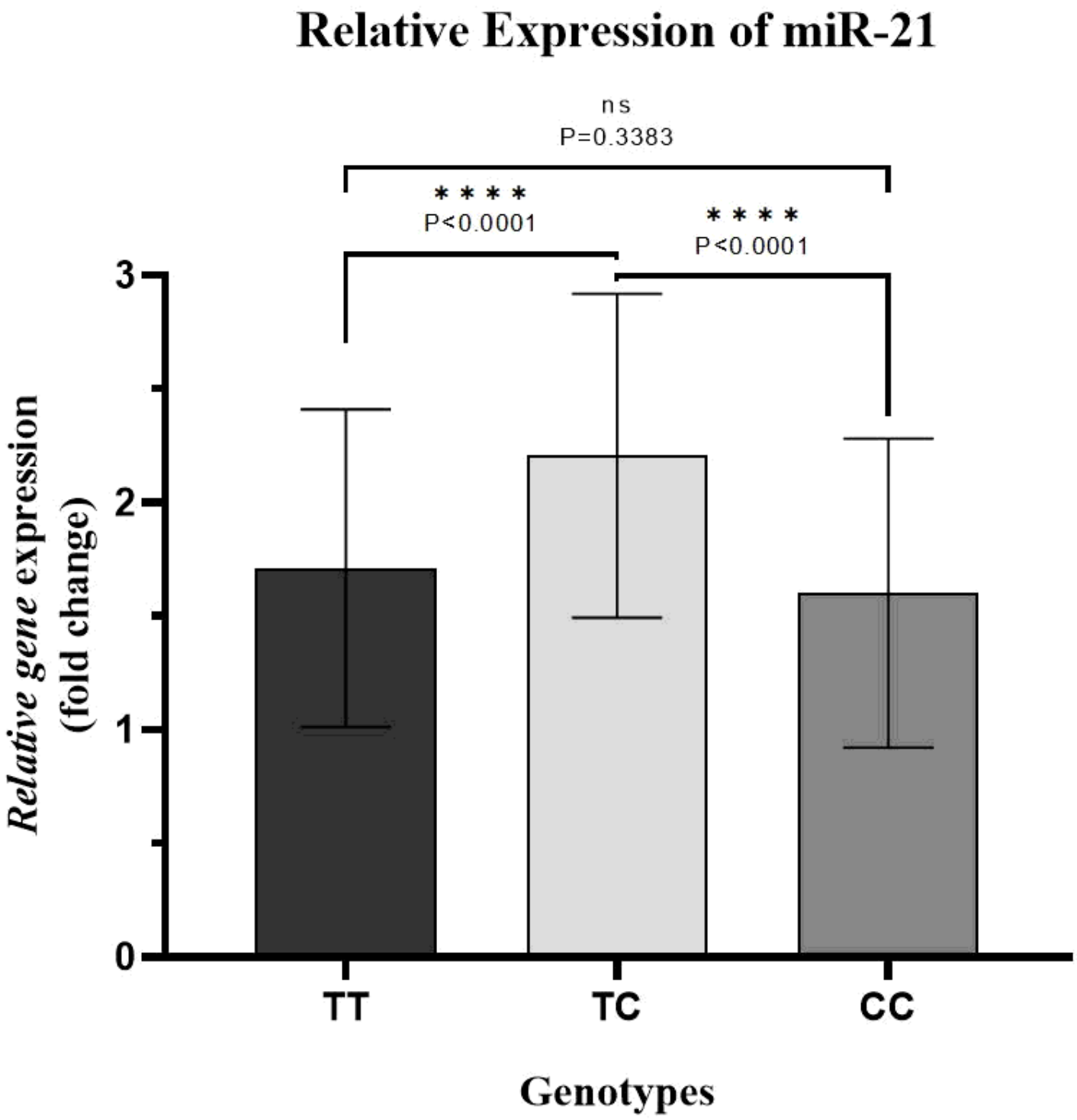

Figure 2

Relative gene expression of miR-21 in subjects carrying the T/T, T/C, or C/C genotypes. As illustrated, subjects with the T/C genotype have the highest significant miR-21 expression.