Exploring Markers to Classify and Evaluate Ketosis Onset Diabetes: a Randomized Clinical Trials

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Research

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Abstract

**Background:** There are many patients, who start with the symptoms of ketosis or ketoacidosis, but their clinical and genetic characteristics are distinct. In this research, we studied the clinical, immune, and genetic characteristics of ketosis-onset diabetes, and the relationship between the clinical characteristics, anti-aminoacyl-tRNA synthetase antibody, and microRNAs.

**Methods:** To study the clinical characteristics, 200 patients with newly onset diabetes mellitus along with ketosis or ketoacidosis were randomly selected. The male population in this study was 60.9%, with ages ranging from 7 to 72 years old, where 27% of the patients' body mass index was $\geq 25 \text{ kg/m}^2$ and the average HbA1c was $10.4 \pm 1.81\%$. Simultaneously, 50 healthy people with similar age and gender were chosen as the control group. Patients were divided into four subgroups based on the existing autoimmune antibodies and the preservation of $\beta$-cell function. The expression of microRNA-191 (miRNA-191) and microRNA-342 (miRNA-342) in plasma was detected by quantitative fluorescence PCR.

**Results:** The frequency of anti-histidyl-tRNA synthetase antibody in A+ $\beta+$ and A+ $\beta$- subgroups was significantly higher than that in the other subgroups. There were no significant differences in the concentration of the anti-glycyl-tRNA synthetase antibody between the groups, but the frequency was higher in the A+ subgroup. The expression of miRNA-191 and miRNA-342 in plasma was up-regulated compared to the normal control group.

**Conclusion:** The anti-histidyl and anti-glycyl-tRNA synthetase antibodies can be used as immunological markers to distinguish the subgroups of ketosis-onset diabetes mellitus, where anti-histidyl-tRNA synthetase antibody was more significant, comparatively. The miRNA-191 and miRNA-342 in plasma are related to ketosis-onset diabetes and can be used as biomarkers to predict ketosis-onset diabetes in the future.

Introduction

At present, the classification of diabetes is based on the concept and classification method of the American Diabetes Association (ADA) and the World Health Organization (WHO). Diabetes can be divided into four types: type 1 diabetes (including two subtypes- autoimmune and idiopathic), resulting mainly from $\beta$ cell dysfunction; type 2 diabetes, resulting from insulin resistance and/or $\beta$ cell dysfunction; Gestational diabetes; and other special types of diabetes [1]. The onset of diabetic ketoacidosis (DKA) is regarded as the clinical characteristic of type 1 diabetes. Therefore, patients with DKA or ketoacidosis are often considered deficient in islet secretion. However, in recent years, some diabetic patients in the epidemiological investigation, at home and abroad, started with ketosis or ketoacidosis but had clinical manifestations, treatment, and the outcome similar to type 2 diabetes [2, 3–8] and were known as obese ketoacidosis patients [4], ketosis-prone type 2 diabetics, or even type 3 diabetics [8] by the researchers. Clinically, type 2 diabetes, adult latent autoimmune diabetes, and idiopathic type 1 diabetes can also start with ketosis or ketoacidosis, but their clinical characteristics, treatment plan, and development of the final
disease are quite distinct. The purpose of this study is to explore the pathogenesis of these patients, find simple and effective classification indexes, clarify the classification attribution, and guide clinicians to diagnose, treat, and predict the outcome of the disease.

**Experimental Subjects And Research Methods**

1.1 Subjects: were patients in China, who were newly diagnosed with diabetic ketosis. Also, a set of healthy people were randomly selected for the control group (the ethics number is 2019276 in Xinxiang Medical College).

1.2 Inclusion criteria for the ketosis-onset diabetic group were as follows [9, 10, 11]:

- The first symptom of the patient was ketosis or ketoacidosis.

Diagnosis of the newly onset diabetes was confirmed by clinical symptoms and laboratory examinations after admission, which was typical polyuria, polydipsia, and also polydipsia within six months, and no more than six months after the onset of ketosis (more than 2+ urinary ketone body) or ketoacidosis.

There were no obvious triggers, such as infection, trauma, inflammation, etc.

Secondary diabetes caused by gestational diabetes, drugs, pancreatic exocrine diseases, and other endocrine diseases were excluded from the study.

1.3 Grouping: was done based on positive glutamic acid decarboxylase antibody (GADA) and Islet Antigen-2 (IA-2) antibody (where A+ means GAD Ab or IA-2 Ab were positive or more than one was positive, and A- means both were negative), and also on the preservation of \( \beta \) cell function (\( \beta+ \) means fasting C peptide > 0.56 µg/L, and \( \beta- \) means fasting C peptide < 0.56 µg/L). Based on this, patients were divided into four subgroups: A+\( \beta^- \)-, A-\( \beta^- \)-, A+\( \beta^+ \)-, and A\( \beta^- \)-group with 50 patients in each group. Simultaneously, 50 healthy people corresponding to the age and gender of the disease group were selected for the control group.

1.4 Specimen collection: when patients were admitted to the hospital, general information, such as age, gender, height, weight, medical history, and family history, were recorded. Also, indexes such as venous blood glucose, the urine ketone body, blood lipid, liver function, and kidney function were detected; samples for HbA1c, GAD Ab, and IA-2 Ab, were collected on an empty stomach, the next day morning. Within 24 h of correcting diabetic ketosis or DKA, fasting blood samples were collected to measure C-peptide or after one week of ketosis correction, islet function was measured.

1.5 Specimen preservation: Plasma: EDTA or heparin was used as an anticoagulant. The samples were centrifuged at 2–8°C at 1000 x g for 15 min within 30 min of blood collection, and stored at −20°C or −80°C, for further use. Repeated freeze-thawing was avoided. For MicroRNA enrichment, plasma (serum) was extracted within 2 h of blood collection, out of which 200 µL was used for the miRNA enrichment.
experiment using The miRcute_miRNA isolation kit. The enriched microRNA was stored at −80°C for subsequent use.

1.6 Detection of anti-aminoacyl-tRNA synthetase (ARS) antibody: was performed according to the instructions of human anti-histidyl-tRNA and human anti-tryptophan-tRNA synthetase ELISA kits.

1.7 miRNA detection:

1.7.1 Total RNA was extracted from whole blood or plasma, and then cDNA was prepared.

1.7.2 The reverse transcription primers were miRNA specific, synthesized by Tiangen biochemical technology (Beijing) Co. Ltd., and U6 was used as the internal reference gene. The gene sequence and primer sequences are as follows: MH-U6 5' ACACGCAAATTCGTGAAGCGTTCC 3', Hsa-miR-191–5p CGGAATCCCAAAGCAGC TG, Hsa-miR-342–3p TCTCACACAGAAATCGCACCC, Hsa-miR-510 GCTACTCAGG AGAGTGGCAATCAC. RNA was denatured at 70°C for 10 min before reverse transcription and then placed on ice for standby.

1.7.3 Quantitative fluorescence PCR: was performed using the miRcute-miRNA fluorescent quantitative kit, where U6 was used as the internal reference gene. This method was used to detect the expression of miR-342, miR-191, miR-510. A 20 µL reaction system was prepared using 1.5 µL cDNA, 1.6 µL of 2.5 nm upstream primer, 0.4 µL of 10 nm downstream primer, 10 µL miR, and 6.5 µL of water. Each sample had three replicates. The reaction conditions were as follows: 94°C for 1 min, then 40 cycles of initial denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

1.7.4 Data analysis: the target miRNA and internal reference (U6) for each sample were subjected to real-time PCR. Relative quantitative analysis was done using the $2^{-\Delta \Delta CT}$ method by analyzing the changes in miRNA expression represented by a ratio of miRNA expression in the patients to the normal group. When detecting relative expression variables of miRNA, U6 were used as an internal reference gene to unify the target genes also ensure the same quantity in the number of samples. The relative expression level of miRNA in plasma (serum) was calculated as follows: $RQ = 2^{-\Delta CT}$, where $RQ$ represents the relative quantity, $\Delta CT = \text{target gene CT} - \text{internal reference gene CT}$. Target gene CT and internal reference gene CT represent the CT value of target genes and internal reference gene in a sample; the miRNA expression level change between group A and group B was calculated by $RQ = 2^{-(\Delta \Delta CT)}$, where $\Delta \Delta CT = (\text{target gene CT} - \text{internal reference gene CT})$ group A - (target gene CT - internal reference gene CT) group B.

1.8 Treatments:

If a patient shows spontaneous ketosis, it is inferred that insulin in the body is very low, which needs exogenous insulin to meet the needs of the body; otherwise, the patient may develop a serious metabolic disorder that can even be life-threatening. In this study, all the patients in the disease group were given a continuous intravenous injection of human recombinant insulin until the urine ketone body was tested negative for 3 consecutive days. Insulin therapy was discontinued for at least one day before analyzing
the data. The target peripheral blood glucose level was 4.4–5.6 mmol/L. All the patients in the study were under a controlled diet during the hospitalization, and the calorie intake was 30 kcal/kg/24 h.

1.9 Statistical methods:

The data were analyzed by SPSS 19.0. Data processing of normal distribution was represented by the mean ± standard deviation (± s). Median and t-test were used for representing non-normal data, and group comparison, respectively. Analysis of variance was used for comparison between the groups. The P-value < 0.05 was statistically significant, and NS indicated no difference. Before statistical analysis, the relative expression level of miRNA, i.e., 2-ΔCT, was logarithmically transformed, and the transformed data was accorded with the normal distribution. The expression levels of miRNA in plasma and serum were compared by paired t-test. The relative expression level of microRNA was expressed by the difference between the threshold cycle number (Δ CT) of microRNA and the internal reference (U6).

Results

Add descriptive characteristics of the patients and controls here. Prepare a table for study characteristics too.

2.1 Ketosis-onset diabetes mellitus and anti-aminoacyl-tRNA synthetase (ARS) antibody

The results of this study showed that there was a significant difference in the concentration of anti-tRNA synthetase antibody between A+ β+ and A- β+, A+ β- and A- β- subgroups. The frequency of anti-tRNA synthetase antibody in the A+ β+ and A+ β- subgroups were significantly higher than that in the other subgroups, especially in the A+ β+ subgroups. There was no significant difference in the concentration of the anti-glycyl-tRNA synthetase antibody between the groups, but the frequency of the antibody was higher in the A+ subgroup. The results showed that both anti-tRNA and anti-glycyl-tRNA synthetase antibodies could be used as immunological markers to distinguish the subgroups of ketosis-onset diabetes mellitus. In addition, the anti-tRNA synthetase antibody group was more significant, comparatively (Table 1–3).
### Table 1
Clinical features of the ketosis-onset diabetes group and the control group

| Group                      | subgroup1 A+ β+ (n = 32) | subgroup2 A+ β- (n = 26) | subgroup3 A- β+ (n = 42) | subgroup4 A- β- (n = 44) | Control group (n = 50) |
|----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|------------------------|
| Average age (year)         | 38 ±11                   | 23 ±8                    | 46 ±8                    | 38 ±13                   | 43 ±9                  |
| Gender (male:female)       | 19:13                    | 10:3                     | 27:15                    | 7:4                      | 16:9                   |
| Diabetes family history (%)| 56%                      | 64%                      | 67%                      | 52%                      | 45%                    |
| Autoimmune disease (Number)| 2                        | 1                        | 0                        | 0                        | 0                      |
| Average BMI (kg/m^2)       | 22.7 ±2.5                | 20.2 ±2.1                | 24.1 ±2.6                | 21.9 ±3.9                | 25.4 ±2.7              |
| FPG (mmol/L)               | 18.9 ±7.1                | 16.8 ±4.8                | 14.1 ±2.8                | 17.8 ±4.5                | 6.5 ±1.1               |
| HbA1c (%)                  | 11.0 ±2                  | 12.4 ±2.9                | 10.3 ±2.0                | 10.8 ±2.1                | 6.6 ±1.1               |

### Table 2
Comparison between anti-histidyl-tRNA synthetases antibodies in the ketosis onset-diabetes group and the control group

| Anti-histidyl-tRNA synthetases antibodies | Subgroup1 A+ β+ (n = 32) | Subgroup2 A+ β- (n = 26) | Subgroup3 A- β+ (n = 42) | Subgroup4 A- β- (n = 44) | Control group (n = 50) |
|------------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|------------------------|
| Positive rate (%)                        | 37.5                     | 11.5                     | 7.1                      | 4.5                      | 1.4                    |
| Gender (male:female)                     | 7:5                      | 1:3                      | 2:1                      | 1:1                      | 3:4                    |
| concentration (ng/ml)                    | 10.7 ± 2.5               | 14.1 ± 2.9               | 13.1 ± 1.7               | 13.8 ± 2.2               | 6.9 ± 3.2              |

Subgroup 1 and 3, subgroup 1 and 4, P < 0.05. No significant differences among other groups.
Table 3
Comparison between anti-glycyl-tRNA synthetases antibodies in the ketosis onset-diabetes group and the control group

| Anti-glycyl tRNA synthetase antibodies | Subgroup1 (n = 32) | Subgroup2 (n = 26) | Subgroup3 (n = 42) | Subgroup4 (n = 44) | Control group (n = 50) |
|---------------------------------------|-------------------|-------------------|-------------------|-------------------|----------------------|
| Positive rate (%)                     | 9.4               | 7.7               | 4.7               | 2.3               | 2.0                  |
| Gender (male:female)                  | 7:5               | 1:3               | 2:1               | 1:1               | 3:4                  |
| concentration (ng/ml)                 | 4.39 ± 2.54       | 5.23 ± 2.93       | 3.31 ± 1.71       | 3.77 ± 2.20       | 3.57 ± 1.95          |

No significant differences in concentrations in each group, but the frequency of the A+ subgroup was higher.

2.2 Ketosis-onset diabetes and microRNA

In this study, the expressions of miRNA-191 and miRNA-342 were detected in the plasma of ketosis-onset diabetic patients along with healthy people. The results showed an up-regulation in the expression of miRNA-191 and miRNA-342 in the disease group. In addition, the expression of miRNA-342 in the A+β+ subgroup was significantly lower than that of the other three subgroups. These results suggest that miRNA-191 and miRNA-342 in plasma may be related to the pathological mechanism of ketosis-onset diabetes, and can be cited as biomarkers to predict ketosis-onset diabetes. Our research result is different from that of Renata Hezova and other researchers who studied the expression of microRNAs in regulatory T cells in type 1 diabetic patients, which showed down-regulation in miRNA-191 and miRNA-342. This also shows that ketosis-onset diabetes is not similar to type 1 diabetes, and its pathogenesis and pathological manifestations have their unique characteristics (Table 4–5, Fig. 1: amplification curve).

Discussion

Endocrinologists have found that diabetes mellitus with ketosis is often difficult to treat, and its classification is a concern. At present, most researchers recommend the “Aβ” classification system to classify ketosis-onset diabetes mellitus, which considers both autoimmune response and the recovery of β cell function. Although, inflammatory response plays an important role in the pathogenesis of ketosis-onset diabetes is still an open question. Hence, all these issues pose a challenge for clinicians to diagnose and treat the disease.

Anti-aminoacyl-tRNA synthetase (ARS) antibodies are often detected in patients with autoimmune diseases, and some of these are particularly abundant in the pancreas [12, 13]. Type 1 diabetes, which occurs in ketosis-onset diabetes mellitus can also be considered an autoimmune disease. Pancreatic β cells produce insulin, which recognizes T cell autoantigen and develops autoimmunity reaction leading to the onset of diabetes with ketosis [14, 15, 16]. So far, there are no reasonable explanations to consider
ARS as autoantigens, but these anti-ARS autoantibodies are certainly related to special autoimmune diseases, such as idiopathic inflammatory myositis, interstitial lung disease, arthritis, Raynaud's phenomenon and fever [17].

At present, anti-histidyl-tRNA synthetase (Jo1) antibodies are mostly used in the diagnosis of polymyositis and dermatomyositis. We detected the plasma anti-histidyl-tRNA synthetase (Jo1) antibodies in patients with newly diagnosed ketosis or ketoacidosis-onset diabetes. The results showed that the concentration of Jo1 was not significantly increased in the A + β + subgroup, but its frequency was markedly increased, and there was a significant difference. This suggests that anti-histidyl-tRNA synthetase antibodies can be utilized together with glutamic acid decarboxylase antibody (GADA), insulin autoantibody (IAA), islet cell antibodies (ICA), and other antibodies to improve the diagnosis rate of type 1 diabetes and other autoimmune diabetes such as latent autoimmune diabetes (LADA). Anti-histidyl-tRNA synthetase antibody can be employed as an immune marker to prove the existence of inflammation in the primary stages of ketosis-onset diabetes. The presence of autoimmune antibodies may also indicate that although there is no infection, the aseptic immune response may trigger the onset of diabetes. The frequency of the Jo1 antibody was higher in A + β + and A + β- subgroups. Our results can assist in understanding the onset of ketosis diabetes in a better way. Perhaps this type of diabetes can be simply classified into two subgroups; A + and A- subgroups, based on the presence of autoimmune antibodies. Currently, glycyl-tRNA synthetase has been considered as related to neurodegenerative diseases. Previous studies have shown that anti-glycyl-tRNA synthetase antibodies are closely linked to the pathogenesis of diabetes. Our results showed that there was no significant difference in the concentration of the anti-glycyl-tRNA synthetase antibody between the groups, but the frequency was higher in the A + subgroup. Thus, anti-aminoacyl-tRNA and anti-glycyl-tRNA synthetase antibodies can be used as immunological markers to distinguish between different subgroups of ketosis-onset diabetes mellitus, where anti-aminoacyl-tRNA synthetase antibody was more significant, comparatively. For the diagnosis and treatment of ketosis-onset diabetes mellitus, we should also consider the existence of immune factors and carry out immune intervention treatment accordingly, which may delay the occurrence and development of the disease, shorten its course, reduce patients’ pain and increase their sensitivity to drugs.

The regulatory effect of microRNAs on an organism involves almost the whole physiological process of cell development, metabolism, cell cycle regulation, cell proliferation and differentiation, cell apoptosis, etc. They regulate about 60% of human genes and participate in the pathological process of tumor development, cardiovascular disease, diabetes, inflammation, and other diseases as well. MicroRNAs are highly tissue-specific, and therefore they directly reflect the physiological and pathological conditions of the body. The expression pattern of microRNAs in pathological tissues is significantly different from that of normal tissues. Clinically, it is often found that late detection of diabetes increases the difficulty in diagnosis and treatment, and the concentration of plasma microRNAs changes with the development of the disease. If microRNAs are utilized as new biomarkers, they may play an important role in the prevention of diabetes.
Current research has proved that microRNAs are present in human body fluids, and under the occurrence of disease and different pathological conditions, changes in microRNAs can stably and specifically reflect the changes of expression in body fluids, especially in plasma or serum [18–22]. These microRNAs in plasma or serum can be amplified and detected by molecular biological methods, and some of them can even be used as biological markers for the early diagnosis of diseases [23, 24].

Zampetaki et al. [25] analyzed the plasma microRNAs expression level in more than 800 diabetic patients, where 80 patients were diagnosed with type 2 diabetes while 19 had normal blood glucose levels during the study period, which ten years later developed into type 2 diabetes. It is suggested that microRNAs can be used as biomarkers for the early diagnosis of diabetes. Zhao et al. [26] confirmed that miRNA-29a, miRNA-132, and miRNA-222 can be used as effective biomarkers to predict gestational diabetes in the early and middle stages. MicroRNAs can exist stably in plasma and are widely involved in the production, secretion, and effect of insulin on target tissues. Compared to healthy people, diabetic patients have specific, stable, and sensitive changes in plasma microRNAs, which are noteworthy. The abnormal changes in the expression of microRNAs often occur earlier than the other proteins. Much before the obvious symptoms of diabetes appear, there may be an anomalous expression of microRNAs. Therefore, plasma microRNAs can be used as a new biological marker for the early diagnosis of diabetes and can be of great significance in studying pathology, classification, early diagnosis, and also delaying and preventing the occurrence of diabetes. Our results showed that the expression of miRNA-191 and miRNA-342 was up-regulated in the disease group, suggesting that their occurrence in plasma may be related to the pathological mechanism of ketosis-onset diabetes and can be further used as biomarkers to predict the disease.

**Declarations**

**Ethics approval and consent to participate:** lot 2019276  Ethics Committee of the First Affiliated Hospital of Xinxiang Medical College

**Consent for publication:** Written informed consent for publication was obtained from all participants.

**Availability of data and materials:** The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions:** Beiyan Liu contributed to the conception of the study; performed the experiment; Lin Li contributed significantly to analysis and manuscript preparation; performed the data analyses and wrote the manuscript; Liwei Bai helped perform the analysis with constructive discussions.

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Tables
Tables 4 and 5 are not available with this version.

**Figures**

**Figure 1**

Comparison between the expression levels of plasma miRNA-342 in the ketosis-onset group and the control group (\(\bar{x} \pm s\), \(2^{-\Delta\Delta Ct}\))
Figure 2

Comparison between the expression levels of plasma miRNA-191 in the ketosis-onset group and the control group (±s), 2−ΔΔCt
Figure 3

Amplification curve of? This figure is not clear, add more high resolution and it’s not well explained in the results and discussion section.