Targeting Mitochondrial Diabetes Through the Use of qPCR

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Diabetes Mellitus, a common health problem worldwide, is a series of chronic metabolic diseases associated with insulin secretion and use. Mitochondrial diabetes, one of the rare subtypes, is a specific type of maternal genetic disease that exists within DNA located in the mitochondria. In all mitochondrial diabetes, the most common diabetes-causing mutant is the target m3242A>G. Mitochondrial diabetes is characterized by large clinical phenotype heterogeneity, atypical symptoms and relatively rare. Consequently, mitochondrial diabetes is frequently misdiagnosed, leading to many problems. Polymerase Chain Reaction (PCR), a method for enzymatic synthesis and amplification of nucleic acid fragments, has evolved into several generations, including real-time PCR (qPCR), a fluorescent-based technique for specific DNA segment replication detection. Overcoming limitations of low DNA content in samples, qPCR quantitatively analyzes DNA sequences by measuring fluorescence during amplification, providing an effective solution compared to early PCR methods. This study aims to detect mitochondrial diabetes using qPCR technology, analyze the proportion of mitochondrial diabetes in the diabetic population and its family heritability. Our results showed that qPCR found that four of 150 diabetic patients had mitochondrial diabetes, and the overall estimate of m3242A > G mitochondrial disease was about 2% of the population of Chinese citizens. Finally, as with previous studies, we demonstrated that mitochondrial diabetes is maternally inherited.

Keywords: Diabetes, qPCR, Mitochondrial gene mutations, Maternal genetic disorder

Introduction

Diabetes is a group of chronic metabolic diseases related to the secretion and utilization of insulin, characterized by hyperglycemia caused by defects in insulin secretion, insulin action, or both. The two main types of diabetes are autoimmune Type 1 diabetes and metabolic syndrome-related Type 2 diabetes. Additionally, there are other rare types, such as mitochondrial diabetes. The pathophysiological mechanisms leading to diabetes may involve inappropriate insulin secretion, insulin resistance in the liver, muscles, and fat, or a combination of these factors. The risk of developing diabetes is influenced by the complex interplay between genetic and environmental factors [1–4].

In the past few decades, numerous gene mutations representing high-penetrance risk genes for diabetes have been identified. Carriers of these gene mutations have nearly a 100% chance of developing diabetes during their lifetime. Mitochondrial diabetes is one type of monogenic diabetes, also known as Maternally Inherited Diabetes and Deafness (MIDD) [5–7]. In the majority of cases, mitochondrial diabetes is associated with the m.3243A>G mutation in mitochondrial DNA (mtDNA). Mutations in mitochondrial DNA affect components of the electron transport chain and ATP synthesis. This results in impaired mitochondrial function, reduced ATP production, and increased production of reactive oxygen species. These abnormalities affect insulin secretion, sensitivity, and glucose metabolism, contributing to the development of diabetes. Mitochondrial diabetes is characterized by its distinct clinical features, including early-onset sensorineural deafness, maternal inheritance pattern, and heterogeneous presentation beyond diabetes, such as neurological symptoms and cardiomyopathy. Unlike type 1 and type 2 diabetes, mitochondrial diabetes can manifest at any age and may exhibit variable responses to standard treatments. Due to the exclusive presence of mitochondrial DNA in the egg cell and its absence in sperm, mitochondrial DNA abnormalities are inherited maternally [8–10].

Mitochondrial diabetes differs in diagnosis, treatment, and subsequent management from other types of diabetes. Diagnosing mitochondrial diabetes presents challenges due to its rarity and heterogeneous clinical presentation. These challenges include the need for genetic testing to identify mutations in mitochondrial DNA, evaluating the clinical phenotype for characteristic features like sensorineural deafness, and reliance on non-specific biochemical markers for diagnosis. Mitochondrial diabetes is often overlooked or misdiagnosed in clinical practice. This results in patients with mtDNA mutations receiving treatments intended for type 1 and type 2 diabetes, leading to various complications [11].

More recently, in genetic diagnosis, the advent of whole exome sequencing (WES) and whole genome sequencing (WGS) hold the potential of higher diagnostic yields due to the universality and unbiased nature of the methods. However, these

approaches are subject to the escalating challenge of variant interpretation. Thus, there is an urgent need to find powerful complementary tools to diagnose patients with mitochondrial diseases [12].

Polymerase Chain Reaction, abbreviated as PCR, is a method developed based on the DNA semi-conservative replication mechanism for enzymatic synthesis and amplification of specific nucleic acid segments in vitro. Over time, PCR technology has evolved, with qPCR, or quantitative real-time PCR, being an extension and innovation of the original PCR method. During the amplification process, fluorescent groups are introduced, and as the DNA quantity increases exponentially, the fluorescence signal in the reaction system also intensifies. By detecting the strength of the fluorescent signal, quantitative analysis of DNA amplification can be performed. This technology effectively addresses the challenge of low DNA content in samples, making it widely applied in various fields, including the diagnosis of genetic diseases, forensic investigations, gene cloning, and DNA sequencing [13]. In comparison to other molecular diagnostic techniques such as next-generation sequencing (NGS) or digital PCR (dPCR), qPCR may have lower sensitivity and specificity, particularly for detecting rare mutations or variants. However, qPCR remains a valuable tool for many applications due to its speed, cost-effectiveness, and ease of use.

In the diagnosis of MIDD, PCR may face challenges. Due to the presence of mitochondrial DNA (mtDNA) in multiple copies per cell and the potential heteroplasmy of mutations (where both mutant and wild-type mtDNA coexist within a cell), PCR may encounter limitations in accurately quantifying and identifying low-level mtDNA mutations. Through accurate and effective molecular diagnostic techniques, such as qPCR, mitochondrial DNA mutations can be detected in patients, thereby confirming or ruling out whether a patient has mitochondrial diabetes. In addition, for pregnant women who carry mitochondrial DNA mutations, molecular diagnostics can also help predict the risk of disease in their offspring. Therefore, through molecular diagnosis, mitochondrial diabetes can be diagnosed more accurately and genetic counseling and preventive measures can be provided to family members to better manage and prevent this inherited disease.

In this study, we focused on 150 diabetic patients from specific hospitals, extracting DNA from their oral cavities and using qPCR for detection. The number of patients with mitochondrial diabetes was calculated, and the proportion of mitochondrial diabetes patients in the Chinese diabetic population was further analyzed. Finally, a patient with a positive test result was chosen, and their family members underwent testing to explore the heritability of mitochondrial diabetes. Accurate detection and prediction of pathogenic genes associated with mitochondrial diabetes could provide greater convenience and hope for the diagnosis and treatment of more patients with mitochondrial diabetes.

Methods

Nucleic Acid Extraction

Preparation: Oral swab was taken out of the fridge to unfreeze. Prepare a 1.5ml Eppendorf tube and label with correct lot number. Set heat bath at temperature 56°C and 95°C.

Extraction and purification of DNA: Liquid oral sample was place on a vortex mixer for 1min, cells inside the oral swab are washed down the to the bottom.

Clean Eppendorf tubes were obtained and labeled with corresponding lot number for every sample. 200ul sample were taken from each Oral swab using a p200 Pipetman into the clean Eppendorf.

The centrifuge was set at 16000rpm for 3 min, and all samples were placed in the centrifuge and started running. After 3 min, the supernatant was discarded from the Eppendorf tubes, leaving the DNA precipitate for later use (Fig 1, 2).

We added 50ul DNA extraction solution and 5ul of Proteinase K into the Eppendorf using pipetman, mixed evenly with resuspend cell pellets (Fig 3).

We heated solution in a 56°C heat pad for 5min then transferred the solution to a 95°C heat pad for another 10min. Through this heating process, cells went through a process of Cell lysis, when cellular structure were broken down, DNA were extracted from the cells and separated with other component such as fats and proteins (Fig 4).

Process of qPCR

Solution preparation

PCR amplification buffer, mixture of MT-TL1 m.3242 A>G mutant primers and probe, mixture of primers and probe from MT-RNR gene segments were taken out of the fridge to unfreeze.

For each 2 ul of DNA sample, 10 ul of PCR amplification buffer, 4 ul of mixture of MT-TL1 m.3242 A>G mutant primers and probe and mixture of primers and probe from MT-RNR gene segments were needed.

According to the number of samples used in the experiment, amount of each component was calculated and added together evenly in an Eppendorf tube. Mixed components were later added in the 8-strip PCR tubes (Fig 5).

Adding samples with component

DNA sample after extraction together with negative control, positive control and H2O were prepared for use. 2ul of each sample were extracted using a pipetman and added into the 8-strip PCR tubes according to the setup.

After all the samples were added in, the 8-strip PCR tubes were sealed and put in vortex mixer to mix samples and component up and wash all liquids to the bottom.

Place 8-strip PCR tubes inside the PCR machine prepare for operation.

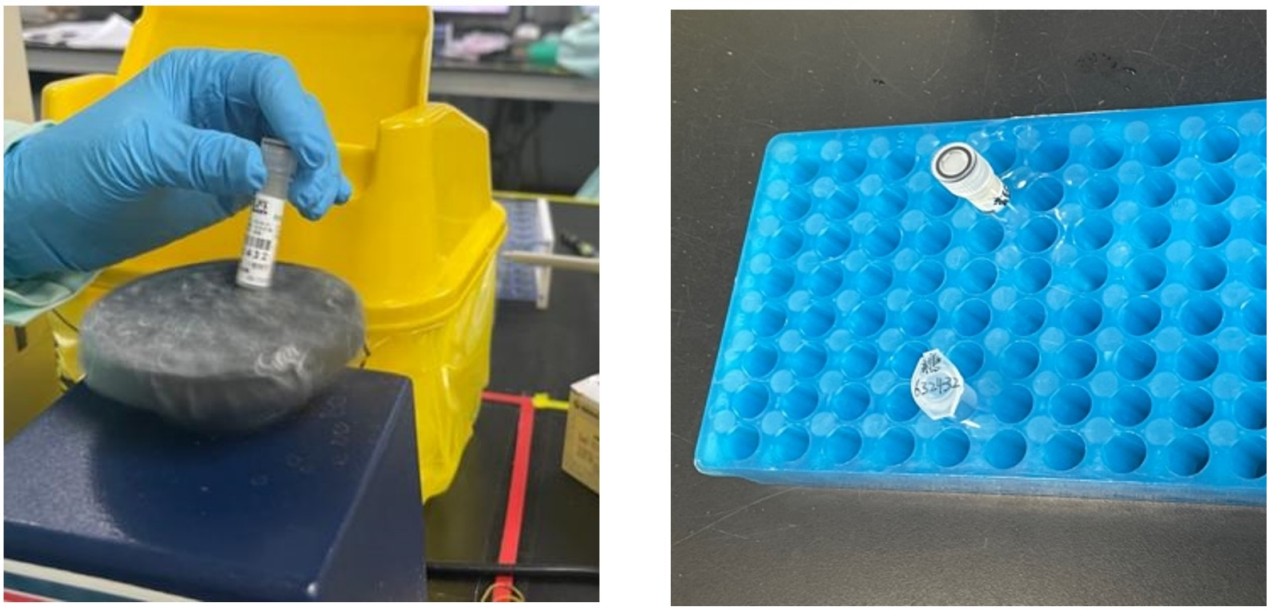

Fig. 1 Sample after centrifuge with DNA principate supernatant

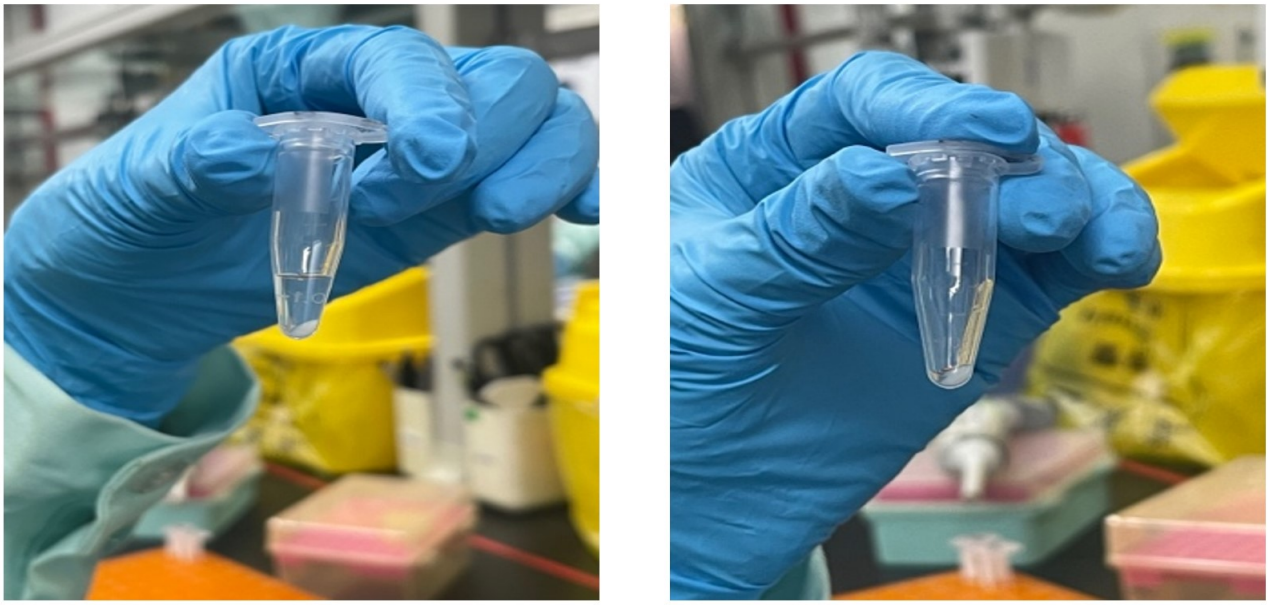

Fig. 2 Sample after supernatant was discard

System setting before running PCR

We put 8-strip PCR tubes into 7500 Fluorescent quantitative PCR instrument and set experiment type as Quantitation-Standard Curve with reagent type TaqMan.

All samples were named with their corresponding lot numbers and the target reporter FAM and VIC values were set for all samples to observe the change in fluorescence values through amplification. The Passive Reference was set as none (Fig 6).

qPCR stage

Data Analysis

Introduction to methodology behind qPCR

Methodology used in this experiment are called TaqMan

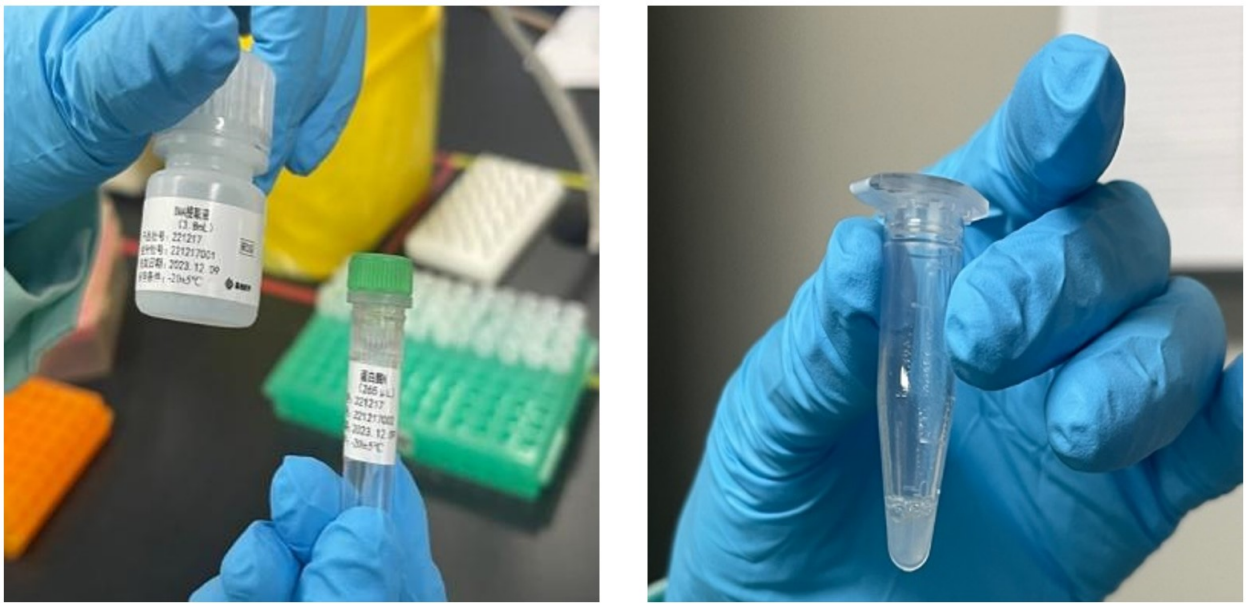

Fig. 3 DNA extraction solution and protease K used to extract DNA from oral sample cell.

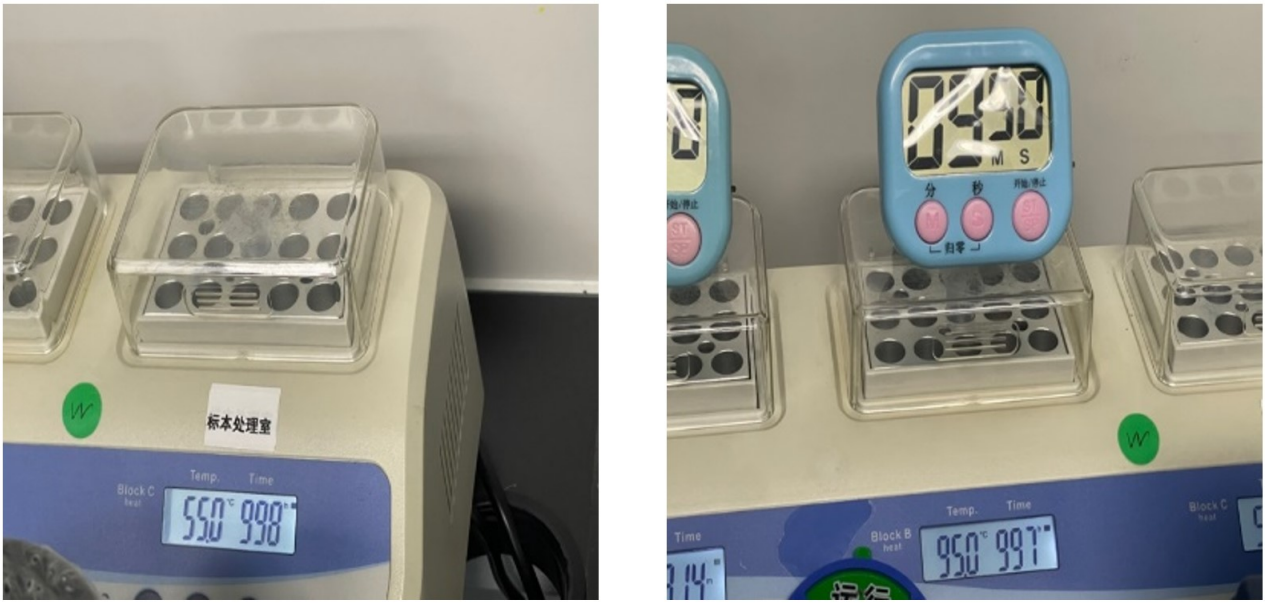

Fig. 4 DNA incubation and heating process

probe method, which is a specific type of way used in gathering florescence during qPCR. During qPCR, the process of denaturation, probe hybridization, extension and fluorescence emission occurs.

TaqMan probes consist of a fluorophore labeled at the 5' end and a quencher labeled at the 3' end and oligonucleotide sequences bound towards the targeting gene sequence. Before DNA amplifies, the fluorescence signal emitted by the fluorophore is captured by the quenching group and therefore not able to be detected by the PCR machine. As DNA extension occurs, probe can be hydrolyzed by DNA when extended to the binding of probe breaking fluorophore and the quencher apart, and florescent will be captured.

Through monitoring of the fluorescence signal during qPCR,

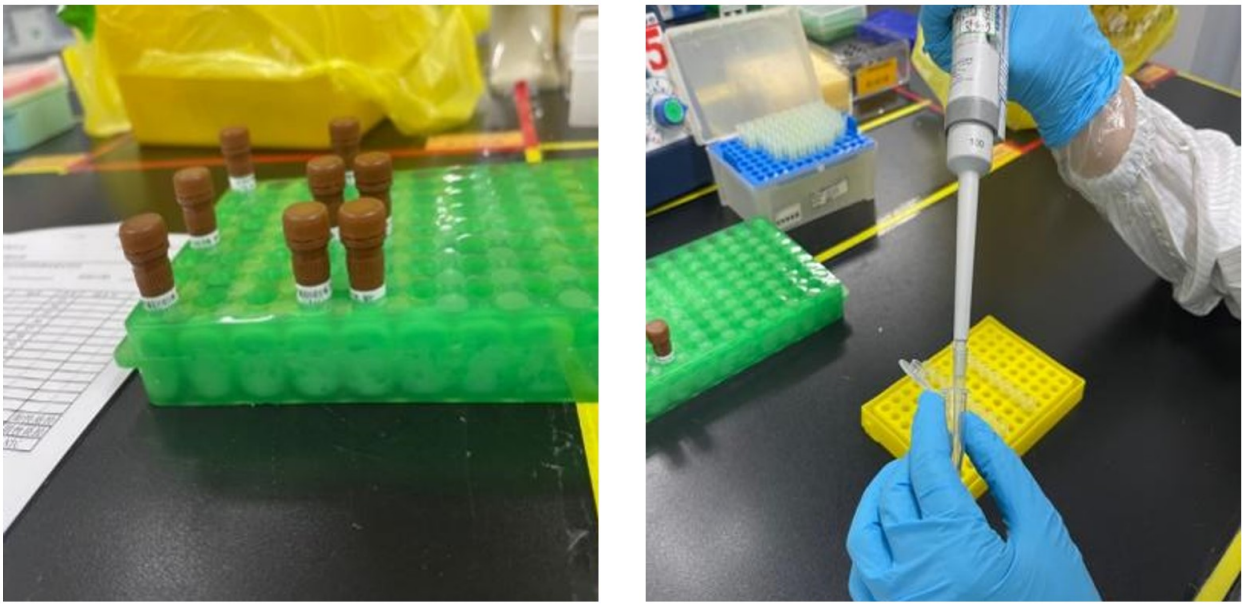

Fig. 5 The 3 samples used to prepare solution and the process of adding the mixed samples from an Eppendorf tube into the 8-strip PCR tubes.

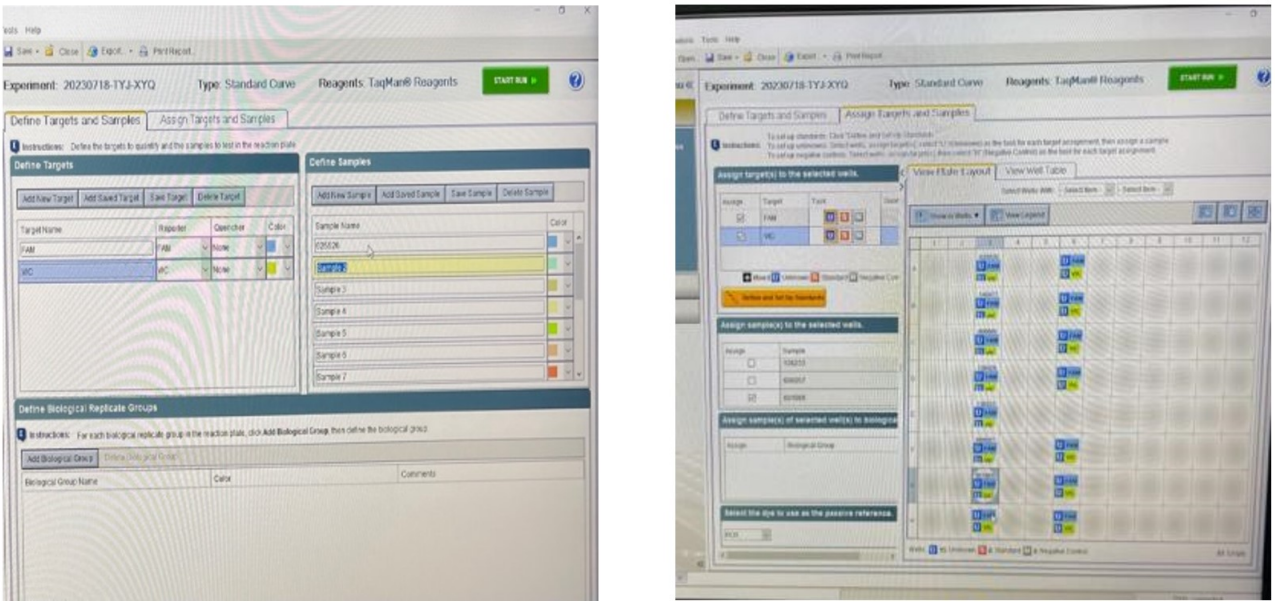

Fig. 6 Settings defining targets FAM and VIC and plates setting with samples and negative positive controls.

the change trend of fluorescence intensity shows an S-shaped curve, which is called the Amplification plot. The number of cycles that the fluorescence signal in each reaction undergoes to reach the set fluorescence threshold is called the Ct value which is called the "threshold cycle".

Identifying CT Value

The CT value of FAM and VIC and the trend of the graph can be used to identify whether the result negative or positive for mitochondrial diabetes according to the table.

According to the table, results can be determined based to the value of Ct of both FAM and VIC. Here are some examples of positive, negative and undetermined outcomes (Table2) (Fig.7,8,9)

Table 1 Temperature and duration for each process of DNA amplification

Steps	Duration	Temperature	Number of Cycles
1. UNG enzyme reaction (Holding stage)	2 min	37°C	1
2. Taq enzyme activation (Holding stage)	30s	95°C	1
3. Denature (Cycling stage)	10s	95°C	40
4. Annealing, extension (Cycling stage)	30s	58°C	40

FAM	VIC	$\Delta CT = \lvert$Ct FAM − Ct VIC$\rvert$	Negative/ Positive	Results
CT value definable, with clear amplification curve	CT value definable	$\leq$14.8	positive	Result shows mutation on target gene m.3243a>G
CT value definable, with clear amplification curve	CT value definable	>14.8	negative	Result shows NO mutation on target gene m.3243a>G
CT value definable, with clear amplification curve	Ct value shows "undetermined"	/	No result	/
CT value definable, but no clear amplification curve	Ct $\leq$ 26	/	negative	Result shows NO mutation on target gene m.3243a>G
CT value definable, but no clear amplification curve OR shows "undetermined"	Ct > 26	/	No result	/
CT value definable, but no clear amplification curve OR shows "undetermined"	CT value definable, but no clear amplification curve OR shows "undetermined"	/	No result	/

Table 2 Positive, negative, and undetermined criteria in qPCR

Results

Detection of Mitochondrial Diabetes using qPCR

Firstly, in order to analyze the proportion of patients with mitochondrial diabetes, we recruited 150 diabetic patients and performed qPCR to detect mitochondrial diabetes. The following is a statistical overview of whether patients are positive or negative, based on CT levels and an overview of trends.

Table 3 Results of qPCR testing in 150 diabetic patients

Total number of patients	Positive	Negative
150	4	146

According to this table, among the 150 diabetic patients, obtained by the qPCR detection method, four patients were diagnosed with mitochondrial diabetes, and the remaining 146 patients were not detected with mitochondrial diabetes. Therefore, we can conclude from a random sample at this hospital that the overall estimate for patients with m3242A> G mitochondrial disease represents about 2% of the total population of Chinese citizens with diabetes. (Table 3, Supplementary Table 1)

Of the four patients with mitochondrial diabetes mellitus detected, we knew the age information of three of these patients. Two of them were in the age range of 34 to 35 years, and the other one had mitochondrial diabetes mellitus detected at 6 years. With no data to support patient age at the initial onset of diabetes symptoms and the data were insufficient to support any additional inferences, we cannot continue to explore the possible link between disease and age.

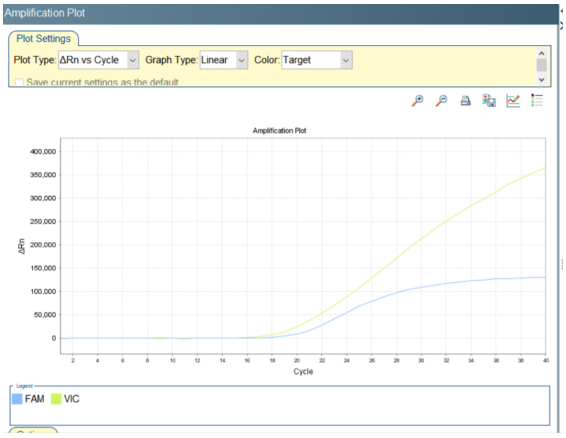

Fig. 7 Settings defining targets FAM and VIC and plates setting with samples and negative positive controls.

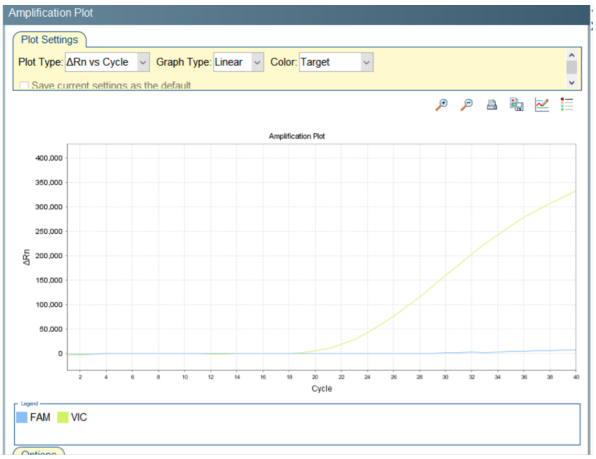

Fig. 8 Settings defining targets FAM and VIC and plates setting with samples and negative positive controls.

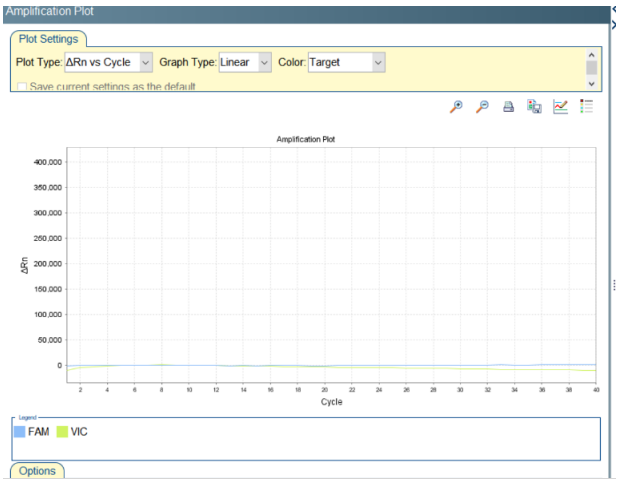

Fig. 9 Settings defining targets FAM and VIC and plates setting with samples and negative positive controls.

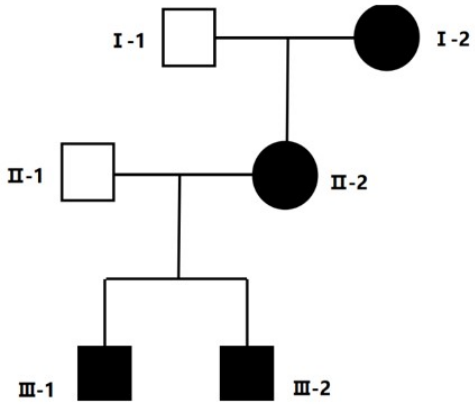

Fig. 10 Family diagram of a positive case
Three generations of test results from a positive patient's family are shown, with circles representing women, squares representing men, black representing positive, and white representing negative.

Familial heritability of mitochondrial diabetes mellitus

Mitochondrial diabetes is a special type of maternal genetic disease that allows the child to inherit the same disease as the mother. In order to understand the familial heritability of mitochondrial diabetes to better predict and treat mitochondrial diabetes, we selected a mother who tested positive by qPCR and performed the same qPCR test on her family members (mother and two sons) to test whether these family members also had mitochondrial diabetes.

Clinical signs: Female patient, diabetes mellitus with multiple complications, folliculitis, diabetic retinopathy, diabetic peripheral neuropathy

Family: Patient, the patient's mother, both sons.

Test results as shown in figure, the pedigree of the circle represents the women in the family, the mitochondrial diabetes test positive for patients is-2, the mother is I-2, the two sons respectively-1 and-2, in the patient, the mother and two sons have been detected m.3243A> G mutation, suggesting that four women in the family have mitochondrial diabetes, which further supports the conclusion of mitochondrial diabetes is maternal inheritance in previous studies. Although her sons did not currently show any symptoms of diabetes, the results showed that they have mitochondrial diabetes and could be treated as early as possible to prevent subsequent effects on their bodies. (Fig.10)

In conclusion, our results show that mitochondrial diabetes is maternally inherited, and therefore, the disease can be treated early according to this characteristic to delay the development of the disease.

Discussion

In this study, using the qPCR method, we tested whether 150 diabetic patients had mitochondrial diabetes. Further analysis of the Chinese diabetic population revealed that among the 150 patients, 4 were diagnosed with mitochondrial diabetes. Based on these results, we estimate that individuals with the m3242A>G mitochondrial mutation constitute approximately 2% of the diabetic population in China. Finally, we selected a female patient with mitochondrial diabetes, and qPCR testing was conducted on her female family members, confirming the maternal inheritance of mitochondrial diabetes.

Mitochondrial diabetes is a rare subtype characterized by mitochondrial gene mutations, predominantly the m.3243A>G mutation in mitochondrial DNA (mtDNA), inherited maternally. Clinical features include significant heterogeneity, atypical symptoms, and rarity, leading to frequent misdiagnosis or confusion with other diabetes subtypes. This results in patients with mtDNA mutations receiving treatments designed for type 1 and type 2 diabetes, creating numerous challenges. Our study emphasizes the higher accuracy of qPCR in detecting mitochondrial diabetes, providing a theoretical foundation for clinical diagnosis. Implementing this method could substantially reduce misdiagnosis rates, facilitating more convenient treatment for mitochondrial diabetes patients. Additionally, our research indicates that approximately 2% of diabetic individuals have mitochondrial diabetes, underscoring the importance of enhancing clinical diagnostic accuracy for targeted interventions. Lastly, the maternal inheritance pattern of mitochondrial diabetes suggests the possibility of early prediction and personalized treatment for asymptomatic female patients in clinical practice.

This study has certain limitations. Firstly, the sample used consisted of 150 diabetic patients from a specific hospital, which may not adequately represent the diversity of the entire Chinese diabetic population. The generalizability of the results could be influenced by sample size and regional constraints. Secondly, the focus on the m.3243A>G mitochondrial gene mutation in this study overlooks other less common mutation types associated with mitochondrial diabetes. Therefore, the study may lack comprehensive observations across various mutations.

In future work, we aim to enhance the representativeness and credibility of research results by expanding the sample size to cover a broader population. Additionally, considering the multifactorial nature of mitochondrial diabetes, we intend to broaden the study scope in future research, encompassing various gene mutation types and including patients of different genders, ages, and regions. Lastly, a more in-depth investigation into other mitochondrial diabetes subtypes, beyond the m.3243A>G mutation, is warranted. Comprehensive and accurate clinical detection of mitochondrial diabetes could pave the way for developing more personalized treatment strategies based on patients' genetic backgrounds, thereby improving treatment efficacy.

In conclusion, our exploration into the genetic detection of mitochondrial diabetes sheds light on the intricate landscape of this rare subtype, emphasizing the significance of accurate diagnostic methods. As we navigate the complexities of mitochondrial gene mutations and their impact on diabetes, it becomes evident that our current understanding is just the tip of the iceberg. This study, with its focus on the m.3243A¿G mutation, provides a foundation for further investigations into the diverse spectrum of mitochondrial diabetes mutations. However, recognizing the limitations of our current research, we envision future studies overcoming these constraints to offer a more comprehensive and nuanced understanding of the genetic underpinnings of mitochondrial diabetes.

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