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Paper Accepted[1] **ISSN Online 2406-0895**

Original Article / Оригинални рад

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Long non-coding RNA signature in beta-thalassemia major associated with prediabetes

Потпис дугих некодирајућих РНК код бета-таласемије мајор удружене са предијабетесом

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Received: April 23, 2025
Revised: September 17, 2025
Accepted: September 19, 2025
Online First: September 26, 2025
DOI: https://doi.org/10.2298/SARH250423076L

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Long non-coding RNA signature in beta-thalassemia major associated with prediabetes

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SUMMARY

Introduction/Objective Functional long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) were investigated in patients with beta-thalassemia major (β-TM) and prediabetes by analysing gene chip expression profiles, experimental evidence and bioinformatics.

Methods Total RNA was extracted after taking blood samples from patients. Microarrays were subsequently used for genetic analysis of patients with β-TM/prediabetes (n = 5) compared with healthy individuals (n = 5). Candidate lncRNAs and mRNAs were randomly validated using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis. Signalling associated with β-TM was identified as being associated with prediabetes based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. **Results** Compared with the healthy controls, 1,511 and 1,932 lncRNAs were up- and downregulated, respectively, whereas 1,128 and 752 mRNAs were up- and downregulated, respectively, in the patients with β-TM/prediabetes. Eight dysregulated lncRNA expressions were confirmed using qRT-PCR analysis, which was consistent with the microarray results. The GO and KEGG analyses showed that the intracellular anatomical structure and multicellular organismal process were the most significantly dysregulated, and the most significantly up- and downregulated pathways were herpes simplex virus 1 infection and vascular smooth muscle contraction, respectively.

Conclusion This study preliminarily determined that the pathophysiology of aberrant glucose homeostasis in β-TM may be linked to the abnormal expression of lncRNAs and mRNAs, deepening the understanding of molecular mechanisms in β-TM complicated by prediabetes.

Keywords: microarray analysis; long non-coding RNAs; beta-thalassemia major; mRNA; prediabetes

САЖЕТАК

Увод/Циљ Функционалне дуге некодирајуће РНК (*lncRNA*) и матричне РНК (*mRNA*) испитиване су код болесника са бета-таласемијом мајор (*β-TM*) и предијабетесом анализом експресионих профила гена добијених помоћу микрочипова, експерименталним доказима и биоинформатиком.

Методе Укупна РНК је изолована након узимања узорака крви од болесника. Микрочипови су затим коришћени за генетичку анализу болесника са *β-TM*/предијабетесом (n = 5) у поређењу са здравим особама (n = 5). Кандидатне *lncRNA* и *mRNA* насумично су верификоване применом *qRT-PCR* анализе. Сигнални путеви повезани са *β-TM* идентификовани су као повезани са предијабетесом на основу анализе *Gene Ontology* (*GO*) и *Kyoto Encyclopedia of Genes and Genomes* (*KEGG*).

Резултати У поређењу са здравим контролама, 1.511 и 1.932 *lncRNA* биле су повећане, односно смањене, док су 1.128 и 752 *mRNA* биле повећане, односно смањене код болесника са *β-TM*/предијабетесом. Осам дерегулисаних експресија *lncRNA* потврђено је анализом *qRT-PCR*, што је било у складу са резултатима микрочипова. Анализе *GO* и *KEGG* показале су да су унутарћелијска анатомска структура и мултићелијски организамски процес били најзначајније дерегулисани, док су најзначајније повећани и смањени путеви били инфекција вирусом *Herpes simplex 1* односно контракција глатких мишића крвних судова.

Закључак Ова студија је прелиминарно утврдила да патофизиологија нарушене глукозне хомеостазе у *β-TM* може бити повезана са абнормалном експресијом *lncRNA* и *mRNA*, чиме се продубљује разумевање молекуларних механизама у *β-TM* компликованој предијабетесом.

Кључне речи: микрочип анализа; дуге некодирајуће РНК; β-таласемија мајор; *mRNA*; предијабетес

INTRODUCTION

Beta-thalassemia (β-TM) is an autosomal recessive haemolytic anaemia disorder caused by genetic defects in the regulation of β-globin, leading to impaired β-globin peptide chain synthesis, which in turn causes haemolysis and ineffective haematopoiesis [1]. Patients require regular blood transfusions to sustain life [2, 3]. The extended extravascular haemolysis caused by β-TM enhances the intestinal absorption of iron; however, long-term massive blood transfusions can result in iron overload, leading to insulin resistance, increasing hepatic glucose production

and reducing insulin secretion, ultimately causing abnormal glucose metabolism [4,5]. Recent studies have revealed that long non-coding RNAs (lncRNAs) can mediate iron metabolism and oxidative stress pathways relevant to diabetes. For example, lncRNAs regulate ferroptosis-mediated β-cell injury and complications of diabetes via modulation of reactive oxygen species, Nrf2 and NF-κB signalling pathways. Moreover, several lncRNAs, including MEG3, H19, MALAT1 and GAS5, have been implicated in insulin resistance and dysregulated glucose or lipid metabolism in metabolic disease contexts. These observations suggest that lncRNAs may have disease-relevant functions in patients with β-TM, potentially linking iron overload, oxidative stress and metabolic dysregulation.

Prediabetes has been identified as a condition lying between normal glycometabolism and diabetes mellitus (DM) [6]. Prediabetes represents a high-risk state for the development of diabetes, with an annual conversion rate of 5%–10% [7]. Although prediabetes does not meet the diagnostic criteria for diabetes, it is strongly associated with multiple diseases as well as an elevated risk of patient death [8, 9, 10]. A meta-analysis indicated that the incidence of impaired glucose tolerance (IGT), impaired fasting glycaemia (IFG) and DM in patients with β-TM was 17.21%, 12.46% and 6.54%, respectively [11]. However, abnormal glucose metabolism in patients with β-TM usually has an insidious onset, and effective biomarkers and diagnostic methods to detect glucose metabolism disorders are lacking [11].

Among gene transcripts, only about 10%–20% of transcripts are messenger RNAs (mRNAs) responsible for encoding proteins, whereas 80%–90% are non-coding RNAs, including miRNAs and lncRNAs [12, 13]. Long non-coding RNAs consist of over 200 bases and lack protein-coding capacity but play key roles in a variety of biological processes [12, 13]. Because lncRNAs are stably present in peripheral blood, they are considered potential diagnostic markers [14]. Studies have shown that diabetes and its associated symptoms are closely related to lncRNAs and mRNAs in the peripheral circulation. In addition, lncRNAs have critical roles in regulating pancreatic β-cell function, glucose metabolism and insulin resistance [15, 16]. Recently, genome-wide analyses of pseudogenes have demonstrated the human-specific essentiality of the lncRNA haemoglobin subunit beta pseudogene 1 in erythropoiesis and its implications in β-TM [17].

However, the role of lncRNAs and mRNAs in the molecular mechanism of β-TM associated with prediabetes remains elusive. By analysing lncRNA compared with mRNA expression profiles in patients with β-TM and prediabetes, potential diagnostic and therapeutic targets may be revealed. In this study, we used microarray technology to detect differentially expressed

transcripts in peripheral blood of these patients to explore their potential role in the pathogenesis of prediabetes in patients with β-TM.

METHODS

Patient information

This study was conducted in Hainan Provincial People's Hospital between January and December 2021 and included a total of five patients with β-TM and prediabetes and five healthy controls. All participants signed an informed consent form before the start of the study. The diagnosis of β-TM was based on haemoglobin electrophoresis and genetic analysis, and the diagnosis of prediabetes was based on the American Diabetes Association 2021 guidelines [18] for determining IFG and IGT through the oral glucose tolerance test (OGTT). The inclusion criterion for the healthy controls was the absence of major disease. The clinical and demographic characteristics of the study participants are detailed in Table 1.

Sample collection

On the day prior to blood collection, the participants were instructed to fast from 8 PM until the completion of blood collection the following day. Blood draws were scheduled for the following morning to ensure that the participants had fasted for more than 8 hours. The median vein or cephalic vein at each participant's elbow was selected as the blood sampling site. The blood collection site was disinfected with a sterile cotton ball with a diameter of approximately 5 cm, starting at the puncture site and moving outwards in concentric circles; the disinfectant was then left to dry naturally. A lancet was inserted into the vein, ensuring that the needle was completely inside the vessel, and an ethylenediaminetetraacetic acid anticoagulant tube was then inserted into the other end of the lancet once blood return was observed. Subsequently, 5 mL of peripheral blood was collected, taking care to avoid blood spillage or bubble generation to ensure smooth blood collection. Following blood collection, the tourniquet was released, gauze was pressed gently onto the site, the lancet was withdrawn and the gauze was fixed with tape to prevent bleeding. The collected blood samples were sent to a laboratory for the following tests: ferritin, fasting plasma glucose, fasting insulin and fasting C-peptide levels. In addition, participants were scheduled for an OGTT, and peripheral blood was collected again 2 hours after glucose administration to determine 2-hour blood glucose levels to complete the OGTT assessment.

RNA extraction

For RNA extraction from blood samples, the blood samples were first treated with TRIzol™ Reagent (Catalogue No. 15596026, Thermo Fisher Scientific, Delaware, DE, USA), mixed thoroughly at a ratio of 5 mL TRIzol™ per 1 mL of blood and left for 5 minutes to facilitate separation of nucleic acid–protein complexes. Chloroform was subsequently added, and the mixture was shaken vigorously and centrifuged to separate the RNA phase from the upper layer. An equal volume of isopropanol was then added, allowed to stand at room temperature and centrifuged to pellet the RNA. The pellet was washed with 75% ethanol, and finally, RNA was solubilised with RNase-free water. Next, RNA was further purified using RNeasy Mini Kit (74104, Qiagen, Germany).

Microarray analysis

Microarray experiments were performed by Kangchen Biotechnology (Shanghai, China). Microarray hybridisations were performed according to the standard protocols of Agilent Technologies Co., Ltd. (California, USA). The extracted RNA was first purified and subsequently transcribed into fluorescently labelled cRNA, and cDNA hybridisation was performed on microarray chips. Following the completion of hybridisation, the chips were scanned using an Agilent microarray scanner to obtain raw image data. Agilent Feature Extraction Software (version 11.0.1.1) was used for data extraction and preprocessing. This software can automatically read and process up to 100 raw image files to complete the determination of feature strength and ratio. Subsequently, expression data were normalised using quantile normalisation with Agilent software and a robust multi-chip averaging technique.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses

Gene Ontology (GO) analysis was used to assess gene enrichment, implemented using the enrichGO function to screen significantly enriched GO terms (set p-value or corrected p-value <0.05). Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was used to explore the involvement of these genes in specific biological pathways, and pathway enrichment analysis was completed with the help of the enrichKEGG function to select significant metabolic or signalling pathways (threshold at p-value or corrected p-value <0.05). Finally, the GO and KEGG analysis results were combined to reveal the potential biological significance of differentially expressed genes at the cellular function and pathway level.

Reverse transcription polymerase chain reaction

In this study, RNA samples were reverse transcribed using the PrimeScript RT kit and gDNA eraser (Takara Biotechnology Co., Ltd.) to remove genomic DNA contamination and synthesise cDNA. Subsequently, quantitative real-time reverse transcription chain reaction (qRT-PCR) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR ™-Green PCR kit (Takara Biotechnology Co., Ltd.) to detect the expression levels of target genes. All qRT-PCR data were quantitatively analysed using the $2^{-\Delta\Delta CT}$ method. The primer sequences are presented in Supplementary Table 1.

Statistical analysis

For data analysis, SPSS 25.0 software was used. The normality of continuous variable data was assessed using the Shapiro–Wilk test, the data were presented as mean ± standard deviation and the t-test was used to compare differences between groups; non-normally distributed data were presented as interquartile ranges and analysed using the Mann–Whitney U test for non-differences. In all statistical tests, $p < 0.05$ was considered statistically significant.

Ethics: The Helsinki Declaration was followed in the conduct of this investigation. Hainan General Hospital's Ethics Committee gave its approval for this research to be conducted (Approval number: NO. 2021-239). All participants gave their permission in writing after being fully informed of the study protocols.

RESULTS

Profile changes of long non-coding RNAs

A total of 26,035 lncRNAs were identified in the lncRNA expression profiles of both the control samples and the samples of β-TM with prediabetes. These were then assessed via a microarray analysis (Figure 1A,C). A total of 3,443 differentially expressed lncRNAs were identified using the criteria of $p < 0.05$ and $|\log2FC| > 2.0$, including 1,511 upregulated and 1,932 downregulated lncRNAs (Figures 1E-H). The 20 lncRNAs with the most significant changes are recorded in Table 2. Among them, the most significant upregulation was in ENST00000496629, which had a fold change (FC) of 82.1860551, whereas the most significant downregulation was in ENST00000581274, which had an FC of 170.1365176. In addition, the taxonomic distribution of dysregulated lncRNAs was summarized. Among the deregulated lncRNAs, there were a total

of 1,799 intergenic lncRNAs, 980 antisense lncRNAs, 409 intronic lncRNAs and 255 bidirectional lncRNAs (Supplementary Figure 1).

Changes in the messenger RNA profile in patients with beta-thalassemia/prediabetes

In this study, we examined mRNA expression patterns in patients with β-TM/prediabetes using microarray analysis. Compared with healthy individuals, the results revealed 17,044 mRNAs (Figure 1B and D), and 1,880 differentially expressed mRNAs were identified using the criteria of $p < 0.05$ and $|\log2FC| > 2.0$. In the patients with β-TM and prediabetes, 1,128 mRNAs were upregulated and 752 were downregulated (Figures 1F and 2H). The 20 most dramatically changing mRNAs are presented in Table 3. The most substantially upregulated and downregulated mRNAs were GDF15 (FC = 100.2322219) and GUCA2B (FC = 143.9714162), respectively.

Validation of dysregulated long non-coding RNAs by quantitative real-time reverse transcription polymerase chain reaction

To further confirm the accuracy of the microarray results, we randomly selected eight dysregulated lncRNAs for validation using qRT-PCR. The qRT-PCR results were consistent with the microarray result trends (Figure 2). Among the validated transcripts, ENST00000496629 and AC090912.2 (lncRNAs) and GDF15 (mRNA) exhibited the most pronounced expression changes. These molecules were therefore considered the most biologically relevant candidates for subsequent functional interpretation.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses

To further explore the function of lncRNAs with substantial changes, we performed GO and KEGG analyses. The GO analysis results showed that upregulated mRNAs were involved in 985 terms and 275 terms were enriched in downregulated mRNAs. Compared with the healthy group, significantly changed upregulated lncRNA functions were mainly concentrated in unfolded protein binding, intracellular anatomy and cellular macromolecular metabolic processes, whereas downregulated lncRNA functions were mainly concentrated in neuropeptide receptor binding, cell periphery and multicellular organismal processes (Figure 3A, B).

The KEGG analysis showed that a total of 31 pathways were upregulated, with the herpes simplex virus 1 infection pathway being the most significantly enriched. Among the 17

downregulated pathways, the vascular smooth muscle contraction pathway was the most significantly enriched (Figure 3C, D). Beyond listing enriched pathways, these findings warrant biological interpretation. Several KEGG pathways enriched in differentially expressed genes are closely related to abnormal glucose metabolism, including protein processing in the endoplasmic reticulum (hsa04141), the cell cycle (hsa04110), autophagy (hsa04140), the NOD-like receptor signalling pathway (hsa04621), the interleukin (IL)-17 signaling pathway (hsa04657), cortisol synthesis and secretion (hsa04927) and pancreatic secretion (hsa04972). Likewise, GO terms such as positive regulation of insulin secretion (GO:0032024), the glucose metabolic process (GO:0006006), negative regulation of gluconeogenesis (GO:0045721) and positive regulation of the endothelial cell apoptotic process (GO:2000353) have direct implications for glucose homeostasis and diabetes-related vascular complications. Notably, enrichment in the herpes simplex virus 1 (HSV-1) infection and vascular smooth muscle contraction pathways has not been previously reported in β-TM or β-TM-associated dysglycemia. Iron overload in β-TM can induce immune dysregulation, and the HSV-1 infection pathway involves immune-modulatory signalling; our preliminary unpublished data indicate elevated circulating IL-1β, IL-6, IL-8, IL-10 and TNF-α levels in β-TM with prediabetes, suggesting possible immune activation through this pathway. Similarly, vascular smooth muscle contraction is relevant to endothelial dysfunction, a hallmark of diabetes-related vascular injury. Whether iron overload in β-TM drives vascular damage through this pathway remains unknown and warrants further mechanistic investigations. These novel pathway associations provide exploratory targets for future research into the biological mechanisms linking β-TM to early glucose metabolism impairment.

DISCUSSION

Beta-thalassemia is an autosomal recessive inherited disorder characterized by reduced or complete loss of β-globin chain production. At present, the molecular pathogenesis between β-TM and prediabetes has not been clarified. To explore the relationship between the two, it is important to detect lncRNA and mRNA expression in patients with β-TM to reveal the etiology and pathophysiology of β-TM complicated with prediabetes. Previously, Fakhr-Eldee et al. [19] found that lncRNAs, MALAT1, MIAT and ANRIL may be closely related to the pathogenesis of β-TM and are expected to be novel molecular biomarkers of β-TM. On this basis, this study is the first to analyse lncRNA and mRNA expression profiles in patients with β-TM complicated with prediabetes using microarray technology. The analysis results showed that differentially expressed lncRNAs may play a key role in the pathogenesis of β-TM, and this conclusion

is basically consistent with the results of previous studies. Notably, ENST00000496629 and AC090912.2 (lncRNAs) together with GDF15 (mRNA) were identified as the most significantly dysregulated transcripts in our study. Moreover, ENST00000496629, which has not been previously reported in β-TM or glucose metabolism, may represent a novel regulatory molecule linking iron overload to metabolic dysregulation. In addition, AC090912.2, although poorly characterised, exhibited marked downregulation and may be involved in pathways affecting glucose homeostasis, and GDF15, a stress-responsive cytokine elevated in various metabolic and cardiovascular disorders, is known to influence insulin sensitivity and β-cell function [20, 21]. These findings suggest that these three molecules could be promising candidates for further mechanistic studies and potential biomarkers for early detection of glucose metabolism impairment in β-TM.

To our knowledge, this is the first study to comprehensively profile lncRNA and mRNA expression specifically in patients with β-TM and prediabetes, as distinct from β-TM without abnormal glucose metabolism or from diabetes alone. Previous transcriptomic or non-coding RNA studies of β-TM have primarily focused on erythropoiesis-related pathways, iron metabolism or diabetic complications individually [22]. Our results identify several lncRNAs (e.g. ENST00000496629, AC090912.2) and mRNAs (e.g. GDF15, GUCA2B) that have not been previously associated with β-TM or prediabetes in the existing literature. These molecules reveal potential mechanistic links between iron overload characteristic of β-TM, oxidative stress and early-stage glucose dysregulation. The co-enrichment of HSV-1 infection pathways and vascular smooth muscle contraction among differentially expressed transcripts also extends current understanding by implicating immune modulation and vascular responses much earlier in glucose metabolism impairment in β-TM than previously recognized. This suggests that gene regulation in β-TM-associated prediabetes may share overlapping inflammatory and endothelial dysfunction components with, but also diverging from, established type 2 diabetes pathogenesis.

Diabetes and its associated complications are closely associated with lncRNAs in the peripheral circulation. Studies have shown that specific lncRNAs play a key role in the occurrence, development and complications of diabetes. For example, MALAT1 showed expression changes associated with insulin resistance in patients with type 2 diabetes, and its expression levels may be affected by resistin and homeostasis model assessment (HOMA-IR) measures [15]; moreover, MALAT1 expression was significantly reduced in serum samples or exosomes from patients with type 2 diabetes [23]. In addition, lncRNA CASC2 is significantly downregulated in

blood and kidney tissues of patients with diabetic chronic renal failure, suggesting that it may be associated with the pathological process of diabetic nephropathy [16]. In the present study, significant changes in lncRNA expression profiles in prediabetes compared with peripheral circulation were further revealed. A large number of significantly up- and downregulated lncRNAs were identified, with FGFR1 and AC090912.2 being the most significantly up- and downregulated. In another study, FGFR1 was demonstrated to be associated with diabetes and metabolic diseases, but the relationship between AC090912.2 and diabetes remains to be further investigated [24].

Located on human chromosome 14q32.3 within the imprinted DLK1–MEG3 locus, MEG3 is a non-coding transcript with β-cell-specific expression in the islets of individuals without diabetes; however, its expression is reduced in the pancreatic islets of patients with type 2 DM. The imprinted DLK1–MEG3 region on chromosome 14q32.2 has also been shown to alter susceptibility to type 1 diabetes. In vivo and in vitro studies in mice have demonstrated that MEG3 expression is enriched in pancreatic islets compared with exocrine tissue in Balb/c mice and is downregulated in the islets of both type 1 and type 2 diabetic mouse models. In MIN6 cells and isolated mouse islets, MEG3 expression is primarily regulated by glucose levels. Inhibition of MEG3 expression in vitro impairs insulin synthesis and secretion and increases β-cell apoptosis. Furthermore, in vivo knockout of MEG3 leads to glucose intolerance, reduced insulin secretion and decreased mRNA as well as the protein levels of Pdx-1 and MafA [25].

A conserved lncRNA located at chr19:5795689-5802671, MALAT1 shows aberrant expression in diabetic retinopathy. Transcription factors play a critical role in regulating cellular processes and signal transduction in response to external stimuli. Analysis using TRANSFAC predicts transcription factor binding sites within the MALAT1 sequence, indicating potential interaction with NF-κB as a cis-acting element. Hyperglycaemia induces MALAT1 upregulation in retinal endothelial cells and in the retinas of rats with diabetes. Silencing MALAT1 expression markedly attenuates diabetes-induced retinal neovascularisation, vascular leakage and retinal inflammation. In vitro genetic knockout of MALAT1 affects key endothelial cell functions, including proliferation, migration and tube formation. Collectively, these findings suggest that MALAT1 may mitigate hyperglycaemia-induced retinal degeneration and improve retinal function, highlighting its broader role in the microvascular complications of diabetes. These dysregulated lncRNAs may influence the pathophysiology of diabetes and its complications by regulating gene expression or participating in signalling pathways. In addition, mRNA expression profiling has revealed significant gene expression changes in patients with prediabetes, with GDF15

and GUCA2B being the most significantly upregulated and downregulated mRNAs [26, 27]. Moreover, GDF15 expression is increased in diabetes and cardiovascular disease and is considered a potential therapeutic target and prognostic indicator, whereas GUCA2B is involved in lipid metabolism and shows protective effects in obesity-associated nonalcoholic fatty liver disease, with increases in its levels associated with improved liver injury [28]. In summary, lncRNA and mRNA expression changes in the peripheral circulation are closely related to the pathophysiological mechanisms of diabetes and its complications, and these molecules may serve as potential biomarkers or therapeutic targets to provide new directions for the early diagnosis and intervention of diabetes.

In patients with β-TM, diabetes and its complications substantially contribute to increased mortality. Understanding the molecular mechanisms driven by hyperglycemia and its related complications is therefore essential for improving clinical strategies and exploring novel therapeutic targets. In recent years, the functional roles of lncRNAs in diabetes and diabetic complications have been increasingly recognized. Notably, a considerable proportion of single nucleotide polymorphisms associated with type 1 and type 2 diabetes are located within lncRNA regions, suggesting their potential involvement in disease pathogenesis. The pancreas senses blood glucose levels and, through pancreatic β cells, secretes insulin to stimulate glucose uptake by peripheral tissues. In type 2 diabetes, hyperglycemia results from β-cell dysfunction combined with insulin resistance, whereas in type 1 diabetes, it arises from the loss of β cells and insulin production. In this study, microarray technology was used to identify differentially expressed lncRNAs and mRNAs in patients with β-TM and prediabetes. Functional enrichment analyses, including pathway, disease and GO annotations, were conducted to investigate their potential roles in disease onset and progression. These findings provide a molecular basis for subsequent functional studies of lncRNAs in β-TM-associated prediabetes and offer novel research directions for biological therapy. As well as potentially serving as early diagnostic biomarkers for patients with β-TM at risk of developing diabetes, the identified lncRNAs may represent promising therapeutic targets for drug development.

In patients with β-TM, iron overload is a common pathological feature, mainly due to abnormal iron absorption resulting from frequent blood transfusions and ineffective erythropoiesis. Accumulation of iron overload in the pancreas may induce oxidative stress in pancreatic β cells, which in turn affects insulin secretion and glucose metabolism homeostasis and increases the risk of prediabetes and diabetes [29]. Studies have shown that lncRNAs play a key role in the regulation of islet function. For example, the lncRNA MEG3 exhibited downregulation in

pancreatic tissue in rat models of type 1 and type 2 diabetes, whereas its silencing further impaired insulin secretion and glucose homeostasis. By contrast, normal MEG3 expression promotes insulin secretion and inhibits apoptosis of diabetic pancreatic β cells, suggesting it has a protective role in maintaining islet function [30]. In addition, the lncRNA MALAT1 is closely associated with resistin and HOMA-IR in type 2 diabetes. In cell models, knockdown of MALAT1 decreased pro-inflammatory cytokine production while improving glucose absorption and intracellular signaling, suggesting it has a potential regulatory role in diabetes pathophysiology [15]. Long non-coding RNAs may be an important molecular mechanism of β-TM complicated by prediabetes, providing a new research direction for the early identification of the disease and prompt intervention.

In this study, through GO functional annotation and KEGG pathway analysis, we investigated the potential role of differentially expressed genes in β-TM complicated by prediabetes in depth. Gene Ontology analysis showed that deregulated mRNAs were mainly involved in metabolic processes, cellular communication and cellular responses to a variety of stimuli, such as inflammatory responses and oxidative stress, and KEGG pathway analysis further revealed multiple significantly enriched pathways, which included protein processing in the endoplasmic reticulum, a process closely associated with glucose metabolism. In addition, key pathways such as the cell cycle, calcium signaling pathway and NOD-like receptor signaling pathway are involved [31]. The results of these analyses suggest that differentially expressed mRNAs are widely involved in inflammation-mediated signaling and oxidative stress responses, reflecting a complex genetic and metabolic regulatory network in prediabetes in patients with β-TM. These dysregulated genes may run through various biological stages of prediabetes development, from metabolic disorders to cell signaling, inflammation and oxidative stress responses, which together drive the development and progression of prediabetes in patients with β-TM.

Summary of findings

In total, 3,443 differentially expressed lncRNAs and 1,880 differentially expressed mRNAs were identified, with ENST00000496629 and AC090912.2 (lncRNAs) and GDF15 (mRNA) emerging as the most significantly dysregulated transcripts. Functional enrichment analyses implicated these transcripts in glucose metabolism, vascular smooth muscle contraction, immune regulation and protein processing in the endoplasmic reticulum. These results suggest novel molecular links between iron overload, oxidative stress and early glucose dysregulation in β-TM.

Interpretation

Previous transcriptomic studies of β-TM have focused on erythropoiesis or iron metabolism. Our findings extend this by identifying transcripts not previously associated with β-TM or prediabetes. In particular, ENST00000496629, upregulated in our cohort, has not been reported in glucose metabolism, suggesting a novel regulatory role in iron-induced metabolic disturbances. Moreover, AC090912.2, although poorly characterised, showed marked downregulation and may affect glucose homeostasis. Finally, GDF15, a stress-response cytokine, is elevated in metabolic disorders, insulin resistance and cardiovascular disease. Enrichment of HSV-1 infection and vascular smooth muscle contraction pathways – previously unreported in β-TM-related dysglycaemia – suggests immune activation and early endothelial dysfunction may contribute to disease. This aligns with our unpublished findings of elevated IL-1β, IL-6, IL-8, IL-10 and TNF-α in β-TM with prediabetes.

Limitations and future directions

This study has several limitations. First, the small sample size (n = 5 per group) limits statistical power and generalizability; these results should be interpreted as exploratory. Second, recruitment was constrained by the rarity of β-TM with prediabetes due to premarital screening and improved management, and the lack of a group of patients with β-TM but without prediabetes prevents separation of thalassemia-related from dysglycaemia-specific transcriptomic changes. Finally, age and sex were not strictly matched, potentially influencing gene expression. Future studies should recruit larger, matched cohorts including patients with β-TM but without prediabetes and perform in-depth functional validation of candidate molecules in cellular and animal models to clarify their roles in disease mechanisms.

CONCLUSION

In summary, the present study revealed a possible close link between substantial changes in lncRNA and mRNA expression profiles and β-TM complicated by prediabetes. These differentially expressed molecules may influence the pathophysiological mechanisms of diseases through complex regulatory networks or participate in specific biological processes. The findings of this study provide an important theoretical basis and research direction for future exploration of potential therapeutic targets, diagnostic markers and prognostic evaluation indicators of β-TM complicated by prediabetes.

ACKNOWLEDGMENTS

The present study was supported by the Natural Research Foundation of Hainan General Hospital (Grant NO. 2021QNXM27) and Research Project of Health Industry in Hainan Province (Grant NO. 22A200210).

Conflict of interest: None declared.

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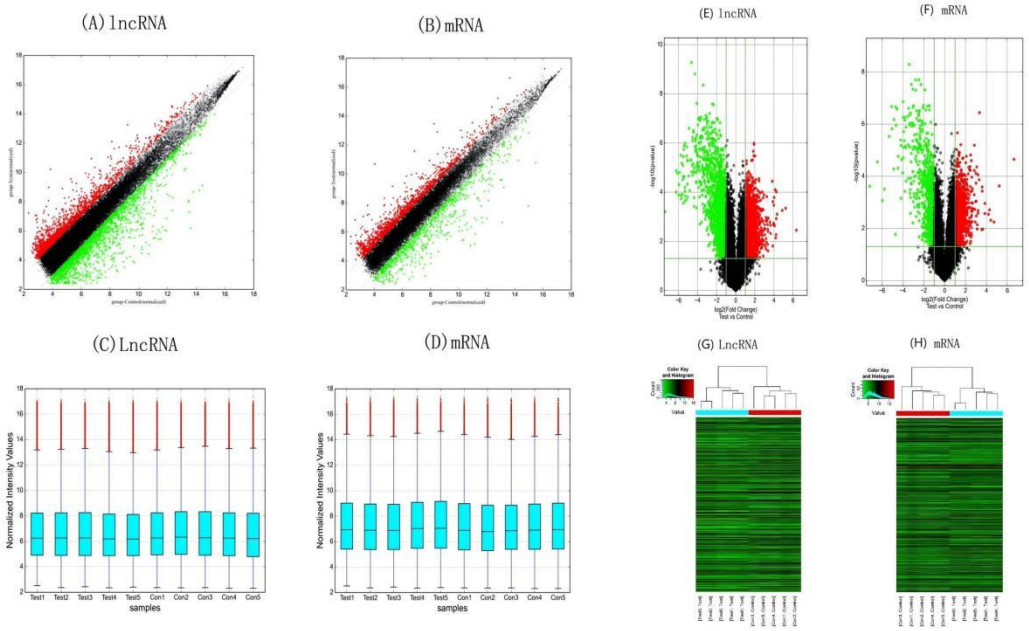


Figure 1. The characteristics of long non-coding RNA (lncRNA) and messenger RNA (mRNA) differential expression and volcano plot and hierarchical clustering of differentially expressed transcripts; a scatter plot shows the differences between lncRNA (A) and mRNA (B) expression in patients with β-thalassemia major with prediabetes (y-axis, group-test) versus healthy controls (x-axis, group-control); red and green represent ≥ 2-fold, $p < 0.05$; the box plots show the lncRNA (C) and mRNA (D) expression levels of each individual sample; (E) volcano plot of differentially expressed long non-coding RNAs (lncRNAs); (F) volcano plot of differentially expressed messenger RNAs (mRNAs); (G) Hierarchical clustering of lncRNA profiles in patients with β-thalassemia major (β-TM) with prediabetes compared with healthy controls (≥ 2-fold, $p < 0.05$); (H) hierarchical clustering of mRNA profiles in patients with β-TM with prediabetes compared with healthy controls (≥ 2-fold, $p < 0.05$)

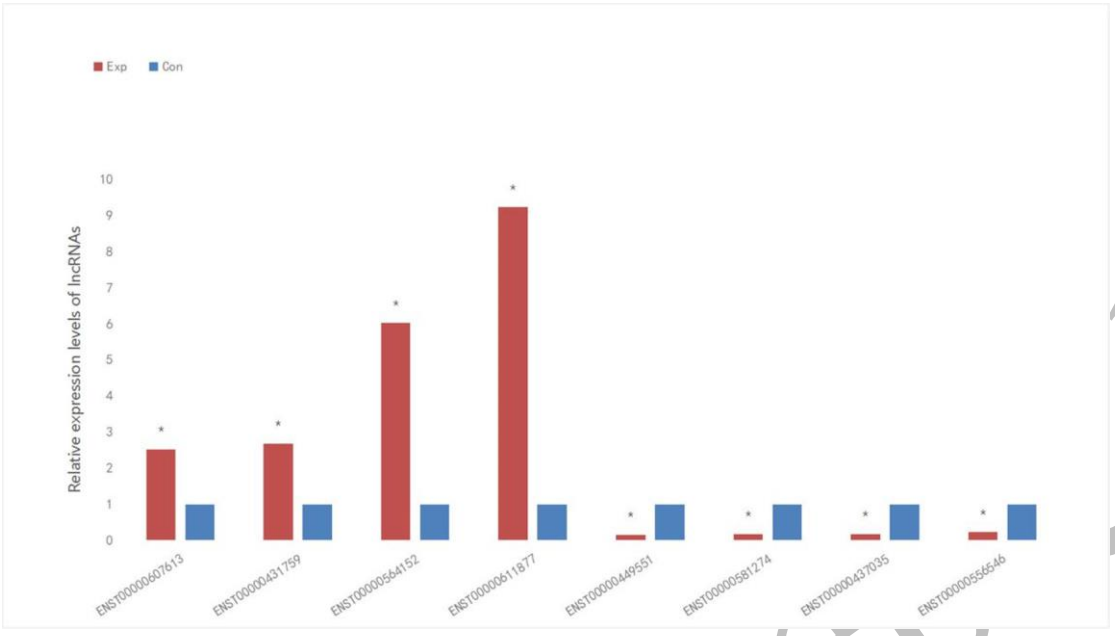


Figure 2. The expression of selected lncRNAs; the expression level detected by qRT-PCR showed that the expression level of lncRNAs ENST00000449551, ENST00000581274, ENST00000437035, and ENST00000556546 was significantly lower and that of lncRNAs ENST00000607613, ENST00000431759, ENST00000564152, and ENST00000611877 was significantly higher in patients with β-TM with prediabetes compared with the controls (*$p < 0.05$); the qRT-PCR analysis was similar in most respects to the lncRNA microarray analysis

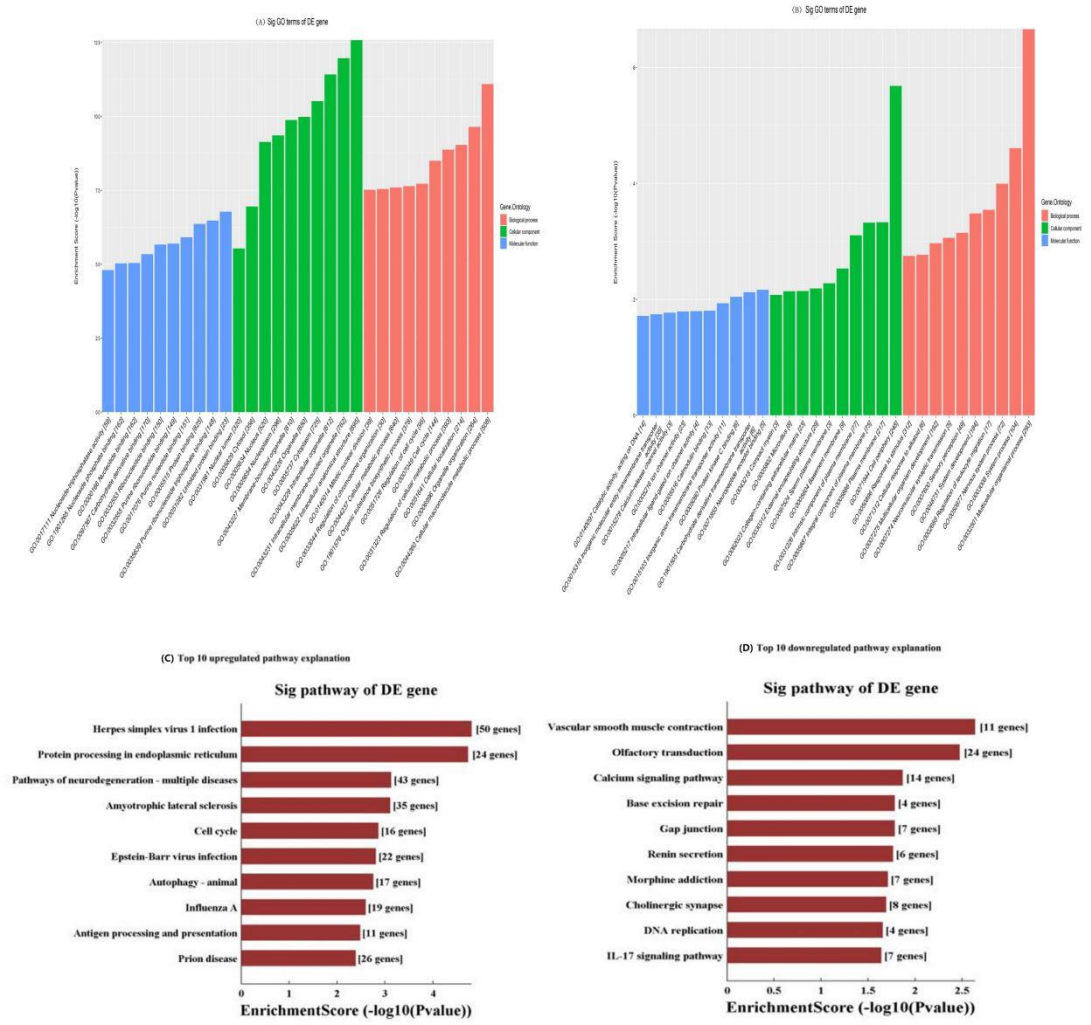
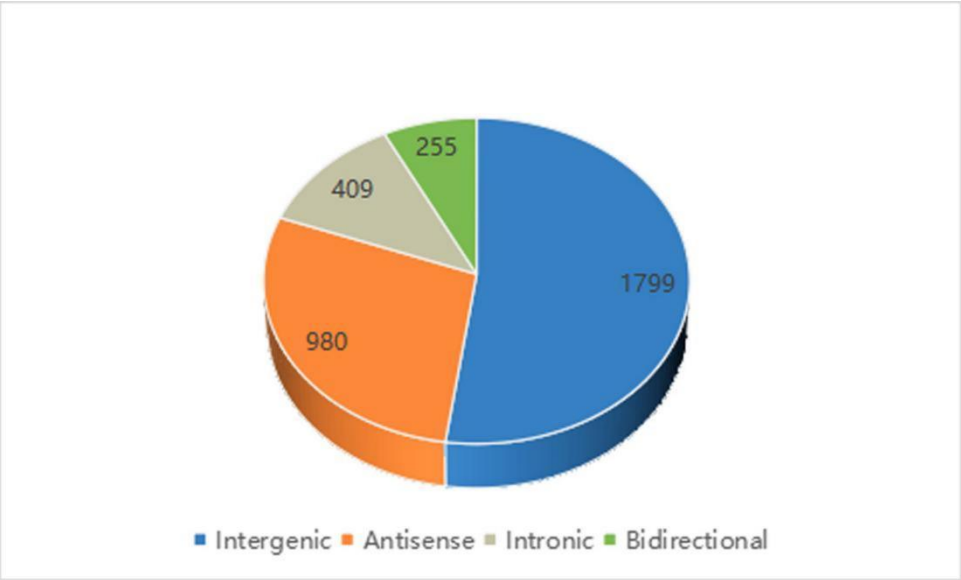


Figure 3. Gene ontology enrichment and genomes pathway analysis of differentially expressed mRNAs



Supplementary Figure 1. Distribution of dysregulated lncRNAs

Table 1. Clinical Characteristics of β-TM patients with prediabetes and healthy controls

Variables	B-TM + prediabetes (n=5)	healthy controls (n=5)	p-value
Age (years)	10.6 ± 1.7	11 ± 1.4	0.78
Sex (male/female)	3/2	2/3	0.65*
Time of blood transfusion (months)	92 ± 5.7	0 (NA)	<0.001*
Ferritin (ng/ml)	7,377.6 ± 1,021.5	146.9 ± 63.8	<0.001*
BMI	15.3 ± 1.7	20 ± 0.9	0.002*
Liver MRI T2 (ms)*	1.64 ± 0.14	-	<0.001*
ALT (U/L)	72.3 ± 46.22	12.94 ± 4.11	0.00286*
Fasting glucose (mmol/l)	6.8 ± 0.48	4.48 ± 0.49	0.00555*
2-hour plasma glucose (mmol/l)	9.04 ± 0.79	5.04 ± 0.60	0.00901*
Fasting insulin (PMOL/L)	66.23 ± 5.09	55.9 ± 8.44	0.00234*
Fasting C-peptide (nmol/l)	0.82 ± 0.17	0.67 ± 0.24	0.0117
HOMA-IRI	2.72 ± 0.3	1.58 ± 0.16	0.00756*
HOMA-ISI	0.62 (0.58–0.68)	0.51 (0.48–0.7)	0.0157*
HOMA-BFI	75.76 ± 14.14	183.84 ± 92.52	0.00258*
HOMA-SC	28.74 (26.02–31.39)	35.85 (27.13–37.35)	0.0157*

BMI – body mass index; HOMA-IRI – insulin resistance index; HOMA-ISI – insulin sensitivity index; HOMA-BFI – β-cell function index; HOMA-SC – secretory capacity;

notes: data presented as mean ± SD or median (interquartile range);

*$p < 0.05$ (significant differences)

Table 2. The 20 most differentially expressed lncRNAs in patients with β-TM with prediabetes relative to matched controls

LNCRNA ID	p-value	FDR	Fold change	Reg.	CHROM	Strand	Relationship	Database
ENST00000496629	0.003526083	0.032526596	82.1860551	up	CHR8	-	bidirectional	GENCODE
ENST00000431759	0.000454682	0.010781106	37.6048365	up	CHR1	+	natural antisense	GENCODE
ENST00000583516	0.000556435	0.012007394	29.3773871	up	CHR3	-	intronic antisense	GENCODE
ENST00000582591	0.000794281	0.014552508	20.1327846	up	CHR18	+	natural antisense	GENCODE
T144753	9.72507E-05	0.004628744	19.1867411	up	CHR17	-	natural antisense	RNA-SEQ: IYER ET AL 2015
HBMT00001229624	0.001197038	0.018044649	19.0708748	up	CHR6	+	intergenic	FANTOM5CAT
T092137	0.005372424	0.041556365	18.7283573	up	CHR13	+	natural antisense	RNA-SEQ: IYER ET AL 2015
ENST00000495240	0.007330467	0.049419663	18.2757294	up	CHR21	-	bidirectional	GENCODE
ENCT00000417654	8.05354E-05	0.004137151	17.5584774	up	CHR7	-	intergenic	FANTOM5CAT
ENST00000523831	0.002313282	0.025628209	16.9052281	up	CHR8	+	intergenic	GENCODE
ENST00000581274	0.000623989	0.012711699	170.1365176	down	CHR18	-	intergenic	GENCODE
T048514	9.98219E-05	0.004660449	76.2685338	down	CHR10	-	intergenic	RNA-SEQ: IYER ET AL 2015
ENST00000421375	1.35499E-05	0.001367337	75.6516897	down	CHR3	-	intergenic	GENCODE
ENST00000556546	1.72633E-05	0.001558422	68.9262261	down	CHR14	-	intergenic	GENCODE
ENST00000608466	0.000131548	0.005419482	63.0881264	down	CHR5	-	bidirectional	GENCODE
ENCT00000192781	4.96226E-05	0.002997505	61.6649287	down	CHR18	+	intergenic	FANTOM5CAT
ENST00000586947	1.57655E-05	0.001487151	58.2341391	down	CHR18	+	intergenic	GENCODE
ENST00000495493	3.16861E-06	0.000598221	54.343005	down	CHR1	-	exon sense-over-lapping	GENCODE
AK024231	0.000182406	0.00656838	52.8044804	down	CHR17	+	intergenic	NRED
ENST00000511474	0.000135322	0.005506699	52.6697045	down	CHR5	+	intergenic	GENCODE

Reg. – regulation

Table 3. The 20 most differentially expressed mRNAs in patients with β-TM with prediabetes relative to matched controls

Gene symbol	p-value	FDR	Fold change	Regulation	CHROM
GDF15	2.26375E-05	0.002411457	100.2322219	up	CHR19
HBG1	0.000236675	0.008984146	37.5558847	up	CHR11
TMEM269	0.005760896	0.048273699	26.6075395	up	CHR1
ST8SIA5	0.004899657	0.045003731	21.4706818	up	CHR18
PAQR9	0.001958959	0.028087005	19.8210477	up	CHR3
TOMM5	0.002893375	0.034055763	18.4003285	up	CHR9
ACSM3	0.001112436	0.020491209	14.4928213	up	CHR16
BBS12	0.002714217	0.032809297	14.4565088	up	CHR4
SFRP2	0.010723854	0.067903233	12.6215505	up	CHR4
IFI44L	0.000374268	0.011411502	12.1742708	up	CHR1
GUCA2B	0.000243604	0.009222062	143.9714162	down	CHR1
B3GALNT2	2.82567E-05	0.002715737	86.152204	down	CHR1
TOX2	0.000854932	0.017757699	62.2958244	down	CHR20
FBXL13	0.000314764	0.010403005	54.406973	down	CHR7
OR10G4	2.96323E-06	0.000742725	39.4923179	down	CHR11
NAA80	2.12895E-07	0.000229889	33.3880965	down	CHR3
CLUH	1.51667E-06	0.000470002	32.7656707	down	CHR17
PCDHA9	5.28901E-05	0.00411625	29.1615088	down	CHR5
BCAP31	6.76081E-07	0.000338915	28.6257891	down	CHRX
CATG00000107414.1	0.01698672	0.088837575	25.8281041	down	CHR9

Supplementary Table 1. Reverse transcription-quantitative polymerase chain reaction primers for randomly selected lncRNAs

Transcript ID	Forward primer (5'–3')	Reverse primer (5'–3')
ENST00000431759	CCACGCAAACTCCTTCTGTA	GCCATTTTTTACCCTTTAGTTC
ENST00000611877	CTTCAGAGTGGGTGGTTTCC	CCTTCGCTGTCCTTTGAGTT
ENST00000564152	GTGGTTGGGTTTCTGAGTTTG	TGTCTGGCTTCCCTCTGTTC
ENST00000607613	TGCTGAGAGGGGTTTAGGAA	GGAATCTGGAAAACTGCCCA
ENST00000581274	ATGACATGGGGAAATGGAAGG	GCTGATCGCACTCAACTCTT
ENST00000449551	TTTCAGAGGAGTGGCTGGTA	TGGAGTGGATCACAGGCTTA
ENST00000437035	ACAAATCTGCCACTCAAGCC	TCTACTCCTGGATGTCTCTTCT
ENST00000556546	CTGCCTCCGATCCAAATTGT	CCTTGGAGAAACGCCATTGA

