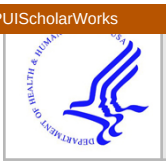

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Nucleic Acid Biomarkers of β Cell Stress and Death in Type 1 Diabetes

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Abstract

Purpose of the review—The purpose of this review is to summarize recent advances in the development of nucleic acid-based biomarkers in type 1 diabetes (T1D).

Recent findings—Recent rodent and human studies have identified new roles for stress pathways intrinsic to the β cell during the development of T1D. As such, methods to identify an authentic nucleic acid signature of β cell stress and/or death may improve our ability to predict T1D at earlier timepoints, allowing for optimal timing of immunomodulatory interventions. To this end, both targeted and unbiased approaches have begun to identify changes in microRNA expression patterns in T1D. Moreover, a number of groups have developed distinct assays that quantitatively detect circulating unmethylated insulin DNA, which is thought to primarily emanate from dying β cells.

Summary—Here we highlight unique blood and urine miRNA signatures identified in T1D cohorts, compare differences between first, second, and third generation assays that detect circulating unmethylated insulin DNA, and review recent technological advances that have the capacity to improve T1D biomarker development.

Keywords

Type 1 diabetes; miRNA; cell-free insulin DNA; biomarkers; unmethylated insulin DNA

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

Introduction

Since 1994, over 260,000 persons have undergone screening to identify cohorts of individuals at risk of developing Type 1 diabetes either as part of the Diabetes Prevention Trial of Type 1 Diabetes (DPT-1) or TrialNet Pathway to Prevention Initiatives. From these efforts, nearly 2000 autoantibody positive or diabetic individuals have participated in NIH-sponsored trials that have tested the ability of immunomodulatory therapies to preserve β cell function in established or pre-clinical T1D. Four agents have shown efficacy in preserving C-peptide secretion and these include a monoclonal antibody against CD20 (rituximab), monoclonoal antibodies against CD3 (tepluzimab), CTLA4-lg-mediated co-stimulatory blockade with abatacept, and alefacept, a fusion protein that binds CD2, and targets CD4+ and CD8+ effector memory T cells (1–6). However, the degree of preservation of β cell function afforded by these drugs has been modest. To date, a therapeutic regimen capable of robustly inducing immune tolerance or insulin independence has not yet been identified. In part, the inability of these treatments to dramatically modify T1D progression may be related to the timing of interventions, since irreparable damage to the β cell pool has already been firmly established by the time of T1D presentation and clinical recognition (7). One potential solution has been to target at-risk individuals before the onset of clinical disease. To this end, oral insulin, abatacept, and tepluzimab are currently being tested in pre-diabetic auto-antibody subjects enrolled in the Type 1 Diabetes TrialNet Pathway to Prevention Cohort. However, to support these efforts, there remains an unmet clinical need centered around improving our ability to more precisely predict risk in the pre-diabetic phase of T1D. Addressing this roadblock represents a critical step towards the development of personalized approaches to T1D prevention and/or reversal.

A number of immune variables including autoantibody (AAb) positivity, HLA status, and T cell signatures have been used to stratify risk (8–10). In clinical trials, measures of β cell function, such as loss of the early insulin response during intravenous or oral glucose tolerance test (OGTT) or changes in the integrated secretion of C-peptide during an (OGTT) have been utilized to document metabolic decompensation (11). However, these paradigms may either fail to reliably differentiate T1D progressors from non-progressors or are cumbersome to measure. Recently, rodent and human studies have begun to identify an increasing role for stress pathways intrinsic to the β cell during the development of type 1 diabetes (12, 13). These data suggest that processes such as β cell calcium dyshomeostasis, misfolded protein accumulation, oxidative stress, and endoplasmic reticulum stress become activated early during the evolution of T1D and act to either initiate or augment autoimmune mediated β cell death and dysfunction (14–17). Thus, directed methods to identify an authentic signature of β cell stress and/or death based on activation of these stress pathways may augment our ability to optimally time immunomodulatory interventions. Here, we highlight recent efforts aimed at the specific identification of nucleic acid biomarkers that may provide insight into the health of the β cell, focusing on microRNA signatures in type 1 diabetes and cell-free DNA based methodologies to measure β cell death.

Differentially expressed miRNAs in type 1 diabetes

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play a central role in the regulation of gene expression. In mammals, miRNAs are generally considered to be negative regulators of gene expression, acting either through translational repression of target mRNAs or by decreasing mRNA stability (18). However, in select cases, miRNAs are also involved in the upregulation of certain mRNAs (19, 20). Currently, upwards of 1800–2000 known human miRNAs have been identified, and it is estimated that miRNAs regulate ~60% of protein-coding genes (21). As such, a role for miRNAs has been demonstrated in a wide variety of biological processes, including development, proliferation, differentiation, cell signaling and cell death. In recent years, several specific miRNAs have also been shown to play important roles in both the regulation of β cell function and the pathogenesis of Type 1 diabetes (22). Although, miRNAs are intracellular origin, they may also be secreted extracellularly, either through microvesicles or exosomes, which can then be taken up by different cells to influence their gene expression patterns (23–25). Compared to mRNA species, miRNAs are invariably more stable in biological fluids, where they have been detected in high abundance in blood, urine, saliva, cerebrospinal fluid, milk, seminal fluid, and amniotic fluid (26). Thus, the identification of circulating miRNAs as biomarkers of specific disease processes is an area of active and intense research.

miRNA-375 is considered to be one of the most abundant miRNAs expressed in the β-cell. Early studies by Poy *et al.,* demonstrated that overexpression of miR-375 in MIN6 cells and primary mouse islets inhibited glucose-stimulated insulin secretion, independent of changes in glucose metabolism and Ca^{2+} signaling (27). Interestingly, mice lacking miR-375 were later found to be hyperglycemic as a result of decreased β-cell mass, increased alpha cell number, and increased glucagon secretion (28). Given the abundance of this miRNA in the β cell, a role for miR-375 as a diabetes biomarker has also been explored. Elevated plasma miR-375 levels have been demonstrated in streptozotocin-treated mice as well as in non-obese diabetic mice prior to the onset of hyperglycemia (29). Human subjects undergoing autologous and allogeneic islet transplantation were found to have elevated levels of miR-375 in plasma samples 7 days after transplantation (30). Moreover, Latrielle et al found that plasma miRNA-375 levels were elevated in C-peptide negative human subjects with Type 1 diabetes. However, this group also showed that β cell-derived miRNA-375 only contributes to about 1% of the total pool of circulating miRNA-375. Thus, while these authors speculated that increases in miR-375 may provide a reasonable assessment of β cell death, they questioned whether miRNA-375 was capable of providing insight into levels of β cell mass (31).

Unbiased approaches to identify other relevant miRNA species involved in the islet or β cell response to diabetic stress and T1D pathogenesis has also been undertaken. Data from global microarray profiling of human islets treated with a cocktail of pro-inflammatory cytokines documented increases in miR-21, miR-34a, and miR-146a. These miRNAs were also found to be increased in islets of NOD mice prior to the onset of hyperglycemia (32). In a subsequent study, the same group performed microarray profiling of islets from pre-diabetic NOD mice and found increased expression of miR-29a/b/c (33).

Comprehensive sequencing analysis of the serum miRNA profiles of subjects with new onset T1D has also revealed differential expression of multiple miRNAs (22). Nielsen and coworkers used global miRNA sequencing analysis to compare pooled sera from two cohorts of pediatric subjects who had been newly diagnosed with T1D within the past month and compared results to a healthy control group. After targeted PCR verification and adjustment for age and sex, the group identified a signature of 12 differently expressed miRNAs in T1D subjects, which included mi52, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR-200a. Interestingly, miR-25 was found to be negatively correlated with HbA1c one month after T1D diagnosis and positively correlated with stimulated C-peptide levels measured three months after diagnosis (34). A recent study by Osipova et.al. investigated circulating levels of three specific miRNAs, miR-21, miR-126 and miR-210, in plasma and urine samples of subjects with established T1D using quantitative RT-PCR. Compared to age-matched non-diabetic controls, levels of miR-21 and miR-210 were significantly increased in plasma and urine samples of subjects with T1D, while miR-126 was decreased in urine from subjects with T1D. Levels of urine miR-126 were also found to be negatively correlated with glycemic control (35).

Studies have also analyzed miRNA expression patterns the immune cell. fraction in individuals with T1D. Sebastiani and colleagues found that expression of miR-326 was increased in lymphocytes isolated from a small cohort of auto-antibody positive subjects with T1D compared to auto-antibody negative subjects with T1D (36). Similarly, Yang and colleagues identified miRNAs that were differentially expressed in PBMCs from subjects with new onset T1D compared to non-diabetic controls. This analysis identified 26 differentially expressed microRNAs. MiR-146 was identified as the mostly significantly changed and levels of miR-146 were decreased by nearly 50% in PBMCs from persons with T1D (37).

Differently methylated β-cell derived DNA as a type 1 diabetes biomarker

The recent identification and characterization of cell-free DNA in bodily fluids and has also emerged as a promising and non-invasive method for disease monitoring, and this approach typically utilizes information regarding the methylation status of a specific gene of interest. DNA methylation occurs through the addition of a methyl group to a cytosine-guanine (CpG) dinucleotide and serves to either silence or promote gene expression in a specific cell type (38). In cells where a gene is highly expressed, the promoter and coding regions are primarily unmethylated. Conversely, in cells where a specific gene is quiescent, the promoter tends to be hypermethylated. Changes in patterns of DNA methylation may be observed during development, entry into cell cycle, aging, as part of disease pathogenesis, and during cell death (39, 40). The use of cell-free DNA as a diagnostic biomarker was pioneered in the cancer field, where cancer cells have been found to exhibit aberrant DNA methylation patterns in oncogenes (41).

In an idea first proposed by Akirav and Herald, the use of assays that detect circulating cell-free DNA has been extended to the field of T1D (42). To date, these efforts have focused on the detection of circulating unmethylated insulin DNA and take advantage of the fact that

certain CpG sites in the insulin gene (Ins1/Ins2 in mice and INS in humans) are specifically unmethylated in pancreatic β-cells and highly methylated in most other tissues (43) Hence, as β-cells die during the evolution of T1D, it is presumed that increased unmethylated insulin DNA emanates from the β-cell and is detectable in the circulation. First generation assays to detect β-cell derived unmethylated insulin DNA were performed using a nested PCR approach, where isolated DNA from serum or plasma was subjected to bisulfite conversion, followed by a methylation insensitive PCR amplification step, manual extraction of the PCR product from a gel, followed by a methylation specific PCR step (42, 44, 45). The major limitation of these methods included potential non- specific amplification of non-target sequences, high background signals, and the inability to perform these assays in a high-throughput manner outside of the research setting.

Second-generation insulin (INS) DNA assays have taken advantage of methylation specific multiplex PCR assays targeting multiple CpG sites on the insulin gene as well as highly sensitive droplet digital PCR-based detection techniques. Indeed, these methodologies appear to have led to improved sensitivity and reduced non-specific background amplifications. Using an assay that detects two distinct methylation-sensitive sites of the human insulin gene at positions +396 and +399 from the transcriptional start site (46), Herold and colleagues showed that autoantibody positive at-risk individuals who progressed to type 1 diabetes while being followed in the TrialNet Pathway to Prevention study had higher ratios of unmethylated/methylated INS DNA compared to healthy control subjects. Furthermore, the ratio of unmethylated/methylated INS DNA was associated with alterations in the insulin secretory pattern (47). Fisher et al reported on a droplet digital PCR based assay targeting differences in the methylation status of the −69 bp position of human INS DNA and showed that pediatric subjects with new onset T1D had higher circulating levels of both methylated and unmethylated insulin DNA compared to age, gender, and weight category matched non-diabetic control individuals (48).

An important distinction between these two assays has centered around normalization methods. The Herold group reports data as a ratio of unmethylated/methylated insulin DNA, while the Mirmira groups reports values for the methylated and unmethylated probes individually. Consistent with the idea that levels of methylated INS DNA may also change dynamically with disease, Fisher and colleagues found that both unmethylated and methylated INS DNA levels were elevated in individuals with new-onset T1D. However, at 8 weeks after T1D diagnosis, levels of the unmethylated INS DNA decreased but methylated INS DNA levels remained elevated. At present, it remains unclear to what extent each species might be independently informative of the underlying disease process. Furthermore, it is not clear precisely which cell populations contribute to circulating levels of methylated INS DNA. Interestingly, a recent report showed that the methylation status of the insulin gene in the β cell becomes progressively more methylated in the non-obese diabetic (NOD) mouse model during the evolution of T1D. These data would suggest that increased levels of methylated INS DNA may also be informative of ongoing β cell death (49).

Yet another distinct approach, or a third generation assay, has been recently described by the Dor group. This approach was predicated on global assessment of tissue specific DNA methylation patterns identified from an Illumina Infinium Human Methylation 450 Bead

Chip array or obtained from publicly available methylome databases. Next, regional tissue-specific patterns of methylation were characterized based on assessment of the methylation status of a CpG marker site combined with the assessment of 4–9 additional CpG sites adjacent to the original marker site. This information was used to design assays that utilized DNA sequencing to quantify the methylation status of a particular region of interest in a tissue specific manner. By example, an assay to detect β cell derived DNA interrogates the methylation status of 6 CpG sites across the insulin promoter region. Using this approach, the authors found that demethylation at all 6 sites was present in ~80% of DNA molecules from β cells compared to less than 0.01% of DNA from other tissues. As proof of principle, samples from persons with recent onset T1D were analyzed, and results compared to healthy controls. The fraction of circulating cell-free DNA in plasma from persons with new onset T1D was 1.9–5.5%, compared to 0.12% in healthy controls (50).

Conclusion: Current roadblocks for robust biomarker development: Charting a path forward

While encouraging progress have been made towards the identification of a miRNA signature of Type 1 diabetes and the development of cell-free DNA assays that detect β cell death, additional refinements are still needed to translate these findings into clinical practice. With a few exceptions, the majority of studies described in this review have utilized cross-sectional analyses of clinical cohorts. Future studies focused on identifying longitudinal changes in biomarker panels in at-risk or autoantibody positive pre-diabetic subjects will likely yield critical new information regarding elevations of biomarkers prior to the onset of clinically detectable T1D. Likewise, efforts to identify novel biomarkers will also be enhanced by technological advancements. For example, Joshi et al recently described the use of label free plasmonic biosensors for detection of circulating miRNAs in plasma samples of subjects with pancreatic ductal adenocarcinoma. This technique does not require RNA extraction and reportedly measures miRNAs levels in the attomolar range (51). Moreover, the identification of exosomes derived specifically from the β cell may increase the sensitivity and specificity of this approach. However, at present, techniques to sort β cell specific exosomes do not exist.

In addition to further refinements focused on detection of circulating INS DNA, the use of methylation specific probes to detect genes other than the insulin gene should increase both the sensitivity and specificity of this approach. Such an approach will require the rigorous and unbiased identification of other genes that are differentially methylated in the pancreatic β cell compared to non β cells. However, these efforts will also need to consider dynamic changes in methylation patterns that occur as part of T1D pathogenesis.

Recently, Snyder and colleagues performed deep sequencing of circulating cell-free DNA to develop genome-wide maps of nucleosome occupancy. In healthy individuals, their analysis showed that the circulating nucleosome footprint most closely matched cells of hematopoietic lineage. These results were then compared to the maps obtained from a small group of individuals with advanced malignancy. Interestingly, their analysis showed that patterns of nucleosome spacing in these individuals revealed contributions to the cell-free

DNA pool that differed from healthy individuals and closely matched the origin of that individual's underlying cancer. This methodology may provide a second method that could be used to infer cells of origin for cell-free DNA analysis (52). However, whether this method could similarly be applied to type 1 diabetes remains to be tested.

In summary, the identification of a robust signature of β-cell stress and death that can be used to accurately predict T1D and guide therapeutic choices remains elusive. Success in this endeavor will most likely require the use of composite biomarker panels that incorporate clinical information, nucleic acid biomarkers discussed here and protein, metabolomics, and lipidomic signatures. While continued discovery efforts will be required, is important that such discovery does not continue in isolation, and efforts to integrate these diverse dataset is rapidly pursued.

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KEY POINTS

1. MicroRNAs are stable in a variety of biological fluids. Unique serum and urine microRNA signatures have been described in human Type 1 diabetes cohorts, suggesting their potential utility as biomarkers of β cell stress and death.
2. Quantitative methods to measure β cell-derived cell free DNA in plasma or serum may help identify β cell destruction in Type 1 diabetes. Recent efforts have focused on the refinement of assays that detect circulating levels of unmethylated insulin DNA.
3. The accurate prediction of β cell stress and death in Type 1 diabetes will likely require the use of composite biomarker panels that incorporate a variety of nucleic acid species.