BRIEF REPORT:

Elevations in Circulating Methylated and Unmethylated Preproinsulin DNA in New-Onset Type 1 Diabetes

Abbreviated title: Elevated circulating preproinsulin DNA in T1D

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Key words: Islet, biomarker, type 1 diabetes, type 2 diabetes

Word count: 1,998

Number of figures/tables: 4
Abstract:

Elevated ratios of circulating unmethylated to methylated preproinsulin \((INS)\) DNA have been suggested to reflect β-cell death in type 1 diabetes (T1D). We tested the hypothesis that absolute levels (rather than ratios) of unmethylated and methylated \(INS\) DNA differ between new-onset T1D subjects and controls, and assessed longitudinal changes in these parameters. We used droplet digital PCR to measure levels of unmethylated and methylated \(INS\) DNA in serum from subjects at T1D onset, and at 8 weeks and one year post-onset. Compared to controls, levels of both unmethylated and methylated \(INS\) DNA were elevated at T1D onset. At 8 weeks post-onset, methylated \(INS\) DNA remained elevated, but unmethylated \(INS\) DNA fell. At one year post-onset, both unmethylated and methylated \(INS\) DNA returned to control levels. Subjects with obesity, type 2 diabetes (T2D), and autoimmune hepatitis exhibited lower levels of unmethylated and methylated \(INS\) compared to T1D subjects at-onset and no differences compared to controls. Our study shows that elevations in both unmethylated and methylated \(INS\) DNA occurs in new-onset T1D and that levels of these DNA species change during T1D evolution. Our work emphasizes the need to consider absolute levels of differentially methylated DNA species as potential biomarkers of disease.
Introduction

The diagnosis of type 1 diabetes (T1D) is made at a time when individuals have lost substantial β-cell mass and function (1,2). Interventions instituted at T1D diagnosis have failed to result in recovery of β-cell function, raising the possibility that earlier detection of β-cell death might provide an opportunity for preventative interventions prior to T1D onset (3). Recently, several groups have proposed the measurement of circulating unmethylated DNA encoding preproinsulin (INS) as a biomarker of β-cell death (4–10), since β-cells have a much higher frequency of unmethylated CpG sites compared to other cell types (6,11,12) and might release this DNA species into the circulation upon death. In these studies, unmethylated INS DNA was expressed as a ratio relative to methylated INS DNA for normalization purposes. However, because β-cells and many other cell types in the islet contain some fraction of both unmethylated and methylated INS (6,12,13), it remains unclear to what extent each species of INS might be independently informative of the underlying disease process in T1D.

Droplet digital PCR (ddPCR) employs the analysis of discrete individual PCR reactions (~20,000/sample) to identify the presence of target DNA, and utilizes Poisson statistics to extrapolate the copy number of target DNA per sample (14). This technology enables direct quantitation of differentially methylated DNA species in serum without the need for normalization. We used ddPCR to analyze serum from individuals with new-onset T1D to test the hypothesis that absolute levels (rather than ratios) of unmethylated and methylated INS differ between new-onset T1D subjects and controls. We also assessed longitudinal changes in INS during T1D evolution and evaluated levels of these DNA species in cohorts with established T2D and autoimmune hepatitis as controls for hyperglycemia and autoimmunity, respectively.

Methods

*Human subjects and islets.* Serum samples were obtained from pediatric T1D subjects at disease onset (within 2 days of diagnosis) and then at 8 weeks and 1 year after onset at Riley Hospital for Children. Subjects were 5-15 years of age and did not present in diabetic ketoacidosis. Banked
serum from healthy pediatric subjects, lean and obese adults without diabetes, adults with T2D (duration of disease 7.1±1.1 years) and adults with autoimmune hepatitis were obtained for comparisons. Protocols were approved by the Indiana University Institutional Review Board. Parents of subjects provided written informed consent and children >age 7 provided assent for their participation. Human islets were obtained from the Integrated Islet Distribution Program.

**Animals.** CD1, NOD, and NOD-SCID mice were maintained under protocols approved by the Institutional Animal Care and Use Committee. Mice were fed regular chow and water ad libitum. Some mice underwent transplant of 200 human islets under the renal capsule, as described previously (15). Blood was collected via tail vein for PCR assays.

**DNA extraction and bisulfite treatment.** DNA was extracted from human islets using the genomic DNA extraction kit (Sigma-Aldrich). DNA was extracted from approximately 20 µl of mouse serum and 30-50 µl of human serum using the ZR serum DNA kit (Zymo Research) or the QIAamp DNA blood mini kit (Qiagen) with 5 µg of poly-A DNA as carrier. DNA recovery was ~85%, with <10-15% variance between samples. All samples then underwent bisulfite conversion using the EZ DNA Methylation Kit or the EZ DNA Methylation-Lightning Kit (Zymo Research), and conversion was verified using a pre- and post-conversion sample in the ddPCR.

**PCR Analysis.** Samples were analyzed by ddPCR utilizing a dual fluorescent probe-based multiplex assay. For human *INS* promoter amplification, the following primers were used: 5’-GGAAATTGTAGTTTTAGTTTATTTGT-3’ (forward); 5’-AAAACCCCATCTCCCCTACCTATCA-3’ (reverse) in combination with probes that detected methylation or unmethylation at bp -69: 5’-ACCCCTACCGCCTAAC-3’ (VIC); 5’-ACCCCTACCACCTAAC-3’ (FAM). We chose this site based on prior studies, which showed that position -69 in *INS* retained its unmethylated state in β-cells even under inflammatory stress of T2D (12). Primers and probes for mouse *Ins2* DNA were described previously (4). Amplified human *INS* PCR products were sequenced to confirm the PCR product
identities. ddPCR was performed using ddPCR Supermix for Probes (Bio-Rad) with the following cycling conditions: 95°C for 10 min, 94°C for 30 s and 57.5°C for 60 s for 40 cycles. Droplets were analyzed by a QX200 Droplet Reader and QuantaSoft Software (Bio-Rad), from which a concentrations (copies/µl) of methylated and unmethylated INS DNA were obtained. This final concentration was extrapolated to copies/µl serum, then log-transformed for parametric statistical analysis.

Statistics. For direct comparisons of methylated and unmethylated INS DNA levels, two-tailed unpaired student’s t-tests were used. For comparisons of longitudinally collected samples, two-tailed paired student’s t-tests were used. P-values <0.05 were considered significant. Statistical calculations were performed using Prism 5.0 Software (GraphPad).

Results

We developed a methylation-specific PCR (MSP) assay to simultaneously quantitate methylation or unmethylation at the CpG at INS position -69 bp, shown in prior studies to be preferentially unmethylated in β-cells (6,11). Control plasmids containing bisulfite-converted methylated or unmethylated INS DNA were used to standardize the MSP assay in ddPCR. Fig. 1A shows the gating strategy (in 2-dimensional ddPCR plots) to distinguish methylated, unmethylated, and double-positive INS-containing droplets. To verify linearity and ability to distinguish simultaneous mixtures of the DNA species, mixtures of plasmids at varying ratios were subjected to ddPCR, as shown in the one-dimensional plots in Fig. 1B and C. These methylation specific plasmids were used to construct linearity curves over the range of DNA copy numbers observed in serum (Fig. 1D).

To test if our MSP assay detects dying human β-cells in vivo, we transplanted human islets into healthy immunocompetent CD1 mice, and allowed the islets to undergo xeno-rejection. Unmethylated human INS peaked in the serum at 6 h post-transplantation, falling to undetectable levels by 48 h (Fig. 2A). By contrast, only a slight (insignificant) increase in methylated INS was detectable at 6 h post transplantation (Fig. 2B). Neither unmethylated nor methylated human INS was
measurable in non-transplanted mice, and neither unmethylated nor methylated mouse Ins2 was altered in transplanted mice (Fig. 2B).

We tested our MSP assay in a mouse model of autoimmune β-cell destruction (NOD mice). Compared to NOD-SCID and CD1 controls, NOD mice exhibit elevated levels of both unmethylated and methylated mouse Ins2 in the pre-diabetic phase, with levels falling at the time of diabetes (Fig. 2C and D). To correlate these findings to humans, we next assessed subjects with new-onset T1D. Serum was obtained from 32 pediatric subjects within 48 hours of T1D diagnosis. Additionally, 24 of these subjects had serum collected 8 weeks post onset and 8 had serum collected one-year post onset. Relevant demographic and laboratory data of these subjects and control groups are presented in Table 1, and representative 2-dimensional and 1-dimensional plots are shown in Supplemental Fig. 1. As shown in Fig. 3A and C, levels of both unmethylated and methylated INS DNA were significantly higher in subjects at T1D onset compared to healthy controls (p<0.0001), similar to NOD mice. At 8 weeks following T1D onset, levels of unmethylated INS decreased significantly (p<0.0001) and were no different than controls (Fig. 3A and B). By contrast, levels of methylated INS DNA remained elevated 8 weeks after T1D onset compared to controls (p<0.0001), falling below control levels one-year post onset (p=0.02, Fig. 3C and D). Unmethylated INS remained at the same levels one-year post T1D onset as at 8 weeks post onset, but was higher than controls (p<0.0001) (Fig. 3A and B).

We asked if elevated levels of unmethylated and methylated INS observed in NOD mice and T1D subjects reflects a generalized response to either autoimmunity or prevailing hyperglycemia. We performed MSP assays using serum from adults with active autoimmune hepatitis, T2D, and lean and obese healthy controls (Table 1). Unmethylated INS levels were not different between lean and obese adults, and methylated INS DNA levels were slightly lower, but with significant overlap, in the obese controls (p=0.04) (Fig. 3A and 3C). Methylated and unmethylated INS in both of these adult control groups were higher than pediatric controls, suggesting that these circulating DNA species may exhibit age-related differences (Fig. 3A and C). Compared to both healthy adult control groups, unmethylated and methylated circulating INS levels were lower or no different in subjects with
autoimmune hepatitis and T2D (Fig. 3A and C). Additionally, circulating unmethylated and methylated INS DNA were lower in autoimmune hepatitis and T2D subjects than T1D subjects at onset (Fig. 3A and C). Collectively, these data suggest that elevations in unmethylated and methylated INS DNA are not observed in T2D or autoimmune disease in general.

Discussion

Elevations of unmethylated INS DNA have been shown to correlate with dying β-cells in both mice and humans (4–6,10). Whereas the ratio of unmethylated to methylated INS was used in prior studies, we employed ddPCR to determine absolute copy numbers of both DNA species in several human cohorts. Our data reveal three important new findings: (1) unmethylated INS is increased at T1D onset, and falls to control levels by 8 weeks post-onset, (2) methylated INS is elevated at T1D onset and 8 weeks post-onset, but falls by one year, and (3) elevations in both methylated and unmethylated INS DNA appear to be specific for new-onset T1D, since concomitant elevation of both species is neither observed at any subsequent time point in T1D nor in other disorders of immunity or glycemia.

Whereas elevations in unmethylated INS are thought to arise primarily from islet β-cells, an unexpected finding in our study was the elevation of methylated INS in T1D, which could arise from any cell type (6). A previous study examined the correlation between cell-free plasma DNA (human β-globin DNA) and severity of illness (sepsis) in humans (16); the authors observed increasing levels of cell-free DNA with sepsis severity. In this regard, our new-onset T1D cohort did not present with underlying infections or ketoacidosis that could have led to elevated methylated INS. Moreover, other concurrent stresses (hyperglycemia and autoimmunity) are unlikely to contribute, since individuals with T2D and autoimmune hepatitis did not present with elevations relative to control populations.

Several scenarios might account for the elevated unmethylated and methylated INS in both human and mouse new-onset T1D. First, it is possible that both species of DNA arise from different cells within the islet affected by T1D immunopathogenesis (β-cells, α cells, T cells, macrophages). However, our results from the mouse transplant studies suggest the source of methylated INS DNA is
extra-islet, since xeno-rejection of transplanted human islets did not produce a significant increase in methylated *INS* DNA. Second, it is possible that neither species arises exclusively from islets, but rather from some other cell type specific for T1D, such as thymic cells or T cells. In blood and spleen cells, up to 10-20% of clones exhibit unmethylated *INS* DNA (6), a finding that is consistent with the relative levels of circulating unmethylated and methylated *INS* in our studies. Finally, it is possible that the elevation in unmethylated *INS* DNA arises only from β-cells, while the elevation in methylated *INS* arises from other cell types related to T1D autoimmunity; this explanation seems most convincing, as xeno-rejection of human islets produced a significant increase in unmethylated *INS*.

Several differences between our study and those previously published should be emphasized. Ours is the first to study a new-onset T1D population. Prior studies have primarily tested recent onset T1D individuals (within 4-18 months of onset) (5,8), in whom insulin administration might have impacted β-cell survival. Also, our assay interrogates methylation at a site different than those of prior studies. It is possible that different sites exhibit different methylation patterns as disease evolves (12), making comparisons between different site-specific assays difficult. Finally, ours is the first study to examine index cases of T1D longitudinally and to compare cases to other relevant populations to exclude confounding effects of concurrent pathophysiologic phenomena. Our study suggests that unique patterns of circulating methylated and unmethylated *INS* DNA may be specific for new-onset T1D, and thereby emphasizes the need to consider absolute levels of differentially methylated DNA species as potential biomarkers of disease. Examination of circulating levels of these DNA species in pre-diabetic populations are needed to determine their utility in predicting eventual disease onset.
Author contributions

MMF, RAW, JB, CEM, LAD, KJM, SAT, and RGM designed research; MMF, RAW, NC, and SAT performed research; MMF, KJM, SAT, and RGM analyzed data; MMF and RGM wrote the manuscript; all authors approved the final draft of the manuscript.

Acknowledgements

This work was supported by NIH grants UC4 DK104166 (to RGM and CEM), T32 DK065549 (to MMF), and K24 DK069290 (to NPC), an American Diabetes Association Junior Faculty Award (to SAT), JDRF grant 3-SRA-2014-41 (to CEM, JB, and LAD), and by grants from the Ball Bros. Foundation and George and Francis Ball Foundation (to RGM and CEM). The authors wish to acknowledge the assistance of Dr. F. Meah and Ms. Jennifer Terrell (both from Indiana University) in data acquisition, and Ms. K. Benninger and Ms. J. Nelson (both from Indiana University) in the Center for Diabetes and Metabolic Diseases Islet Core and Translation Core for transplantation of human islets and for the performance of ddPCR assays. The authors would also like to thank Dr. Susan Ragg, the INbank® biorepository, and Fairbanks Institute, who provided banked serum samples.

MMF and RGM are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

The authors have no conflicts of interests to disclose
References


Table 1
Demographic and Laboratory Evaluation of Subject Cohorts

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<th></th>
<th>Pediatric Control</th>
<th>T1D at onset</th>
<th>T1D at 8 weeks</th>
<th>T1D at 1 Year</th>
<th>Lean Adult Control</th>
<th>Obese Adult Control</th>
<th>Adult T2D</th>
<th>Adult Autoimmune Hepatitis</th>
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<tr>
<td><strong>Age (yrs)</strong></td>
<td>9.5 ± 3.6</td>
<td>10.8 ± 3.0</td>
<td>10.7 ± 3.1</td>
<td>11.6 ± 3.1</td>
<td>51.3 ± 9.0</td>
<td>49.3 ± 5.6</td>
<td>48.6 ± 7.5</td>
<td>47 ± 14</td>
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<td><strong>Female/Male</strong></td>
<td>14/13</td>
<td>14/18</td>
<td>11/13</td>
<td>1/7</td>
<td>12/3</td>
<td>11/10</td>
<td>5/12</td>
<td>12/2</td>
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<tr>
<td><strong>BMI Z-Score</strong></td>
<td>-0.08 ± 0.7</td>
<td>+0.07 ± 1.4</td>
<td>+0.54 ± 0.9</td>
<td>+0.16 ± 1.0</td>
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<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
<td></td>
<td>22.9 ± 1.2</td>
<td>32.7 ± 3.5</td>
<td>35.9 ± 7.4</td>
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<tr>
<td><strong>Hgb A1c (%)</strong></td>
<td>11.3 ± 1.7</td>
<td>7.6 ± 0.8</td>
<td>8.3 ± 1.4</td>
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<td>8.4 ± 1.6</td>
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<tr>
<td><strong>C-Peptide (pM)</strong></td>
<td>150 ± 167</td>
<td>303 ± 166</td>
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Figure Legends:

Figure 1. MSP assay specificity and validation. (A), 2-dimensional plots using plasmid standards for unmethylated and methylated INS DNA, and for a 1:1 mixture of the two plasmids. Arrows identify the unmethylated, methylated, and unmethylated + methylated (double-positive) INS DNA-containing droplets. (B) and (C), dilutions of plasmids containing bisulfite-converted unmethylated and methylated INS DNA were subjected to ddPCR; 1-D plots from ddPCR are shown for fluorescent probes specific for unmethylated INS DNA (B) and methylated INS DNA (C). In panels (B) and (C), the positive, negative, and overlap (FAM probe overlapping into the VIC channel, and vice-versa) signals are identified. (D), quantitation of plasmid dilution curves, presented as copies/µl; r²=0.9818 for unmethylated INS DNA; r²=0.9685 for methylated INS DNA.

Figure 2. Circulating unmethylated and methylated human INS and mouse Ins2 DNA levels in transplanted immunocompetent mice and NOD mice. (A) and (B), CD1 mice (n=4) were either transplanted (Transplant) or not (No Transplant) with 200 human islets beneath the kidney capsule. Serum was collected at the time points indicated and processed for MSP assay. Circulating unmethylated (A) and methylated (B) INS and Ins2 DNA levels were measured by ddPCR. (C) and (D), serum from NOD, NOD-SCID, and CD1 mice (n=3 per group) were collected at the ages indicated and at the age NOD mice developed diabetes (12-14 weeks, Diabetes), and processed for MSP assay. Circulating unmethylated (C) and methylated (D) Ins2 DNA levels were measured by ddPCR. *p<0.05 compared to time 0 in panels (A) and (B), and *p<0.05 compared to CD1 mice at the corresponding age in panels (C) and (D).

Figure 3. Circulating unmethylated and methylated INS DNA levels in human cohorts. (A), circulating unmethylated INS DNA levels in human cohorts depicted as Log(copies/µl). (B), longitudinal change in circulating unmethylated INS DNA levels in pediatric T1D subjects at onset, 8 weeks following onset, and 1 year following onset. (C), circulating methylated INS DNA levels in
human cohorts depicted as Log(copies/µl). (D), longitudinal change in circulating methylated INS DNA levels in pediatric T1D subjects at onset, 8 weeks following onset, and 1 year following onset. 
*p<0.05, ***p<0.0001, **p<0.001, ns = not significant (p>0.05).
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239x357mm (300 x 300 DPI)
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144x146mm (300 x 300 DPI)
Supplemental Figure 1. Representative 2D and 1D ddPCR plots from Control and T1D subjects. (A), 2D plot from a representative Control subject. (B), 2D plot from a representative new-onset T1D subject. Arrows (A) and (B) identify the respective unmethylated, methylated, and unmethylated + methylated (double-positive) INS-containing droplets. (C), representative 1D plots for methylated INS DNA from two control and two new-onset T1D subjects. (D) representative 1D plots for unmethylated INS DNA from two control and two new-onset T1D subjects. In panels (C) and (D), the positive, negative, and overlap (FAM probe overlapping into the VIC channel, and vice-versa) signals are identified.