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ORIGINAL ARTICLE

Novel method utilizing bisulfite conversion with dual amplification-refractory mutation system polymerase chain reaction to detect circulating pancreatic β-cell cfDNA

Asami Okada[1†], Misuzu Yamada-Yamashita[2†], Yukari Tominaga[2], Kyoka Jo[2], Hiroyasu Mori[2], Reiko Suzuki[2], Masashi Ishizu[2], Motoyuki Tamaki[2], Yuko Akehi[2], Yuichi Takashi[2], Daisuke Koga[3], Eisuke Shimokita[4], Fuminori Tanihara[5], Kiyoe Kurahashi[6], Sumiko Yoshida[6], Yukari Mitsui[6], Shiho Masuda[6], Itsuro Endo[6,7], Ken-ichi Aihara[6,8], Shoji Kagami[1], Masahiro Abe[6], Kevin Ferreri[9], Yoshio Fujitani[10], Munehide Matsuhisa[2], Akio Kuroda[2*]

[1]Department of Pediatrics, Tokushima University, Tokushima, Japan, [2]Diabetes Therapeutics and Research Center, Institute of Advanced Medical Sciences, Tokushima University, Tokushima, Japan, [3]Diagnostic Division, Department of Research, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan, [4]Department of Anatomy and Cell Biology, Tokushima University Graduate School of Medical Sciences, Tokushima, Japan, [5]Animal Resource Laboratory, Center for Development of Advanced Medical Technology, Jichi Medical University, Shimotsuke, Japan, [6]Department of Hematology, Endocrinology and Metabolism, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan, [7]Department of Chronomedicine, Tokushima University Graduate School of Medical Sciences, Tokushima, Japan, [8]Department of Community Medicine and Medical Science, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan, [9]Department of Translational Research & Cellular Therapeutics, City of Hope, Duarte, California, USA, and [10]Developmental Biology and Metabolism, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan

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***Correspondence**
Akio Kuroda
Tel.: +81-88- 633-7587.
Fax: +81-88-633-7589.
E-mail address:
kurodaakio@tokushima-u.ac.jp

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ABSTRACT

Aims/Introduction: Several research groups have reported methods for quantifying pancreatic beta cell (β-cell) injury by measuring β-cell-specific CpG unmethylation of the insulin gene in circulation using digital droplet PCR or next-generation sequencing. However, these methods have certain disadvantages, such as the need to consider the background signal owing to the small number of target CpG sites and the need for unique equipment.

Materials and Methods: We established a novel method for detecting four CpG unmethylations of the insulin gene using two-step amplification refractory mutation system PCR. We applied it to type 1 diabetes (T1D) patients with a wide range of disease durations and to healthy adults.

Results: The assay showed high linearity and could detect a single copy of unmethylated insulin DNA in experiments using methylated and unmethylated plasmid DNA. The unmethylated insulin DNA level in the type 1 diabetes group, whose β-cell mass was considerably reduced, was similar to that of healthy adults. An inverse correlation was observed between copy number and disease duration in patients with unmethylated insulin DNA-positive type 1 diabetes.

Conclusions: We developed a novel method for detecting unmethylated insulin DNA in circulation that can be performed using a conventional real-time PCR system. This method would be useful for analyzing dynamic profiles of β-cells in human disease such as type 1 diabetes.

INTRODUCTION

Type 1 diabetes (T1D) is characterized by the progressive destruction of insulin-secreting beta cells (β-cells) due to autoimmune attacks by T cells and other immune cells. Symptoms of hyperglycemia appear when 70–80% of β-cell function is impaired in type 1 diabetes[1]. If the onset of type 1 diabetes can be predicted in the early stages, it may be possible to intervene and prevent disease progression. Biomarkers that can quantitatively assess β-cell death are required.

†These authors contributed equally to this work

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Recently, the novel biomarker, differentially methylated circulating cell-free DNA (cfDNA), has become the focus of substantial research[2]. cfDNA is a DNA fragment released by dying cells into circulation. Each tissue has a unique DNA methylation pattern; therefore, it is possible to determine the tissue of origin by detecting tissue-specific methylation markers[3].

DNA methylation, the addition of a methyl group at the fifth position of cytosine in a CpG dinucleotide, is a relatively stable component of the epigenome. DNA methylation establishes and stabilizes cellular phenotypes by maintaining the gene expression status[4]. While methylated cytosines are unaffected by bisulfite treatment, unmethylated cytosines are converted to deoxyuracil and participate in subsequent primer annealing and amplification as thymines[5–7]. The human insulin gene is predominantly expressed in pancreatic β-cells, and CpG sites in the promoter and exon regions of the insulin gene are tissue-specifically unmethylated in pancreatic β-cells[3,8–10]. This feature is useful, as the extent of β-cell injury can be estimated by measuring unmethylated insulin cfDNA in circulation. Advanced technologies are used to quantify cfDNA methylation, including digital droplet polymerase chain reaction (ddPCR) with a probe that recognizes one or two CpG sites[11–13] and next-generation sequencing (NGS), which distinguishes six CpG sites at a time[3] using bisulfite-converted cfDNA. However, ddPCR recognizes a small number of CpG sites, rendering it insufficiently specific to distinguish β-cell-derived from non-β-cell-derived cfDNA. NGS is more reliable than conventional clone-based bisulfite sequencing; however, it is expensive and requires a large amount of cfDNA and complicated output data.

The amplification refractory mutation system (ARMS) PCR was developed to detect single nucleic acid mutations[14,15]. The principle of ARMS PCR is that oligonucleotides with a mismatched 3′-residue do not function as PCR primers. PCR proceeds if there is a single mismatch at the 3′ end of the primer, however, it does not proceed if there are more than two mismatches. Using this method, the unmethylated CpG sites were identified after bisulfite conversion. No reports have described the application of ARMS PCR after bisulfite conversion.

We developed an ARMS PCR to detect unmethylated insulin DNA after bisulfite conversion in this study. We used specific and quantitative unmethylation-specific ARMS PCR in patients with type 1 diabetes using an ordinary real-time PCR system.

MATERIALS AND METHODS

Genomic DNA from human tissues

Genomic DNA from human tissues such as pancreas, liver, lung, adipose, muscle were obtained from BioChain Institute Inc., Newark, NJ, USA. Genomic DNA from human blood was obtained from Promega, Tokyo, Japan.

Patients

We enrolled 114 patients with type 1 diabetes and 31 healthy volunteers in this study. Written informed consent was obtained from all participants. This study was approved by the Tokushima University Hospital Institutional Review Board (ID: 2320) and registered with the University Hospital Medical Information Registry (ID: 000033501). This study was conducted in accordance with the guidelines of the International Clinical Trials Registry Platform the Declaration of Helsinki.

cfDNA sample preparation and bisulfite conversion

Blood samples from each participant were left to sit at room temperature for 30 min. Serum was prepared by two consecutive centrifugation steps (2000 *g*, 12 min and 2,650 *g*, 12 min) at 4 °C. cfDNA was isolated from 1 mL of serum using a NextPrep-Mag cfDNA Isolation Kit (BIOO Scientific Corporation, Austin, TX, USA) and eluted in 24 μL. The double-stranded cfDNA concentration was measured using Quantus (Promega, Tokyo, Japan) with 2 μL of cfDNA. Twenty microliters of eluted cfDNA were treated with an innuCONVERT Bisulfite Basic Kit (AJ Imnuscreen GmbH, Berlin, Germany) and eluted in 50 μL, as indicated.

Quantitative real-time PCR reaction and conditions (Unmethylated and methylated control DNA)

Neiman *et al.* reported that CpG sites in the promoter region of the insulin gene are highly unmethylated in β-, alpha, and delta cells, and that CpG sites downstream of the transcription start site (TSS) are unmethylated more specifically in β-cells[10]. Therefore, we selected the coding regions (+331, +367, +374, and +404 bp from TSS) as targets. The human insulin gene was obtained from the human liver genomic DNA (BioChain Institute Inc., Newark, NJ, USA). Methylated CpG sites become unmethylated after PCR amplification because methyltransferases are absent. Liver genomic DNA was amplified with the #1 primer set (Table 1), and the PCR amplicon was bisulfite-converted using the kit described above. This DNA fragment was amplified with the #2 primer set (Table 1) and served as unmethylated control DNA, in which the four CpG target sites were converted to 'TG'. The bisulfite-converted human liver

Table 1 | PCR primers

PCR Name	Direction	Primer sequence
#1	Forward	ATGGCCCTGTGGATGCGCCTC
	Reverse	ACAGGGAGCTGGTCACTTTTAGGACGT
#2	Forward	TTTTGGGGATTTGATTTAGT
	Reverse	ACTCACCCTACAAATCCTCTAC
Nested	Forward	AGTTGTAGTTTTTGTGAATTAATATTTG
	Reverse	TCACCCTACAAATCCTCTACC
First ARMS	Forward	TAGTTTTTGTGAATTAATATTTGTTTG
	Reverse	CCTACAAATCCTCTACCTCCGAA
Second ARMS	Forward	TTTGGTGGAAGTTTTTTATTTAGTGTCTG
	Reverse	AATCTTAAATATATAAAAAAAACCTGAT

Red characters indicate mismatches at the 3′ end of primers.

genomic DNA was amplified with the #2 primer set (Table 1) and served as methylated control DNA, in which the converted four CpG sites were left as 'CG'. These four CpG sites in the unmethylated and methylated control DNA were confirmed by DNA sequencing (Figure S1).

Nested PCR

Nested PCR was designed to amplify a fragment (128 bp) in exon 2 of insulin gene (Table 1) using the HotStarTaq *Plus* Master Mix Kit (QIAGEN, Hilden, Germany). Amplification consisted of initial denaturation at 95°C for 5 min, followed by 15 cycles at 94°C for 30 s, 50 °C for 30 s, and 72 °C for 30 s using nested primer sets (Table 1). The amplicon was cleaned using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and eluted in 25 μL. Clear LightCycler 8-Tube Strips (Roche, Basel, Switzerland) using a LightCycler96 Instrument (Roche) were used for all qPCR measurements.

Two-step ARMS PCR

The principle of ARMS PCR and part of the insulin gene coding region sequence downstream of TSS to polyA is shown in Figure 1. ARMS PCR is a simple detection method based on the use of sequence-specific PCR primers that allow amplification of the test DNA only when the target allele is contained within a sample and will not amplify non-target alleles[15]. One base of a point mutation and subsequent thymidine was inserted into the 3′ end of the primer, which corresponds to each target CpG site, creating two mismatches if the CpG site is methylated. PCR amplifies template DNA if there is one mismatch at the 3′ end of the primer but does not amplify if there are more than two mismatches[14]. The 3′ end of each ARMS PCR primer will recognize 'TG' or 'CA' in the forward or reverse primer. In addition, there were three different types of mismatches for each forward and reverse primer at the 3′ end. We selected primer sets that exhibited the largest Ct value difference between the unmethylated and methylated controls for each ARMS PCR assay.

The first ARMS PCR assay was designed to amplify the exon fragments of insulin gene when CpG sites at +331 bp and +404 bp were simultaneously unmethylated using the first ARMS primer set (Table 1). In the first ARMS PCR assay, amplification was performed using 1 μL of the cleaned up DNA. Amplification consisted of initial denaturation at 95°C for 10 min, followed by 15 cycles at 95°C for 10 s, 56 °C for 10 s, and 72°C for 10 s. The PCR product was purified using the above-mentioned kit and eluted in 25 μL, as indicated. The second ARMS PCR assay was designed to amplify the exon fragments of insulin gene when CpG sites at +367 bp and +374 bp were simultaneously unmethylated using the second ARMS primer set (Table 1). The amplification protocol was the same as that of the first ARMS PCR assay, except for amplifying the 35-cycle of the procedure. The calibration series containing methylated and unmethylated control DNA at 10^{4-7} copies/mL was run for quantitation in the second ARMS PCR assay. There was no internal control for qPCR because it is an absolute quantification method using a standard dilution series. The melting curve was observed to verify that the result was not due to non-specific reactions.

The unmethylated insulin DNA copy number(/mL) was calculated using the following formula:

$$250 \times 10^{(\text{y\_intercept-Ct value})/(-\text{slope})} \text{ of 2nd ARMS PCR} \times 25/(\text{efficiency of 1st ARMS PCR})^{15}/(\text{efficiency of nested PCR})^{15}.$$

Ct indicates threshold cycles. The amplicon was confirmed with the DNA sequence.

Clinical characteristics of human study

Clinical parameters were collected from the medical records of the 114 patients with type 1 diabetes and 31 healthy volunteers. The BMI %tile score, and not the absolute score, was used to assess physical size because BMI fluctuates dynamically with age in childhood.

Statistical analyses

Continuous variables are presented as medians (lower quartile, upper quartile). Categorical variables are presented as numbers. The Mann–Whitney *U* test was used to compare differences between the two groups. Data distribution was evaluated using the Shapiro–Wilk test. The correlation between the positivity of unmethylated insulin DNA and the clinical characteristics was analyzed using Spearman's rank correlation coefficient. Statistical analyses were performed using SPSS Statistics 22 (IBM Japan, Tokyo, Japan) and Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical tests were two-sided, and statistical significance was set at $P < 0.05$.

RESULTS

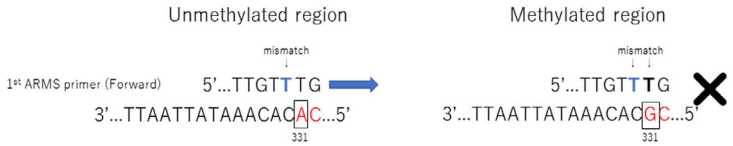
Bisulfite sequencing

Genomic DNA from several human organs was bisulfite-treated to amplify insulin gene fragments and was sequenced (Figure 2a). Genomic DNA from β-cells could not be obtained; therefore, pancreatic DNA was used instead. All four target CpG sites were unmethylated only in a part of pancreatic DNA. Bisulfite conversion efficiency, calculated using cytosine, which is not associated with CpG sites as controls, was 99.6% in genome DNA.

Spike-in ARMS PCR

To evaluate the linearity of our assay, spike-in PCR was performed. Unmethylated control DNA was spiked into 4 ng of human blood genome in different amounts (10^0–10^5 copies) by serial dilution, and nested PCR and two-step ARMS PCR were performed. The copy number was calculated and found to linearly increase with the input of the unmethylated control DNA (Figure 2b).

(a)



(b)

TSS

CGACAGGTAGGGGAGATGGGCTCTGAGACTATAAAGCCAGCGGGGGCCCAGCAGCCCTCAGCCCTCCAGGACAGGCTGCATCAGAAGAGGCCATCA
TGATAGGTAGGGGAGATGGGTTTTGAGATTATAAAGTTAGTGGGGGTTTAGTAGTTTTTAGTTTTTTAGGATAGGTTGTATTAGAAGAGGTTATTA

AGCAGGTCTGTTCCAAGGGCCTTTGCGTCAGGTGGGCTCAGGGTTCCAGGGTGGCTGGACCCCAGGCCCCAGCTCTGCAGCAGGGAGGACGTGGCT
AGTAGGTTTGTTTTAAGGGTTTTTGTGTTAGGTGGGTTTAGGGTTTTAGGGTGGTTGGATTTTAGGTTTTAGTTTTGTAGTAGGGAGGATGTGGTT

GGGCTCGTGAAGCATGTGGGGGTGAGCCCAGGGGCCCCAAGGCAGGGCACCTGGCCTTCAGCCTGCCTCAGCCCTGCCTGTCTCCCAGATCACTGT
GGGTTTGTGAAGTATGTGGGGGTGAGTTTAGGGGTTTTAAGGTAGGGTATTTGGTTTTTAGTTTGTTTTAGTTTTGTTTGTTTTTTAGATTATTGT

#1-F
CCTTCTGCC**ATG**GCCCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGGGACCTGACCCAGCCGCAGCCTTTGTGAACCAACAC
TTTTTTGTTATGGTTTTGTGGATGTGTTTTTTGTTTTTGTTGGTGTTGTTGGTTTTTTGGGGATTTGATTTAGTTGTAGTTTTTGTGAATTAATAT
#2-F
1st ARMS-F
nested-F
CTGTGCGGCTCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCCAAGACCCGCCGGGAGGCAGAGGACCTG
TTGTGTGGTTTATATTTGGTGGAAGTTTTTTATTTAGTGTGTGGGGAATGAGGTTTTTTTTATATATTTAAGATTTGTTGGGAGGTAGAGGATTTG
2nd ARMS-F
2nd ARMS-R
1st ARMS-R
#nested-R
CAGGGTGAGCCAACCGCCCATTGCTGCCCCTGGCCGCCCCCAGCCACCCCCTGCTCCTGGCGCTCCCACCCAGCATGGGCAGAAGGGGGCAGGAGG
TAGGGTGAGTTAATTGTTTATTGTTGTTTTTGGTTGTTTTTAGTTATTTTTTGTTTTTGGTGTTTTTATTTAGTATGGGTAGAAGGGGGTAGGAGG
#2-R
#1-R
CTGCCACCCAGCAGGGGGTCAGGTGCACTTTTTTAAAAAGAAGTTCTCTTGGTCACGTCCTAAAAGTGACCAGCTCCCTGTGGCCCAGTCAGAATC
TTGTTATTTAGTAGGGGGTTAGGTGTATTTTTTTAAAAAGAAGTTTTTTTGGTTATGTTTTAAAAGTGATTAGTTTTTTGTGGTTTAGTTAGAATT

TCAGCCTGAGGACGGTGTTGGCTTCGGCAGCCCCGAGATACATCAGAGGGTGGGCACGCTCCTCCCTCCACTCGCCCCTCAAACAAATGCCCCGCA
TTAGTTTGAGGATGGTGTTGGTTTTGGTAGTTTTGAGATATATTAGAGGGTGGGTATGTTTTTTTTTTTATTTGTTTTTTAAATAAATGTTTTGTA

GCCCATTTCTCCACCCTCATTTGATGACCGCAGATTCAAGTGTTTTGTTAAGTAAAGTCCTGGGTGACCTGGGGTCACAGGGTGCCCCACGCTGCC
GTTTATTTTTTTATTTTTATTTGATGATTGTAGATTTAAGTGTTTTGTTAAGTAAAGTTTTGGGTGATTTGGGGTTATAGGGTGTTTTATGTTGTT

TGCCTCTGGGCGAACACCCCATCACGCCCGGAGCGCCCGGAGGAGGGCGTGGCTGCCTGCCTGAGTGGGCCAGACCCCTGTCGCCAGGCCTCACGG
TGTTTTTGGGTGAATATTTTATTATGTTTGGAGTGTTTGGAGGAGGGTGTGGTTGTTTGTTTGAGTGGGTTAGATTTTTGTTGTTAGGTTTTATGG

CAGCTCCATAGTCAGGAGATGGGGAAGATGCTGGGGACAGGCCCTGGGGAGAAGTACTGGGATCACCTGTTCAGGCTCCCACTGTGACGCTGCCCC
TAGTTTTATAGTTAGGAGATGGGGAAGATGTTGGGGATAGGTTTTGGGGAGAAGTATTGGGATTATTTGTTTAGGTTTTTATTGTGATGTTGTTTT

GGGGCGGGGGAAGGAGGTGGGACATGTGGGCGTTGGGGCCTGTAGGTCCACACCCACTGTGGGTGACCCTCCCTCTAACCTGGGTCCAGCCCGGCT
GGGGTGGGGGAAGGAGGTGGGATATGTGGGTGTTGGGGTTTGTAGGTTTATATTTATTGTGGGTGATTTTTTTTTTAATTTGGGTTTAGTTTGGTT

GGAGATGGGTGGGAGTGCGACCTAGGGCTGGCGGGCAGGCGGGCACTGTGTCTCCCTGACTGTGTCCTCCTGTGTCCCTCTGCCTCGCCGCTGTTC
GGAGATGGGTGGGAGTGTGATTTAGGGTTGGTGGGTAGGTGGGTATTGTGTTTTTTTGATTGTGTTTTTTTGTGTTTTTTTGTTTTGTTGTTGTTT

CGGAACCTGCTCTGCGCGGCACGTCCTGGCAGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGG
TGGAATTTGTTTTGTGTGGTATGTTTTGGTAGTGGGGTAGGTGGAGTTGGGTGGGGGTTTTGGTGTAGGTAGTTTGTAGTTTTTGGTTTTGGAGGG

GTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGACGCAGCCCGCAGG
GTTTTTGTAGAAGTGTGGTATTGTGGAATAATGTTGTATTAGTATTTGTTTTTTTTATTAGTTGGAGAATTATTGTAATTAGATGTAGTTTGTAGG

CAGCCCCCCACCCGCCGCCTCCTGCACCGAGAGAGATGGAATAAA
TAGTTTTTTATTTGTTGTTTTTTGTATTGAGAGAGATGGAATAAA

Figure 1 | (a) Schematic illustration of the human ARMS PCR. The figure shows the first ARMS primer. One base of a point mutation shown in red and subsequent thymidine shown in blue was inserted into the 3′ end of the primer, creating two mismatches if the CpG site is methylated. PCR amplifies template DNA if there is one mismatch at the 3′ end of the primer but does not amplify if there are more than two mismatches. (b) Part of the insulin gene coding region sequence downstream of TSS to polyA signal with indications of all primers. The sequence before bisulfite conversion is shown in the upper row, and the sequence after bisulfite conversion is shown in the lower row. The TSS is shown in bold, and the four targeted CpG sites are shown in red. F represents forward and R represents reverse.

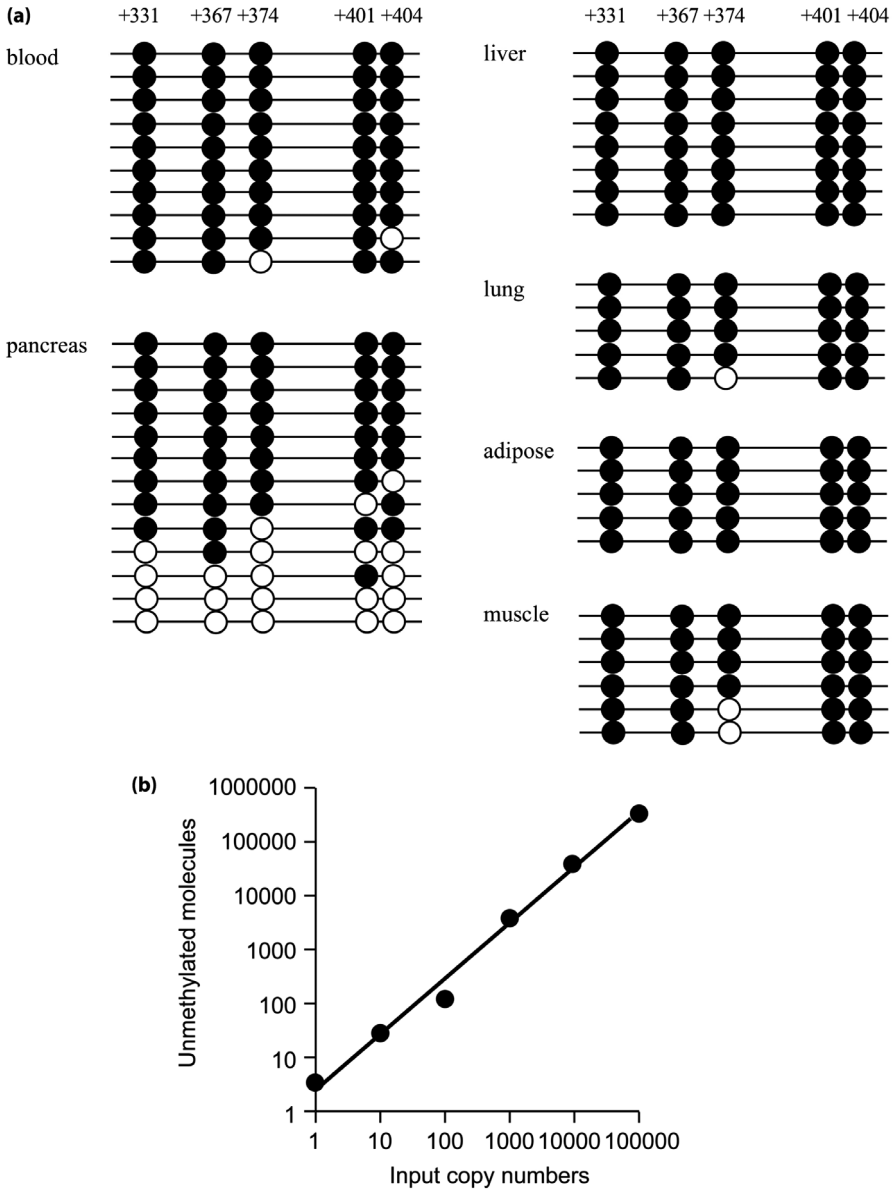


Figure 2 | (a) Bisulfite sequencing of five CpG dinucleotides in exon 2 of human insulin gene, from +331 to +404. (b) Spike-in ARMS PCR. Unmethylated control DNA was mixed with human blood genome DNA in the indicated amounts (10^0–10^5 copies), and the copy number of each sample was determined.

Table 2 | The clinical characteristics of the subjects with type 1 diabetes

	Type 1 diabetes (n = 114)	Healthy control (n = 31)
Male/Female	42/72	21/10
Age (years)	41.5 (19.9, 54.8)	35.0 (31.5, 44.8)
Duration (years)	8.3 (3.1, 18.0)	
BMI (kg/m^2) percentile <18 years old	65.7 (49.1, 81.5)	
BMI (kg/m^2) ≥18 years old	21.8 (20.7, 24.0)	22.1 (20.4, 24.5)
HbA1c (%)	7.3 (6.7, 8.1)	
Serum CPR (ng/mL)	0.05 (0.00, 0.07)	
TDD (U/kg/day)	0.64 (0.49, 0.90)	

BMI, body mass index; CPR, C-peptide; TDD, total daily dose of exogenous insulin.

Detection ability of ARMS PCR

Next, to evaluate the sensitivity, the minimum detectable copy number was checked. We prepared 16 samples that were unmethylated control DNA diluted to 0 or 1 copy into 10^5 copies of methylated control, and nested PCR and two-step ARMS PCR were performed. In 7 of the 16 samples, a single copy of unmethylated control DNA was detected and no methylated control DNA was detected; therefore, one copy was determined to be the minimum detectable copy number of unmethylated insulin DNA in this assay.

Clinical results of ARMS PCR

The clinical characteristics of the 114 patients with type 1 diabetes and 31 healthy volunteers are shown in Table 2. The duration of type 1 diabetes was <1 year in 10 of 114 patients. The serum C-peptide level was preserved (≥0.17 nmol/L) in 41 of 114 patients. The total cfDNA levels were significantly higher in type 1 diabetes patients (Figure 3a). There was no correlation between the total cfDNA levels and age, disease duration, BMI, HbA1c, or serum C-peptide immunoreactivity (CPR; Table 3). There was no correlation between unmethylated insulin DNA copy number and total cfDNA level in the unmethylated insulin DNA-positive type 1 diabetes group ($\rho = 0.243$, $P = 0.154$). Unmethylated insulin DNA was detected in approximately 30% of the cases in both patients with type 1 diabetes and controls (Figure 3b). There were no significant differences in the positive rate and copy number of unmethylated insulin DNA between patients with type 1 diabetes and controls. Copy number was inversely correlated with age ($\rho = -0.458$, $P = 0.005$) and disease duration ($\rho = -0.403$, $P = 0.015$) in the unmethylated insulin DNA-positive type 1 diabetes group (Table 4, Figure 3c). There was also an inverse correlation between unmethylated insulin DNA copy number and BMI in patients over 18 years of age ($\rho = -0.481$, $P = 0.003$), and a similar trend was observed in patients under 18 years of age. There was no significant correlation between β-cell copy number and HbA1c, serum C-peptide levels, or total daily insulin dose.

DISCUSSION

Noninvasive monitoring of pancreatic β-cell injury in patients with type 1 diabetes is useful for understanding the pathogenesis of the disease. It may also help screen the onset of the disease and determine the timing of treatment initiation to prevent disease progression in high-risk individuals, such as those who have close relatives with type 1 diabetes or are positive for islet autoantibodies. Furthermore, it may be possible to quantify injuries, such as rejection after islet transplantation. The assay quantifying pancreatic β-cell injury by measuring unmethylated insulin DNA in circulation was first reported in 2011[16] and has been reported by several research groups using ddPCR and NGS. There is variability in the measurement results between assays due to differences in the type and amount of optimized blood samples, collection methods, cfDNA isolation techniques, and target CpG sites[17]. The detection of high levels of unmethylated insulin gene DNA after islet transplantation is a common finding in several assays[3,17]. In studies of patients with type 1 diabetes, high levels of unmethylated insulin DNA in patients with onset stage and high-risk individuals was reported[3,11,12], while there is also a report

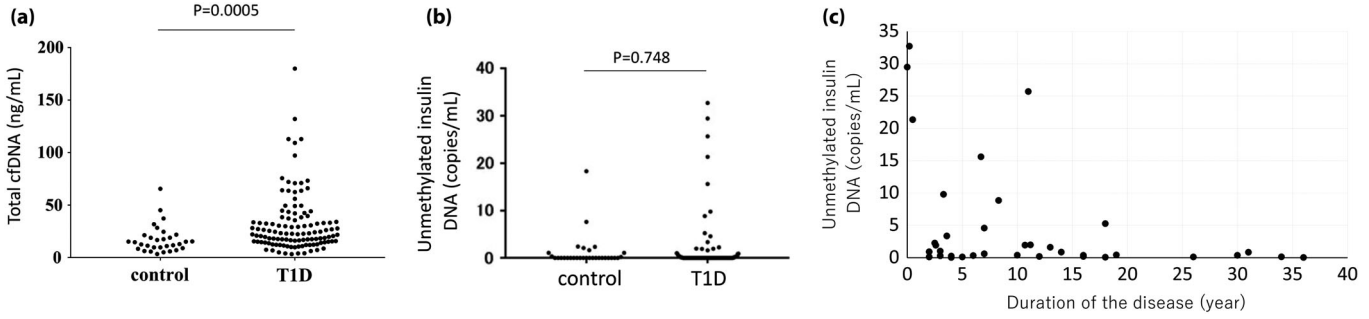


Figure 3 | (a) Total cfDNA levels in healthy controls and type 1 diabetes patients. (b) The copy number of unmethylated insulin DNA in the circulation of healthy controls and type 1 diabetes patients. (c) Correlation between the copy number of unmethylated insulin DNA and the duration of the disease in unmethylated insulin DNA-positive type 1 diabetes patients. ($P = 0.015$).

Table 3 | The correlation between total cfDNA level and clinical parameters in type 1 diabetes patients

	ρ	*P*
Age (years)†	−0.129	0.173
Duration (years)†	−0.140	0.139
BMI (kg/m^2) percentile <18 years old†	−0.110	0.591
BMI (kg/m^2) ≥18 years old†	0.128	0.236
HbA1c (%)†	−0.059	0.541
Serum CPR (ng/mL)†	−0.081	0.446
TDD (U/kg/day)†	0.151	0.118

CPR, C-peptide; TDD, total daily insulin dose. †Spearman's rank correlation coefficient analysis.

Table 4 | The correlation between unmethylated insulin DNA positivity and clinical parameters in unmethylated insulin DNA-positive type 1 diabetes patients

	ρ	*P*
Age (years)†	−0.458	0.005
Duration (years)†	−0.403	0.015
BMI (kg/m^2) percentile <18 years old†	−0.357	0.358
BMI (kg/m^2) ≥18 years old†	−0.481	0.003
HbA1c (%)†	0.194	0.273
Serum CPR (ng/mL)†	0.200	0.290
TDD (U/kg/day)†	0.243	0.114

CPR, C-peptide; TDD, total daily insulin dose. †Spearman's rank correlation coefficient analysis.

showing no difference in patients with a long disease duration compared to healthy adults[18]. We developed a novel method for detecting unmethylated insulin DNA in circulation using ARMS PCR, which was able to detect a single copy of unmethylated insulin DNA, and applied it to patients with type 1 diabetes and healthy adults, both of which can be detected in approximately 30% of cases. Our method costs only $30 per sample. The procedure time was 6 h; we could obtain results without outsourcing by using a regular real-time PCR system. Up to 96 samples can simultaneously be analyzed using a Lightcycler96. Thus, our method has advantages in terms of cost, procedure time, and the number of samples analyzed simultaneously.

Our ARMS PCR method is specific to unmethylated PCR primers; therefore, it is sensitive to bisulfite efficiency. Bisulfite treatment fragments DNA as a side effect of the low pH and high-temperature setting for the conversion of unmethylated cytosine to uracil. Further fragmentation is a concern when bisulfite treatment is applied to cfDNA, which is highly fragmented. Werner *et al.* treated plasma cfDNA with bisulfite, visualized it on agarose gel, and quantified it using qPCR[19]. They reported that cfDNA fragmentation during bisulfite treatment was slight and did not contribute to the loss of sensitivity in the methylation analysis. We calculated bisulfite conversion efficiency in bisulfite sequencing of the target insulin gene region using cytosine, which is not associated with CpG sites in cfDNA as well as genome DNA, and there was no difference between the two. In addition, we applied our amplification method without bisulfite treatment to four surplus samples of patients with type 1 diabetes who were positive for unmethylated insulin DNA, and the results were all negative.

Unmethylated insulin DNA in circulation may reflect β-cell destruction by autoimmune attacks as well as physiological turnover. Up to approximately 20 copies/mL of unmethylated insulin DNA was detected in approximately 30% of healthy adults, which may represent turnover. The high level of unmethylated insulin DNA in non-diabetic subjects may be due to active turnover at a young age[20] or increased β-cell mass due to obesity[21], and may also be reflected in the pre-diabetic stage. It is necessary to increase the number of non-diabetic patients by expanding the range of age and body size and to conduct a longitudinal analysis of cases with a high copy number of unmethylated insulin DNA. The high level of unmethylated insulin DNA in patients with type 1 diabetes may be associated with a high amount of residual pancreatic β-cells and/or strong β-cell destruction, and it may be necessary to consider the balance of these factors. In our study, unmethylated insulin DNA was negative in multiple cases, which was likely owing to the small number of residual pancreatic β-cells. The level of unmethylated insulin DNA in patients with type 1 diabetes was not different from that in healthy adults in this study. Most of beta cell volume in type 1 diabetes is supposed to be smaller compared with healthy controls. There may be a certain amount of beta cell death due to phenomena other than turnover may also occur in patients with type 1 diabetes. Moreover, the copy number was inversely correlated with the duration of the disease in unmethylated insulin DNA-positive type 1 diabetes patients, suggesting that the residual β-cells may continue to be gradually destroyed by autoimmune attacks.

This study has certain limitations. First, the duration of the disease ranged from early onset to long-standing. To verify whether our method can contribute to the development of a treatment that prevents disease progression in type 1 diabetes, it is necessary to analyze a large number of patients in the onset phase. Second, endogenous β-cell function was not adequately assessed. Urinary CPR excretion or glucagon challenge tests were not performed because the samples were collected during regular outpatient care. Third, because cfDNA is fragmented as multiples of 166 bp, we designed PCR product as small as possible including 4 CpG sites. If cfDNA fragmentation happens between the 4 CpG sites, we have the possibility of underestimating the result. We could not verify the effect of cfDNA fragmentation because we used plasmid DNA and not cfDNA as a control. Finally, there are numerous steps involved in our amplification method.

Our results of tissue methylation pattern of human insulin gene exon 2 differ from those of Husseiny *et al.* that reported

to be unmethylated in the liver as well as in β-cells. The reason for this is unclear, but might be due to the different sources of genomic DNA. In addition, Moss *et al.* predicted that 1% of the plasma cfDNA of healthy adults originates from hepatocytes in the report regarding the origins of cfDNA using comprehensive human cell-type methylation atlas[22]. If our target CpGs of liver-derived insulin DNA are unmethylated, they would be detected in the most of our samples from health subjects and insulin-depleted patients with type 1 diabetes. However, unmethylated insulin DNA was not detected in many cases of those subjects.

In conclusion, we have developed a novel method for detecting β-cell-derived insulin DNA by combining bisulfite conversion and ARMS PCR, which can be performed using a conventional real-time PCR system. The unmethylated insulin DNA level in the type 1 diabetes group, whose β-cell mass was considered to be substantially reduced, was similar to that of healthy adults, and there was an inverse correlation between copy number and disease duration in unmethylated insulin DNA-positive type 1 diabetes patients, indicating that this method would be useful for analyzing dynamic profiles β-cells in human diseases such as type 1 diabetes.

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DISCLOSURE

AK is a member of the Speakers Bureau for Sanofi, Novo Nordisk Inc., and Eli Lilly Japan K.K. MM receives research support from Novo Nordisk Inc., and is a member of the Speakers Bureau for Sanofi, Novo Nordisk Inc., and Eli Lilly Japan K.K. The other authors do not have any potential conflicts of interest relevant to this article.

Approval of the research protocol and approval date of registry is June 22nd 2015 and the registration No. is 2320 of The Ethics Committie of Tokushima University Hospital.

Informed consent is obtained from all the participants.

Animal studies: N/A.

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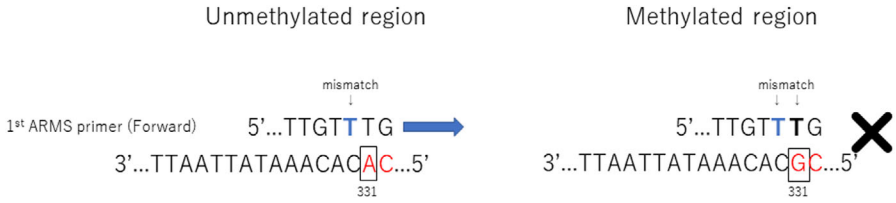
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1| Bisulfite sequencing of unmethylated (a) and methylated (b) control plasmid DNA. Nested primers are highlighted. Targeted four CpG sites were shown in red.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main.



To detect pancreatic beta cell death from the circulating cell-free DNA. We detect DNA methylation pattern using bisulfite conversion DNA. Also, we can detect one mismatch of DNA sequence very efficiently using amplification-refractory mutation system PCR with ordinary PCR machine. We have utilized these two techniques together to detect pancreatic beta cell specific sequence in the circulation.