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Glycogen Storage Disease Type Ia Screening Using Dried Blood Spots on Filter Paper: Application of COP-PCR for Detection of the c.648G>T *G6PC* Gene Mutation

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Glycogen storage disease type Ia (GSDIa, OMIM #232200) is an autosomal recessive metabolic disease characterized by impaired glucose homeostasis and has a long-term complication of hepatocellular adenoma/carcinoma. GSDIa is caused by deleterious mutations in the glucose-6-phosphatase gene (*G6PC*). Recent studies have suggested that early treatment by gene replacement therapy may be a good solution to correct the glucose metabolism and prevent serious late complications. Early treatment of the disease needs an early disease detection system. Thus, we aimed to develop a screening system for GSDIa using dried blood spots (DBS) to detect the c.648G>T mutation in *G6PC*, which is a frequent mutation in the East Asian population. In this study, a total of 51 DBS samples (50 healthy controls and one patient with c.648G>T) were tested by modified competitive oligonucleotide priming PCR (mCOP-PCR). In control DBS samples, the c.648G allele was amplified at lower Cq (quantification cycle) values (<11), while the c.648T allele was amplified at higher Cq values (>14). In the patient DBS sample, the c.648T allele was amplified at a lower Cq value (<11), and the c.648G allele was amplified at a higher Cq value (>14). Based on these findings, we concluded that our mCOP-PCR system clearly differentiated the wild-type and mutant alleles, and may be applicable for screening for GSDIa with the c.648G>T mutation in *G6PC*.

Glycogen storage disease type Ia (GSDIa), or von Gierke disease (OMIM #232200), is a glycogen metabolic disorder with an autosomal recessive inheritance trait, caused by glucose-6-phosphatase (G6Pase) deficiency [1]. The incidence of GSDIa has been estimated to be about 1 in 100,000 births in the general population, although its prevalence is higher (about 1 in 20,000 births) in the Ashkenazi Jewish population [2]. The incidence of the disease has not yet been clarified in the Japanese population.

The gene responsible for the disease, *G6PC*, maps to chromosome 17q21 [3], and more than 100 mutations in *G6PC* have been reported to date [Human Gene Mutation Database: http://www.hgmd.cf.ac.uk]. The c.648G>T mutation is a common mutation among GSDIa patients in East Asia, including Chinese, Japanese and Korean populations, accounting for more than 85% of cases in the Japanese and Korean population [4–6]. This may suggest a founder effect of the mutation in these countries. However, the mutation is not as frequent in other ethnicities, such as Ashkenazi Jewish [2]. The mutation is termed c.648G>T based on nomenclature with nucleotide numbering from the translation initiation site, although it has also been called “the G727T mutation” in nomenclature with nucleotide numbering from the transcription initiation site.

The disease is characterized by hypoglycemia, lactic acidosis, hepatomegaly, dyslipidemia, and hyperuricemia [1]. More specifically, patients present with various symptoms including hypoglycemic seizures in infancy, short stature throughout life, polycystic ovaries in females, gout due to long-term hyperuricemia, pancreatitis due to severe dyslipidemia, increased bleeding due to impaired platelet aggregation, frequent bone fractures due to osteopenia, benign or malignant hepatic tumors, renal failure, and pulmonary hypertension [7]. In particular, malignant hepatic tumors, renal failure, and pulmonary hypertension are serious late complications [8–10].

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A recent development in treatment demonstrated that adeno-associated virus vector-treated GSDIa mice maintained glucose homeostasis, and that the treatment could potentially prevent hepatocellular carcinoma [11,12]. These results led to the idea that early diagnosis and treatment could allow normal glycogen metabolism to be maintained and prevent serious late complications.

These studies suggested that early treatment by gene therapy may be a good solution to correct the glucose metabolism and prevent serious late complications. However, early treatment of the disease requires an early disease detection system. Thus, we aimed to develop a screening system for GSDIa using dried blood spots (DBS) to detect the c.648G>T mutation in *G6PC*, a frequent mutation in East Asian populations.

To achieve early detection of GSDIa, real-time polymerase chain reaction (PCR)-based technology is preferable, for example, modified competitive oligonucleotide priming-PCR (mCOP-PCR). A nested PCR system with mCOP-PCR technology has been previously established to clearly detect a difference of one nucleotide [13,14]. Here, a total of 51 DBS samples (50 healthy controls and one patient with c.648G>T) were tested by a nested PCR system with mCOP-PCR technology.

MATERIALS AND METHODS

1. Patient and control samples

One DBS sample from a patient with a c.648G>T *G6PC* gene mutation and 50 control DBS samples were analyzed in this study. A total of 50μl whole blood was spotted onto the Flinders Technology Associates (FTA) Elute Cards® (GE Healthcare, Boston, MA, USA) and was dried at room temperature for at least one hour. The DBS was stored in a dark room at room temperature until used. The storage period of the DBS ranged from 1 week to 2 years. Prior to analysis, informed consent was obtained from all study participants. The study was approved by the Ethics Committee of Kobe University Graduate School of Medicine (reference number 1210, approved on August 10, 2014) and was conducted in accordance with the World Medical Association Declaration of Helsinki.

The patient was a 50-year-old female. She presented in childhood with hypoglycemia, lactic acidosis, hepatomegaly, and short stature, and she was clinically diagnosed as having GSDIa. She underwent resection of an ovarian cyst at 31 years old, plasmapheresis at 44 years old, and liver transplantation at 45 years old. A nucleotide sequencing analysis using DNA extracted from freshly-collected blood showed that she was homozygous for a c.648G>T *G6PC* gene mutation.

2. Detection system for c.648G>T *G6PC* gene mutation using DBS

2.1. Outline of detection system for c.648G>T *G6PC* gene mutation

The two steps in our system were as follows: (1) the first PCR step with non-allele-specific amplification of *G6PC* exon 5 using conventional PCR. A small, punched circle from the DBS was used directly for PCR, without any DNA extraction or purification procedures from the DBS; and (2) the second PCR step with allele-specific amplification of *G6PC* exon 5 using real-time mCOP-PCR. The two alleles, c.648G (wild type) and c.648T (mutant), were differentially amplified in this step.

2.2. The first PCR: amplification of *G6PC* exon 5 using DBS

Amplification of the sequence containing *G6PC* exon 5 from the DBS was performed by conventional PCR using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). A punched circle, 2 mm in diameter (equivalent to ~15 μL of whole blood), from the patient DBS sample or the control DBS samples, was directly added to the reaction mixture with DNA polymerase KOD FX Neo (TOYOBO, Osaka, Japan). The following primers were used to amplify the target sequence containing *G6PC* exon 5: Int4-forward (5′-TATCTCTCACAGTCATGC-3′) and Ex5-reverse (5′-TCCAGAGTCCACAGGAGGTC-3′) [15].

The PCR conditions for the 50 μL reaction mixture were: (1) initial denaturation at 94ºC for 2 min; (2) 30 cycles of denaturation at 94ºC for 30 sec, annealing at 62ºC for 30 sec, and extension at 72ºC for 1 min; (3) additional extension at 72ºC for 7 min; and (4) hold at 10ºC. The first PCR products were then subjected to agarose gel electrophoresis.

2.3. The second PCR: allele-specific amplification of *G6PC* exon 5

Allele-specific amplification of *G6PC* exon 5 by real-time mCOP-PCR was performed using the LightCycler® 96 system (Roche Applied Science, Mannheim, Germany). The PCR product from the first reaction was diluted 100-fold, and then 2 μL was added to a final reaction mixture of 50 μL containing DNA polymerase, FastStart Taq (Roche Diagnostics, Mannheim, Germany), and EvaGreen® Dye (Biotium, Hayward, CA, USA). The primer set for c.648G detection consisted of Int4 forward primer and COP-G (5′-CTGAACAGGAAG-3′) reverse primer; the primer set for c.648T detection consisted of Int4-forward primer

and COP-T (5′-CTGAAAAGGAAGA-3′) reverse primer. The wild-type and mutant allele detection were done in separate tubes.

The PCR conditions for both detection reactions were: (1) initial denaturation at 94ºC for 7 min; and (2) 15 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 1 min. Melting curve analysis was performed after PCR amplification, with 10 s of denaturation at 95ºC, 1 min of renaturation at 65ºC, and then continuous fluorescence reading from 65ºC to 97ºC at the rate of five data acquisitions per ºC. Post PCR analysis was done using LightCycler® 96 software version 1.1.0.1320 (Roche Applied Science, Mannheim, Germany).

3. Nucleotide Sequencing

The first PCR products were purified using NucleoSpin® Gel and PCR Clean-up (Takara Biomedical, Tokyo, Japan) and were submitted for sequencing. All sequencing analyses were conducted by Fasmac Co., Ltd. (Atsugi City, Kanagawa, Japan).

4. Statistical analysis

To compare the PCR amplification efficiency between the c.648G-specific primer (COP-G) and the c.648T-specific primer (COP-T), Student's t-test was used. For these analyses, we used Microsoft Excel with the add-in software Statcel 3 (The Publisher OMS Ltd., Tokyo, Japan). A p-value less than 0.05 was considered to be statistically significant.

RESULTS

1. The first PCR: amplification of *G6PC* exon 5 using DBS

DBS samples from 50 control individuals and one GSDIa patient were analyzed. The first PCR directly amplified DNA from one punch of the DBS using Int4-forward and Ex5-reverse primers (Figure 1A).

Both control and patient DBS samples showed a single band of 191 bp in the agarose gel electrophoresis, because the primers targeted a common region of *G6PC* (Figure 1B).

We sequenced the PCR product to confirm the genotype of the samples (Figure 1C). Control DBS samples carried the wild-type sequence of *G6PC* (c.648G), while the patient DBS sample carried a c.648G>T point mutation.

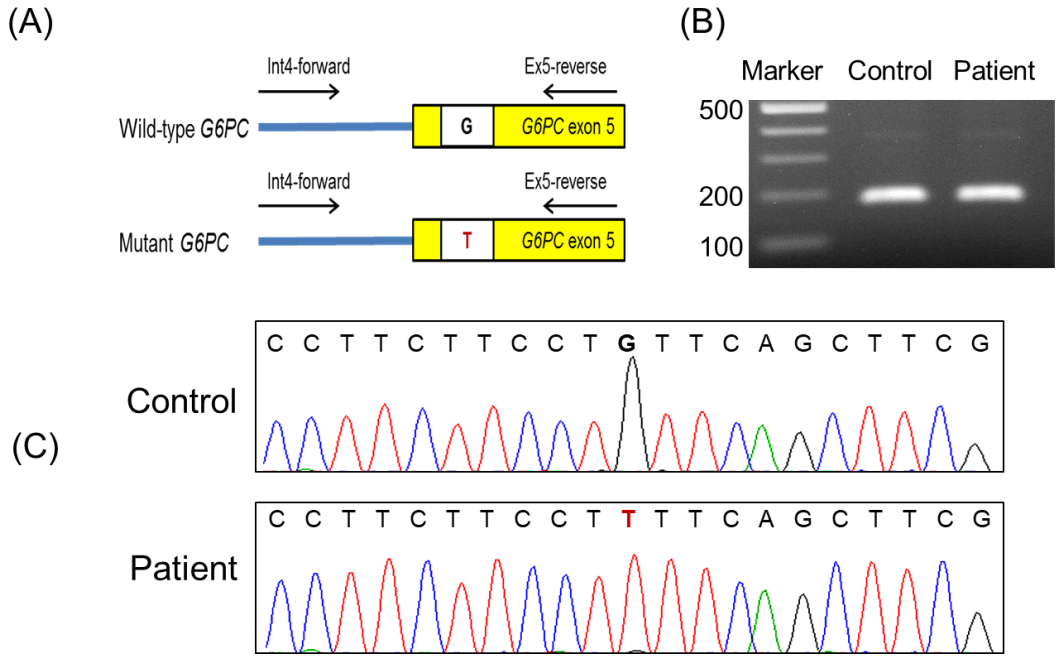

Figure 1. Molecular genetic analysis. (A) Primer locations used in this study. Arrows indicate direction of the primer. The location of wild-type (black letter) and mutant (red letter) nucleotides are indicated in the box. (B) Agarose gel electrophoresis indicates a single band (191 bp) for the first PCR product. (C) G6PC exon 5 sequence electropherogram shows the wild-type sequence (blue letter) in healthy controls and the point mutation (red letter) in the patient.

2. The second PCR: allele-specific amplification of *G6PC* exon 5

The first PCR product was used as the template DNA for the second PCR experiments using two different primer sets, Int4-forward and COP-G, or Int4-forward and COP-T. The products of the second PCR were all 123 bp in size.

Figure 2 shows the amplification curves of the COP-G and COP-T primers. All control DBS samples showed amplification with the COP-G primer at a lower cycle number (Figure 2A) and amplification with the COP-T primer at a higher cycle number (Figure 2B). The mean quantification cycle (Cq) values of the control DBS sample were 9.63 ± 1.03 (mean ± SD) and 14.89 ± 0.28 (mean ± SD) for the COP-G and COP-T primers, respectively (Table I). The high Cq value difference between the COP-G and COP-T primers was statistically significant ($p < 0.05$), suggesting clear differentiation of the wild-type and mutant sequences (Table I).

Conversely, the patient DBS displayed the opposite result to the control DBS, with amplification with COP-G at a higher cycle number (Figure 2A) and amplification with COP-T at a lower cycle number (Figure 2B). The mean Cq values for the patient DBS were 14.46 (assay 1, 13.83; assay 2, 15.08) and 10.04 (assay 1, 8.97; assay 2, 11.1) for the COP-G and COP-T primers, respectively (Table I).

Here, we determined a Cq value > 13 as the cut-off point for a negative result in both the COP-G and COP-T amplifications. Thus, we concluded that we detected a positive COP-T amplification in the patient DBS sample, but not in any of the 50 control DBS samples. Similarly, we detected COP-G amplification in the 50 control DBS samples, but not in the patient DBS sample.

Our method with allele-specific amplification can detect heterozygous carriers as well. A heterozygous carrier will be detected when the sample showed significant amplification with COP-G and COP-T primers, because it means the presence of both wild-type sequence (c.648G) and mutant sequence (c.648T). To test this, we created a carrier model sample by mixing DBS pieces from a control with only c.648G and a patient with only c.648T. As expected, the carrier model was amplified with both COP-G and COP-T primers.

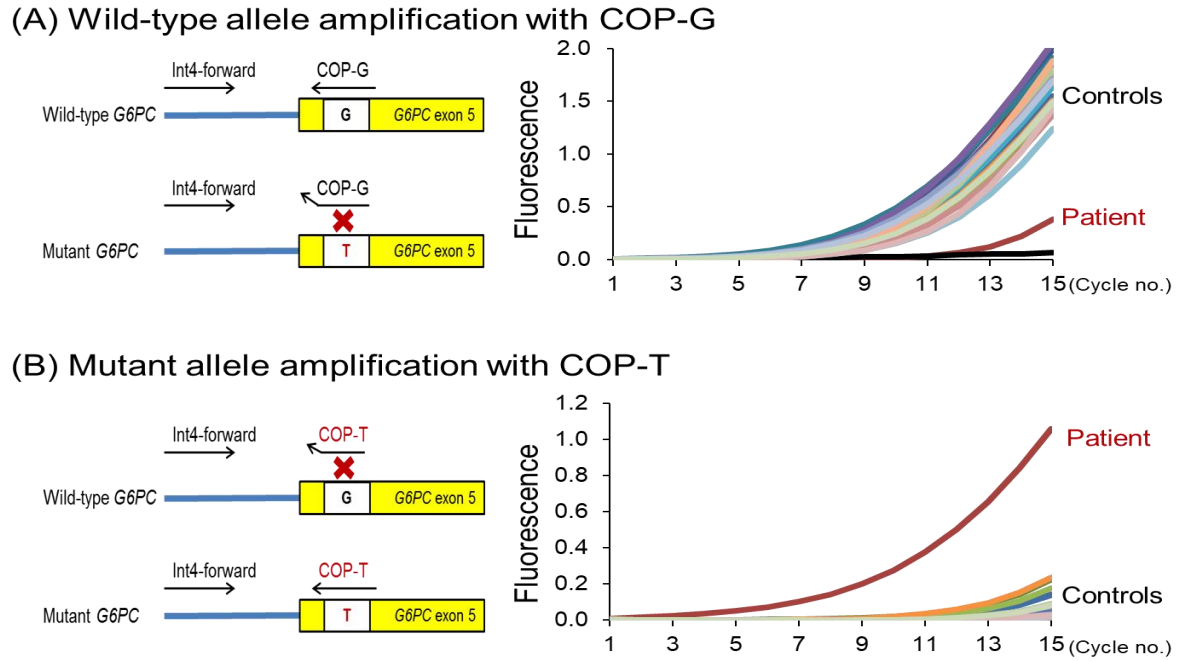

Figure 2. The real-time mCOP-PCR detection of the wild-type allele with COP-G (A) and mutant allele with COP-T (B). The annealing sites of the COP-G (black) and COP-T (red) primers are indicated. The COP-G amplified the target fragment in the controls, but not in the patient (× symbol), while the COP-T amplified the target fragment in the patient, but not in the controls (× symbol). The horizontal axis indicates the quantification cycle (Cq) value and the vertical axis indicates the fluorescence amplitude.

Table I. Cq values of real-time mCOP-PCR analysis

	COP-G	COP-T	*p*
Control (n=50) mean ± SD	9.63 ±1.03	14.89 ± 0.28	< 0.01
Patient (n=1) (Results of Two assays)	14.46 (13.83, 15.08)	10.04 (8.97, 11.1)	

Student's t-test with significant difference of $p < 0.05$.

DISCUSSION

1. GSD diagnosis based on mutation detection

Accurate diagnosis is an important factor for proper treatment of a metabolic disease such as GSDIa. However, it remains challenging, because there are many GSD subtypes that share similar clinical presentations, such as hepatomegaly, hypoglycemia, and hyperlipidemia [16]. The gold standard for GSDIa diagnosis still relies on an invasive procedure, requiring an evaluation of liver enzyme deficiency and pathological findings on liver biopsy [16,17].

Even so, invasive diagnostic procedures are being replaced by mutation detection in the GSD-causing genes. Such mutation detection is directly linked to the diagnosis of GSD subtypes. Various methods for mutation detection have been developed, ranging from simple conventional methods to more advanced methods. Each method has advantages and disadvantages.

For instance, single-strand conformation polymorphism (SSCP) combined with heteroduplex analysis is a simple method to screen for any unknown mutation [18]. However, the sensitivity is low and some mutations may not be detected [19]. Another method, denaturing gradient gel electrophoresis (DGGE), has been developed to identify any unknown mutation in GSD patients from a single nucleotide change, but it may be laborious [19,20]. Alternatively, restriction fragment length polymorphism PCR (RFLP-PCR) is another example of a simple method to accurately detect known mutations in GSDIa patients, but this requires a large amount of DNA and is time-consuming [21]. Lastly, a more advanced method, allele-specific PCR combined with fluorescent probes using real-time PCR apparatus, is being used to screen for known mutations [22,23]. Allele-specific amplification to detect the c.648G>T point mutation in *G6PC* is favorable for the GSDIa screening system in the Japanese population. Allele-specific amplification is rapid, reliable, and considered a high-throughput method. However, fluorescent probes are not always inexpensive.

2. GSDIa Screening system using DBS on filter paper

In this study, we established a GSDIa screening system using DBS on filter paper. Our system had four features: easy handling of DBS on filter paper, no procedures of DNA extraction, robustness of nested PCR, highly specific amplification of mCOP-PCR, and economical efficiency of the total system.

Genetic analysis for disease diagnosis usually requires a relatively large amount of DNA extracted from fresh whole blood. Here, we used DBS on filter paper, which meant a very small amount of non-fresh DNA. Genetic analysis using DBS also offers many other advantageous aspects for clinical practice. The patients (or caregivers of the newborn patients) can collect blood samples by finger-prick (or heel-prick for newborn patients) and easily ship the DBS under ambient temperature from remote areas to the analysis center. A DBS is easy to handle and is stable for a long storage period. Some types of filter paper (FTA Elute card) can also deactivate pathogenic microorganisms, making the handling of blood less hazardous [24].

For our PCR assay, the DNA extraction procedures were to be skipped, because DNA polymerase KOD FX Neo was able to amplify crude DNA samples. We put a punched circle of DBS to the reaction mixture and directly amplified a common region of *G6PC* in the first round, being followed by allele-specific amplification with mCOP-PCR in the second round. Nested PCR, which was consisted of the first and second round amplification, gives our system robustness; it helps to overcome the low quantity and quality of the DNA template within the DBS [13]. In addition, nested PCR also enhances the sensitivity and specificity of real-time PCR-based detection [25].

To detect a point mutation, we adopted the mCOP-PCR technology in the second round of the nested PCR. The mCOP-PCR technology is also based on the principle of allele-specific amplification, where the short primer contains a specific nucleotide in the middle of the primer that binds only to the target allele [26]. This technology has been used extensively to differentiate *SMN1* exon 7 from *SMN2* exon 7 in SMA screening from DBS [13,14]. This proved that allele-specific amplification using mCOP-PCR may reduce the cost but maintain comparable results to fluorescence probe-based PCR method. Therefore, we adopted this method to detect a point mutation in *G6PC* of GSDIa patients.

3. Necessity of neonatal screening for GSDIa

Gene replacement therapies are being developed for inborn errors of metabolism, including GSDIa, and are showing promising results in maintaining blood glucose levels and preventing late complications, including the development of hepatocellular carcinoma/adenoma [11,27–29]. In fact, a phase I/II clinical trial and long-term study are currently underway for treatment of GSDIa (NCT03517085, NCT03970278) with adeno-associated virus serotype 8-mediated *G6PC* gene transfer (AAV8*G6PC*) to evaluate dose-finding and safety in adult GSDIa patients. Although no results have yet been reported, we anticipate a promising result that would justify a screening system.

In gene replacement therapy, the introduced viral vector or target gene may produce "non-self" proteins in the patients. To avoid a host-side immune response to these proteins, it is desirable that gene replacement therapy be performed in early infancy (the neonatal period) when immune tolerance is induced [30,31].

In addition, the neutralizing antibody titer against the virus from which the viral vector is derived increases with age [32]. Neutralizing antibodies to the virus can determine the success or failure of gene replacement therapy [33]. Thus, gene replacement therapy should be done early in infancy.

Early diagnosis and early treatment of the disease are necessary, both to avoid the side effects (particularly immune disorders) of gene replacement therapy for inborn errors of metabolism, and to maximize the therapeutic effect. Considering the ongoing clinical trials, we believe that gene replacement therapy for the treatment of GSDIa will be available in the near future, and that implementation of our DBS screening method will therefore be significant for successful treatment.

4. Conclusion

To our knowledge there has been limited use of DBS for GSDIa screening, although DBS screening for GSD type II or Pompe disease has been extensively performed [reviewed in Sawada 2020] [34]. There is growing research into GSDIa treatments, including gene therapy, which shows a better outcome when administered at an early stage [11,12]. This supports the importance of a screening system for GSDIa. Considering the advantages of DBS and robustness of mCOP-PCR, this study provides alternative options for GSDIa screening in the Japanese population.

However, this method only covers GSDIa patients who carry a c.648G>T mutation. Although GSDIa is a pan-ethnic disease, unique *G6PC* mutations have been identified in specific ethnic groups [16], making allele-specific amplification-based detections only applicable for particular populations.

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CONFLICT OF INTEREST STATEMENTS

The authors declare no competing interests.

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