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NEONATAL BUCCAL CELL COLLECTION: A NON-INVASIVE STRATEGY TO PROVIDE A RELIABLE SOURCE OF DNA FOR HIGH RESOLUTION MELTING ANALYSIS

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Abstract

Background: The low yield and quality of buccal-derived genomic DNA have reduced its applicability in various genetic research. The aim of this study was to assess the quantity, purity and genotyping efficiency of genomic DNA isolated from neonatal buccal swabs. **Methods:** Paired buccal swabs and whole blood samples were collected from 60 neonates with the mean age 5 days (SD=1.57). The genomic DNA quantity and purity were measured by using Infinite[®] 200 PRO NanoQuant reader and agarose gel electrophoresis. High-resolution melting (HRM) analysis was used to analyse the sequence variants present in uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1* c.211G>A) and nuclear receptor subfamily 1, group I, member 3 (*NR1I3* IVS8+116T>G) genes. **Results:** Buccal swabs provided lower mean genomic DNA concentration (18.78 ± 8.39 ng/ μ l versus 40.02 ± 13.03 ng/ μ l), yield (2.63 ± 1.17 μ g versus 8.00 ± 2.61 μ g). The purity of buccal samples however were inconsistent with 16 samples (26.7%) having A260/280 ratios below 1.8 which indicated protein contamination. Genomic DNA purity for all blood samples were within the ideal range with average absorbance ratios of 1.8–2.0. However, all buccal genomic DNA demonstrated 100% genotype call rates for all variants. A complete genotype concordance was also observed between paired genomic DNA samples. **Conclusion:** Despite related to a reduced quantity and purity, neonatal buccal genomic DNA could generate reliable HRM genotyping results. Therefore, buccal swab collection is a promising alternative to the invasive blood sampling to provide genomic DNA for genetic analysis involving paediatric population.

Keywords:

Buccal, Non-invasive, High-Resolution Melting, Neonate, *UGT1A1*, *NR1I3*

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Introduction

Sampling of genetic materials for molecular analysis is paramount in understanding the genetic basis of a disease as it determines the reliability of research findings and further affects the downstream clinical decisions. Epidemiologic studies, candidate gene analysis and genome-wide association studies typically involve whole blood samples due to the critical demand for high-quantity and -quality DNA [1,2,3]. However, the collection of blood sample has disadvantages: a qualified practitioner is required to perform the venipuncture, risk of exposure to blood pathogens and expensive processing. Painful sensation and trauma from venipuncture could

also reduce the participation rate and render the procedure unsuitable especially among paediatric population [4,5].

As an alternative or supplement to blood sampling, the non-invasive approach of obtaining genetic materials from the buccal cells has overcome some potential drawbacks of the blood sampling [6,7]. Buccal cells can be collected from the mouth wash, saliva, swab and cytobrush to

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provide DNA for genetic analysis [8,9,10,11]. Although the sampling method of genomic isolation from buccal cells is cost-effective, uncomplicated and appropriate especially in paediatric setting, the DNA quantity and quality inconsistency have discredited the buccal cells-derived DNA isolation method [9].

The present study was undertaken to evaluate the total yield, purity and high-resolution melting (HRM) genotyping efficiency (genotype call and concordance rates) of neonatal buccal genomic DNA in comparison with that of the paired blood samples. To the best of our knowledge, there are no studies have compared the genotyping efficiency between buccal and blood DNA from paediatric population using HRM analysis.

Methods

Subjects

Our study subjects comprised of 60 neonates admitted to Hospital Universiti Sains Malaysia in the year 2014. The subjects constituted part of the genetic association study on neonatal hyperbilirubinaemia. All subjects were term neonates with gestational age ≥ 37 weeks and birth weight ≥ 2500 g. Informed consent was obtained from the parents of eligible neonates prior to collecting the samples. This study was approved by Human Research Ethics Committee of Universiti Sains Malaysia (No. USMKK/PPP/JEPeM [263.3(12)]).

Genomic DNA isolation

Two buccal swabs and whole blood samples were collected from each neonate during their first week of life. Buccal swabs were collected at least two hours after feeding. Sterile cotton swabs (Goodwood, China) were gently rubbed against the inside of both cheeks for about 20 s and kept in a 2-ml microcentrifuge tube. Whole blood samples (200 μ L) were obtained simultaneously with routine blood investigation and kept in a K2EDTA tube (Becton Dickinson, USA). Buccal and blood genomic DNA were isolated within three days by using ExogeneTM blood SV mini kit (GeneAll Biotechnology Co. Ltd., Korea) with some modifications in the protocols. For buccal genomic DNA extraction, 400 μ L of 1X PBS was added to the buccal swab. Proteinase K (20 μ L) and 400 μ L of buffer BL were then added. For blood genomic DNA extraction, 20 μ L of proteinase K was added to 200 μ L of whole blood

and 200 μ L of buffer BL was added subsequently. The mixture was then incubated at 56°C for 1 h (Thermomixer, Eppendorf Model 5436, USA) after vortexing and centrifugation steps. Then, the mixture was added with absolute ethanol (400 μ L and 200 μ L for buccal and blood samples respectively). Transfer, centrifugation, discard and reinsert steps were repeated until all mixture had been applied to the SV column. For buccal samples, mixtures from two buccal swabs were combined in one SV column. The amount of buffers BW and TW used were 600 μ L and 700 μ L followed by centrifugation in every step. The amounts of elution buffer used to derive the buccal and blood genomic DNAs were 140 μ L and 200 μ L respectively. The isolated genomic DNA were stored at -20°C until HRM analysis.

Assessment of DNA quantity and purity

Genomic DNA concentration was determined spectrophotometrically by using Infinite[®] 200 PRO NanoQuant reader (Tecan Group Ltd., Switzerland) which measured the absorbance at 260 nm. The genomic DNA purity was provided by the ratio of absorbance at 260 and 280 nm (A260/A280). Absorbance ratios of 1.8–2.0 indicated good-quality genomic DNA [12]. The quality of buccal and blood genomic DNA was also evaluated via agarose gel electrophoresis using 1% agarose gel which was run at 80 Volt for 60 min.

High-resolution melting and DNA sequencing analyses

HRM analysis of the uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1* c.211G>A) and nuclear receptor subfamily 1, group 1, member 3 (*NR1I3* IVS8+116T>G) sequence variants were performed using buccal and blood genomic DNA [13,14]. The primer pairs were shown in Table 1. HRM assays were conducted on PikoReal 96 real-time PCR system (Thermo Fisher Scientific Inc., United States) using previously described protocols [15]. To assess the sensitivity, specificity and ability of buccal genomic DNA to provide reproducible HRM genotyping results, six paired buccal and blood genomic DNA samples were randomly selected for re-genotyping. Genotyping efficiency of the buccal genomic DNA was evaluated by comparing the genotype call and concordance rates of paired and duplicate samples. Random paired samples (n = 20) were subjected to DNA sequencing analysis to validate the genotypes.

Table 1. HRM primer pairs for genotyping *UGT1A1* and *NR1I3* variants

Sequence variants	Primer sequence (5' to 3')	Amplicon size (bp)	T _a (°C)
<i>UGT1A1</i> rs4148323; c.211G>A	F: GCAGCAGAGGGGACATGAAA R: GCACAGGGTACGTCTTCAAG	95	56
<i>NR1I3</i> rs4073054; IVS8+116T>G	F: GGTGAAACATTGAGCTTGGG R: GCTGAAACGATGTGAGACAGG	114	58

T_a, annealing temperature; F: forward; R: reverse.

Results

Quantity and purity of buccal and blood genomic DNA

Buccal genomic DNA was isolated from 60 neonates and compared with blood samples for the genomic DNA yield and quality, and HRM genotyping efficiency. Although wide ranges of genomic DNA concentration were obtained from both buccal and blood samples (Table 2), the

mean buccal genomic DNA concentration (18.78 ng/μl) was lower than that of the blood samples (40.02 ng/μl). Buccal genomic DNA concentration of as low as 4.36 ng/μl was isolated while the minimum blood genomic DNA concentration was 15.38 ng/μl. Meanwhile, the maximum amount of buccal genomic DNA concentration was 47.60 ng/μl and 71.33 ng/μl was isolated from blood genomic DNA.

Table 2. Buccal and blood genomic DNA concentration and total yield

Genomic DNA extraction	Paired DNA samples (n = 60 pairs)	
	Buccal swabs	Whole blood
Genomic DNA concentration, ng/μl, mean ± SD (range)	18.78 ± 8.39 (4.36–47.60)	40.02 ± 13.03 (15.38–71.33)
Average elution volume, μl	140	200
Total DNA yield, μg, mean ± SD (range)	2.63 ± 1.17 (0.61–6.66)	8.00 ± 2.61 (3.08–14.27)

For all blood samples, acceptable genomic DNA purity was obtained with average absorbance ratios within the ideal range of 1.8–2.0. However, buccal samples had inconsistent purity with 16 samples (26.7%) having A260/280 ratios below 1.8 which suggested protein contamination of the buccal genomic DNA. Gel electrophoresis of some random buccal genomic DNA samples exhibited evidences of genomic DNA degradation by the presence of smeared genomic DNA bands (Supplementary 1).

Supplementary 1: Gel pictures of blood and buccal genomic DNA

High-quality gDNA bands (lanes 1 and 2) of the blood samples were >10 kb in size. Buccal gDNA exhibited evidences of DNA degradation by the presence of smeared bands and less intense bands of >10 kb (lanes 3 and 4). Agarose gel electrophoresis (1%) of gDNA were performed at 80 V for 60 min. Lane M1 = 1 kb DNA ladder; lane M2 = 100 bp DNA ladder; lanes 1 and 2 = blood gDNA samples; lanes 3 and 4 = buccal gDNA samples.

Concordant HRM genotyping results between paired DNA

High Resolution Melting analysis was successfully optimised. The quantification cycle (Cq) values of both buccal and blood samples were in the range of 25–29 cycles although only 1 ng of genomic DNA was used in each reaction. An optimal real-time PCR amplification graph suitable for HRM analysis is shown in Figure 1. HRM analysis of the sequence variants showed that all buccal and blood genomic DNA were successfully genotyped

and achieved 100% genotype call rates. Buccal genomic DNA generated HRM genotyping results comparable with that of the blood samples. The melting patterns of wild type, heterozygous and homozygous variant genotypes were clearly distinguishable from each other, hence allowing straightforward determination of the genotypes (Figure 2). Besides, each genotype produced consistent and distinct melting patterns, thereby generating reproducible results that facilitated the genotype calling of unknown samples.

Figure 1. Real-time PCR amplification graph of c.211G>A using buccal DNA

Cq values (26–28 cycles) of buccal DNA samples were adequate to produce reliable HRM genotyping results. Samples amplified to the plateau phase following real-time PCR indicated that sufficient and comparable amounts of amplicons were available for the subsequent melting in HRM assays. The plateau phase also ensured that the HRM analysis would not be affected by variable amplicon amounts. RFU, relative fluorescence units.

Figure 2. HRM analysis of *UGT1A1* c.211G>A and *NR1I3* IVS8+116T>G

HRM analysis demonstrated distinguishable melting patterns of wild type, heterozygous and homozygous variant genotypes of (A) c.211G>A and (B) IVS8+116T>G using buccal DNA as template. The buccal DNA

samples generated consistent melting patterns for each genotype. DNA sequencing analysis of random samples confirmed the genotypes.

The A260/280 ratios between DNA from buccal swab and blood samples were not significantly different thus the excellent concordance in genotyping (100%) was achieved between genotypes and HRM melting patterns. Paired samples demonstrated the same genotypes for all variants that were evaluated. Table 3 shows the fully compatible HRM genotyping results

between buccal and blood DNA samples. Besides, duplicate samples of paired buccal and blood DNA exhibited 100% genotype call and concordance rates, indicating 100% sensitivity and specificity of the HRM analysis. Therefore, highly accurate and reproducible HRM genotyping results could be produced by the buccal DNA samples.

Table 3. Fully compatible buccal and blood HRM genotyping results

		Blood DNA (<i>n</i> = 60 pairs), <i>n</i>		
Buccal DNA (<i>n</i> = 60 pairs), <i>n</i>	<i>UGT1A1</i> c.211G>A	GG	GA	AA
	GG	56	0	0
	GA	0	3	0
	AA	0	0	1
	<i>NR1I3</i> IVS8+116T>G	TT	TG	GG
	TT	42	0	0
	TG	0	16	0
	GG	0	0	2

The frequencies (*n*) of each genotype were shown for the paired buccal and blood DNA samples. There was no discrepancy between the buccal and blood genotyping results.

Discussion

DNA from buccal swab samples was significantly lower in concentration and yield but not quality. Our study has successfully demonstrated that the buccal genomic DNA samples generated satisfactory HRM genotyping results. Using buccal DNA, HRM assays of the *UGT1A1* and *NR1I3* sequence variants showed that the melting patterns of different genotypes were distinguishable from each other, thus facilitating genotype determination of all buccal samples. Besides, 100% genotype call and concordance rates obtained with duplicate and paired buccal and blood samples indicated the accuracy and reproducibility of using buccal DNA in HRM analysis. Hence, our study suggests that the buccal swab could provide a reliable alternative source of DNA that is suitable for molecular genetic analysis.

A growing number of clinical and genetic studies utilising buccal DNA samples have been conducted. The successful use of buccal DNA has been documented in numerous applications including the short tandem repeat analysis, TaqMan assay, PCR-restriction fragment length polymorphism, whole exome sequencing and Illumina Beadchip arrays [16,17,3]. Similarly, the present study and our previous report which employed HRM analysis have demonstrated that the buccal DNA could generate clearly defined melting patterns for different genotypes of the variants [15]. In addition, comparable genotyping results between paired buccal- and blood-derived DNA implied that the buccal DNA could produce accurate genotype calls thereby confirming the reliability of applying buccal DNA in HRM analysis.

Buccal cell collection is increasingly used as an alternative to blood sampling mainly due to its non-invasive, cost-effective, easy re-sampling and convenient system. Particularly, the collection method is suitable for paediatric population, healthy subjects and in case-control studies which require large number of participants where the request for blood drawing may reduce the participation rate [18]. Besides, additional blood drawing from neonates or children for research purposes is undesirable and the sampling procedure may result in pain or infection associated with venipuncture. Since the primary concern is on the care of subjects, the collection of buccal cells is thus more suitable for the susceptible group in comparison with blood sampling.

Despite the advantages of buccal cell collection, buccal DNA is commonly associated with some limitations. In this study, the DNA yield obtained from buccal swabs was lower than that of the whole blood samples. Likewise, several studies have also reported low DNA yields isolated from the buccal cells [9,19]. Besides, a wide range of buccal DNA yield was found in our study which could be due to the lag time between the sample collection and DNA isolation. To accommodate some applications requiring high yield of DNA, buccal DNA precipitation could be performed but the DNA samples might be exhausted rapidly. An interesting solution is by employing whole genome amplification (WGA). Notably, a study has revealed an improved buccal DNA yield with WGA while another study has uncovered that WGA could enhance the efficiency and validity of genotyping by using buccal DNA [20,21]. The collection of two buccal swabs in our study is estimated to provide DNA samples sufficient for at least 150 HRM reactions without the need for WGA.

Studies have suggested that there is potential for the contamination of neonatal buccal cells with breast milk from the mother [22,23]. Residual breast milk carrying small but quantifiable amount of maternal DNA may be obtained together during the collection of neonatal buccal cells [23]. However, such small amount of maternal DNA is insignificant in modulating the

genotyping results which are mostly qualitative in nature [24]. The justification is consistent with our findings which found comparable genotyping results between paired buccal and blood DNA despite the possible contamination of neonatal buccal samples with maternal DNA from the breast milk. On the other hand, bacteria present in the oral cavity have been a significant element related to the buccal-derived genomic DNA considering the possible interference on the human buccal DNA quantity and quality [25]. However, amplification and HRM genotyping of our buccal samples were still achievable suggesting that the buccal samples contained adequate quantity of human DNA. Further, refrigeration of the buccal samples may be useful in restricting the growth of bacteria while reducing the degradation of human DNA [26].

There are several limitations in our study. We did not control the time of day during buccal cell collection and medications consumed by the mother. These factors may affect buccal DNA yield obtained from neonates and further studies are required to evaluate the relationship among these parameters. Nevertheless, the HRM genotyping results exhibited reliable detection of variants even by using a small amount of human DNA. However, future studies are still needed to evaluate the purity consistency of buccal DNA in a large cohort paediatric population.

Conclusion

Our approach of analysing buccal DNA through HRM analysis has demonstrated a simple, sensitive, rapid and an overall low-cost system. HRM analysis of paired buccal and blood DNA has exhibited comparable and accurate genotyping results. Hence, our study has successfully validated the use of buccal DNA as a promising alternative to blood and suitable for research involving paediatric population.

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