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DNA methylome profiling using neonatal dried blood spot samples: A proof-of-principle study

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ABSTRACT

DNA methylation is the most common DNA modification and perhaps the best described epigenetic modification. It is believed to be important for genomic imprinting and gene regulation and has been associated with the development of diseases such as schizophrenia and some types of cancer.

Neonatal dried blood spot samples, commonly known as Guthrie cards, are routinely collected worldwide to screen newborns for diseases. Some countries, including Denmark, have been storing the excess neonatal dried blood spot samples in biobanks for decades. Representing a high percentage of the population under a certain age, the neonatal dried blood spot samples are a potential alternative to collecting new samples to study diseases. As such, neonatal dried blood spot samples have previously been used for DNA genotyping studies with excellent results. However, the amount of material available for research is often limited, challenging researchers to generate the most data from a limited quantity of material.

In this proof-of-principle study, we address whether two 3.2 mm disks punched from a neonatal dried blood spot sample contain enough DNA for genome-wide methylome profiling, measuring 27,578 loci at the same time. We selected two subjects and carried out the following with each: 1) collected an adult whole-blood sample as reference, 2) spotted a fraction of the whole-blood sample onto a similar type of filter paper as used in the newborn screening and stored it for 3 years to serve as a dried blood spot reference, and 3) identified the archived neonatal dried blood spot samples, stored for 26–28 years, in the Danish Newborn Screening Biobank as a representative of the archived samples. For comparison, we used two different kits for DNA extraction.

The DNA, extracted using the Extract-N-Amp Blood PCR kit, was analyzed, and no statistically significant differences were observed ($P<0.001$) when we compared the methylation profile of the reference whole-blood samples to the dried blood spot references. This indicates that two 3.2 mm disks contain enough material for reliable methylome profiling and that storing the whole-blood sample on neonatal dried blood spot filter paper for 3 years does not interfere with the outcome of the analysis.

Furthermore, we compared the adult DNA methylation profile to the neonatal dried blood spot sample profile. Approximately 50 sites in the subjects were significantly ($P<0.001$) different in the newborn sample compared with the adult sample. Both being healthy adults and the high quality of the DNA methylation array led to the conclusion that the archived neonatal dried blood spot samples can be used for methylome profiling, despite decades of storage and DNA degradation.

In conclusion, we show that reliable methylome data can be obtained from old neonatal dried blood spot samples, by using a reasonable amount of the limited resource. This further adds to the use of neonatal dried blood spot samples in genetic research and screening and paves the way for unique population-based studies of epigenetic modifications after birth.

1. Introduction

Epigenetic processes are important for cellular differentiation and development. DNA methylation is perhaps the best understood epigenetic adaption and the most common DNA modification. It has been shown to be involved in genomic imprinting (parent-of-origin specific expression), tissue differentiation, gene silencing, chromosome stability, and the regulation of gene expression [1–5]. Aberrant

Abbreviations: DBSS, dried blood spot samples; DS, differential score; ENA, Extract-N-Amp Blood PCR kit; CS, ChargeSwitch Forensic DNA Purification kit; QC, quality control.

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DNA methylation profiles have been observed in diseases such as schizophrenia, bipolar disorder [6], and different types of cancer [1]. In most cases, it occurs by the addition of a methyl group to the 5′-position of the cytosine residue within a CpG dinucleotide. The CpG sites are often located in large islands, defined as regions of at least 200 bp with a GC content greater than 50% and an observed CpG ratio greater than 60% [7]. CpG islands are found in the 5′ regulatory (promoter) regions of many genes and housekeeping genes in particular, and they are not typically methylated. However, hypermethylation of CpG islands in the promoter regions is seen in many diseases, such as cancer, and can result in down-regulation of the transcriptional expression of the gene or gene silencing. Approximately 45% of human genes do not contain CpG islands, and the role of these non-CpG-island promoter regions is unclear and not yet fully understood [8,9]. The DNA methylation profile can be determined in numerous ways and on many different platforms. Platforms such as the Pyrosequencer or the Sequenom are often used if the number of targets is low. However, if the methylation status of the whole genome – the methylome – is the target, either arrays (as in this study) or high-throughput sequencing can be used.

Many DNA methylation studies of human diseases use DNA extracted from peripheral blood leukocytes, and often the sample material is collected after or at the onset of the disease or during a period of time in which the individual is being monitored. As an alternative or additional possibility, a blood sample collected shortly after birth could be a good indicator of a relatively unexposed individual, only influenced by in-utero factors. Interestingly, dried blood spot samples (DBSS), also known as Guthrie card samples, are routinely collected in many countries as a part of their neonatal screening programs. In Denmark, neonatal screening for metabolic disorders began in 1975, and since 1982 the access neonatal DBSS have been stored in the Danish Newborn Screening Biobank. Today, the biobank contains almost 2 million neonatal DBSS, representing almost every Dane born since 1982 [10]. In combination with a well-functioning public registration system that makes it possible to combine data from practically all public databases, researchers can study Denmark as a cohort [11]. Due to this, the Danish Newborn Screening Biobank has become an invaluable resource for biological material for research studies and is frequently used for genetic and biomarker studies [12–16].

The aim of this study was to evaluate if a realistic amount of material extracted from stored neonatal DBSS can be used for reliable methylome profiling, thereby leading to future early-life epigenetic profiling of whole-blood samples in human diseases.

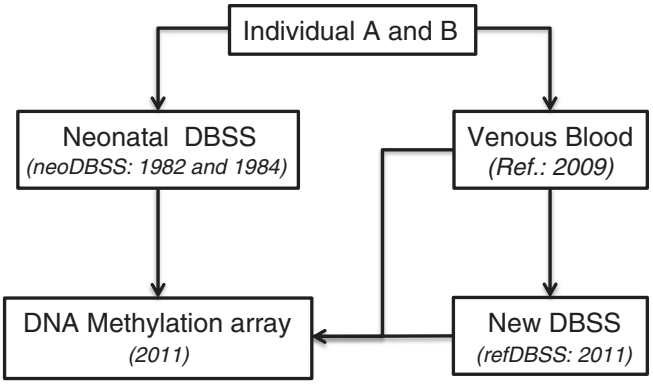

Fig. 1. Study overview. At birth, the DBSS of the two individuals A (born 1982) and B (born 1984) were collected and archived in the Danish Newborn Screening Biobank (neoDBSS). In 2009, a venous whole-blood sample (Ref.) was drawn from both individuals A and B, aged 28 and 26, respectively. A fraction of the venous blood was spotted on filter paper (refDBSS) similar to the type used for the neonatal screening (Whatman 903® Specimen Collection Paper) and stored at −20 °C until analysis was carried out in 2011.

2. Material and methods

2.1. Sample overview and DNA extraction

Both individuals A and B (siblings) were informed volunteers. Being a potent future newborn screening method, this study was categorized as a category 2 study ("development of new methods for newborn screening analyses," Nørgaard-Pedersen, et al. 2007), and permission from the ethical committee was not necessary, as per Danish law [10].

Venous blood from each individual was drawn in a heparin collection tube (Ref.). Fifty milliliters was transferred to Whatman Specimen 903® Collection Paper (refDBSS), dried for 3 hours at room temperature, and stored at −20 °C for 3 years. The neonatal DBSS (neoDBSS) from each individual was isolated from the Danish Newborn Screening Biobank. DNA from the venous blood sample was extracted using the Maxwell® 16 LEV Blood DNA Kit (Promega) according to the manufacturer's instructions. Four 3.2 mm disks were punched from the individual's refDBSS and neoDBSS. Two disks were used for a standard protocol DNA extraction with the Extract-N-Amp Blood PCR kit (ENA) (Sigma), and the other two were used with the ChargeSwitch Forensic DNA Purification kit (CS) (Invitrogen). See Fig. 1 for a study overview.

2.2. Bisulphite conversion and Infinium arrays

The DNA samples were bisulphate converted using the EZ-96 DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. For quality control purposes, we profiled positive and negative control samples from the whole genome amplified DNA of individual A using the CpGenome Universal Methylated DNA (Millipore) and REPLIg (QIAGEN) kits, respectively. We used 1 μg of DNA from the Ref. samples and positive and negative controls. The DNA extracted from the refDBSS and neoDBSS, approximately 30 ng, was used in its entirety. The bisulphite converted Ref. DNA (input 1 μg) was eluted in 15 μL, and 4 μL was used for Infinium HumanMethylation27 (Illumina Inc.) labeling. For the refDBSS and neoDBSS, the bisulphite converted elute was vacuum centrifuged before using the entire volume for labeling.

2.3. Differential methylation analysis

The methylation level per CpG site was estimated by measuring the signals of the methylated (signal B, green channel) and unmethylated (signal A, red channel) probes. Each of the intensities was used

Table 1
Methylation QC probe evaluation. Probe *P*-value was set to 0.01. Number: the number of detected probes with a *P*-value<0.01. Percentage: the percentage of detected probes (*P*<0.01) of total 27,578 probes on the HumanMethylation27 Beadchip. For positive control we used the CpGenome Universal Methylated DNA (Millipore) and for negative control we whole-genome amplified individual A before bisulphite treatment. For DNA extraction we used the Extract-N-Amp (ENA) and ChargeSwitch (CS) kits. Average is the average β-value of the detected probes (*P*-value<0.01). Minimum/maximum: the minimum and maximum β-value of the sample.

		Probe		β-Value		
Sample	Individual	Number	Percentage	Average	Minimum	Maximum
Controls	Pos.	27,567	100.00%	0.889	0.3386	0.9865
	Neg.	27,247	98.80%	0.1327	0.018	0.8345
Ref.	A	27,557	99.90%	0.287	0.011	0.9823
	B	27,564	99.90%	0.2889	0.0096	0.9843
refDBSS	A	27,359	99.20%	0.2781	0.0115	0.9807
(ENA)	B	27,391	99.30%	0.2833	0.011	0.9811
neoDBSS	A	27,213	98.70%	0.2824	0.0116	0.9835
(ENA)	B	27,509	99.70%	0.2934	0.0111	0.9821
refDBSS	A	25,214	91.40%	0.2721	0.0096	0.9847
(CS)	B	23,251	84.30%	0.2767	0.0098	0.9842
neoDBSS	A	24,010	87.10%	0.2701	0.0085	0.9869
(CS)	B	26,365	95.60%	0.2822	0.0091	0.9843