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Storage conditions and stability of global DNA methylation in placental tissue

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Abstract

Aim—The placenta is an informative and easily available tissue for many epidemiological studies. We analyzed the extent to which storage delay affects DNA methylation.

Material & methods—Biopsies from two placentas were sequentially stored at -80°C after standing at room temperature for 30 min, 1 h, 2 h, 6 h and 24 h. Global DNA methylation was measured by bisulfite pyrosequencing of repetitive elements and the luminometric methylation assay.

Results—Small changes in global DNA methylation in relation to time-to-storage were observed by pyrosequencing, with a coefficient of variation (COV) of 2.49% (placenta 1) and 2.86%

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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(placenta 2), similar to the mean technical variation observed for pyrosequencing (COV: 1.91 and 1.51%, respectively). A luminometric methylation assay yielded more variable results in the two placentas analyzed, both among time points (COV: 9.13 and 10.35%, respectively) and technical replicates (COV: 11.60 and 9.80%, respectively).

Conclusion—Global DNA methylation is stable at room temperature. However, some techniques to measure methylation might be confounded by DNA degradation caused by a delay in storage.

Keywords

cohort study; DNA methylation stability; luminometric methylation assay; placenta; pyrosequencing; variability

Increasing evidence from animal and human studies has revealed that epigenetic marks, such as DNA methylation, both global and gene specific, are modified following prenatal exposure to several environmental factors [1–3].

Much epidemiological research is focused upon studying the link between altered DNA methylation patterns and later health outcomes and diseases of complex etiology in humans, including cancer, hypertension, ischemic heart disease, lung function and chronic obstructive pulmonary disease [4–8].

The exponential increase in DNA methylation studies is highly dependent upon the feasibility of analyzing available biological archives. Previous studies have shown that DNA samples stored for several years at -80°C are amenable to use for further applications, including DNA methylation analysis [9]. An additional critical step in the creation of biological archives is the collection and preprocessing of biospecimens prior to freezing. RNA and proteins have been shown to be strongly affected by the time between collection and processing. A study analyzing the effect on RNA expression profiles of the time elapsed from delivery to collection and sample storage of full-term placentas found significant decreases in the expression of two transcripts after just 25 min [10], while others report very small amounts of RNA in placentas 2 h after delivery [11]. Autopsy studies have evidenced that RNA extracted from brain samples is highly sensitive to post-mortem delay, storage temperature and hypoxia [12]. Storage temperature also affects degradation of proteins and post-translational modifications, such as protein phosphorylation in post-mortem samples [9,13,14]. In epigenetic studies, H3 histone methylation levels in the human cerebral cortex have been shown to be maintained across wide ranges of post-mortem intervals (5–30 h) and tissue pH (0–6) [15]. However, little is known about the stability of DNA methylation following storage delays and, to our knowledge, only one previous study has analyzed DNA methylation stability and sampling distribution variability by repeatedly sampling a single placenta over a 24-h period in different sites and assessing gene-specific and long interspersed nuclear element (LINE)-1 methylation changes by bisulfite pyrosequencing [11].

In human studies, standardization of timing of collection and processing is often constrained by participant- and center-related logistics and is often balanced against the need to ensure optimal participation rates, therefore, resulting in heterogeneous recruitment conditions, including delays of up to several hours before sample storage.

Collection of placental tissues in particular – although of high interest as they can be non-invasively obtained from virtually any birth and may have potential importance in identifying fetal programming of human disease – is subject to further challenges owing to the variable timing and type of delivery. Therefore, assessing the variability of DNA

methylation measures in placental tissues derived from variable collection conditions is of critical importance to epidemiological studies of DNA methylation in relation to environmental exposures and later health effects.

Two techniques commonly used in epidemiological investigations to assess global DNA methylation are the analysis of genomic repetitive elements by bisulfite pyrosequencing [6,16–19] and the luminometric methylation assay (LUMA) [20].

Retrotransposons are one type of repetitive element, which, as a whole, account for approximately 42% of the human genome [21], and have the ability to copy themselves into an RNA intermediate and insert back into the genome as a new cDNA copy [22]. These elements are rich in CpG repeats and tend to be methylated in order to suppress their expression [23]. The most well-studied families are the LINEs, short interspersed nuclear elements and long-terminal repeat retrotransposons.

LUMA is a cost-effective and easy-to-perform assay [24], based upon an enzymatic digestion of DNA using a pair of methylation sensitive endonucleases, HpaII and MspI (New England Biolabs, MA, USA), which cleave 5'-CCGG-3' sites that are dependent and independent of methylation of the internal cytosine, respectively, following a single nucleotide extension using pyrosequencing [25]. The CCGG sequence accounts for only 8% of CpG sites in the genome [26], but is enriched 15-fold in CpG islands, which are potentially important regulatory sequences in the genome [27].

The purpose of this study was to assess changes in DNA methylation in placental tissue in relation to time intervals at room temperature (RT) prior to storage by using two different techniques. In addition, we analyzed methylation at three different regions within the villous parenchyma to determine the possible variability in methylation attributable to the histological heterogeneity of term placentas.

Our results may be useful to on-going or future epidemiological studies, especially for those birth cohorts that have collected placental tissue [28].

Materials & methods

Sample collection

Two placentas of similar weights (580 and 570 g) from vaginal spontaneous deliveries were selected for this study. Both placentas were from healthy male births with normal weights and gestational ages, no major complications during pregnancy, and both had optimal Apgar scores at birth (9/10/10). Mothers were contacted upon admission for delivery and informed consent was signed before placenta collection. The study protocol was approved by the Institutional Review Board Committee of the Hospital del Mar (Barcelona, Spain). Samples were collected during May 2012 and biopsies were performed within 20 min of delivery.

The fetal side of the placenta was recognized by physical examination and cord insertion location at the placental disk. We divided each placenta into three equal parts, named regions A, B and C according to the scheme in Figure 1. From each of these three regions, using a sterile scalpel, we performed six biopsies of approximately 0.5 cm³ of the parenchyma villous of the fetal side (~5 cm away from site of cord insertion), avoiding chorionic plate contamination (amnion and chorion membranes) by biopsying 1.0–1.5 cm below the fetal membranes. Three of these biopsies, one from each region (A, B and C) were immediately immersed in liquid nitrogen, kept on dry ice and frozen at –80°C in less than 5 min (time 0 condition). The other biopsies were frozen sequentially at –80°C after staying at

RT for 30 min, 1 h, 2 h, 6 h and 24 h, respectively (Figure 1). A total of 36 biopsies were obtained from the two placentas.

DNA extraction

Genomic DNA from placental biopsies was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, CA, USA) in narrow time windows (same week) in order to minimize technical and operator variations. None of the samples presented visual signs of DNA degradation (smear bands or bands below 10,000 bp) as observed after running 100 ng of DNA on a 1.3% agarose gel. The isolated genomic DNA was stored at -80°C .

Global DNA methylation analysis

DNA methylation of repetitive elements was analyzed in a total of ten different subfamilies, including four LINEs (L1PA5, L1PA2, L1HS and L1Ta), three Alu elements (AluSx, AluYa8 and Alud6) and three long-terminal repeats (MLT1D, ERV1 and ERV9) using highly quantitative PCR amplification followed by pyrosequencing (PSQ MD 96 pyrosequencing system; Qiagen). A total of 1 μg of genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA). A total of 1 μl of bisulphite-converted DNA was used for PCR amplification using the GoTaq® Hot Start Polymerase (Promega, WI, USA). The PCR conditions and pyrosequencing primer sequences for L1HS, AluSx and AluYb8 have been previously published by Choi *et al.* [29] and Yang *et al.* [30], and additional assays specific for three LINE-1 subfamilies (L1PA5, L1PA2 and L1Ta), for the AluYd6 subfamily and for three HERV subfamilies (MLT1D, ERV1 and ERV9) were developed in our laboratory (primer sequences and PCR cycling conditions are shown in Supplementary Table 1; see online at www.futuremedicine.com/doi/suppl/10.2217/EPI.13.29). Between one and five CpG sites were analyzed, depending on the repetitive element. Samples were run in duplicate on the same day, and non-CpG cytosines in the analyzed sequence were used to determine the efficiency of the bisulfite conversion.

LUMA is a quantitative luminometric-based method used to analyze global genomic DNA methylation. The approach involves enzymatic digestion of the DNA using a pair of methylation-sensitive isoschizomer enzymes, HpaII and MspI, as described in detail by Karimi *et al.* [25]. An improved LUMA protocol was used that minimizes the effects of DNA degradation on quantification by adding additional measurements for free DNA ends [27]. Owing to the greater technical variability of the LUMA technique, each experiment was repeated five-times.

Data analysis

Methylation results are presented as the percentage of 5-methylcytosines. For the repetitive elements, we averaged the methylation of all CpG sites analyzed for a given element.

The coefficient of variation (COV), calculated as the ratio of the standard deviation to the mean, is a useful statistical measure for comparing the dispersion of data points (degree of variation) from one data series to another, and can be used even when the means show considerable differences between groups. We compared the COV of DNA methylation (percentage of 5-methylcytosines) between pyrosequencing (repetitive elements) and the LUMA among experiment replicates (technical variability), villous parenchyma regions in the placenta (regional variability) and time-to-storage at -80°C (time variability).

Results

Mean global DNA methylation analyzed in ten different repetitive element subfamilies ranged from 16 to 87% (Figure 2A–C).

Pyrosequencing duplicates presented similar methylation values in the ten repetitive elements, with technical differences in 5-methylcytosines below 5.54% and a COV of 1.91% in placenta 1 and 1.51% in placenta 2. We were, therefore, able to average technical duplicates (Supplementary Tables 2–4).

Variability in global methylation between the placenta regions A, B and C (regional variability) was also relatively small in all of the different repetitive element subfamilies, ranging from 0.01 to 3.58%, with a COV of 2.07% in placenta 1 and 2.72% in placenta 2. This did not change in relation to time-to-storage (Supplementary Tables 2–4).

Mean global methylation (average of technical duplicates and placental regions) in the ten repetitive elements among the different storage conditions showed changes in percentage methylation no greater than 2.80% from 0 to 24 h in the two placenta samples analyzed (range: 0.21–2.80%) (Figure 2). The COV due to time-to-storage from time 0 to 24 h at RT was 2.49% for placenta 1 and 2.86% for placenta 2.

Using the LUMA, the mean global DNA methylation level in the analyzed placentas was approximately 60% (Figure 2D).

Compared with pyrosequencing, a higher COV among technical replicates (taking into account all the time points) was observed with the LUMA of 11.60% in placenta 1 and 9.80% in placenta 2 (Figure 2D & Supplementary Table 5). The highest technical variability was observed at time 24 h. When this time point was excluded, the LUMA COV of technical replicates decreased to 9.96 and 8.64% in placenta 1 and 2, respectively.

The COV across all time points between the three villous parenchyma regions analyzed by LUMA (regional variability) was 5.73% for placenta 1 and 5.79% for placenta 2. Again, time 24 h showed the highest levels of regional variability in percentage methylation, and excluding this time point decreased the placental region-associated COV to 5 and 4.26% for placenta 1 and 2, respectively.

When analyzing changes in global DNA methylation levels following different storage time conditions using the LUMA in both placentas, we observed changes in global methylation of no more than 4% after 2 h at RT, compared with baseline (time 0), and no more than 6.5% from 0 to 6 h at RT. A higher decrease in global methylation levels was observed after tissue had spent 24 h at RT (~15%) (Figure 2 & Supplementary Table 5). The COV due to time-to-storage across the three regions from 0 to 24 h was 9.13% for placenta 1 and 10.35% for placenta 2. When the 24 h time point was excluded, the time-to-storage COV (from 0 to 6 h) was 4.32% in placenta 1 and 4.80% in placenta 2.

Supplementary Figure 1 summarizes the technical, time-to-storage (0–24 h) and placental region-associated variabilities using the two different techniques (pyrosequencing and LUMA) in the two analyzed placentas.

Discussion

We observed a small variation in DNA methylation levels in relation to time at RT in the ten repetitive elements analyzed, which was similar to the technical variability observed for the bisulfite pyrosequencing technique, which is among the most reliable PCR-based methods to assess DNA methylation owing to its quantitative nature and high reproducibility [31]. Elsewhere COV ranging from 1.8 to 2.82% have been reported for the pyrosequencing technique when used to interrogate methylation of the LINE-1 element [20,32], similar to the COV we observed for the time-to-storage variability. Therefore, our results suggest that a delay of storage at RT for tissue biopsies does not affect the levels of DNA methylation,

confirming results by Avila *et al.* on LINE-1 methylation and storage delay in placenta using the same technique [11].

Higher technical variability in global methylation was observed using the LUMA compared with the bisulfite pyrosequencing, especially at 24 h. This is perhaps because LUMA involves prior digestion with CpG methylation-sensitive restriction enzymes, which may be a source of variability for reasons other than DNA methylation [33]. In a recent study by Virani *et al.* on cord blood from 400 newborns, LUMA and LINE-1 tests were run in duplicate on each sample and measures that showed a difference in methylation levels of more than 15% among duplicates were removed [20]. Consistent with our findings, 4% of LUMA measures (16 out of 392) and only 0.25% of the LINE-1 measures (one individual out of 407) were removed. However, in our study we only observed differences above 15% between LUMA replicates at 24 h. The degree of change in DNA methylation in relation to storage time was also greater in the LUMA analysis, especially after 6 and 24 h at RT.

It is known that LUMA performance is sensitive to DNA degradation, similar to other techniques that are based on endonuclease digestion of DNA, such as reduced representation bisulfite sequencing [33,34]. Therefore, the decrease in global DNA methylation observed after 24 h and also the high variability in technical replicates at this time point could be dependent on DNA degradation rather than on an actual change in DNA methylation. In fact, the technical variability for the LUMA observed in our data up to 6 h (8.64 and 9.96% for each placenta, respectively) was slightly higher than the previously reported COV for the LUMA, which typically ranges from 4.9 to 6.2% [16], suggesting that there might already be DNA degradation at this time point in our samples, which becomes greater at time point 24 h (COV of 9.80% for placenta 1 and 11.60% for placenta 2).

In addition, when there is DNA degradation, it is plausible that the ratio of HpaII:MspI used to calculate the percentage of global methylation with the LUMA is skewed toward increased hypomethylation, as suggested by our data. However, we observed no signs of DNA degradation when samples were run on agarose gels.

Tissue heterogeneity and differences in perfusion and the degree of hypoxia are major sources of within-placenta variability, at least in relation to gene expression [35]. In our study, the biopsies collected from the different regions represented the 'inner' region of the placenta (closest to the cord insertion point), while the 'outer' part of the placenta at term typically presents an increase in the number of small villi, fibrin deposits and common syncytial knots (all signs of lower perfusion). For the ten subfamilies of repetitive elements analyzed, we observed similar levels of global DNA methylation among the three biopsies of the villous parenchyma analyzed from each placenta. Differences in methylation levels between placental regions analyzed using LUMA were larger, but not above 5%, with the exception of the 24 h time point. A study analyzing DNA methylation in five different genes related to trophoblast differentiation and migration across placental sites (from the cord insertion to the peripheral region of the placenta) and placental depths (from the fetal to the maternal side) observed no significant differences [11], although it is known that the physical location of cells may affect the levels of expression of certain hypoxia-related genes [35]. Similarly, a microarray-based gene expression study of 303 genes in normal placenta, comparing different regions of the villous parenchyma among individuals, found that interindividual variation in expression was greater than intraindividual variation [36].

A limitation of this study is that no specific villous parenchyma cell types were isolated from the biopsies, such as mesenchymal cells and trophoblasts. Although separation of different cell types to study methylation patterns would have been a more accurate approach, it may be less of a concern when studying global rather than gene-specific methylation

changes owing to the cell type-specific expression of several genes observed in placental tissue [37]. A study that analyzed global methylation in LINE-1 in different extraembryonic tissues from a set of 14 placentas found no differences in methylation levels between mesenchyme and trophoblast, and similar mean methylation values to our data for this repetitive element [11].

Conclusion

Our study is limited to assays of global DNA methylation. Future studies are warranted to evaluate the effect of the length of storage at RT or placental region on DNA methylation in specific genes, including those in pathways that may be more susceptible to temperature, hypoxia and/or apoptosis.

Based on our results, we conclude that DNA methylation can be studied in placenta tissue from placentas biopsied several hours after birth. In particular, the methylation levels measured by bisulfite pyrosequencing demonstrated more robust results, which were more accurate and less dependent upon time-to-storage. Methods based on enzymatic digestion, such as the LUMA, may be suboptimal and lead to biased methylation levels when DNA degradation – even if low grade – is suspected. In summary, compared with labile molecular signals, such as those in proteins or RNA, DNA methylation – as expected based on its chemical structure featuring covalent binding of a methyl group to cytosines – is an epigenetic mark that appears to be stable at RT.

Future perspective

The field of epigenetics and its potential involvement in the cause of chronic diseases in humans has expanded enormously in recent years. However, the study of epigenetic modifications – especially linking environmental exposure to DNA methylation alterations – presents a number of methodological challenges important for epidemiological studies. In addition, novel CpG dinucleotide modifications with potential regulatory roles, such as 5-hydroxymethylcytosine, have been described, and future studies analyzing the impact of collection and storage conditions of biological samples upon these marks would be of great interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- of considerable interest

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Executive summary

Background

- There is growing interest in the effects of *in utero* environmental factors on DNA methylation and how this may be linked to disease outcomes in adult life.
- The exponential increase in DNA methylation studies is highly dependent upon the feasibility of analyzing available biological archives.

Materials & methods

- The current study investigated the effect of sample storage delay at room temperature on global DNA methylation stability in placental tissue and the variability associated with different areas of the villous parenchyma.
- Global DNA methylation was measured using two different techniques: bisulfite pyrosequencing of repetitive elements; and the luminometric methylation assay.

Results & conclusion

- Global DNA methylation was stably preserved on DNA double strands at room temperature and was homogeneous across villous parenchyma areas when studied using bisulfite pyrosequencing.
- Our data suggest that enzymatic-based methods to measure DNA methylation may be less reproducible and seem to be affected by DNA quality, which is in turn dependent on storage conditions of the biological specimens.

Future perspective

- Methodologies to assess global DNA methylation should be selected according to sample storage conditions. Stability of additional epigenetic marks, such as 5-hydroxymethylcytosine, requires further research.

Figure 1. Placenta collection

Sampling scheme of the biopsies obtained for each region of the placenta.

Figure 2. DNA methylation and time to storage

Changes in mean DNA methylation over different intervals of time (h) at room temperature in placenta 1 and placenta 2 analyzed by pyrosequencing of repetitive element subfamilies: (A) LINES; (B) Alu elements; and (C) LTRs. (D) Changes in mean DNA methylation using the LUMA.

LINE: Long interspersed nuclear element; LTR: Long terminal repeat; LUMA: Luminometric methylation assay.