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Short communication

The effects of pH on DNA methylation state: *In vitro* and post-mortem brain studies

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ABSTRACT

Assessment of methylation state of DNA extracted from brain is becoming one of the most investigated issues in the study of epigenetics and psychopathology. pH effects in brain are known to affect gene transcription, though pH effects on DNA methylation state are unknown. We demonstrate *in vitro* using an artificially methylated plasmid that DNA methylation state remains stable, even under extreme pH conditions. Next, using two different genomic regions from human DNA, we assess methylation state from both cortical and sub-cortical brain regions using subjects with varying pH levels. No correlation was found between DNA methylation state and pH. These results suggest that DNA methylation state is stable in post-mortem brain.

1. Introduction

A wealth of epigenetic studies in brain is currently under way (Abdolmaleky et al., 2004; Mill and Petronis, 2007), and methylation is one epigenetic mechanism that affects gene transcription which could mediate the interaction of genes and environment (Weaver et al., 2004). Methylation refers to the process of the addition of a methyl group to DNA and a number of proteins are known that bind methylated DNA or that add methyl groups to DNA. These protein/DNA interactions can have important repercussions on gene expression (Amir et al., 1999).

Of particular interest to psychopathological research is the methylation status of DNA isolated from post-mortem brain, but a better understanding of the potential effect of confounding factors, such as pH, is needed before associations between methylation state and certain illnesses are made. pH effects in post-mortem brain are a major caveat of gene expression studies (Vawter et al., 2006), though the effect of pH on methylation state of DNA extracted from post-mortem brain is unknown. It is possible that DNA exposed to more acidic conditions even in the absence of any biological function, could affect DNA methylation state.

This study addresses the effects of pH in post-mortem brain on DNA methylation state. Using both *in vitro* and post-mortem brain experiments, we find that DNA methylation state is stable in post-mortem brain.

2. Materials and methods

2.1. In vitro analysis of pH effects on DNA methylation

We treated the pGL3 plasmid (Promega) with SSSI methyltransferase, an enzyme that methylates all cytosine nucleotides in a CpG dinucleotide. To ensure that this step was effective, we took two sub-samples of the methylated plasmid (pGL3-CH3) solution and exposed them to two restriction enzyme digestions: HPAII and MSPI. Both of these enzymes recognize the same site (CCGG), but HPAII is blocked from cutting DNA when the internal C is methylated. The pGL3 plasmid is 4800 bp long and has 25 CCGG sites. MSPI is insensitive to the methylation status of the internal C. The *in vitro* methylated pGL3 was purified by standard phenol–chloroform extraction procedures.

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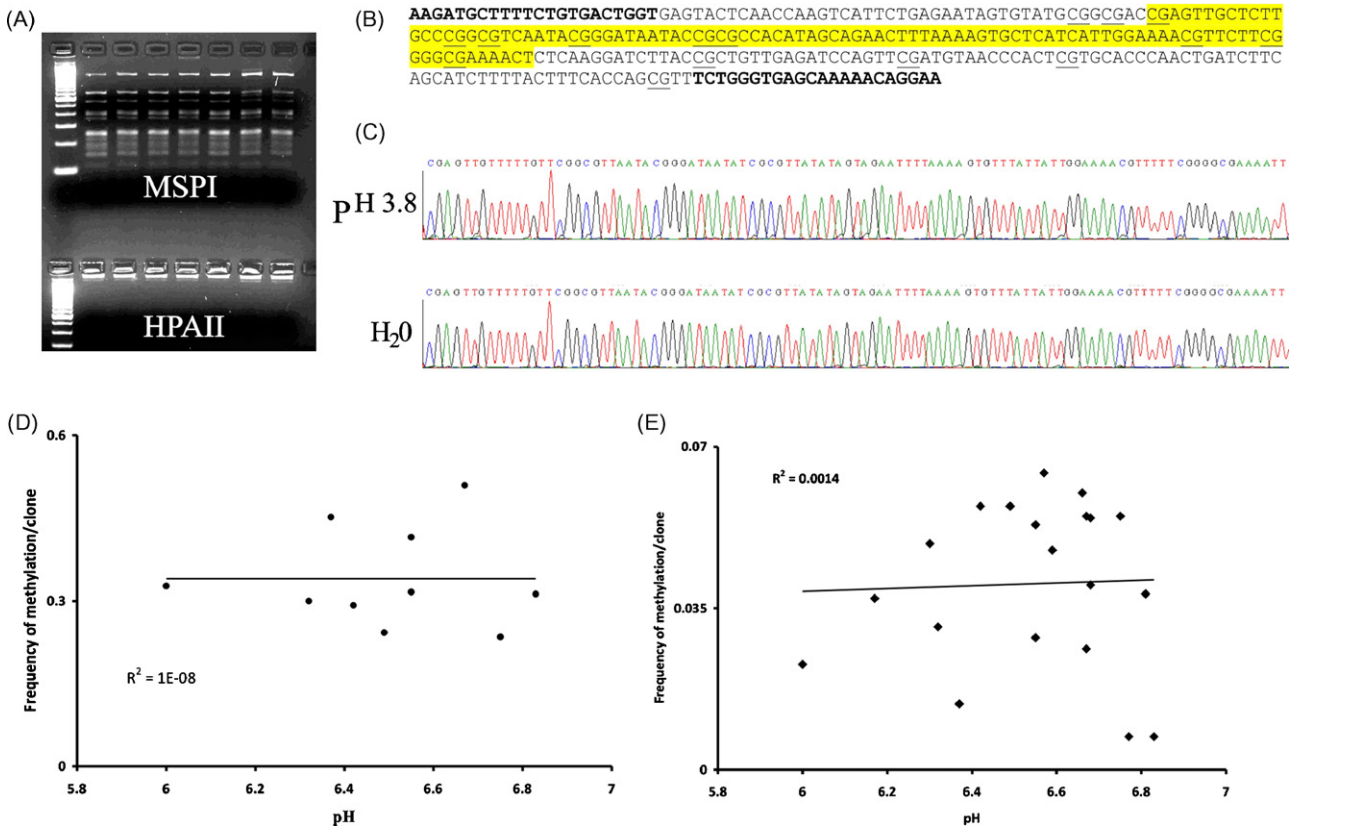

Fig. 1. pH does not affect methylation status of DNA *in vitro* or in post-mortem tissue. (A) Successful methylation of the pGL3 plasmid. (B) Tested sequence from pGL3 plasmid for the described experiment. pGL3 primers specific for bisulfite-treated plasmid are emboldened. CpG sites are underlined. Yellow highlight demonstrates area where sequence traces in 1C are taken. (C) Sequences from pure water and pH 3.8. Note that all CpG sites are methylated and C sites not in a CpG dinucleotide are detected as thymidine. (D) pH versus frequency of methylation, rRNA, and (E) NTRK2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Following ethanol precipitation the plasmid DNA was re-suspended and incubated for 48 h at 22 °C in pure water and a series of solutions that differed in pH (3.8, 6.1, 6.62, 7.2, 10 and 12). pH solutions were made using HPLC-grade water with NaOH and HCl. Following incubation, the DNA samples were treated with sodium bisulfite following the manufacturer's protocol (Qiagen EpiTec Bisulfite Kit).

Bisulfite treatment converts all cytosine residues to uracil, but methylated cytosines remain intact (Clark et al., 1994). This treatment creates a sequence difference between methylated and unmethylated cytosines which enables mapping of the methylation pattern at single base resolution. Primers specific for pGL3-CH3 were designed using Methyl Primer Express (Fig. 1B; forward: 5′-AAGATGTTTTTTTGTGATTGGT-3′; reverse: 5′-TTCCTATTTTTACTCACCCAAA-3′). Using these primers in a PCR reaction, a product of 278 bp was generated.

2.2. pH measurements from human brain

We followed the protocol used by Vawter et al. (2006) to take pH measurements. 80–120 mg of cerebellar tissue was homogenized in Chromosolv water (ultrapure water normally used for high performance liquid chromatography—Sigma–Aldrich) at a 10:1 water to tissue ratio. All tissue was taken from previously frozen brains. Tissue was homogenized with a TissueTearor (Biospec Productions Inc.), on ice, until no brain fragments were visible. After re-equilibration to room temperature, solutions were measured with a Corning pH meter.

2.3. Subjects

All subjects in this study were recruited at the Montreal Morgue as part of on-going recruitment of subjects for the Douglas Hospital Brain Bank. All subjects were male and did not die in an extreme agonal state, according to medical charts and/or informant reports. After death and permission from next-of-kin, brains were extracted, sectioned based on Brodmann region at 4 °C and snap frozen in isopentene at −80 °C. Brains were then stored at −80 °C. DNA was extracted from the dorsolateral prefrontal cortex (BA 9) and hippocampus from each subject and bisulfite treated.

2.4. Post-mortem analysis of pH effects on DNA methylation state

Two different primer pairs were used to assess methylation status in CpG rich promoter regions: ribosomal RNA gene regulatory region (U13369)—forward: 5′-GTT TTT GGG TTG ATT AGA-3′; reverse: 5′-AAA ACC CAA CCT CTC C-3′. DNA used was from hippocampus. NTRK2 promoter (NM_000346)—forward: 5′-GAGAGTGGGTATATTGGTGGTTTTA-3′; reverse, 5′-CCAACTTATCAAAAACTAAACTAATCC-3′. DNA used was from BA 9. The amplified products were extracted from the gel, ligated into a pDrive vector, and transformed into competent *Escherichia coli* cells (Qiagen PCR Cloning*Plus* Kit). Incorporation of the correct DNA fragment was verified by restriction enzyme digestion. All sequencing was done at the Genome Quebec Innovation Centre. At least 8 clones were used for each subject and for each primer pair.

3. Results

To test the effects of pH *in vitro* we first needed a fully methylated DNA sequence with which to perform the experiment. We selected the pGL3 plasmid due to plasmid availability and presence of a number of CpG dinucleotides inside of an easily amplifiable region. We first exposed the plasmid to SSSI methyltransferase to methylate all CpG dinulceotides. To test the effectiveness of this step, we exposed the experimentally methylated plasmid (pGL3-CH3) to two restriction enzymes: The first (HPAII), an enzyme incapable of cleaving methylated CpG dinucleotides and the second (MSPI), an enzyme fully capable of cleaving methylated CpG dinucleotides. Both enzymes recognize CCGG site for cleavage. Fig. 1A demonstrates the resulting gel from the pGL3-CH3 plasmid being treated with each of the two enzymes.

We next used the pGL3-CH3 plasmid to assess the effects of pH on DNA methylation patterns *in vitro*. We made a wide range of pH solutions (3.8, 6.1, 6.62, 7.2, 10 and 12) and incubated pGL3-CH3 for 48 h with differing pH solutions and pure water. After incubation, we extracted pGL3-CH3 from the solutions, bisulfite treated the extract, and amplified a small region of DNA within the plasmid. The amplified product was then cloned into a pDrive vector (at least 8 clones per solution) and sequenced. We found that the sequences from plasmids incubated in varying pH solutions were indistinguishable from those incubated in pure water (Fig. 1B and C). All cytosines residing in the dinucleotide CpG sequence remained methylated under all conditions while all cytosines found in other sequence contexts were detected as thymidine bases, indicating lack of methylation.

We next tested the effects of pH on DNA methylation status in post-mortem brain. All subjects used in this study underwent full psychological autopsy procedures at the McGill Group for Suicide Studies (Dumais et al., 2005). We used two different primer pairs and two different brain regions for this study.

We first analysed ribosomal RNA (rRNA), a gene known to have a heavily methylated promoter region (Ghoshal et al., 2004). After sequencing clones from 10 individuals using DNA extracted from hippocampus, we found no significant correlation between pH and methylation state of the rRNA gene (Fig. 1D; PCR product size of 250 bp with 27 CpG dinucleotides). We next analysed the promoter of a gene that has been investigated as a candidate in psychiatric disorders (NTRK2 (Dwivedi et al., 2003)) in 20 subjects (10 of whom were the same as for the rRNA analysis) in frontal cortex. No correlation was found between pH and methylation state (Fig. 1E; PCR product size of 440 bp with 35 potential CpG dinucleotides).

4. Discussion

This study has demonstrated that DNA methylation state is a stable phenomenon, at least in regards to acidity and alkalinity in post-mortem brain and *in vitro*. We first demonstrated this under extreme pH conditions (i.e. pH conditions outside of physiological range for human brain) *in vitro* and then under physiological pH conditions in post-mortem brain tissue.

This study did not investigate *in vivo* effects of pH on DNA methylation. The purpose of this study was to understand whether pH in post-mortem tissue is a relevant confounding factor in experiments where DNA is extracted from brain to be used for methylation analyses. If DNA methylation is an active process, as has been suggested (Kangaspeska et al., 2008; Metivier et al., 2008), than pH changes may affect DNA methylation *in vivo*.

One time point of interest that could not be addressed by this study is the point from death to brain preservation in cold storage, i.e. methylation state changes during the post-mortem interval. Immediately after death, some brain cells are still alive and lactic acidosis may occur (Alafuzoff and Winblad, 1993; Ravid et al., 1992). This potential change in pH as brain cells die is accompanied by a host of other physiological conditions (e.g. hypoxia, apoptosis and necrosis) each of which is a variable that could alter DNA methylation state. Studying only pH effects on DNA methylation status during brain death, even in a controlled laboratory setting using animals, is technically very challenging.

What this study can conclude, however, is that once pH is set (once the brain is in cold storage) there is no correlation across subjects between pH and methylation state of DNA. This could be directly assessed using DNA incubated in solutions that differed in pH. This allowed the examination of pH effects on DNA methylation state without the interference of any other biological factors. We caution that our post-mortem correlation results apply only to the range of pH reported in this study (6.0–7.0).

This study suggests that pH does not affect methylation state, either in post-mortem brain or under experimentally induced extreme pH conditions *in vitro*. These findings should be of use to studies examining methylation state of DNA extracted from any human tissue where pH could be a factor.

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