ORIGINAL ARTICLE

Determination of epigenetic age through DNA methylation of NPTX2 gene using buccal scrapes: A pilot study

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Abstract

Context: DNA methylation (DNAm) age can be used to evaluate the chronological age of individuals often called "epigenetic age." In this study, buccal scrape samples were used for the determination of epigenetic age. **Aims:** To examine if epigenetic age could be determined using neuronal pentraxin 2 (NPTX2) gene in buccal cells. **Setting and Design:** This cohort study was designed to validate the use of buccal cells for epigenetic age estimation. Sanger sequencing was used to determine the genetic sequence of the gene of interest postamplification. Nucleotide base sequence for NPTX2 gene was obtained for each case using this protocol. **Subjects and Methods:** The study was conducted on buccal scrapes obtained from 26 subjects of both genders, whose age varied from 1 to 65 years. The samples, collected by wooden spatulas, were placed in cell suspension buffer and stored at 4°C until transported to the laboratory. **Results:** Methylation levels of 5'-C-phosphate-G-3' located in the gene NPTX2 of 26 subjects were studied and analyzed by bisulfate sequencing. The percentage of methylation in this study falls in the range between 15% and 51%. **Conclusion:** In this study, a sufficient amount of gDNA was retrieved from the buccal cells, thus confirming that buccal scrape was a feasible technique to obtain ample DNA. This study also showed that DNAm-polymerase chain reaction method was a feasible method for the evaluation of methylation pattern of NPTX2 gene.

Key words: Age determination, buccal scrape, DNA methylation, epigenomic age, forensic science

Introduction

Age determination plays an important role in forensic medicine in the identification of bodies as well as in connection with crimes.[1] Forensic anthropology is continually evolving and expansion of the discipline, combined with rapidly improving technology, has seen an increase in the development of new as well as refinement of existing methods.[2]

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DNA is present in the flakes of the skin, drops of blood, hair, and saliva that can be used to identify a human being. The study of forensics relies on these small bits of DNA to link criminals to the crimes they commit. With the help of DNA, the gender and age of a person in question can be determined.[3] DNA present in blood, hairbrush, clothes, cervical smear, or biopsy sample provides a good source. The extracted DNA can be analyzed by various methods such as restriction fragment length polymorphism, polymerase chain reaction (PCR), and microarrays.[4]

The epigenetic landscape provides new perspectives for use as biomarkers with the development of experimental technologies mediated by high-throughput sequencing and genomic data mining. One of these epigenetic modifications, DNA methylation (DNAm), has been shown to closely correlate with age, that is, the global level of methylated genomic DNA decreases or increases as a person ages depending on the gene chosen for age determination.[5]

The estimation of human age is important in forensic science in cases of a crime investigation or a mass disaster.[6] Blood, saliva, and hair recovered at crime scenes help determine the owner's age.[7] Buccal cells can serve as a better substitute to collect DNA when blood and hair samples become difficult to obtain or are nonviable.[8]

Recent predictor of age is known as epigenetic or DNAm age.[9] Methylation patterns of neuronal pentraxin 2 (NPTX2) gene shows a progressive increase with age.[1] Our aim was to check if buccal cells were a viable source to calculate DNAm age and its correlation to chronological age.

Subjects and Methods

The study was conducted on buccal scrapes obtained from healthy individuals aged 1–65 years and collected by the Department of Oral and Maxillofacial Pathology, Krishnadevaraya College of Dental Sciences and Hospital, Bengaluru, after obtaining appropriate ethical clearance from the Institutional Ethics Committee to carry out this study. The total number of subjects taken for the study was 26, i.e., 13 male and 13 female subjects with no history of oral habits such as smoking, tobacco chewing, or alcohol use. The age of these individuals varied from age group 1–5 years to 60–65 years. One male and one female subject were taken from each lustrum. The details of each subject were taken along with their chronological ages.

The subjects were briefed about the procedure, a brief history was noted down and informed consent was obtained from all the individuals. It was ensured that the person providing the sample had not consumed any food or drink for 30 min before sample collection. Sterile wooden spatulas were used for buccal scrape; the stick was scraped against the inside of each cheek about five times. To dispense the cells, the sample collected was put into cell suspension buffer taken in a centrifuge tube. The samples collected were stored at 4°C in a refrigerator until transported to the laboratory within 24 h of sample collection in a cooler.

For DNA analysis (in laboratory)

Once the samples were received in the laboratory, the collection tubes with buccal cells were centrifuged at room temperature for 5 min at 10,000 rpm. This step settled the buccal cells. After this, the supernatant was discarded and extraction of DNA with cell pellet was carried out.

The gDNA was extracted with the help of gDNA extraction kit. The gDNA of the samples is shown in Figures 1 and 2. After gDNA was extracted, it was stored at −20°C overnight. This was followed by bisulfite conversion of the samples. Bisulfite conversion is used to deaminate unmethylated cytosine to produce uracil in DNA and is considered the "gold standard" for downstream applications to assess DNAm status. Methylated cytosines are protected from the conversion to uracil, allowing the use of direct sequencing to determine the locations of unmethylated cytosines and 5-methylcytosine at single-nucleotide resolution.

Before the direct PCR sequencing, purification of PCR products is necessary to remove the residue of the PCR reaction that might interfere with the outcome of sequencing results. Chromous gel extraction kit was used for the purification of PCR products. DNA obtained was purified DNA and it was then sent for PCR without any precipitation step.

Sanger sequencing was done for the bisulfite-converted samples. PCR amplicons (for NPTX2 region) were used for sequencing. Routine PCR was carried out consisting of a series of 35 repeated temperature changes, called cycles, with each cycle commonly consisting of three discrete temperature changes. One of the exon regions (Exon 1) that had been previously shown to be hypermethylated with aging was studied. After the PCR amplification, the sequence data showing nucleotide base sequence for the NPTX2 region for each case were obtained. The methylation pattern for the region under study was also analyzed. The bisulfite conversion pattern of each case obtained was then compared to the reference sequence. This was followed by bisulfite conversion and PCR amplification of the NPTX2 gene. PCR amplification of NPTX2 region from converted genomic DNA is shown in Figure 3.

Once the PCR-amplified exon region of NPTX2 gene to be studied for each case was obtained, a reference sequence was obtained from a patent where the gene had been studied in relation to age changes. Based on the comparison of the number of methylated CpG islands in sample and reference sequence, the percentage of methylation for each case was calculated.

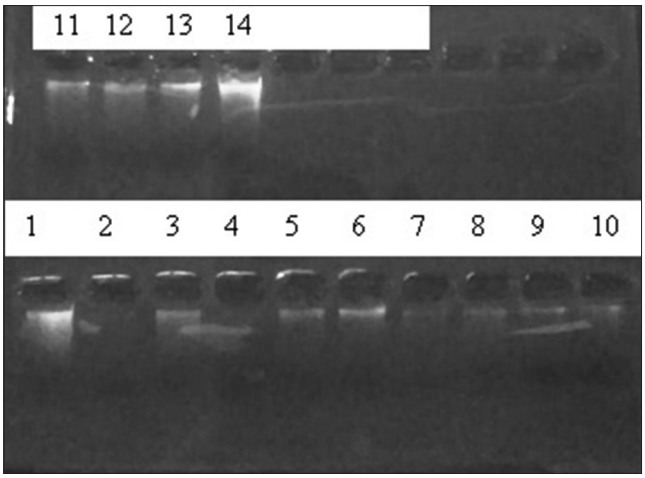

Figure 1: Genomic DNA extracted from 16 buccal samples loaded on 1% agarose gel

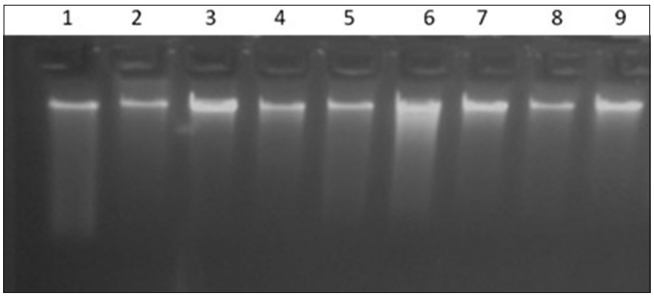

Figure 2: Reextraction of genomic DNA from 9 buccal samples loaded on 1% agarose gel

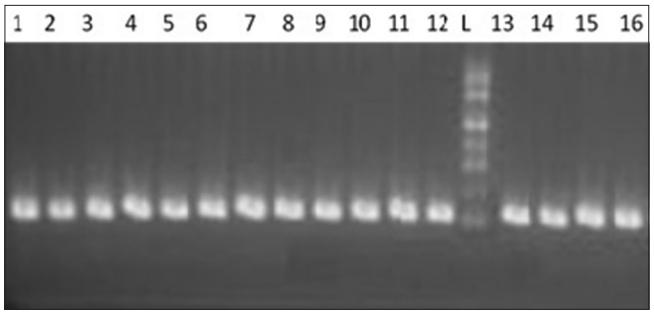

Figure 3: Polymerase chain reaction amplification of neuronal pentraxin 2 region from converted genomic DNA. The size of polymerase chain reaction-amplified product is ~ 500 bp

The reference sequence is given below:

The reference sequence

CGACCCTCGAGACGACAGCGCGGCTACTGCCAGCA
GCGAAGGCGCCTCCCGCGGAGCGCCCCGACGGCGC
CCGCCGCCCATGCCGAGCTGAGCGCGGCAGCGGC
GGCGGGATGCTGGCGCTGCTGGCCGCCAGCGTGG
CGCTCGCCGTGGCCGCTGGGGCCCAGGACAGCCC
GGCGCCCGGTAGCCGCTTCGTGTGCACGGCACTG
CCCCCAGAGGC GGTGCACGCCGGCTGCCCGCTGC
CCGCGATGCCCATGCAGGGCGGCGCGCAGAGTCCC
GAGGAGGAGCTGAGGGCCGCGGTGCTGCAGCTGCG
CGAGACCGTCGTGCAGCAGAAGGAGACGCTGGG
CGCGCAGCGCGAGGCCATCCGCGAGCTCACGGGCA
AGCTAGCGCGCTGCGAGGGGCTGGCGGGCGGCA
AGGCGCGCGGCGCGGGGGCCACGGGCAAGGACA
CTATGGGCGACCTGCCGCGGGACCCCGGCCACGTC
GTGGAGCAGCTCAGCCGCTCGCTGCAGACCCTCA
AGGACCGCCTGGAGAGCCTCGAGGTAGCGGCCCGC
GGGGAGCGCGGGGGACCTGGAATGGGGACGCTCC
CGAGTCGGGGGCGGAAG.

Observations and Results

In this study, methylation status of 60 CpG islands of NPTX2 gene was investigated. The number of sites that were completely methylated was calculated after conversion and compared to the reference sequence. The number of CpG in query sequence postconversion in all cases ranged between 9 and 31.

For each of the 26 cases, the subject sequence was compared to the reference sequence. The fluorogram for the NPTX2 gene sequence for one case is given in Figure 4. Similarly, observations for each case were recorded.

Ample genomic DNA (approximately 2 μg) was obtained from buccal epithelial cells of all cases. After bisulfite conversion was done for all samples, purification of PCR products was done which is necessary to remove the residue of the PCR reaction that might interfere with the outcome of sequencing results. DNA obtained was then sent for PCR, followed by Sanger sequencing which helped in obtaining the data for all cases. After the PCR amplification, the sequence data showing nucleotide base sequence for the NPTX2 region for each case were obtained. The sequenced data obtained were compared to the reference sequence. Based on the comparison of the number of methylated CpG islands in sample and reference sequence, the percentage of methylation for each case was calculated. The percentage of methylation obtained was in the range of 15%–51%. Previous studies had shown an increase in the epigenetic difference with age, suggesting increased epigenetic drift. However, we were unable to replicate these methylation changes. The methylation pattern in this study was random, with some younger subjects showing more methylation percentage than older subjects.

Discussion

Numerous studies have focused on the relationship of epigenome state and age, especially DNAm and chronological age. Heyn *et al*. in their study reported that centenarians exhibit lower DNAm levels. Their study also found that throughout the entire genome of centenarians compared to newborns, the correlation between neighbor CpGs is weaker. Horvath accurately predicted age based

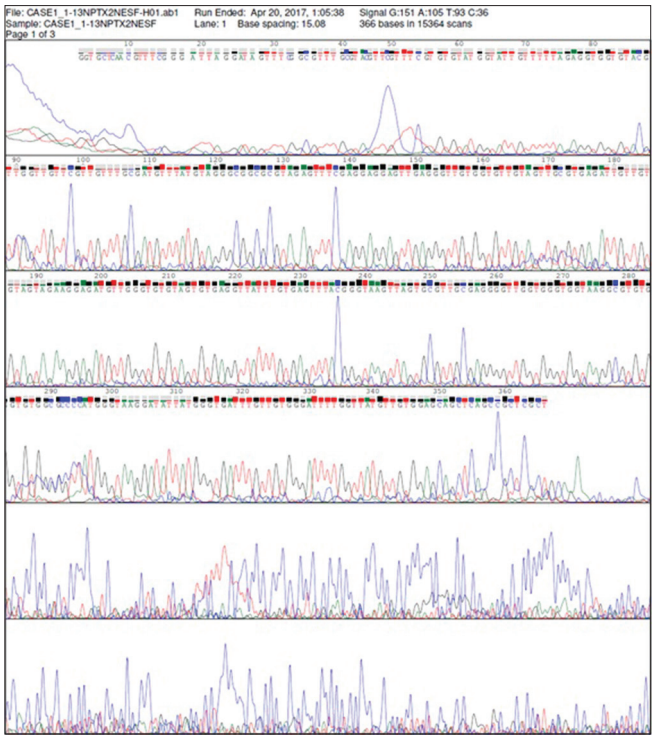

Figure 4: Fluorogram of raw sequences

on DNAm and identified human biological age markers to institute what is known as an epigenetic clock.[9]

DNAm has shown a relationship with age; the overall level of methylated genomic DNA decreases as a person ages. According to some reports, some specific CpG sites, 5-methyldeoxycytidine (5 mC) modification increases with age, whereas at other CpG sites, 5 mC level decreases with age. Age prediction models with an average error of 2.9–5.2 years have been built using DNAm.[5]

The different organs that have been studied for the comparison of methylation changes with age include small intestine, lung, kidney, liver, spleen, whole blood, saliva, and buccal cells.[10-14]

ADAR, AQP11, ITGA2B, and PDE4C are the four genes that have shown relation to age; ten other reports have shown methylation of ITGA2B and PDE4C (cAMP-specific 3′,5′-cyclic phosphodiesterase 4C) to be the age-associated marker by ADAR, a RNA editor.[5]

Whole blood leukocytes of different age populations have shown 9 DNAm genes (EXHX1, TSP50, GSTM1, SLC5A5, SPI1, F2R, LMO2, PTPN6, and FGFR2) to be associated with age. More than 15% decrease of the CG methylation was seen in four genes (TRIP6, ABCB4, ABCC2, and GML).[15]

Other genes that have been associated with age include MSX1, MYL5, MPG, ACTN3, NAGS, DYDC1-DYDC2, SCN4B, HYDIN, thiosulfate sulfurtransferase, LFT, CTSZ, EMP3, and NET1. Some other genes such as ANK1, HOXA9, TMEM61, ATP8A2, TEX264, and TINAGL1 may also show age-related methylation changes. Several CpG sites were involved in the classification of a wide range of ages. For example, the sites associated with NPTX2, CHRNB4, RAB42, and ESRP2 were reported as significant for all ages between 19 and 60 years.[16]

Other genes that have been shown to be associated with age include TBOX3, GPR137, ZIC4, ZDHHC22, MEIS1, UBE2E1, PTDSS2, and UBQLN1.[10]

A linear correlation of methylation of three sites of EDARADD, TOM1 L1, and NPTX2 genes was seen with age over a range of five decades. A regression model was able to predict the age of an individual with an average accuracy of 5.2 years. Among these genes, NPTX2 methylation status was shown to be linked with age in blood as well.[17]

Bocklandt *et al*. in their study of EDARADD gene showed a linear correlation with age over a 5-decade period by studying 88 sites in saliva samples.[4,18]

Based on these published data, where NPTX2 gene was studied for its correlation with age, we decided to study this one age-related marker, using quantitative Sanger sequencing. We based the selection of our gene on shortlists published by various other research groups. Here, we examined a series of CpG sites, selected based on their overall rankings in multiple studies, for application of these loci for use as an age-specific epigenetic marker.

Several different assays are available for studying the DNAm of specific genes/regions. Bisulfite sequencing, the first step for many downstream methods, is considered to be the "gold standard" method in DNAm studies.[19]

We used the method of bisulfite treatment and PCR followed by sequencing based on the fact that it allows a quantitative analysis. Furthermore, this is not too tedious. The main benefit of bisulfite treatment for interrogating DNAm status is that the location of the 5-mC modification can be determined at single-nucleotide resolution, meaning that the precise cytosine residue that is methylated can be pinpointed.

In the present study, we studied the age-associated differences in DNA from the buccal epithelial cells of 26 individuals aged 1–65 years. The sample size in the previous studies done ranged between 44[20] and 80 years.[21] Genomic DNA was extracted from the buccal epithelial cells. Buccal cells have been previously used for studying DNAm changes with age as demonstrated from studies by Gustafson[12] van Dongen *et al*.,[22] and Kaminsky.[23] The bisulfite conversion of the extracted DNA was done

followed by PCR treatment. This was followed by Sanger sequencing. This was done in accordance with the study done by Christensen *et al*.[24] and Silva *et al*.,[20] in which bisulfite modified DNA Sanger sequencing was performed. The gene that we studied was NPTX2, based on the fact that numerous other studies had studied its methylation pattern with age.[11,16-18,20] The same studies showed an increase in the percentage of methylation of this gene with age.[11,16-18,20] The percentage of hypermethylation was calculated manually. This was done in contrast to different studies that used software to do the same.[17,20] Previous studies have shown an increase in the epigenetic differences with age, suggesting increased epigenetic drift.[11,16-18,20] We were unable to replicate these methylation changes.

The lack of epigenetic drift within this study contrasts with the previous studies done on NPTX2 gene. The main difference between the two studies is that we focused on the small sample size. Another important question related to age markers is how reproducible the results are when the analysis is done with different cell types. It has been noted that many age-associated DNAm changes are tissue specific. It was observed that a correlation between methylation values and age was found when analyzing both saliva and blood samples.[20] In this study, we used buccal cells and as such the difference in the composition of the samples can result in differences in their gene expression and as such change in methylation pattern should be expected.

The other reason can be the fact that only one gene (NPTX2) was studied in comparison to studies that have taken into consideration at least two genes.[18] The small number of loci of CpG islands being studied can be yet another reason. The NPTX2 gene studied has five exons – we had chosen to study one of these. The methylation changes in the gene might have been seen in other exons. This can be the reason why methylation changes with age could not be observed with age in our case.

As far as the technique used is concerned, bisulfite conversion can lead to DNA fragmentation, which makes amplification of long fragments difficult and can also result in the generation of chimeric products. To overcome the problem of unspecific amplification, nested PCR is often required. Amplification of long fragments from bisulfite-treated DNA is difficult. Another important point to be taken into consideration is to ensure complete conversion of nonmethylated cytosines. This is important as the estimated level of DNAm depends on it and as such it is necessary to incorporate controls for bisulfite reactions.[19]

Conclusion

In this study, the buccal scrape samples from 26 individuals ranging from 1 to 65 years were evaluated. A sufficient amount of gDNA to study hypermethylation status of NPTX2 gene was retrieved from the samples. Thus, we could confirm that buccal scrape was a feasible technique to obtain ample DNA.

The gDNA was bisulfite treated followed by PCR amplification of NPTX2 gene followed by Sanger sequencing. This method helped obtain the sequence data for the NPTX2 gene. Thus, small samples of DNA (2 μg) obtained from epithelial buccal cells are ample for routine DNA analysis.

After Sanger sequencing, the sequenced data for NPTX2 gene were calculated. Based on the comparison of the number of methylated CpG islands in sample and reference sequence, the percentage of methylation for each case was obtained. This study showed that DNAm-PCR method was a feasible method for the evaluation of methylation pattern of NPTX2 gene.

In age-related DNAm studies, the challenges include (a) finding the more sensitive and specific mechanism that will provide results with better reproducibility and (b) to identify principal genes and loci for which methylation patterns can be observed. Further research needs to be conducted to determine the age-related methylation changes in genes associated with age and its ability to predict chronological age with more accuracy. In this regard, studying all exons/larger gene sequence/full gene sequence/promoter regions or multiple age related genes can be helpful.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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