Review Article

DNA Methylation: An Approach to Forensic Age Prediction by Molecular Mechanism

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Abstract

Epigenetic processes have an important role in gene expression which is affected by living environments conditions. Occurrence of epigenetics is conciliated by two major molecular mechanisms which includes histone modification and DNA methylation. DNA methylation is an epigenetic channel action, this is a natural process where transmission of a methyl group on the C5 position of the cytosine to form 5-methylcytosine at genome. So, epigenetic change is normal and continual fact that may be influenced by many factors including age, the environment / lifestyle, and disease state. The normal process of aging causes a span of transformation of tissues and organs which gathered over life time. Now this could be possible to be examined through molecular based method. In view of this methylation levels of age associated marker have been used for highly accurate age prediction in this area and depicted that an age associated methylation marker on specific gene location found to be useful with marginal lapse. In Indian scenario there are no such study published on epigenetic markers for age estimation. In future for forensic cases work Molecular biomarkers may be significant to estimate age.

Keywords: DNA methylation, Epigenetics, Age prediction, Age associate CpGs

Introduction

DNA methylation is an epigenetic action that regulate gene expression and involves adding methyl group to a cytosine base. The study of inheritable modification in gene activity or its function due to alteration of point mutation, deletion, insertion and translocation of base on DNA sequence is called genetics. In contrast of this epigenetic is also a constant heritable change in gene activity or function that is not associated with any modification of DNA sequence itself. In this phenomenon all cells in an organism contains the same genetic information that means not all genes are expressed simultaneously by all cell types.

Unlike genetic, epigenetics is the study of inheritable alteration in gene expression which do not modify DNA sequence rather change in phenotype without a change in genotype. Epigenetic alteration are those changes which affects directly gene expression by means of DNA methylation, histone modifications, acetylation and chromatic remodelling. An epigenetically re-programming of the gene is express by removal or addition of methyl group, the process of a removal of methyl group from DNA is known as demethylation where agene can be activated by removal of methyl group and leads to gene expression by promoting transcription. In non-coding region of DNA, GC (Guanine-Cytosinebases) rich DNA is highly
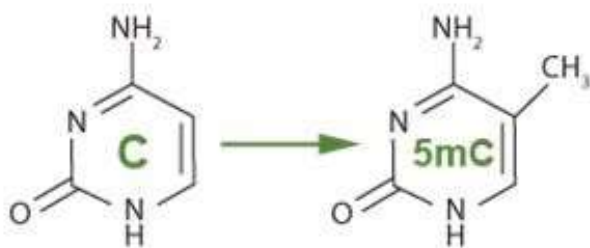
Fig 1. Conversion of Cytosine to 5-methylcytosine

methylated where 5th carbon of cytosine base is more prone to methylation which convert into the 5-methylcytosine (5mC). Basically, where these 5mC commonly occurs in CG rich region called CpG island in somatic cells and in embryonic cells these 5mC is occurred at non-CpG region. At post replication modification in which addition of methyl group happened to the CpG island then this process deactivates the gene activity. In this phenomenon an active gene can be deactivated by additional methyl group on its promoter region, by doing this it inhibits the binding of proteins and enzymes involved in regulate gene expression. Promoter region of gene are non-coding CG rich region of DNA which are more prone to DNA methylation.

In multicellular organism epigenetic action are determined in the diversified gene expression profiles in different types of cells and tissues.[1] So, epigenetic change is normal and continual fact that may be affected by many factors including age, environmental conditions, heredity, lifestyle, treatment history, autoimmune diseases and certain disorders. So, DNA methylation is the genetic process by which methylation of DNA molecule can differ the activity of DNA segment without changing in the sequence. DNA methylation is necessary for normal development and is related with many crucial processes including genomic imprinting, X-chromosome inactivation, repression of transposable element, aging and carcinoma. When this present on gene's promoter region it supresses the gene transcription. An abnormal methylation pattern has been affiliated with many conditions and disease.[2,3] After the development of whole genome bisulfide sequencing, this technique has been applied to epigenetic studies. In this article, age associated DNA methylation marker are reviewed for future aspect of investigation in forensic field.

Role of DNA Methylation

DNA methylation involves the addition of a methyl group (CH3) at 5'position of cytosine residue, which occurs mainly in CpG dinucleotide. The CpG is the region of genomic DNA where a cytosine nucleotide is coming after guanine nucleotide in the linear form of bases at 5' → 3' direction and that CpG is shorthand for this nucleotide sequence order, when CpG present in higher frequencies in a genomic region than they know as CpG island. The whole human genome contains 30 million dinucleotides, which exist in methylated and unmethylated state. It is estimated that 30-90% of CpGs are methylated in mammals and these methylated CpGs found in CpG Island in human genome.[4,5] Advancement in research is consistently finds the role of epigenetics in a various fatal disease and genetic disorders of humans. In this regard various research studies have shown that the age-associated CpGs are specific in tissue, and DNA methylation patterns present widely divergent among tissues. Therefore, over the last decade it has become clear that epigenetic markers can be significant for age estimation in forensic investigation.[6,7]

Human tissues change as grow older and this age process is regulated by DNA, many researchers have been successfully used this ageing process to predicts individual's age. The determination of potential change in DNA methylation forms could help in various forensic investigations, such as differentiating monozygotic twins, identifying the tissue source or determining the age of tissue donors.[8] Age prediction is an important part in forensic field. Traditionally age determination is used by morphological method based on bone ossification, chemical analysis of tooth dentine and radiocarbon analysis. Age estimation based on such method has very less accuracy i.e estimation error margin >10 years, these techniques require expert medical experienced anthropologist. The normal process of aging causes a range of alteration of tissues and organs which accumulate over life time.[9] Now this is possible to be examined at molecular level from small remains of biological samples.

Methodology for Methylation

It is an essential task to developing an accurate, sensitive and robust method which could be analyses the age associated CpG sites in forensic samples. Though predicting age across a broad spectrum of human tissue and cell types might be very challenging task but it would be very advantageous to translate and observed age associated DNA methylation differences in the chronological age of an individual. In view of this, for analysis of DNA methylation, currently

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researcher have used methodologies of gene specific number of CpG sites on cell types.[10]

Most of the researcher have calculated age, based on targeted methylation detection by the molecular technology such as Bisulfited conversion pyrosequencing, qPCR, EpiTYPER system and massive parallel sequencing. Among all Pyrosequencing found to be gold standard for DNA methylation. Apart from this base on genome-wide profiling has lad more extensive followed for gene regulation epigenetic mechanism. Illumina's Human methylation bead Chip technology is one of the most commonly used method for methylation which allows for simultaneous measurements of the methylation status of 27,578(27K chip) or 482421(450K Chip) CpG site in the genome at single resolution. To minimise prediction errors and increased accuracy, other statistical software has also been used together for regression analysis.[11]

Age associated – methylation marker on specific gene location have been reported useful with marginal lapse of 3.75 years in blood samples and 4.86 years from teeth.[12] Such techniques may be significant in police investigations to determine the age of criminal and unidentified bodies that can leads to identification. In Indian scenario there are no such study published on epigenetic markers for age estimation. Extensive research has been done on the effects of age upon DNA methylation by various working groups in order to address forensic investigation and its importance.[13]

Analysis of DNA methylation from Age-associated CpGsin various tissues

Bocklandt et. al 2011 reported first age predictive model base on DNA methylation data of saliva samples using Illumina 27K from 34 pairs of twin subject aged ranged 21 to 55 years. The model was composed of three age associated CpGs of the EDARADD, NPTX2, and TOM1L1 genes and showed an average precision of 5.2 years.[14]

Koch and Wagner 2011 identified epigenetic age signature which is applicable for various tissue to predict age of donor. The Illumina's Human Methylation 27 Bead Chip array (Illumina 27K) were used which represents 27,578 CpG sites. They used 19 CpG hyper-methylated sites with aging from five public data sets, which were generated from 20-30 samples dermis, epidermis, uterine cervical smear, T-lymphocyte, and monocyte. Among 19 CpG sits four CpGs sites in the NPTX2, TRIM58, GRIA2, and KCNQ1DN genes, and another hypo-methylation site in the BIRC4BP gene were chosen to carried out an age prediction.

The developed predictive model that showed mean absolute deviation from chronological age of 12.7 years in eight validation sets with an accuracy. In that study total of 766 samples were collected for validation which includes mainly peripheral blood (PB) leukocytes, saliva, breast tissue, CD34 + hematopoietic progenitor cell, PB monocytes, PB lymphocytes, buccal epithelial cells and cord blood monocytes. DNA methylation were found to be lower in breast tissue in comparison to other cell types used at the CpG site of the BIRC4BP gene and the prediction accuracy increased by11.4 years. The workers of this study were performed and focused on most significant CpG sites in the KCNQ1DN, NPTX2, and GRIA2 for age prediction.[15]

DNA methylation were investigated at the specific locus (IGF2/H19) by C. pirazzini etal. in 2012 for age of homogeneous individuals (males of restricted age range between 30-50 years) who belonging to four Italian districts as well as representative of the major genetic clans. Similarly in a companion of monozygotic (MZ) and dizygotic (DZ) twins of different ages (22-97 years). The analysis of twins' personal life histories suggests that the all-twin pairs difference is likely the result of the aging process, because they sharing a same environment for long periods had no effect on DNA methylation divergence. He reported aging more than population genetics is responsible for the inter-individual variability in DNA methylation patterns in humans; DNA methylation variability appears to be highly region-specific.[16]

The study of Johansson A 2013 reported that age alters DNA methylation at around one third (29%) of the sites (Bonferroni adjusted P-value <0.05), of which 60.5% turns into hypomethylated and 39.5% hypermethylated with growing age. They examined DNA methylation in genome of white blood cells from a community group (N=421) age ranging from 14 to 94 years at 476,366 sites throughout the genome. Their study suggested that the methylation sites on DNA are more often convert into hyper-methylated within CpG islands in compared to sites outside an island. It appears that the change in DNA methylation partially overlap with segment that change histone modifications with age. Naturally happened epigenetic correction and change in gene expression throughout the time generally reflects normal process of aging and variation between individuals. It indicating an interaction between two major epigenetic mechanisms which aid to the elaborate of age-related phenotypes and diseases such as cardiovascular, type II diabetes and autoimmune disease.[17]

Bekaert B 2015. have studied the age-associated genes (ASPA, ELOVL2, EDARADD and PDE4C) from the published literature and demarcated CpG methylation levels from 206 blood samples of both living and deceased personal's (age upto 91 years) and to understand chronological age prediction they used both linear and non-linear regression models with an accuracy. Their report suggested that methylation levels of ELOVL2 showed highest accuracy with quadratic regression model with a Mean Absolute Deviation (MAD) between chronological age and predicted age of 3.75 years. There was no difference in accuracy among both living and deceased personals including among two genders. They also analysed 29 teeth samples from other personals whose age ranged 19-70 years, using same set of age associated markers resulting MAD between age 4.86 years. On validation of both types of samples, results from blood demonstrated the more powerful and reproducible assay. They suggested that set of these 4 CpG DNA methylation markers is capable of producing highly accurate age predictions for blood samples in both living and deceased individuals.[18]

Giuliani. C et. al. 2016 have conducted a study to analyze DNA methylation status at specific CpGs location in FHL2, ELOVL2 and PENK gene and corelates with age in modern teeth (cementum, dentine and pulp) by using Mass spectrometry. They considered 21 modern teeth samples from 17-77 years old subjects to get methylation data and develop a mathematical model that able to utilize DNA methylation values to predict age of an individuals. They reported that the methylation status of the examined regions in the FHL2, ELOVL2 and PENK genes could be used to predict age from modern teeth. Given the accuracy of such approach, these biomarkers promise to be highly informative also when applied to paleo-epigenetics investigations, where teeth are commonly available. This study has suggested DNA methylation is a robust method to predict age for anthropological applications. Based on their work cementum and pulp has 1.25 ± 2.5 years, cementum is 2.45 ± 3.3 years and dentine age 7.07 ± 7 years.[19]

A study was carried oud out by Naue J et.al 2017 using massive parallel sequencing (MPS) and random forest regression on chronological age prediction based on DNA Methylation. This has reported that MPS allowed accurate DNA methylation determination of pre-selected markers and neighbouring CpG sites are best age predictive markers for the age prediction tool. From 208 individuals blood samples were used for training of the algorithm and another 104 individual's samples were used for model evaluation with known age ranged 18-69 years. For this prediction fifteen age-dependent markers of different loci were chosen form publicly available 450K microarray data, and then thirteen were finally selected for the age tool based on MPS which includes DDO, ELOVL2, F5, GRM2, HOXC4, KLF14, LDB2, MEIS1-AS3, NKIRAS2, RPA2, SAMD10, TRIM59, and ZYG11A. This study reported that the validation of the training set leads to a mean absolute deviation (MAD) of 3.21 years and a root-mean square error (RMSE) of 3.97 years. On further evaluation of model were conducted using the test set showed a MAD 3.16 years and RMSE 3.93 years respectively. On considering a reduced model based on only 4 markers (ELOVL2, F5, KLF14, and TRIM59) the result was found RMSE of 4.19 years and MAD of 3.24 years for the test set whereas for cross validation training set: RMSE were 4.63 years and MAD 3.64 years were observed. In this model of study,no statistical difference was found for accuracy of age prediction.[20] Similarly in another study M. Spólnicka et al have analysed DNA methylation profile,for calculation of forensic age estimation in three groups of individuals who diagnosed with three types of medical conditions by observing five markers from five different genes (ELOVL2, C1orf132, KLF14, FHL2, and TRIM59). A converted DNA methylation profile and adjusted age prediction accuracy were reported in all medical conditions. The results of their work demonstrated that the preferred age related CpG sites have found unaffected age prediction capacity in Alzheimer's disease subjects at late onset. Abnormal hyper-methylation and weakened prediction accuracy were found for TRIM59 and KLF14 markers in the group of early onset Alzheimer's disease suggesting increased aging of patients. In the Graves' disease subjects, aberrant hypermethylation observed on TRIM59 and FHL2 for the former and aberrant hypomethylation for the latter. Their study has emphasized high efficacy of the ELOVL2 and C1 or f132 markers for estimation of chronological age in forensics by demonstrating unchanged prediction accuracy in affected individuals by three systematic diseases. Their study also demonstrated that artificial neural system could be a suitable alternative for the forensic predictive DNA analyses.[21] In 2019 a study was conducted by Jung SE considering that the age associated DNA Methylation marker are tissue specific, in view of this a total of 448 samples of various tissue types (blood, buccal swab, saliva) were examined for age prediction at 5CpG sites from ELOVL2, C1 or f132/MIR29B2C, FHL2, KLF14 and TRIM59 gene. DNA

Table1: Age -Related DNA methylation based other studies for age prediction

Author	Age-related CpG marker	Sample types	Age rang	Technique	Findings
Zbieć-Piekarska R et.al.2015 (27)	ELOVL2	Blood	2-75	pyrosequencing	Accuracy are ±5.75 years, high correlation with increasing age.
Park JL et.al 2016 (28)	ELOVL2,ZNF 423, CCDC102B	Blood	11-90	pyrosequencing	Accuracy ±3.156 years & markers ae useful for analysis in Asian population
Eipel M et.al. 2016 (29)	PDE4C, ASPA, ITGA28, CD6, SERPINB5	Complete blood, Buccal epithelium	1-85	Bisulfite-converted pyrosequencing	Accuracy are ±4.3 years in training set and ±7.03 years in validation set and ±5.09-5.12 years in specific cell combination
Freire-Aradas A et.al. 2016 (30)	ELOVL2 ASPA PDE4C.FHL2, CCDC102B C1 or f132, chr16:85395429	Blood, Peripheral blood, cell lines	Control:19-101, Peripheral blood: 18-104, Twin blood: 42-69	Flanking Sequencing Epi TYPER	Accuracy with ±3.07 years
Freire-Aradas A et.al.2018 (31)	SDS, PGLYRP2, HKR1, TOM1L1, KCNAB3, PRKG2, EDARADD, FL146365, ITGA2B, ELOVL2 ASPA, PDE4C, FHL2, CCDC102B MIR29B2CG, chr16:85395429	Blood	2-18	Flanking Sequencing Epi TYPER® DNA methylation analysis system	Accuracy are ±0.94 years. KCNAB3 has strongest correlation with age of childhood & adolescences
Shi L et.al 2018 (32)	DDO, PRPH 2, DHX8, ITGA2B, Unknown gene (Illumina ID 22398226)	Blood	6-15	Bisulfite Sequencing	Accuracy are ±0.47 years. in boys & ±0.33 years in girls.
Aliferi A et.al 2018 (33)	VGF, TRIP10, KLF14, CSNKID, FZD9, C21ORF63, SSRP1, NHLRC1, ERG, FXN, P2RXL1, SCGN	Blood, Saliva Semen	Blood:11-93 Saliva:16-90.5 Semen:23-50	Bisulfite conversion, Massive parallel sequencing (Illumina MiSeq)	Accuracy between 4.13 - 4.9 years in blood,7.3 -11.1 in saliva & no DNA methylation in semen samples.
Feng L et.al 2018 (34)	TRIM59, RASSF5 Clorf132, CSNK1D, ELOVL2, PDE4C, chr 17 21452808	Blood	15-75	pyrosequencing	Accuracy with ±2.89 years
Peng F 2019 (35)	TRIM59, RASSF5 Clorf132, PDE4C, chr 10:22334463/65, CCDC102B, ELOVL2	Blood stain	18-66	Bisulfite Sequencing EpiTYPER	Accuracy with ±2.94-3.55 years
XuY et.al.2019 (36)	SALL4, MBP, C17OR76, B3GALT6, NOC2L, SNN, NPTX2, SLC22A18, TMEM106, ALEP, SCAP, C16ORF30, FLJ25410	Non blood tissue (Taking form platform Human Methylation 27 Beadchip DNA & Human Methylation 450 Beadchip DNA	0-90	Pyrosequencing	Accuracy are ±4.66 years by MAD. and ±6.08 years by Gradient Boosting Regressor MAD
Fleckhaus J & Schneider PM 2020 (37)	ELOVL2, CDC102B, KLF14, FHL2, C1 or f132, SST, PDE4C, EDARADD	Blood DNA	-	pyrosequencing	ShowedSignificant correlation with age by all marker except FHL2
Correia Dias H et.al 2020 (38,39)	ELOVL2, FHL2, EDARADD PDE4C, C1 or f132,	Blood (deceased) Blood (living)	24-86 1-95	Bisulfite Sequencing	Accuracy are ±6.08 years by MAD in deceased Accuracy are ±5.35 years by MAD in living

methylation were measured by SNaPshot assay and age prediction models were built separately for each samples type. Their results showed high accuracy for age prediction with Mean absolute deviation from chronological age of 3.478 in blood, 4.293 years in buccal swab and 3.552 years in saliva. This study supports their model is useful for forensic analysis.[22]

Correia Dias H etal in 2020 have reported DNA methylation in blood samples of living and deceased subjects for age estimation by SNaPshot assay. They used 5CpG sites located on 5genes (ELOVL2, FHL2, KLF14, C1 or f132 and TRIM59) to compare DNA methylation status between two populations(Portuguese and Korean individuals) previously investigated apart from living and deceased subjects. For that 59 blood samples includes male & females with age ranged 1-94 years, and 62 deceased subjects age ranged 28-86 years from both male & females were examined. They used linear regression model for relationship between methylation level and chronological age. The results showed that for living subject 3CpG sites at ELOVL2, FHL2 and C1 or f132 genes were 4.25 years with mean absolute deviation from chronological age (96.3% of age variation). In deceased subjects 4CpG site at ELOVL2, FHL2 and C1or f132 and TRIM59genes were 4.97 years with MAD (92.5% of age variation). In comparison of Korean and Portuguese population there were some differences found in the extent of age association at targeted locations. Their study suggested usefulness of multiplex methylation by SNaPshot assay for forensic analysis in blood samples. In case of population there is possibility that markers can be population specific.[23]

In 2021 a study was conducted by Zapico SC and co-workers to identify methylation pattern from the erupted third molar dental pulp samples of healthy individuals and correlated this pattern with age. They wanted to understand the current age-at-death estimation for Forensic anthropological point of view as age estimation is less accurate in adult individuals based on degenerative changes in bones and teeth. They collected samples from individuals whose age were 22-70 years. The ELOVL2, FHL2, NPTX2, KLF14, and SCGN marker at CpG sites of genes were used to predict chronological age by different multivariate regression models and reported that mean absolute error (MEA) of 1.5-2.13 years for age-at-death estimation in adult personals. This study suggested excellent accuracy and potential maker for age estimation.[24]

DNA methylation is the most usable and described part of the chromatin marks that formed epigenome. It is a specific chemical conversion of highly stable biological macromolecule. The DNA methylation is an attractive detection and diagnostic biomarker on chosen gene that can serving as an ideal target for epigenetic studies in human populations.[25] The age associated CpGs have been located in a specific tissue as well as across tissues, as DNA methylation profiles are highly dissimilar in different tissues.Among all possible age dependent marker, ELOVL2 is found to be most promising age predictive marker at CpG sites of genes in blood samples, which showed high association of age with DNA methylation by using the 450K Bead Chip array.[26]

Conclusion

Determination or prediction of age of individuals in forensic investigation is an important and difficult aspect. DNA analyses have facilitated scientists to accomplish particular genetic profiles of individuals from DNAs isolated from biological specimens, apart from identify, predictions of age and gender also important part in the investigation. Current approach for age prediction relies on examining the structure and composition of teeth and bone,resulting imprecise estimation. DNA methylation presents another sensitive molecular approach to age prediction.Scientist has identified genomic regions whose DNA methylation patterns is age sensitive. Molecular biomarkers may be significant to estimate age in future for forensic cases solving. Epigenetic approach provides new perspective for using biomarkers. Using such loci will attribute more precise biological age prediction.

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