ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Methylation Status of KLF4 Associated with Chronological Age

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Abstract

The expression of Krüppel-like factor 4 has associated with various biological processes including stem cell reprogramming and tumorigenesis. Although these advances in the epigenetic role of KLF4 expression in cancer and other physiological disorders, the methylation data of the KLF gene extracted from the previous studies that are not enough to coverage of non-coding of the KLF gene. In particular, the epigenetic mechanism is an essential regulator and the multiple studies reported DNA methylation level of many CPG sites in the human genome has a correlation with aging. This study highlights epigenetic changes of the KLF4 gene with aging. The present study included 92 blood samples from healthy persons, Arabic Iraqi population; age ranged 18-93 years, blood DNA samples subject to bisulfate DNA modification-pyrosequencing method. The statistical analysis detected a correlation between each CpG sites of the KLF4 gene, and age adopted the linear regression model using correction coefficient R^2. Methylated 10 CpG sites of the KLF4 gene showed weakly correlated with age. R-values ranged from 0.1 to 0.2 and the highest value appeared in the CpG site 7 (Chr9: 107489111) R was 3.0. The fitting process using the simple regression model provided weak correction coefficients (R^2 <80%) of the methylation levels of steady during the lifetime. The variance analysis suggested that there was no significant difference between the methylation levels of 10 CpG sites of intron 1 KLF4 gene and gender (P-values >0.05).

Keywords: *KLF4 gene, DNA methylation, Age, Pyrosequencing.*

Introduction

Krüppel-like factor 4 (KLF4) gene is an evolutionarily conserved zinc finger-containing transcription factor that regulates various cellular processes such as cell growth through control G1/S transition phase of the cell division [1,2]. The KLF4 gene has dual functions as a tumor suppressor possible that considered as a prognostic tumor biomarker may lead to valuable clinical management and as an oncogene, which considered a promising biomarker for several types of cancer [3].

The expression of KLF4 gene has strongly associated with the methylation level in certain CpG islands, it dramatically changes which in various physiological types of negative and positive functions [4]. The epigenetic modifications of KLF gene have essential roles in the significant way of development, cellular differentiation, and maintain cell cycle integrity and programmed apoptosis of somatic cells. In the opposite direction, there are different mechanisms for methylation of KLF 4 that contribute significantly to cancer [5]. An increasing group of evidence suggests that many expressions of aging are epigenetic. Age-related DNA methylation including the addition of the methyl group to cytosine (position 5) of the cytosine-guanine (CpG) covers what happens to result in the host of phenomena correlated with age and what strategies can slow the biological activities [6].

Beginning at birth, developmental stages result in age-related changes to gene expression, growth, and cellular functions in human. In particular, the previous studies focus on epigenetic KLF4 gene in cases of cancer and metabolic disorders within a limited range of age [7, 9]. Therefore, the most results have predominantly toward to the promoter regions and involved a limited age range, while in this study suggested that determination of methylation status of KLF4 gene for a broad range of the age that will be

a data set of the KLF4 gene subject in further studies.

Methods and Materials

DNA Extraction

Peripheral blood samples were collected from 92 healthy individuals from the Iraqi population between 18- 93 years of age. Then, blood 200µl was immediately extracted by using Quick Blood Genomic DNA Extraction Kit (DSBIO, china), the concentration and purification of DNA yield were detected by using Nanodrop-1000 UV-VIS spectrophotometer (Thermo Scientific, Loughborough, UK).

Bisulfate Sodium Conversion of genomic DNA

For DNA methylation, 500 ng g DNA was sodium bisulfate treated using the Methyl Edge Bisulfite Conversion kit according to the manufacturer's protocol (Promega, USA) and then, eluted to the final volume of 46 µL.

PCR

The amplification assay requires two independent primer sets for PCR amplification, one primer labeled by biotin designed to recognize the methylated templates and other primer is to the unmethylated versions of the bisulfite-modified sequence. PCR primers (ADS187FP and ADS187RPT) for KLF4 gene were designed and purchased from EpigenDx Company. The methylation assays were designed to interrogate the DNA methylation status of CpG sites in the Intron 1 region of the KLF4 gene.

The CpG loci location or coordinates are based on Ensembl Gene ID (ENSG00000136826), Ensembl transcript ID (ENST00000374672), and GRCh38/hg38 genomic build. PCRs were performed using 1 µL of bisulfite treated DNA, 0.75 U of Qiagen Hot Star Taq Polymerase and 0.2 µM of each primer (ADS187FP and ADS187RPT, EpigenDx, USA), 3 µL of 1X PCR buffer, 200 µM of dNTPs and adjusted to a final volume 30 µL.

Thermal cycling conditions performed using TC-9600-G (Labnet, USA) included a 15min at 95°C initial denaturation followed by 45 cycles of the 30s at 95°C, 30s at 53°C, and 30s at 72°C, ending in a 5min 72°C final extension and 4°C ∞.

In order to purify the final PCR product using sepharose beads by HPLC purification.

PCR Bias Testing

In vitro methylated DNA and unmethylated DNA as an internal control to detect the efficiency of bisulfite conversion. PCR bias testing was performed by mixing at different ratios (0%, 5%, 10%, 25%, 50%, 75%, and 100%), followed by bisulfite modification, PCR, and Pyrosequencing analysis.

Pyrosequencing

PCR product was bound to Streptavidin Sepharose HP (GE Healthcare Life Sciences), after which the immobilized PCR products were purified, washed, denatured with a 0.2 µM NaOH solution, and rewashed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen), followed by adding 0.5 µM of sequencing primer was annealed to the purified single-stranded PCR products. 10 µL of the PCR products were sequenced by Pyrosequencing on the PSQ96 HS System (Pyrosequencing, Qiagen).

The pyrosequencing analysis was performed the protocol according to the manufacturer's instructions. The methylation status of each CpG site was determined individually as artificial C/T SNP using Q CpG software (Pyrosequencing, Qiagen). The methylation level at each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of measured 10 CpG sites within the targeted region of the KLF 4 gene.

Statistical Analysis

In statistical processes were used the Pearson correlation coefficients (r). For comparison purposes, we showed the linear regression model to check the fitting degree of each CpG site that belongs to the KLF 4 gene using the correction coefficient (r^2) as well as p values. The correlation between CpG sites of the genes and chronological age were analyzed with the paired t-test. All statistical processes were performed by SPSS software.

Results and Discussion

This study targeted to understand the DNA methylation changes with aging of healthy persons, which provides control data of DNA

methylation status of the KLF4 gene in a wide spectrum of age 18 - 93 years. The bisulfate DNA pyrosequencing assay was interrogating the DNA methylation status of intron 1 region chr9: 107489051-107489167, that intron is 177 bp of the KLF4 gene contains a relatively high CG content span in intron 1 at chr9: 107489124-107489055 (GRCh38/hg38). Epigenetic studies related to the KLF4 gene locus have focused on DNA methylation status of CpG islands which including its promoter and the coding regions, which predominantly have the important role of controls the G1/S phase transition, the integrity of the G2 to M checkpoint of cell cycle upon DNA damage and cancer cells [10, 2]. In vitro methylated DNA and unmethylated DNA control of PCR bias testing mixed different ratios followed by bisulfite modification, PCR, and pyrosequencing analysis. The percent methylation obtained from the mixing study was highly correlated with expected methylation percentages with an R^2 was 0.9809, as shown in Figure (1).

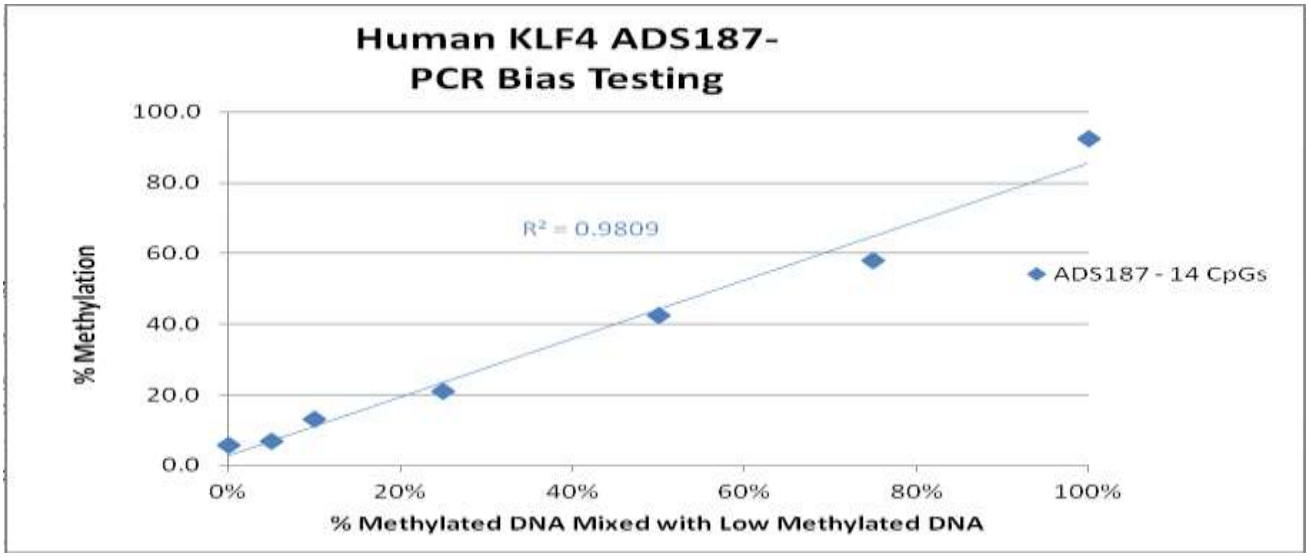

Figure 1: Efficiency of bisulfite conversion of input DNA and detection of PCR bias. The value of R^2 was 0.9809 that shows the strong correlation between DNA methylation percentages and mixed DNA methylation ratios

This study result detected the methylation levels of 10 CpG sites within the intron 1 of the KLF4 gene in blood samples of the normal individuals (Supplementary data-SA) reported a correlation between each methylated CpG site and the chronological age. The R-values ranged from 0.1 to 0.2 and the highest value appeared in the CpG site 7 (Chr9: 107489111) R was 3.0, as shown in the Table (1). However, the correlation was detected between each CpG sites of the KLF4 gene, and age adopted the linear regression model using correction coefficient R^2 (Figure 2).

Table 1: Correlation between CpG sites of the KLF4 gene and the chronological age

Sites	GRCh38/hg38	Pearson Correlation	P-values	R^2
CpG 1	Chr9:107489055	0.224*	0.032	5.7%
CpG 2	Chr9:107489071	0.229*	0.028	6.2%
CpG 3	Chr9:107489092	0.248*	0.017	4.9%
CpG 4	Chr9:107489099	0.261*	0.012	10.5%
CpG 5	Chr9:107489103	0.170	0.105	3.5%
CpG 6	Chr9:107489106	0.178	0.090	4.5%
CpG 7	Chr9:107489111	0.300**	0.004	13.8%
CpG 8	Chr9:107489114	0.196	0.062	3.0%
CpG 9	Chr9:107489116	0.154	0.143	4.0%
CpG10	Chr9:107489124	0.242*	0.020	9.4%

From the results shown in the Table (1) and Figure (1), 10 methylated CpG sites of the KLF4 gene have not appeared any persuaded linear relationship with age. The fitting process using the simple regression model illustrated in the Table (1) and Figure (1) which provided weak correction coefficients (R2 <80%) of the methylation levels of steady during the lifetime. The variance analysis suggested that there was no significant difference between the methylation levels of 10 CpG sites of intron 1 KLF 4 gene and gender (P-values >0.05).

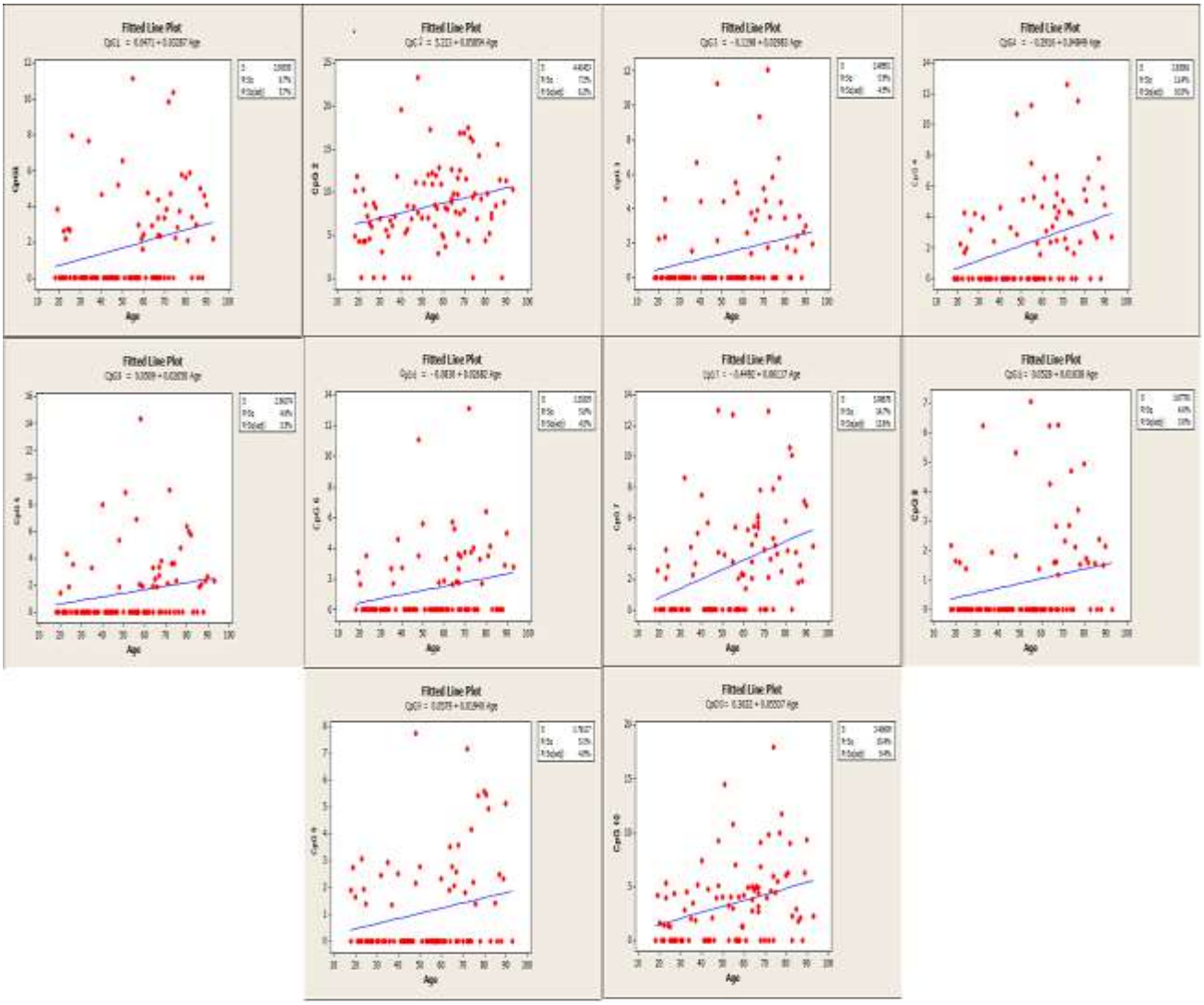

Figure 1: Regression line drawn on the scatter diagram regarding 10 **CpG sites of KLF4 gene and chronological age**

In general, KLF4 is organize at a translation level through various condition dependent mechanisms that have not yet been fully elucidated, but there is an important role of non-coding regions in regulating KLF 4 gene expression. Malignant cancer originated from a normal cell shared to the abnormalities epigenetic changes of some CpG islands of genes cause to changes in gene expression patterns, therefore, result to the dysregulation of a gene. It not only causes cancers as results abnormal patterns and levels of DNA methylation over the human genome but extend to cardiovascular, neurologic disease and several disorders as developmental abnormalities are linked to gene expression changes [11].

The KLF4 gene has an important role in cellular processes that are essential to maintain the homeostasis of cells. In a normal cell, epigenetic patterns of Klf4 stabilized compared with induced competent induced pluripotent stem cells, in case induced competent induced pluripotent stem cells, the KLF 5 gene is silent and hyper methylated [12]. The relationship between KLF4 and hTERT are keys to maintain the stemness and immortality of cancer cells, since hTERT regulates the key hallmarks of cancer, an insight of the molecular mechanism of its regulation, that help to better understand a mechanism of the ambiguous nature of KLF4 in carcinogenesis [13].

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