

EVALUATION OF SECRETED FRIZZLED-RELATED PROTEIN-2 METHYLATION STATUS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

Mohammed Hassan Tahir^a, Dlnya Asad Mohamad^a, and Mohammad Hamid^b



Submitted: 16/10/2022; Accepted: 15/3/2023; Published: 21/9/2023

ABSTRACT

Background

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder defined by the chromosomal translocation (t) (9;22) encoding for the BCR-ABL fusion gene. Tumor development and progression may be affected by hypermethylation of promoter regions of certain genes. Secreted Frizzled-Related Proteins (SFRPs) genes produce Wnt signaling antagonists, and their epigenetic silencing has been detected in multiple types of leukaemia. While epigenetic silencing has been broadly reported for SFRPs, SFRP2 is a family member less studied in leukaemia.

Objectives

To evaluate SFRP2 gene methylation patterns in CML patients compared with healthy individuals.

Materials and Methods

Sodium bisulfite was applied to the extracted DNA from blood samples of 75 CML patients and 25 healthy subjects who served as the control group. The DNA was then analyzed by methylation-specific high-resolution melting (MS-HRM) using specific primers for the SFRP2 gene in promoter sequence.

Results

Forty-five out of 75 CML patients investigated were shown to have SFRP2 methylation percentages between 50% to 100%, whereas 19 out of 25 healthy subjects had a methylation level of less than 50%.

Conclusion

The current study demonstrated that SFRP2 promoter hypermethylation exists in CML, as in several other solid tumours. Hence, methylation of this gene may play a part in initiating leukemogenesis.

Keywords: *Chronic myeloid leukemia, Wnt signaling, SFRP2, DNA methylation.*

^a Department of Biology, University of Sulaimani, Kurdistan Region, Iraq.

Correspondence: mohammed.tahir@univsul.edu.iq

^b Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

INTRODUCTION

Chronic myeloid leukaemia (CML) is a haematological disorder characterized by an abnormal proliferation of bone marrow stem cells, with an annual frequency of 15 per 1,000,000 around the world with a male-to-female ratio of 1.34, women are less likely than men to be impacted and have slightly greater survival rates^(1, 2). CML is identified by the existence of the Philadelphia chromosome, the derivative 22 of the reciprocal translocation between chromosomes 9 and 22. It was the first genetic aberration to be linked to a particular cancer type. The BCR-ABL gene's protein product which arises from this translocation is an extensively active tyrosine kinase (TK) that stimulates or blocks signaling pathways such as MAPK/ERK, JAK/STAT, RAS, PI3K/AKT, and Wnt, all of which are responsible for unregulated cellular growth and apoptosis resistance in cancer cells⁽³⁾.

In all metazoans, the Wnt signaling pathway is essential for controlling developmental processes, stem cell proliferation, and tissue homeostasis⁽⁴⁾. Moreover, many studies displayed that the Wnt pathway plays a role in the self-renewal of multiple different adult stem cell types⁽⁵⁾. Thereby, any disruption in the Wnt pathway results in pathological conditions such as birth defects, cancer, and other diseases. The crucial function of the Wnt pathway in cancer was first described in CML⁽⁶⁾. One mechanism seen in many malignancies that leads to abnormal Wnt signaling is the epigenetic silencing of Wnt inhibitors⁽⁷⁾. Epigenetics comprises heritable gene expression alterations, such as DNA methylation, deprived of any corresponding changes in DNA sequence⁽⁸⁾. It has been shown that promoter methylation is a very efficient method for suppressing Wnt antagonists in a variety of human malignancies, including leukaemia⁽⁹⁾. Functionally, Wnt antagonists are divided into two groups: the Dickkopf (DKK) and SFRP families.

SFRPs are a family of five secreted glycoproteins, namely, SFRP1-5, they are extracellular signaling molecules that antagonize the Wnt pathway. Owing to the loss of expression of SFRPs in many human malignancies, they have been classified as tumour suppressor genes. The epigenetic silencing of SFRP expression might lead to abnormal cell proliferation, incursion, and migration, which eventually lead to cancer cell formation⁽¹⁰⁾. DNA hypermethylation is a common epigenetic silencing mechanism for the SFRP gene family. In tumour tissue from colorectal cancer, the

SFRP2 promoter was demonstrated to be methylated^(11, 12). Moreover, tumour tissue with low levels of SFRP2 appeared in patients with non-small cell lung cancer^(13, 14). One of the least studied SFRP family members in leukaemia is SFRP2. In this research, we reveal extensive promoter methylation of the SFRP2 gene in CML. Furthermore, significant methylation and silencing of SFRP2 in CML-studied samples suggest its potential role in leukemogenesis.

MATERIALS AND METHODS

Patients and healthy donors

Seventy five peripheral blood samples from patients with a preliminary diagnosis of CML and positive for the BCR-ABL gene product were analyzed. The presence of the BCR-ABL fusion product was confirmed by real-time PCR in each patient. The Median age of patients was 53.6 years (range 23-85 years), and 45 (60%) patients were female. All patients consented to participate in the study. Peripheral blood samples from 25 healthy subjects were selected to act as controls. Healthy control samples were taken from people whose medical examinations and routine laboratory tests were normal and who had not used any specific medication.

DNA isolation and sodium bisulfite treatment

Genomic DNA isolation was achieved by using the standard salting out method⁽¹⁵⁾. The genomic DNA Purity and integrity were calculated by 260/280 nm absorbance using an Epoch microplate spectrophotometer (BioTek, Vermont, USA). Unmethylated cytosines were converted to uracil by the bisulfite treatment. Genomic DNA samples were modified via EZ DNA Methylation Kit (Zymo Research Corp. Irvine, CA, USA) according to the manufacturer's instructions. In all steps, samples were stored at -20°C immediately.

Methylation specific-high resolution melting

To evaluate methylation alterations between patients and healthy controls, the methylation specific-high resolution melting (MS-HRM) method was applied. MS-HRM is a technique that detects the methylation alteration of bisulfite-treated DNA semi-quantitatively⁽¹⁶⁾. According to the principles of designing primer for MS-HRM, specific primer pairs were designed for GC-rich segments of promoters using Meth primer (<http://www.urogene.org/methprimer/>) (Table 1). The PCR amplifications by real-time PCR (Applied Biosystems, StepOnePlus,) were performed in a total volume of

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10 µl containing master mix 5X HOT FIREPol® Eva Green HRM Mix (no ROX) (2 µL, final concentration 1X), primers with 10 picomole concentration (0.1 µL forward, 0.08 µL Reverse), nuclease-free water (Sinacolon Co, Iran) (6.82 µL), and bisulfite-treated DNA template (1 µL). Reactions were carried out in duplicate. The PCR condition was optimized for the SFRP2 gene at 95°C for 12 minutes for initial denaturation, followed by 40 cycles of 15 seconds at 95°C for denaturation, 60 seconds at 57°C for annealing and 35 seconds at 72°C for extension. Heteroduplex formation was done after PCR amplification at 95°C for 10 seconds and subsequently at 60°C for one minute. MS-HRM analysis was performed immediately after heteroduplex formation by 0.3°C increments, and temperature was held for 15 seconds of each increment. The increments were done from 60°C to 95°C. A no template control and set of serial percentage control

(0%, 10%, 25%, 50%, 75%, and 100%) that is provided by mixing standard controls fully methylated (EpiTect Control DNA methylated 100, Germany) and fully unmethylated (EpiTect Control DNA unmethylated 100, Germany), in duplicate were included in each experimental run. The melting curves were normalized relative to two normalization districts before and after major fluorescence reductions. The resulting plots from the samples with unknown methylation statuses were displayed relative to the standard curves. Statistical analysis

Statistical analysis

Statistical analyses were accomplished using R Project version 4.2.1 (version 4.2.1, Vienna, Austria). The methylation status of the SFRP2 promoter sequence in CML patients and healthy subjects was compared using the Compare proportions test.

Table 1. Primers used for MS-HRM.

Gene	Direction	Sequence (5'- 3')	Tm	Product size
SFRP2	forward	GCGGTTAGGTTTTTTGTTTGTG	57.6°C	131bp
	reverse	ATCTAATTTTACGTTAAAAATACCCCTC	57.8°C	

RESULTS

A total of 75 CML patients (including 40 males and 35 females) and 25 healthy subjects were tested to determine the methylation status of SFRP2 promoter regions using MS-HRM (Figure 1). The SFRP2 promoter methylation percentage frequencies in healthy controls and CML patients were subdivided into three categories, namely 0-24%, 25-49%, and 50-100%. In more than half of healthy controls (n=14), promoter methylation levels were between 0% to 24%, whereas less than a third of CML patients (n=15) had the same methylation percentage range (p<0.001). Moreover, 60% (45 out of 75) of CML patients had a methylation level between 50% - 100%, nevertheless, only 24% (6 out of 25) of healthy controls had the same methylation percentage (p<0.003), as demonstrated in (Figure 2)

(Figure 1) Comparison of aligned melt curves of serial controls with melt curve of patient samples with methylation percentage (S1: range 25-50%, S2: range 50-75%, 50%, 75%, and, 100%). 0 (unmethylated): yellow line, 10% purple line, 25% pink line, S1: range 25-50% blue line, 50% green line, S2: light brown range 50-75%, 75% dark blue line, 100% fully methylated light green line. (Figure 2) 60% (45 out of 75) of patients had methylation levels between (50%-100%), whereas 76% (19 out of 25) of healthy controls had a methylation level below 50%. ns: not significant, **P<0.01, ***P<0.001.

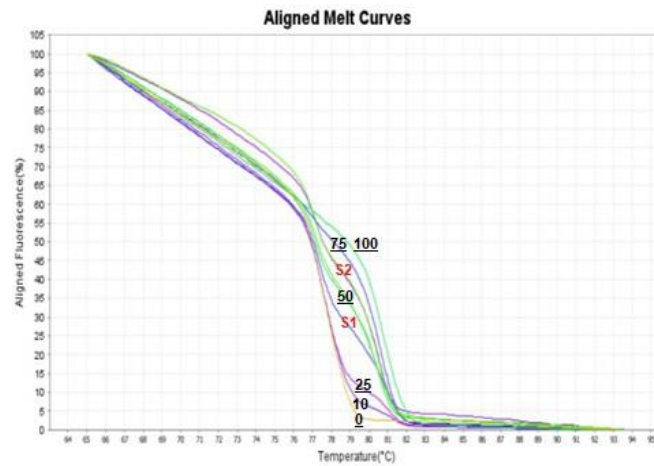


Figure 1. MS-HRM curve for SFRP2 gene.

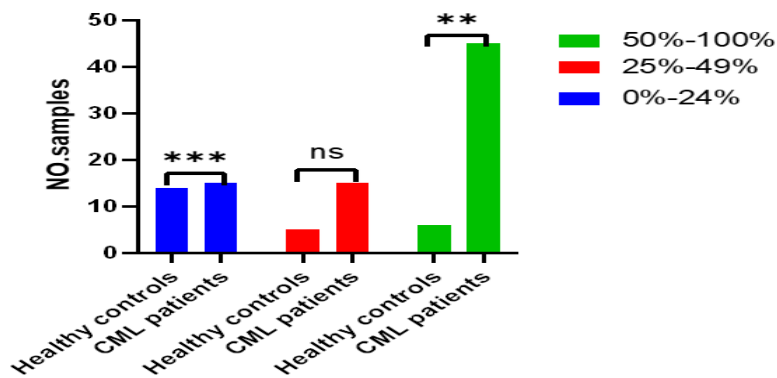


Figure 2. Methylation percentage frequencies of SFRP2 gene in CML patients and healthy controls.

DISCUSSION

The Wnt signaling pathway has been associated with many biological processes including cell adhesion, apoptosis, migration, polarity, development, proliferation, and organogenesis ⁽¹⁷⁾. Moreover, the abnormal activity of the Wnt pathway can precede tumorigenesis ⁽⁷⁾. The possibility that the Wnt pathway may be involved in leukemogenesis, was developed as a result of knowing the functions of Wnt signaling in the survival, proliferation, and differentiation of hematopoietic stem cells⁽¹⁸⁻²⁰⁾. The first identified haematological cancer with aberrant Wnt signaling was CML ⁽²¹⁾. Numerous human malignancies have been confirmed to have pathogenesis that involves silencing of Wnt antagonists' genes through hypermethylation of their promoters ⁽²²⁻²⁴⁾. SFRPs are tumour suppressor genes that work as Wnt/antagonists. These proteins can

attach to the Wnt protein and thus inhibit its binding to the Wnt-frizzled receptor. The result is the inactivation of the Wnt pathway. The SFRPs gene family is often the target of epigenetic silencing by promoter hypermethylation. Downregulation of SFRP genes in leukaemia and a range of cancers has been linked to hypermethylation of CpG islands in the promoter region ^(9, 23, 25, 26).

Various quantitative and qualitative methods have been utilized to detect alterations in methylation of DNA. MS-HRM is an easy and reproducible technique that detects the methylation alteration of bisulfite-treated DNA semi-quantitatively ^(16, 27).

The study of Jun et al ⁽²⁸⁾, has shown that SFRP1 and SFRP2 promoter hypermethylation were 8.48, and 8.21 higher, respectively, in patients with cancer compared with that in healthy controls. They also highlighted

the significant correlation between promoter methylation of SFRP1 with leukaemia. Moreover, they revealed that promoter methylation of SFRPs was associated with ovarian cancer, colorectal cancer, hepatocellular carcinoma, lung cancer, esophageal cancer, gastric cancer, renal Adenocarcinoma, cervical cancer, uterine cancer, bladder cancer, leukaemia. Epigenetic downregulation of SFRPs in hematopoietic malignancies is believed to have an impact on disease pathogenesis⁽²⁹⁾. SFRP1 silencing has been announced in nearly all types of leukaemias studied to date^(30, 31).

In Lymphocytic Leukemia, Te-Hui et al⁽⁹⁾ showed that promoter methylation of SFRP1 and SFRP2 were 100%, and 55%, respectively, in 20 CLL samples. Revealing that the epigenetic silencing of certain SFRPs, particularly SFRP1, may play a vital function in the progress of Chronic lymphocytic leukaemia (CLL). Another study revealed that promoter methylation of SFRP1 was 51% in 50 ALL patients, using Methylation-specific polymerase chain reaction (MSP), suggesting that SFRP1 is crucial for leukemogenesis⁽³²⁾. Moreover, Promoter methylation and reduced expression levels of the SFRP1 gene were noted in 121 patients with Acute myeloid leukaemia (AML), Acute lymphocytic leukaemia (ALL), and myelodysplastic syndrome (MDS)⁽³¹⁾. These findings are supported by further studies that show downregulation and promoter methylation of SFRP1 and SFRP2 in primary CLL cells⁽³³⁾.

Different studies have displayed that SFRP1 and SFRP2 promoter methylation were higher in AML patients compared to healthy controls, suggesting that SFRP1 and SFRP2 may take a part in the onset of AML^(30, 34).

In the current study, methylation levels of the SFRP2 gene were studied by using MS-HRM in CML patients. To the best of our knowledge, this is the first study that analyzes methylation alterations of the SFRP2 gene in CML patients compared to healthy controls by using the MS-HRM method. Results of this study showed that SFRP2 promoter methylation levels were higher in patients with CML compared to healthy controls. In 60% (45 out of 75) of CML patients, SFRP2 methylation percentages were more than 50%, whereas, in 76% (19 out of 25) of healthy subjects, methylation levels were less than 50%. Hence, it could conceivably be hypothesized that aberrant methylation of Wnt pathway antagonists namely the SFRP2 gene may have a vital role in the onset or progression of CML. This finding agrees with Ziyi Li and Jianmin Luo's⁽³⁵⁾ findings

which showed that the methylation status of SFRP2 was higher in CML patients compared with healthy subjects.

In conclusion, the purpose of the current study was to evaluate the methylation status of the SFRP2 gene as one of the Wnt pathway antagonists in CML patients compared to healthy individuals. We revealed that SFRP2 methylation was higher in patients with CML compared to healthy subjects, suggesting that methylation of SFRP2 may be implicated in the onset or progression of CML. However, it is recommended to conduct more studies to uncover the role of hypermethylation of the SFRP2 gene in the pathogenesis of CML and other hematologic malignancies.

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