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EVALUATION OF SECRETED FRIZZLED-RELATED PROTEIN-4 METHYLATION PROFILES IN PATIENTS WITH BCR/ABL POSITIVE CHRONIC MYELOID LEUKEMIA

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Abstract – *Objective:* *Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder characterized by the chromosomal translocation t(9;22) encoding for the Bcr/Abl fusion gene. Secreted Frizzled Related Protein (sFRP) gene products are antagonists of Wnt signaling and their epigenetic silencing has been reported in different types of leukemia. While epigenetic silencing has been extensively reported for sFRP, sFRP4 is a family member less studied. We aimed to study epigenetic silencing of sFRP4 in CML patients.*

Patients and Methods: *Epigenetics alterations in the promoter region of the sFRP4 gene were analyzed in 43 CML patients. DNA methylation of CpGs in the sFRP4 promotor region was investigated using methylation specific PCR. Conventional cytogenetic analyzes were performed directly and after 24 h short cultures from bone marrow samples of patients with a preliminary diagnosis of CML.*

Results: *42 out of the 43 CML patients investigated were shown to be unmethylated at the sFRP4 promoter region, while in 1 patient hemimethylation was observed. This patient was the only one in which cytogenetic progression with additional chromosomal abnormalities were identified. All others achieved major or complete cytogenetic remission.*

Conclusions: *We observed that sFRP4 methylation is a very rare CML patients' phenomenon. Yet when observed it may implicate disease progression and therapy resistance. A large study needs to clarify the implication of methylated sFRP4 in CML progression.*

KEYWORDS: *sFRP4, CML, DNA methylation, Wnt signaling, Cytogenetics.*

Chronic myeloid leukemia (CML) is a myeloproliferative disease originating from myeloid CD34+/CD38- hematopoietic stem cells in the bone marrow[1]. The Philadelphia chromosome (Ph) is the hallmark of disease, formed by the translocation between the Abelson (ABL1) gene on the chromosome 9 and the Breakpoint Cluster Region (BCR) on chromosome 22 [t(9; 22 (q34; q11))]. The Bcr-Abl fusion oncoprotein resulting from this translocation encodes a deregulated tyrosine kinase (TK) that activates different survival pathways, leading to uncontrolled proliferation and resistance to cell death signals and an increase in the number of leukemic stem cells [2]. Wnt signaling is a highly conserved developmental signal transduction pathway involved in various cellular processes such as proliferation, cell fate, migration, polarity and cell death. There are 19 different Wnt genes that encode secreted glycoproteins that act as ligands for Frizzled (Fzd) receptors [3]. Deregulated Wnt signaling is one of the most observed phenomena in the development of many different types of cancer [4]. Canonical Wnt pathway activation leads to hypophosphorylation and nuclear translocation

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of β-catenin. Nuclear β-catenin interacts with the transcription factors TCF (T-cell factor) and LEF (Lymphoid enhancer-binding factor) to regulate the expression of target genes such as c-Myc and cyclin-D [5]. Wnt signaling is tightly regulated and one of the major modes of regulation is by secreted antagonists. These antagonists exert their effects by disrupting Wnt ligand-Fzd receptor interactions. The functional loss of Wnt antagonists leads to deregulated activation of Wnt pathways resulting in disruption of proliferation and/or differentiation processes, which in turn, may contribute to carcinogenesis. Epigenetic suppression of Wnt antagonists by promoter methylation has been shown to be quite effective in many human malignancies including leukemia [6]. Functionally, Wnt antagonists can be divided into two classes: sFRP and Dickkopf (DKK) families. The secreted frizzled-related protein (sFRP) family is one of these Wnt antagonists' families that is known to be suppressed epigenetically. sFRPs expressed in various embryonic and adult tissues are soluble regulators of the Wnt signaling pathway [7,8].

In humans, the sFRP family consists of five members sFRP1, sFRP2, sFRP3, sFRP4 and sFRP5 [9]. In terms of sequence homology, the sFRP gene family consists of two closely related subgroups. sFRP1, sFRP2 and sFRP5 form the first subgroup (subfamily 1 Sarp), while sFRP3/FRZB (bone development-related frizzled motif) and sFRP4 are members of the second subgroup (subfamily 2 FrzB) [7,10]. The sFRP4 gene is localized at 7p14-p13, contains six exons and encodes mRNA transcripts of 2974 bp and 1041 bp. The first exon contains the translation initiation codon [9,10]. Structurally, the C-terminal of sFRP4 contains netrin-like domains (NTR) and 9 potential serine/threonine phosphorylation sites and is considered as a potential target for protein phosphorylation by serine/threonine kinases [11]. sFRP4 is expressed in a variety of tissues, including endometrial stroma, pancreas, stomach, colon, lung, skeletal muscle, testis, ovary, kidney, heart, brain, mammary gland, cervix, bone, prostate, liver and eye [10-13].

Recent studies in acute/chronic leukemias have provided abundant information on the role Wnt signaling plays in malignant hematopoiesis. Nevertheless, there is a lack of reported mutations that may be the cause for deregulated Wnt signal transduction in leukemias and other hematopoietic malignancies[14]. Promoter hypermethylation of Wnt antagonists, such as the sFRP family members, has gained attention in this context. In 20 Chronic lymphoid leukemia (CLL) patient samples, promoter methylation rates for the sFRP family members-analyzed by Combined Bisulfite Restriction Analysis (COBRA) assay and bisulfite sequence analysis- were reported to be 100% for sFRP1, 55% for sFRP2, 30% for sFRP4 and 15% for sFRP5 [6]. Similarly, in Acute Myeloid Leukemia (AML), the expression of Wnt antagonists was reported to be downregulated. sFRP1, sFRP2, sFRP3, sFRP4 and DKK1 were epigenetically inactive due to CpG island hypermethylation of their promoters; and their inactivation correlated with poor prognosis [15-17]. One of the least studied sFRP family member in leukemia is sFRP4. The extent of inactivation due to promoter methylation of the sFRP4 gene has not been reported for leukemias, including CML. In this study we aimed to study the promoter methylation status of the sFRP4 gene in CML patients.

PATIENTS AND METHODS

Patients

Bone marrow (BM) samples of 43 patients positive for the Bcr/Abl chimeric gene product and with a preliminary diagnosis of CML were analyzed. The presence of the Bcr/Abl (p210) fusion product was confirmed by both conventional Polymerase Chain reaction (PCR) and Real-time quantitative PCR (qPCR). Average age of patients is 44.13, and gender rates are 1.0-1.2 (male-female). Bone marrow of healthy individuals (NBM) was used to determine a methylation status of the sFRP4 gene promoter region in normal hematopoietic tissue and to compare it with patients. NBM donors, 3 male and 1 female, have an average age of 56 years, and BM samples were taken from the sternum during open-heart surgeries with patient consent.

Ethical Declaration and Informed Consent

This study was performed in line with the principles of the Declaration of Helsinki. Informed consent was obtained from all NBM donors and CML patients and this study has been approved by the Dokuz Eylul University, Faculty of Medicine, Institutional Drug and Clinical Investigations Ethics Committee and Clinical and Laboratory Research Ethics Committee.

Cytogenetic Analysis

Conventional cytogenetic analyses were performed directly and after 24 h short cultures from BM samples of patients with a preliminary diagnosis of CML. Analyses were performed at diagnosis and after a year follow-up. Staining of the metaphase chromosome was done by convention-

TABLE 1. PCR primers used for methylated and unmethylated MS-PCR.

Primers	*Sequence (5'-3')*
sFRP-4 Met S	GGGTGATGTTATCGTTTTTGTATCGAC
sFRP-4 Met U	CCTCCCCTAACGTAAACTCGAAACG
sFRP-4 Unmet S	GGGGGTGATGTTATTGTTTTTGTATTGAT
sFRP-4 Unmet U	CACCTCCCCTAACATAAACTCAAAACA

al G banding. The karyotypes were described according to guidelines proposed in the International System for Human Cytogenetic Nomenclature 2016 (ISCN 2016)[18].

Genomic DNA Isolation

Genomic DNA isolation was performed using the "DNeasy Blood and Tissue Isolation kit" (Qiagen, 69504, Hilden, Germany) for both NBM and patient's bone marrow samples, in accordance with the manufacturer's protocol. Purity and integrity of genomic DNA was calculated by 260/280 nm absorbance using a T60 UV-Visible spectrophotometer (PG Instruments, Lutterworth, UK).

Bisulfite DNA Modification

Promoter region of the *sFRP4* gene (NCBI, Ref Seq: NC 000007.14) was determined by "Promotor 2.0". Primers specific for this region were designed by "Oligos v.9.4" software. Bisulfite treatment converts unmethlyated cytosines to uracil. Genomic DNA samples were modified by using "The Methyl Detector Bisulfite Modification Kit" (Active Motif, 55001, Rixensart, Belgium), following manufacturer's instructions.

Methylation Specific Polymerase Chain Reaction (MS PCR)

DNA methylation of CpGs in the sFRP4 promotor region was analyzed using MS PCR. Two separate PCR reactions were designed from same genomic DNA sample using methylated and unmethylated PCR primers. Primer sequences are given in Table 1.

0.5 µg of bisulfite converted DNA was added to each PCR mixture. PCR cycling conditions were 95°C for 5 min (preincubation), followed by 33 cycles at 95°C for 45 s (denaturation), 60°C for 45 s (annealing) and 72°C for 45 s (extension). Samples were analyzed on 4% nusieve agarose gel stained with ethidium bromide.

Statistical Analysis

Statistical analyses were performed using IBM Statistics version 25.0 (SPSS Inc., Armonk, NY, USA). sFRP4 promoter methylation status in CML patients and healthy donors were compared by nonparametric Mann-Whitney test.

RESULTS

Methylation status of the sFRP4 promoter in bone marrow samples from CML patients and NBM

sFRP4 promotor methylation status was evaluated by methylation specific PCR in bone marrow samples of CML patients and NBM samples. From the total of 43 patients, 42 patients displayed unmethylated promoter regions while hemimethylation was observed in only one patient. All 4 NBM samples were unmethylated at the sFRP4 promoter region (Figure 1). There was no statistically significant association between CML patients and healthy donors (p>0.05).

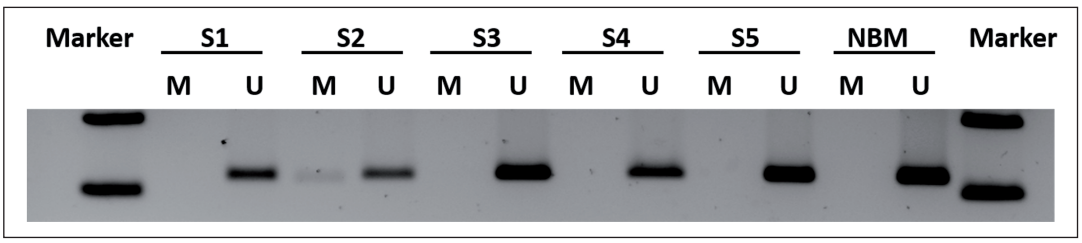

Fig. 1. Methylation status of the sFRP4 promoter region. Samples 1, 3, 4, 5 and NBM are unmethylated. Sample 2 is hemimethylated at the sFRP4 promoter. *Abbreviations:* M, methylated; U, unmethylated; NBM, normal bone marrow.

Relationship of sFRP4 methylation status and cytogenetic analysis results

All 42 patients observed to be unmethylated at the sFRP4 promoter region and they achieved complete or major cytogenetic remission after 1 year of tyrosine kinase inhibitor (TKI)-imatinib therapy. Cytogenetic analyses for the hemimethylated patient at the time of diagnosis was 46, XX, t (9;22) (q34; q11) [10]. This patient displayed cytogenetic progression. A second Ph chromosome, in addition to complex structural anomalies, was observed after 12 months (46,XX,t(9;22) (q34;q11), +13, +der(22) t(9;22) (q34;q11), +mar[8] /46,XX, t(9;22) (q34;q11)[2]).

Statistical analysis

The frequencies of methylation were not significantly different between sFRP4 promoter methylation status in CML patients, and healthy donors were compared ($p>0.05$).

DISCUSSION

Chronic myeloid leukemia is a myeloproliferative disease in mature or maturing granulocyte cells. The t(9; 22) translocation is a specific and diagnostic cytogenetic feature observed in these patients. The Bcr/Abl chimeric gene product resulting from this translocation possesses unregulated protein tyrosine kinase activity and is detected in the bone marrow and peripheral blood of 95% of CML patients [19]. The Bcr/Abl oncoprotein has been reported to act as a negative regulator of the Wnt signaling pathway in CML [20].

The Wnt signaling pathway plays an active role in many developmental processes, including cell proliferation, cell fate determination, apoptosis, migration, cell polarity, differentiation and stem cell maintenance [21]. Therefore, it is not surprising that changes in the Wnt signaling pathway have serious consequences in both development and adult tissue homeostasis [22].

Recent studies have focused on Wnt antagonists functioning as regulators of the Wnt signaling pathway. An important group of Wnt antagonists are sFRPs, in which promoter methylation status has been of interest. sFRP methylation rates were compared in primary multiple myeloma patients and human multiple myeloma cell lines. Patient promoter methylation rates for sFRP1, sFRP2, sFRP4, sFRP5 were reported to be 27%, 52%, 7%, 6% respectively; while in cell lines these rates were observed to be 78%, 56%, 56%, 67%, respectively [23]. There are no CpG islands in the promoter region of sFRP3/FRZB; therefore, it is not included in the methylation studies[6]. Studies on AML patients and Kasumi-1, KG1a, HL-60 and THP-1 cell lines, methylation rates of promoter regions of sFRP1, sFRP2, sFRP4 and sFRP5 genes in 184 AML patient samples, were reported to be 41%, 31%, 22% and 4% respectively. sFRP1, sFRP2 were found to be methylated in all cell lines; sFRP5 methylated in Kasumi-1, KG1a and THP-1 cell lines; and sFRP4 was found to be methylated only in the HL-60 cell line [16]. Both these studies show that the methylation status of sFRP genes in cell lines is greater than that of patients, in addition to the observation that sFRP4 is less prone to promoter methylation when compared to other sFRPs.

Rush et al [24] reported that abnormal CpG island methylation is common in CLL by genome-wide methylation screening using Restriction Landmark Genome Scanning and sFRP4 was identified as an important methylation target in CLL [24]. Another study with CLL patients, observed a 30% methylation rate of the sFRP4 promoter. In addition, the same study showed that in the WaC3D5 cell line, CpG islands in the promoter regions of sFRP1, sFRP2 and sFRP4 genes were densely methylated, while sFRP5 displayed a lower level of aberrant methylation. sFRP4 expression was found to be downregulated in these CLL patients. In some patients in which the sFRP4 promoter region was unmethylated, sFRP4 expression was not observed, implying mechanisms other than CpG methylation are responsible for the downregulation of sFRP4 expression [8]. Nevertheless, these results suggest that sFRP4 downregulation is important in CLL pathogenesis.

In the present study, the sFRP4 promoter region of 4 NBM and 43 CML patient bone marrow samples, was examined for methylation status. The 4 NBM samples were found to be unmethylated at the sFRP4 promoter. No significant difference was observed in 42 of the patients when compared to NBMs. Only one CML patient sample was determined to be hemimethylated at their sFRP4 promoter region.

We previously reported that sFRP4 mRNA expression was not observed in 15 CML bone marrow samples and 8 out of 10 NBM samples studied [25]; whereas we were able to detect sFRP4 mRNA expression in the CML cell line K562. In this present study, we aimed to investigate whether suppressed the sFRP4 expression observed in CML patients is due to promoter methylation. Our data reflects that sFRP4 promoter methylation is not common in CML. Interestingly, the only

hemimethylated sample was from a patient that showed cytogenetic progression with complex structural abnormalities, implying that promotor methylation of the sFRP4 gene may correlate with disease progression and therapy resistance.

CONCLUSIONS

We conclude that the lack of sFRP4 expression in CML patients may result from mechanisms other than promoter CpG methylation. In addition, it is plausible that sFRP4 promoter methylation may proceed CML progression. Although rare in chronic phase CML, whether sFRP4 methylation status is associated with cytogenetic progression and resistance to CML treatment is yet to be determined and is a question that would need a large sample size to investigate.

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Ethical Declaration and Informed Consent:
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Conflict of interest:
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