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Methylated SFRP1,2 and CD25 Expression in Acute Myeloid Leukemia Play an Important Role in the Pathogenesis of the Disease and in Turn in its Treatment

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Abstract

Objectives: Recently, hyperactivation of the Wnt signaling pathway has been implicated in leukomogenesis, so we studied the epigenetic dysfunction of SFRP1,2 and expression of interleukin2 receptor α chain (IL2Rα, also known as CD25) and its prognostic impact in acute myeloblastic leukemia (AML).

Methods: We studied the methylation profile of SFRP1,2 in AML cells by methylation-specific polymerase chain reaction (MSP) and the hyper expression of IL2Rα (CD25) by flowcytometry.

Results: We analyzed the methylation profile of SFRP1,2 in 40 de novo AML patients. The percentage of hypermethylation in the patient samples were 37.5% for SFRP1, 12.5% for SFRP2. CD25 was positive in 12(30%) of 40 patients AML. We found that in patients whom 60 years and younger with intermediate risk cytogenetics in de novo AML, hypermethylation of SFRP1 and CD25 were accompanied with relapse (P=0.024).

Conclusion: Our data indicates that in a subgroup of AML patients, hypermethylation of SFRP1 and high expression of CD25 predict relapse.

Introduction

Acute myeloid leukemia is a clonal hematopoietic disease associated by aberrant renewal of hematopoietic stem cells, differentiation arrest at blast cells, peripheral blood infiltration of blasts. It is found that pathogenesis of AML is accompanied by chromosomal translocations and genetic disorders [1]. Recently, epigenetic disorders, like promoter hypermethylation has been integrated in the pathogenesis of leukemia [2]. Wnt signaling pathway is important in the development of different organ systems [3,4] and also a very important factor in cell self renewal, cell proliferation, maturation and hematopoietic stem cell survival [4,5]. Prolonged activation of the Wnt pathway has been detected in AML [6]. SFRP is a tumor suppressor protein that regulates the Wnt signaling pathway. SFRP binds to Wnt protein and in turn inhibits its binding to Wnt-frizzled receptor and in order result in inhibition of Wnt signaling pathway. Thereafter there may be an association between methylation of Wnt signaling antagonist genes (SFRPs) and the activation of this pathway in leukemia [7]. Wnt signaling composed of the canonical pathway with b-catenin as a central factor and the non-canonical pathways including planar cell polarity and calcium ions [8]. If Wnt ligands absent, b-catenin is phosphorylated by glycogen synthase kinase-3b (GSK3b) which leads to its ubiquitination and degradation [9]. Attachment of Wnt to the Frizzled receptor leads to an inhibition of the GSK3b by the protein Dishevelled activation. This leads to b-catenin stabilization and its translocation into the nucleus where it binds to a number of transcription factors, like T-cell factor (TCF) and lymphoid enhancing factor-1 (LEF1) [10]. Elevated b-catenin has been found to block myeloid, lymphoid and erythroid cell maturation associated by an increase of blast cells [11]. In a case of hypermethylation of SFRP genes, its inhibitory effect on wnt singnaling pathway was lost which leads to elevation of cytoplasmic and nuclear levels of β- cathenin and in turn β-cathenin as a transcription factor increases expression of cyclin D and Myc genes that are included in cell cycle regulation [12]. Abnormal Wnt signaling has been detected in AML patients with normal karyotype and in patients with fms-like tyrosine kinase -3(Flt3) mutations [13] CD25 (α chain of interleukin 2 receptor) expression on AML cells has a role in the differentiation and proliferation of myeloid stem cells, cell-to-cell interactions, and the chemoresistant condition of the malignant cells [14]. We aimed to study the prognostic impact of hypermethylated SFRP1,2 and overexpression of CD25 in AML patients.

Patients and Methods

Patients

Twenty five healthy persons as a negative control group and 40 patients with de novo AML were involved in this study. These patients were admitted to Hematology unit, clinical pathology department and south Egypt cancer institute, Assiut University, Egypt, and were grouped depending on morphology and immune phenotyping (FAB classification).

The clinical data consisted of age and sex, laboratory data consisted of hemoglobin level, white blood cell count, platelets count and the recovery after induction chemotherapy were taken from patients' medical records.

Flowcytometric analysis

A panel of monoclonal antibodies was designed against myeloid lineage specific antigens including CD13, CD33, MPO, Cyto MPO,

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CD15, CD14, CD41 and and non-specific antigens including CD45, CD34, CD 11C, CD 117 and HLA-DR. In addition to, CD25 (IL-2Rα) were analyzed on 4 color BD FACSCALIBUR.

Cytogenetic study

Depending on the recommendations of the International System for Human Cytogenetic, cytogenetic analysis was done using short term cultures [15]. At least 20 metaphases were examined. Cytogenetic risk groups were categorized as follows: high risk, –7/ del(7q), –5/ del(5q), abn 3q, complex aberrations (X3 independent aberrations), t(6;9) and t(9;22); low risk, inv(16) and t(8;21); inter- mediate risk, normal karyotype or all other karyotypic abnormalities.

DNA extraction and bisulfite treatment

Genomic DNA was extracted from peripheral blood samples at diagnosis using an Invitrogen PureLink™ Genomic DNA Mini Kit, USA. Then, bisulfite conversion was done with the Invitrogen MethylCode™ Bisulfite Conversion Kit, USA using manufacurer's protocol. By this, methylated cytosine stayed as it where unmethylated cytosine converted to uracil.

Molecular analysis

In FLT3-internal tandem duplication (flt3/ITD) mutation analysis, we added 200 ng of DNA, 50 mM KCL, 10 mM Tris-HCL, pH 8.3, 1.5 mM $MgCL_2$, 200 µM dNTPs, 0.4 µM of forward primer 5'GCAATTTAGGTATGAAAGCCAGC-3'and 0.4 µM reverse primer 5'CTTTCAGCATTTTGACGGCAACC-3', and 1U of polymerase, in a volume of 50 µl [16]. The PCR consisted of an initial incubation step at 95°C for 10 minutes followed by 35 cycles at 94°C for 30 seconds, 57°C for 60 seconds, and 72°C for 90 seconds. The final extension step was at 72°C for 10 minutes. PCR products were analyzed on standard 3% agarose gels. Normal amplification produced a 330 bp product; whereas, FLT3 ITD mutations showed longer PCR products.

MSP methylation analysis

We used MSP (Methylation specific PCR) method to analyze the methylation profile of SFRP1 and SFRP2 genes. MSP is a PCR type used to analyze the CpG islands methylation state. In this technique, we used 2 pairs of primers specific for the methylated or unmethylated residue. Table 1 showed these primers sequences accompanied with product values [17].

In methylation testing, we added 2 µl of bisulfite-treated DNA, 4.5 µl of dH_2O, 12.5 µl of Master mix , 0.5 µl of forward primer(0.91 µM for SFRP1,0.88 µM for SFRP2) and 0.5 µl of reverse primer(0.95 µM for SFRP1,0.87 µM for SFRP2), and in unmethylation testing we added 2 µl of DNA, 8.5 µl of dH_2O, 12.5 µl of master mix , 0.5 µl of forward primer(0.91 µM for SFRP1,0.88 µM for SFRP2), 0.5 µl of reverse primer(0.95 µM for SFRP1,0.87 µM for SFRP2), and 1 µl of $MgCl_2$. In the first step of unmethylated status testing, we put reaction components in pre-thermal condition including 98°C for 1 minute and 96°C for 3 minutes then 40 cycles including 99°C for 10 seconds, 97°C for 20 seconds, 54°C for 30 seconds, while for methylated status testing, we did as previous steps except for the last step (64°C for 30 seconds), then 72°C for 7 minutes (extension). ddH_2O was used as the negative control. Finally, electrophoresis using a 2.5% agarose gel was done for MSP product identification.

We studied the prognostic impact of hypermethylated SFRP1,2 and overexpression of CD25 on the outcome in AML patients (complete remission(CR) which was defined as presence of <5% of blast cells in the bone marrow, Relapse was defined as bone marrow blast ≥ 5%, reappearance of blasts in peripheral blood and/or extramedullary disease and death) [18].

Results

Of the 40 patients, 29 (72.5%) were males and 11 (27.5%) were females (Table 2). Mean age was 35.5 years old (18-60 years). SFRP1 gene was completely unmethylated in 18 patients (45%), hemi-methylated in 7 patients (17.5%), while completely methylated in 15 patients (37.5%), however, SFRP2 gene was completely unmethylated in 29 patients (72.5%), hemi- methylated in 6 patients (15%), and completely methylated in 5 patients (12.5%) (Figure 1). All the control group showed negative methylation in SFRP1 and SFRP2 genes.

In our study, hypermethylation percentage of SFRP1 and SFRP2 genes was 37.5% (15 out of 40 patients) and 12.5% (5 out of 40 patients), respectively (Figure 2). Hypermethylation of SFRP1,2 was found in all FAB classifications of AML, except for M6. Hypermethylation of SFRP1 (P=0.021) gene was found significantly in subtype M0 (Table 3). No significant relationship was found between hypermethylation of SFRP1,2 and clinical data of patients including age, sex, laboratory data including Hb level , white blood cells and platelets count (Table 3). In addition, there was no effect of the SFRP metylation patterns on CR, relapse or death.

Among the 15 patients with hypermethylated SFRP1 there were 5 patients with favorable risk cytogenetics, 8 patients with intermediate risk cytogenetics (5 of 8 patients with normal karyotype P=0.01 and 3 of 8 patients had flt3/ITD mutation P=0.44) (P=0.025), and 2 patients with adverse risk cytogenetics. However, there was no significant relationship between hypermethylated SFRP2 and risk cytogenetics (Table 3). However, we found that there was a significant relationship between methylated SFRP1 and flt3/ITD mutation in AML patients

Primer	Sequence	Annealing temperature	Product size (bp)
SFRP-1 MF	TGTAGTTTTCGGAGTTAGT GTCGCGC	65	126
SFRP-1 MR	CCTACGATCGAAAACGACG CGAACG		
SFRP-1 UF	GTTTTGTAGTTTTTGGAGT TAGTGTTGTGT	54	135
SFRP-1 UR	CTCAACCTACAATCAAAAA CAACACAAACA		
SFRP-2 MF	GGGTCGGAGTTTTTCGGAG TTGCGC	62	138
SFRP-2 MR	CCGCTCTCTTCGCTAAATA CGACTCG		
SFRP-2 UF	TTTTGGGTTGGAGTTTTTT GGAGTTGTGT	64	145
SFRP-2 UR	AACCCACTCTCTTCACTAA ATACAACTCA		

MF: Methylated Forward, MR: Methylated Reverse, UF: Unmethylated Forward, UR: Unmethylated Reverse

Table 1: SFRP-1 and SFRP-2 gene primers sequences, annealing temperature and product size for MSP Assays.

Parameters	AML patients	Control group	P
Number of patients (%)	40	25	
Age(years),(Mean ± SD)	35.3 ± 11.55	36.1 ± 11.3	0.643
Sex (%)			
Male	29 (72.5%)	17 (68%)	
Female	17(68%)	8 (32%)	0.589
Rural/Urban (%)	16(40)/24(60)	9(36)/16(64)	0.732

Table 2: Demographic characteristics of AML patients and control group.

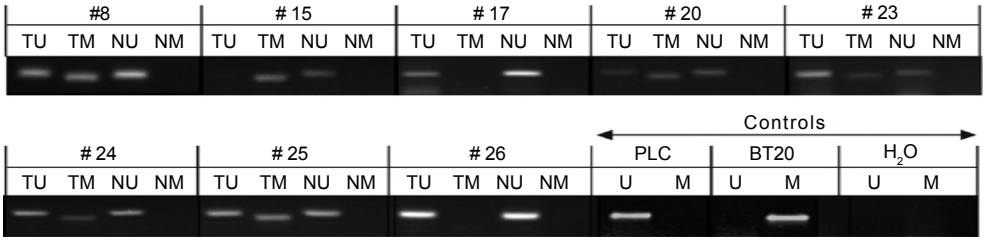

Figure 1: Methylation analysis of SFRP1 AML patients. SFRP1 Methylation-specific PCR (MSP) was performed on bisulphite-treated DNA from AML (T) and matching normal subjects (N). MSP results from eight representative patients are shown. DNA bands in lanes labelled with U indicate PCR products amplified with primers recognising the unmethylated promoter sequence. DNA bands in lanes labelled with M represent amplified products with methylation-specific primers. Human placenta (PL) and *in vitro* methylated DNA (IVD) served as positive controls for the U and M reaction, respectively. Water was used as template in the negative control (NTC). Patient number 15: methylated SFRP1, Patients number 8,20,23,24,25: hemimethylated SFRP1, Patients number 17,26: unmethylated SFRP1.

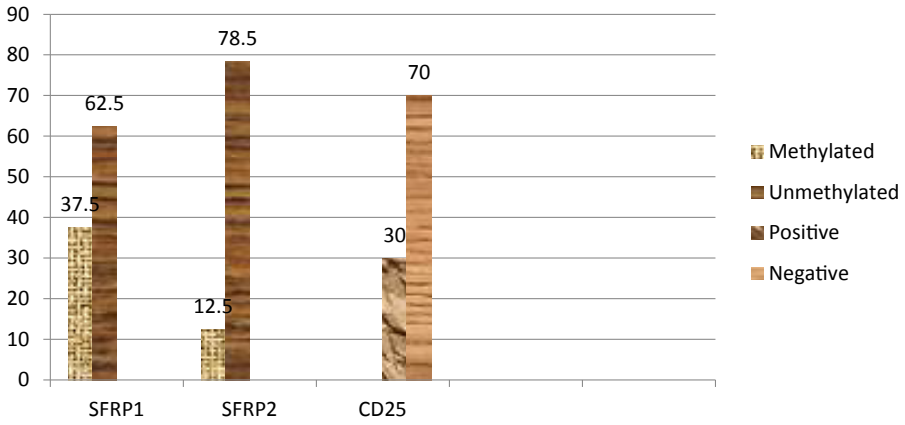

Figure 2: Frequency of promoter methylation of SFRPs and CD25 expression in 40 patients with AML.

Parameters	SFRP1			SFRP2		
	M	U	P	M	U	P
Number of patients (%)	15(37.5)	25(62.5)		5(12.5)	35(87.5)	
Age(years), (Mean ± SD)	34.5 ± 11.8	36 ± 11.3	0.411	33.8 ± 13	35 ± 11.9	0.565
Sex (%)						
Male	12(30)	17(42.5)	0.532	10(25)	19(47.5)	0.443
Female	2(5)	9(22.5)	0.435	1(2.5)	10(25)	0.307
WBCs (Mean ± SD)10^3/µl	145.3 ± 113.3	136.9 ±109	0.526	143 ± 110	150 ± 114	0.639
Hb level (Mean ± SD) g/dl	7.1 ± 1.4	8 ± 1.6	0.732	8.3 ± 1.1	8.7 ± 1.6	0.892
Platelet (Mean ± SD) 10^3/µl	45.8 ± 15.6	47.3 ± 14.6	0.699	46.7 ± 13	48.2 ± 14	0.807
FAB, n (%)						
M0	3(20.0)	0	0.021	0	1(2.8)	0.438
M1	2(13.3)	5(20)	0.657	1(20)	8(22.8)	0.876
M3	1(6.6)	2(8)	0.865	0	6(17.1)	0.054
M4	3(20)	5(20)	0.986	1(20)	8(22.8)	0.876
M5	2(13.3)	4(16)	0.812	1(20)	3(8.6)	0.564
M6	0	2(8)	0.557	0	1(2.8)	0.438
M7	0	1(5.5)	0.732	1(20)	0	0.021
Karyotype, n						
Favourable	5(33.3)	14(56)	0.343	2(40)	17(48.6)	0.877
Intermediate, (normal karyotype)	8(53.3), 5(33.3)	4(16), 1(4)	0.025, 0.01	1(20), 1(20)	11(31.4), 5(14.2)	0.673
Adverse	2(13.3)	7(28)	0.310	2(40)	7(20.0)	0.562
Outcome, n (%)						
CR	4(26.7)	11(44)	0.531	1(20)	14(40)	0.356
Relapse	7(46.7)	8(32)	0.642	2(40)	12(34.3	0.761
Relapse	4(26.7)	6(24)	0.858	2(40)	9(25.7)	0.603

Table 3: Clinical and laboratory characteristics in AML patients in relation to methylation profile of sFRP.

(20% of methylated SFRP1 patients had flt3/ITD compared to 4% of unmethylated SFRP1 patients) (Figure 3).

The patients 60 years and younger with intermediate risk cytogenetics, Multivariate analysis indicated that the methylation status was independent adverse prognostic factor for relapse (P=0.024).

Monoclonal antibodies against CD25(IL2Rα) was positive (cutoff of positivity is >20%) in 12 patients (30%). 9(60%) of hypermethylated SFPR1 patients were positive for CD25 and only one Hypermethylated SFPR2 was positive for CD25. There was significant relationship between high expression of CD25 and hypermethylation of SFRP1 (P=0.023). There was significant relationship between Hypermethylated SFPR1 gene and increased expression of Cyto MPO (P=0.046) and CD117 (P=0.044) (Table 4). With regards to CD25, we analyzed the relationships between the level of CD25 expression and clinical (age, gender), laboratory (WBCs, Hb and platelets at diagnosis), FAB classification, cytogenetic risk groups, flt3/ITD and the outcome in patients with de novo AML. Regarding the clinical data there was no significant relationship with high expression CD25, there was 6 patients with overexpressed CD25 had flt3/ITD mutation. With regards to FAB classification and cytogenetic risk groups, there was significant relationship between high expression CD25 and M1 AML patients (P=0.043) and intermediate risk cytogenetics (P=0.026). Furthermore, significant relationship (P=0.022) was found between Relapse and high expression of CD25 (Table 5).

In multivariate analysis hypermethylated SFRP1 with intermediate risk cytogenetics were predictors for relapse in patients showed high expression of CD25 and flt3/ITD mutation (Table 6), the results given as odds ratio {95% Confidence interval}: 2.53{1.38-5.89}P=0.031, 3.1{1.81-6.1}P=0.028 respectively. Furthermore, hypermethylated SFRP1 with intermediate risk cytogenetics were predictors for relapse in patients with increased WBCs (leukocytosis>20 10^3/μl) 2.1{0.89-4.9} P=0.041.

Discussion

Hypermethylation of tumor suppressor genes is a common epigenetic event in cancers [19]. In our study, we analyzed the methylation profile of SFRP 1 ,2 genes promoters in 40 de novo AML patients and its correlation with karyotying, immunophenotypic features of blast cells, complete remission and relapse in addition to CD25 (IL2αR) expression. As over expression of CD25 was listed before as a standard poor prognostic factor in AML, so we studied the correlation between it and hypermethylated SFRP1,2 in AML patients. In this study we stated that hypermethylation of SFRP1,2 genes occurs with a percentage of 37.5% (15 out of 40 patients) and 12.5% (five out of 40 patients) in de novo AML patients, respectively. While none of the control samples showed hypermethylation. Veeck et al. stated that hypermethylation of SFRP-1 associated with poor prognosis in breast cancer patients [20,21]. SFRP gene promoter hypermethylation has been demonstrated in different hematopoietic malignancies like acute lymphoblastic leukemia (ALL) and Multiple myeloma (MM). Cooper et al. demonstrated that recombinant SFRP1 may be a

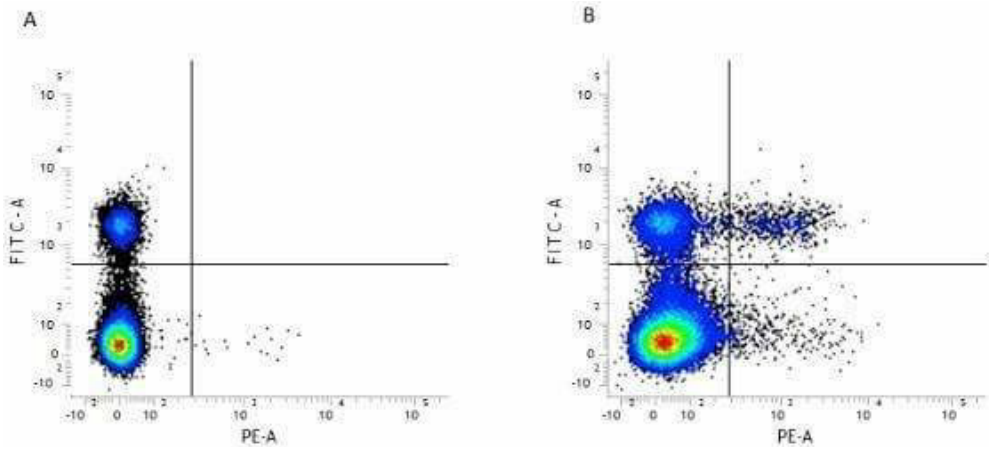

Figure 3: Double immunostaining of leukemia cells using anti-IL-2Rα and anti-CD33 antibodies in a representative patient with AML. The horizontal axis indicates the logarithmic scale of PE- conjugated anti-CD33 binding, and the vertical axis FITC-conjugated anti- IL-2Rα (CD25)binding. A- negative case. B- positive case (cut off of positivity is >20%).

Parameters	SFRP1				SFRP2	
	M	U	P	M	U	P
Number of patients,%	15(37.5)	25(62.5)	5(12.5)		35(87.5)	35(87.5)
Antigens						
HLA DR	11(73.3)	20(80)	0.945		32(91.4)	0.872
CD13	12(80)	23(92)	0.864	4(80)	35(100)	0.648
CD33	15(100)	24(96)	0.921	5(100)	31(88.5)	0.611
CD14	5(33.3)	6(24)	0.873	1(20)	5(14.2)	0.754
CD41	1(6.6)	1(4)	0.932	1(20)	2(5.7)	0.081
Cyto MPO	10(66.6)	7(28)	0.046	2(40)	15(42.8)	0.952
CD117	8(53.3)	6(24)	0.44	3(60)	13(37.1)	0.76
CD11c	4(26.6)	8(32)	0.84	2(40)	11(31.4)	0.625

Table 4: Immunophenotyping characteristics of AML patients in relation to methylation profile of sFRP.

Parameters	Po CD25	Negative CD25	P
Number of patients (%)	12(30)	28(70)	
Age(years), (Mean ± SD)	32.1 ± 12	34.6 ± 12.1	0.839
Sex, %			
Male	8(66.6)	18(64.3)	0.974
Female	5(33.3)	10(35.7)	0.878
WBCs (Mean ± SD) 103/µl	143.5 ± 115.2	144.8 ± 113	0.745
Hb level (Mean ± SD)g/dl	8.3 ± 1.5	8.4 ± 1.4	0.834
Platelet (Mean ± SD) 103/µl	46.3 ± 14.7	48.2 ± 13.8	0.73
FAB, n (%)			
M0	2(16.6)	1(3.6)	0.671
M1	5(41.6)	2(7.1)	0.043
M2	1(8.3)	6(21.4)	0.783
M3	1(8.3)	8(28.5)	0.531
M4	1(8.3)	8(28.5)	0.531
M5	1(8.3)	2(7.1)	0.911
M6	1(8.3)	1(3.6)	0.832
M7	0(0)	0(0)	0.993
Methylated SFRP1	9(60%)	6(30%)	0.023
Karyotype, n			
Favorable	4(33.3)	15(53.6)	0.064
Intermediate (normal karyotype)	6(50) , 4(33.3)	6(21.4) , 2(7.1)	0.026 , 0.01
Adverse	2(16.7)	7(25)	0.837
Flt3/ITD	6(50)	9(36)	0.083
Outcome, n(%)			
CR	4(33.3)	15(53.6)	0.068
Relapse	6(50)	6(21.4)	0.022
Death	2(16.6)	7(25)	0.713

Table 5: Clinical and laboratory characteristics in AML patients in relation to CD25 expression.

Parameters	Relapse				Death		
*Selected patient							
	Odds Ratio	95% CI	P		Odds Ratio	95%CI	P
Age	0.84	0.35-1.9	0.748		0.34	0.04-1.56	0.332
Positive CD25	2.53	1.38-5.8	0.031		1.06	0.46-2.48	0.713
WBCs	2.1	0.89-4.9	0.041		0.97	0.44-2.13	0.933
Flt3/ITD	3.1	1.81-6.1	0.028		0.83	0.38-1.9	0.811

Table 6: Multivariate analysis for relapse and death in patients with hypermethylated SFRP1 and intermediate cytogenetics risk.

therapeutic policy for patients with suppressed SFRP1 expression [22]. Follow up of hypermethylation status of SFRP genes may help in risk stratification and relapse prediction [23] and as epigenetic changes are pharmacologically reversible, so the demethylating factors may help in reactivation of SFRP1,2 function in patients with hypermethylation induced inhibition of those genes. And so SFRPs could used as biomarkers in monitoring the *in vivo* effects of demethylating factors [24,25].

Because of the 40 patients studied, 7 patients (17.5%) for SFRP1 gene and 6 patients (15%) for SFRP2 gene had one methylated allele, hemi-methylated patients may develop with time and it is an important poor prognostic factor. Hypermethylation of SFRP genes has been found in hematologic malignancies, as Pehlivan et al. stated that hypermethylated SFRP1 can lead to drug resistance in chronic myeloid leukemia(CML) patients treated with imatinib mesylate by inhibition of imatinib mesylate effect on BCR-ABL signaling pathway [26]. Wang found that hypermethylation of SPRP genes in myelodysblastic syndrome (MDS) patients is accompanied with bad prognosis and low survival rate [8]. In this study, we did not found any significant association between hypermethylation of SFRP genes and other prognostic parameters in AML, like WBC count and age. Also no significant relationship was found between hypermethylation of these genes and other parameters like hemoglobin, platelets count and sex. Our results showed that hypermethylation of SFRP genes occurs in all FAB subgroups involving M0, M1,M2, M3, M4 and M5, except M6 and M7. While the highest percentage of hypermethylation of SFRP-1 (P=0.021) was shown in M0 subgroup. Hou et al. observed that FAB M0 subtype patients had the highest percentage of hypermethylation of SFRPs genes, whereas patients with M4, M5 subtype had the lowest percentage [27]. Recently, it has been demonstrated that in AML patients with flt3/ITD mutation are associated with aberrations in Wnt signaling pathway and so we found that there was a significant relationship between patients with hypermethylated SFRP1 and flt3/ITD mutation (P=0.042). In the present study, the high percentage of hypermethylated SFRP1 gene (P=0.025) was found in patients with intermediate risk karyotyping. However, no significant association was found between hypermethylation of SFRPs and complete remission or death after induction therapy.

We also observed that hypermethylation of SFRPs has a poor prognostic factor in 60 years and younger patients with intermediate risk karyotyping. AML is a heterogeneous disease with divergent clinical characteristics, of which karyotyping is the most important prognostic factor. Unfortunately, the intermediate risk group includes a different group of patients either of normal karyotypes or with a variety of other mutations whose prognostic impact is uncertain. Therefore, better discovery of other prognostic factors in this group has been of specific interest in the recent years.

CD25+ CD34+ leukemic cells have been found to be chemotherapy resistant and initiate AML in xenograft models, suggesting a leukemic stem cells (LSC) biology [28]. IL2 increases survival, proliferation and chemotherapy resistance in CD25+ chronic lymphocytic leukemia (CLL) *in vitro* [29]. Regarding the results of CD25 expression in our results, twelve (30%) AML patients were CD25 positive. When comparing CD25+ and CD25- patients, there was a significant relationship between positive CD25 AML patients and intermediate risk karyotype(P=0.026). Moreover, expression of CD25 at diagnosis was a predictor of relapse 6 (50%) CD25+ patients have relapse compared with 6(21.4%) CD25- patients (P=0.022). Jan Cerny, 2013 stated that over expression of CD25 was a strong predictor of induction failure and relapse. Terwijn l 2009 reported that over CD25 expression (>10%) in young AML patients (<60 years) in retrospective study associated with a significant shorter survival and relapse free survival [30].

Our results found that the methylation status of SFRP1 together with CD25+ is a poor prognostic impact in the subgroup of 60 years and younger patients with intermediate risk karyotyping (also only normal karyotyping). And the combination of both markers (hypermethylated SFRP1 and CD25 over expression) together in the same patient may play a stronger role in the poor prognosis for relapse (P=0.031).

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