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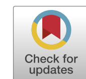
Epigenetic changes in FOXO3 and CHEK2 genes and their correlation with clinicopathological findings in myelodysplastic syndromes



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Abstract

Objectives/background: Myelodysplastic syndromes (MDSs) are a heterogeneous disease in terms of clinical course and response to therapy. Epigenetic changes are the primary mechanism of MDS pathogenesis. *FOXO3* and *CHEK2* genes play significant roles in normal cellular mechanisms and are also known as tumor suppressor genes. We aimed to clarify the correlation of epigenetic changes in these genes with clinicopathologic findings in MDS.

Methods: A total of 54 newly diagnosed MDS patients referred to Shariati and Firouzgar Hospitals (Tehran, Iran) were included in the study from 2013 to 2015, comprising the following cases: 26 with refractory cytopenia with unilineage dysplasia, 10 with refractory cytopenia with multilineage dysplasia, four refractory anemia with excess blasts-1 (RAEB-1), 11 refractory anemia with excess blasts-2 (RAEB-2), and three MDS associated with isolated deletion (5q-). Risk

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groups were determined according to the Revised International Prognostic Scoring System (IPSS-R). The methylation status of *CHEK2* and *FOXO3* promoters were determined by methylation-sensitive high-resolution melting analysis of sodium bisulfite-converted DNA. Expressions of *CHEK2, FOXO3,* and *GAPDH* were measured by quantitative real-time polymerase chain reaction and fold changes were calculated using the ΔΔCT method.
Results: Statistical analysis revealed no promoter methylation of *CHEK2* and *FOXO3* in healthy control specimens. *FOXO3* promoter methylation was associated with high-risk World Health Organization subgroups (p = .017), high-risk IPSS-R (p = .007), high-risk cytogenetics (p = .045), and more than 5% blasts in bone marrow (p = .001). *CHEK2* promoter methylation was correlated with more than 5% blasts in bone marrow (p = .009).
Conclusions: Promoter methylation of *CHEK2* and especially *FOXO3* is associated with adverse clinicopathological findings and disease progression in MDS.

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Introduction

Myelodysplastic syndromes (MDSs; OMIM 614286) represent a heterogeneous group of myeloid neoplasms defined by peripheral cytopenia, ineffective hematopoiesis, morphologic dysplasia in hematopoietic lineages, and genetic instability with a potential transformation to secondary acute myeloid leukemia (AML; OMIM 601626) [1]. The pathogenesis of MDS and its transformation to AML is not well determined yet. Epigenetic changes play the central role in MDS pathogenesis, such as in self-renewal of MDS-initiating cells, impaired differentiation of MDS cells, progression to AML, and apoptotic defects in MDS cells. The epigenetic changes, mostly promoter hypermethylation of tumor suppressor genes, are well known as a mechanism that contributes to the malignant phenotype of neoplasms. Recently, most studies have focused on epigenetic impairments of tumor suppressor genes in MDS [2,3]. Cell cycle checkpoint kinase 2 (CHK2, also known as ‘‘CHEK2") is a tumor suppressor protein encoded by the CHEK2 gene (OMIM 604373), located on the long arm of chromosome 22 (locus 22q12.1). CHEK2 is a multifunctional kinase that is responsible for monitoring cell cycle checkpoint control and DNA damage response [4]. The activated checkpoints delay cell cycle progression to facilitate DNA repair or induce cell death, and thereby impede the development of cancers [5]. Somatic mutations of the *CHEK2* gene have been found in subsets of diverse types of human cancers including breast, lung, vulva, colon, ovary, osteosarcoma, MDS, and lymphomas [4,5].

Forkhead box O3, also known as FOXO3 or FOXO3a, is a human protein encoded by the *FOXO3* gene (OMIM 602681) on chromosome 6 (6q21) and belongs to the O subclass of the Forkhead family of transcription factors. The FOXO family is a direct downstream target of AKT (Protein kinase B (PKB)) that has an essential regulatory role in diverse biological processes [6]. In the absence of active AKT, FOXOs localize to the nucleus where they regulate the transcription of genes involved in cell cycle arrest, apoptosis, and tumor suppression [7]. Many studies have revealed that post-translational modifications including phosphorylation, acetylation, ubiquitination, and arginine methylation have essential roles in the regulation of FOXO's activity [8—10].

Hypermethylation has been implicated as an essential and causal factor in the pathophysiology of several cancers. Despite this, the role of promoter hypermethylation of *FOXO3* has been studied rarely in hematologic neoplasms.

This study focused on whether the methylation of *FOXO3* and *CHEK2* changes their expression in patients with MDS. Correlations with clinicopathological findings, MDS subgroups, and risk stratification system (Revised International Prognostic Scoring System-[IPSS-R]) were also evaluated. We applied high-resolution melting (HRM) analysis and real-time polymerase chain reaction (RT-PCR) to assess methylation of *FOXO3* and *CHEK2* and their expression changes, respectively.

Materials and methods

Case selection

A total of 54 patients with MDS referred to Shariati and Firouzgar Hospitals (Tehran, Iran) were included in the study after obtaining informed consent (from 2013 to 2015). The diagnosis of MDS was made by peripheral blood and bone marrow findings, according to the World Health Organization (WHO) classification, as well as by conventional cytogenetic analysis. Clinical features of the patients are listed in Table 1. The study population included 54 primary MDS cases comprising 26 cases of refractory cytopenia with unilineage dysplasia, 10 cases of refractory cytopenia with multilineage dysplasia (RCMD), four cases of refractory anemia with excess blasts-1 (RAEB-1), 11 cases of refractory anemia with excess blasts-2 (RAEB-2), and three cases of MDS associated with isolated deletion (5q-).

Risk groups were determined according to the IPSS-R for all 54 cases (Table 1).

Conventional cytogenetic analysis

Conventional cytogenetic investigation was carried out for 54 patients. Chromosomes were prepared by a culture of bone marrow cells. Karyotypes were investigated on banded metaphases according to what has been previously described. Chromosome abnormalities were reported

Table 1 Patient demographics (n = 54).

Characteristics	Median (interquartile range)	*n*
Age (y)	60 (21.25)	
Sex		
Male		29
Female		25
Absolute neutrophil count ($\times10^9$/L)	2.33 (2.2)	
Hemoglobulin (g/dL)	9.45 (1.9)	
Platelets ($\times10^9$/L)	105 (115)	
Bone marrow blast (%)		
<5%		39
5%–9%		4
10%–19%		11
Cytogenetic risk category (IPSS-R)		
Very good		4
Good		36
Intermediate		8
Poor		3
Very poor		3
IPSS-R		
Very low		20
Low		13
Intermediate		8
High		4
Very high		9
WHO		
RCUD		26
RCMD		10
RAEB-1		4
RAEB-2		11
5q-		3

5q- = MDS associated with isolated deletion; IPSS-R = Revised International Prognostic Scoring System; RAEB-1 = refractory anemia with excess blasts-1; RAEB-2 = refractory anemia with excess blasts-2; RCMD = refractory cytopenia with multilineage dysplasia; RCUD = refractory cytopenia with unilineage dysplasia.

according to the International System for Human Cytogenetic Nomenclature [11].

Genomic DNA isolation

Genomic DNA was isolated from all samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations.

Bisulfite conversion and analysis of promoter methylation by methylation-sensitive HRM

To convert DNA, EpiTect Bisulfite Kit (Qiagen) was used following the manufacturer's specifications; 1 μg of DNA from each sample was used for conversion.

The methylation status of *CHEK2* and *FOXO3* promoters was determined by methylation-sensitive HRM (MS-HRM) of sodium bisulfite–converted DNA. The obtained modified DNA was analyzed by MS-HRM using two sets of primers for *CHEK2* and *FOXO3*. The MS-HRM was carried out as previously described [11].

We included in each assay 20 normal blood samples and EpiTect PCR Control DNA (Qiagen) as control.

RNA extraction and quantitative RT-PCR

RNA from mononuclear cells of all samples was extracted and converted to complementary DNA. The *CHEK2*, *FOXO3*, and *GAPDH* expressions were measured by quantitative RT-PCR (qRT-PCR) in triplicate on Rotor-Gene 6000 (Corbett Research, Mortlake, Australia). The fold changes were calculated using the $\Delta\Delta$CT method.

Statistical analysis

Statistical analysis was performed using SPSS version 17 (IBM, New York, NY, USA). Normality of variables was evaluated by the Kolmogorov–Smirnov test. The associations of methylation/unmethylation status with WHO subgroups, IPSS-R risk stratification, and cytogenetic risk subgroups were examined by the chi-square test. Associations of bone marrow blast percentage with methylation frequency and gene expression with promotor methylation/unmethylation were analyzed by the Mann–Whitney *U* test. For all analyses, the p values were two tailed, and all p values ≤ 0.05 were considered statistically significant.

Results

Demographics of patients

A total of 54 patients with a median age of 61.56 were included in this study. Demographic and hematologic findings are presented in Table 1.

Methylation analysis

Promoter methylation of *FOXO3* and *CHEK2* genes

The methylation profile of *FOXO3 and CHEK2* genes in patients with MDS and controls was studied by MS-HRM on bisulfite-treated DNA. All melting curves were compared individually with methylated/unmethylated commercial controls (EpiTect; Qiagen).

The number of methylated cases in the MDS group was 30 (55.6%) and eight (14.8%) for *CHEK2* and *FOXO3*, respectively. Both genes were determined to be unmethylated in all normal samples.

In our patients, all the subtypes included in this study showed the *CHEK2* methylation pattern (p = .368), whereas only RCMD, RAEB-1, and RAEB-2 subgroups showed the *FOXO3* methylation pattern (p = .017). However, a higher frequency of the two-gene hypermethylation was encountered in patients with RAEB-1 and RAEB-2 subtypes as compared with other subtypes (Table 2; chi-square analysis).

CHEK2 methylation was studied in different IPSS-R subtypes (chi-square analysis). Aberrant methylation of *CHEK2* in different subtypes showed a higher incidence of methylated *CHEK2* in the IPSS-R high-risk subgroup and a lower incidence of methylated *CHEK2* in the IPSS-R very low and low subgroups, but these results were not statistically significant (p = .45). This analysis showed a significant association between *FOXO3* promoter methylation and IPSS-R high-risk subgroups (p = .007; Table 3). Data also illustrated a significant association between cytogenetic and *FOXO3* methylation (p =.045) and bone marrow blast percentage and *FOXO3* methylation (p = 0.001) (Table 4 and Fig. 1).

Statistical analysis comparing *CHEK2* and *FOXO3* methylation with clinical factors revealed a significant association between methylation and bone marrow blast percentage of more than 5% (p = .009 and 0.001, respectively; Fig. 1).

Messenger RNA expression by RT-PCR

To investigate the association between DNA methylation and messenger RNA (mRNA) expression, mRNA levels were detected using qRT-PCR in the MDS and control samples (Fig. 2).

Discussion

MDS is a highly heterogeneous disease in terms of pathogenesis and response to treatment. Epigenetic changes in tumor suppressor genes is likely to play a role in the pathogenesis and progression of MDS. The *FOXO3A* gene product is a transcription factor which exhibits its effects by regulation of expression of proapoptotic genes such as FasL and TRAIL [12,13]. Our study revealed *FOXO3* promoter methylation in 14.8% of patients, which significantly differs among WHO subgroups (p = .015). The more aggressive the subgroups, the more promoter methylation were detected. Promoter methylation frequency decreased from RAEB-2 > RAEB-1 > RCMD, respectively. Other subgroups showed no evidence of *FOXO3* promoter methylation. Like the correlation with WHO subgroups, there was also a significant statistical association between *FOXO3* promoter methylation levels and IPSS-R stratification subgroups, especially in patients with *FOXO3* promoter methylation. High-risk IPSS-R patients (poor and very poor) were associated with higher promoter methylation (p = .022). Levels of promoter methylation are well correlated with diminished gene expression in patients who demonstrate *FOXO3* promoter methylation (p = .001). However, this result should be accepted with caution because its effect size is minimal (−0.48) and 95% confidence interval for effect size contained the 0 (statistical nonsignificance). Unexpectedly, 12 of 18 patients with low levels of *FOXO3* mRNA showed no evidence of promoter methylation. This could be a result of genetic deletions and/or effects of noncoding RNAs. There were no associations of *FOXO3* mRNA levels with WHO subgroups, IPSS-R stratification, and other clinicopathological findings in the total cohort. To the best of our knowledge, this is the first report of the association between *FOXO3* promoter methylation and high-risk MDS. In line with our findings, other tumors such as hepatocellular and ovarian carcinomas with low expression of *FOXO3* were associated with poor prognosis and high-risk disease [14,15]. However, Santamaria and

Table 2 *CHEK2* and *FOXO3* methylation/unmethylation status in WHO subgroups.

Gene	WHO subtypes (n)					Chi square/p value
	RCUD (26)	RCMD (10)	RAEB-1 (4)	RAEB-2 (11)	5q- (3)	
FOXO3						0.017
Met (%)	0	20	25	45.45	0	
Unmet (%)	100	80	75	54.55	100	
CHEK2						0.368
Met (%)	42.3	70	75	72.7	33.3	
Unmet (%)	57.7	30	25	27.3	66.7	

5q- = MDS associated with isolated deletion; RAEB-1 = refractory anemia with excess blasts-1; RAEB-2 = refractory anemia with excess blasts-2; RCMD = refractory cytopenia with multilineage dysplasia; RCUD = refractory cytopenia with unilineage dysplasia; WHO = World Health Organization.

Table 3 *CHEK2* and *FOXO3* methylation/unmethylation status in the IPSS-R prognostic category.

Gene	IPSS-R prognostic category (*n*)					Chi square/*p* value
	Very low (20)	Low (13)	Intermediate (8)	High (4)	Very high (9)	
FOXO3						0.007
Met (%)	0	7.7	12.5	50	44.4	
Unmet (%)	100	92.3	87.5	50	55.6	
CHEK2						0.455
Met (%)	45	46.1	75	75	66.6	
Unmet (%)	55	53.9	25	25	33.4	

Note: IPSS-R = Revised International Prognostic Scoring System.

Table 4 *CHEK2* and *FOXO3* methylation/unmethylation status in cytogenetic risk subgroups.

Gene	Cytogenetic risk subgroups (*n*)					Chi square/*p* value
	Very good (4)	Good (36)	Intermediate (8)	Poor (3)	Very poor (3)	
FOXO3						0.045
Met (%)	0	8.3	25	66.6	33.3	
Unmet (%)	100	91.7	75	33.4	66.7	
CHEK2						0.731
Met (%)	50	50	50	66.6	66.6	
Unmet (%)	50	50	50	33.4	33.4	

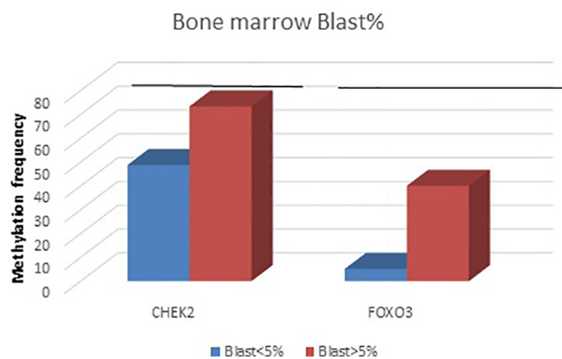

Fig. 1 Association of methylation frequency of *CHEK2* and *FOXO3* genes with bone marrow blast percentage.

Fig. 2 *CHEK2* expression is reduced in patients with promotor methylation ($p < .001$). Met; methylation. Unmet; unmethylation.

colleagues reported that high *FOXO3* mRNA expression is associated with adverse prognosis and increased relapse risk in AML patients with normal cytogenetics [16]. Although *FOXO3* is a tumor suppressor, reasons for paradoxical effects of *FOXO3* overexpression and adverse prognosis in AML are not well known, and further investigations are needed. High expression of *FOXO3* did not mean higher activity of the FOXO3 protein. Some studies show that FOXO3 hyperphosphorylation and subsequent abnormal intracellular localization and functional disruption in AML and MDS cell lines and patient-derived leukemic cells can be corrected by treatment with low-dose hypomethylating agents [8,9]. In the case of MDS, hypomethylating agents also induced expression and promoter demethylation of the *FOXO3* gene [9]. Collectively, these data emphasize the role of *FOXO3* aberrations in the pathogenesis and progression of MDS.

CHEK2 belongs to the DNA damage response (DDR) system (ATM/CHEK2-p53 pathway), which is activated by ATM and results in cell cycle arrest or apoptosis due to DNA damage [17,18]. CHEK2 showed tumor suppressor activities, and some studies demonstrate epigenetic changes in the *CHEK2* gene during MDS progression [19]. Our findings revealed promoter methylation in 55.6% of MDS specimens. Hopfer and colleagues also demonstrated *CHEK2* promoter methylation in 34+ progenitor cells of 13 patients with MDS [19]. However, analysis of non-Hodgkin lymphoma patients by Tort et al. revealed no evidence of *CHEK2* promoter methylation [20]. Some studies demonstrated the role of *CHEK2* promoter methylation in tumor progression [21,22]. A recent study by Popp and colleagues revealed abnormal DNA damage response and distorted and diminished expression of CHEK2 protein in MDS and AML samples. They suggest the main role for the Ataxia-telangiectasia-mutated (ATM) and

ataxia telangiectasia and Rad3-related (ATR) pathway and its downstream proteins in the progression of MDS [23]. In our study, the bone marrow blast infiltration (>5%) was associated with *CHEK2* promoter methylation ($p = .009$). There was also a considerable association between *CHEK2* promoter methylation and reduced gene expression ($p < .001$). Other clinicopathological findings showed no associations with *CHEK2* promoter methylation and expression.

In conclusion, our results provide evidence for epigenetic silencing of *FOXO3* and *CHEK2* genes in MDS samples when compared with healthy specimens. These alterations in *FOXO3* are associated with MDS progression and risk subgroups, whereas *CHEK2* promoter methylation is associated with blast percentage in the bone marrow. Further evaluations are needed to elucidate the exact role of epigenetic changes in *FOXO3* and *CHEK2* genes with respect to pathogenesis and progression of MDS.

Declaration of Competing Interest

The authors report no conflicts of interest.

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