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Activation-induced cytidine deaminase expression in patients with myelodysplastic syndrome and its relationship with prognosis and treatment

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Background/aim: Activation-induced cytidine deaminase (AID) enables antibody diversity in B lymphocytes. It may also have an effect on MDS pathogenesis by causing somatic mutations and by inducing epigenetic changes in myeloid cells. This study aimed to compare AID expression of MDS patients with healthy controls, of MDS patients in different risk groups, and of MDS patients according to their treatment.

Materials and methods: Total RNA was isolated and complementary DNA (cDNA) was transcribed from the peripheral blood samples of MDS patients and healthy controls. AID and the reference gene HPRT1 were analyzed using quantitative real-time PCR (QRT-PCR). AID expression relative to HPRT1 was calculated. Patients were classified into "lower risk" and "higher risk" subgroups according to their initial IPSS and IPSS-R scores and their MDS subtypes at the time of study. Patients were also divided into two groups based on receiving treatment with hypomethylating agents. AID expressions of different groups were compared using the Mann–Whitney U test.

Results: Thirty MDS patients and thirty healthy controls were included. AID expression in MDS patients was significantly higher compared to healthy controls ($p < 0.001$). There was no significant difference in AID expression of "lower risk" and "higher risk" subgroups of patients. Patients that received hypomethylating agents did not have a significant difference in AID expression compared with patients that did not receive hypomethylating agents.

Conclusion: AID expression is increased in the peripheral blood of MDS patients compared to healthy controls. However, AID expression is not significantly different in "lower risk" and "higher risk" subgroups and in patients treated with hypomethylating agents. Increased AID expression may be an early step in MDS pathogenesis.

Key words: Activation induced cytidine deaminase, hypomethylating agents, myelodysplastic syndrome

1. Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of disorders characterized by ineffective and impaired hematopoiesis in one or more myeloid cell lineages of bone marrow. It is associated with cytopenias in the peripheral blood and an increased risk of transformation to acute myeloid leukemia (AML) [1,2]. Myelodysplastic syndrome can arise de novo (primary) or secondary to ionizing radiation, toxin, or chemotherapeutic drug exposure [1].

Somatic mutations and epigenetic changes such as DNA methylation play a role in MDS pathogenesis [3]. Abnormal DNA methylation was detected in the promoters of tumor suppressor genes in MDS [4]. DNA methyltransferase (DNMT) 3A mutations were detected in MDS patients [5]. These mutations were associated with downregulation of hematopoietic stem cell differentiation, poor prognosis and rapid progression to AML [6,7]. One of the ten eleven translocation (TET) family proteins, TET2 catalyzes conversion of 5-methylcytosine to 5-hydroxymethylcytosine and its loss of function mutations are associated with DNA hypermethylation and gene silencing [8,9]. TET2 mutations were detected in 20%–25% of MDS patients [10,11]. While some studies associated TET2 mutations with a better prognosis, their prognostic significance was unproven in other studies [12,13].

Activation-induced cytidine deaminase (AID), an enzyme which catalyzes conversion of cytosine to uracil[1], was originally described as a B lymphocyte specific factor [14]. AID enables generation of antibody diversity in B lymphocytes by the mechanisms of somatic hypermutation, isotype switching, and gene conversion [15,16]. However, AID may also facilitate tumorigenesis by inducing proto-oncogene mutations, chromosome breaks and translocations in other cell lineages [17].

[1] Activation-Induced Cytidine Deaminase (2000). Online Mendelian Inheritance in Man [online]. Website http://omim.org/entry/605257. [accessed 18.02.2016]

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A study by Rai et al. demonstrated that AID could catalyze conversion of 5-methylcytosine to thymine by deamination which could lead to DNA demethylation in zebrafish [18]. Another study by Popp et al. found increased DNA methylation in AID deficient primordial mouse germ cells and hypothesized the possible function of AID in epigenetic reprogramming [19]. Another study by Kumar et al. demonstrated AID's possible role in deletion of epigenetic memory of pluripotent stem cells, by its potential function in DNA demethylation. According to this study, AID seemed to have a fundamental role in the stabilization and reprogramming of these cells [20].

Thus, we hypothesized that AID expression could have a role in the pathogenesis of MDS by inducing point mutations and chromosomal translocations and/or by interacting with epigenetic mechanisms of DNA methylation and demethylation. The aim of this study is to compare AID mRNA expression levels of MDS patients with healthy controls, AID expression levels of MDS patients in different risk groups and AID expression levels of patients that received hypomethylating agents with those that did not receive this treatment.

2. Materials and methods

2.1. MDS patients and healthy control group

We enrolled 30 MDS patients who visited the outpatient clinic or who were admitted to the inpatient ward of Istanbul University, Istanbul Faculty of Medicine, Division of Hematology between December 2016 and March 2017 in this study. We took their blood samples. We also obtained blood samples of an age-matched healthy control group of thirty people. All participants in both the patient group and healthy control group provided informed consent in the format required by the institutional research committee.

We recorded the history, physical examination findings, complete blood count, bone marrow biopsy, and cytogenetic findings of MDS patients at the time of diagnosis. We analyzed MDS subtypes both at the time of diagnosis and at the time of sample collection because transformation to other subtypes occurred in some patients. We determined MDS subtypes according to the 2008 classification of World Health Organization (WHO) [21]. We also recorded the International Prognostic Scoring System (IPSS) and the Revised International Prognostic Scoring System (IPSS-R) scores of the patients at the time of diagnosis [22,23]. We then determined the patients that were treated with hypomethylating agents (azacitidine and decitabine). Next, we classified the patients into "lower risk" and "higher risk" subgroups according to the IPSS and IPSS-R scores at the time of diagnosis. Patients in "low" and "intermediate-1" categories according to IPSS were classified as "lower risk" and patients in "intermediate-2" and "high" categories according to IPSS were classified as "higher risk". Patients in "very low", "low", and "intermediate" categories according to IPSS-R were classified as "lower risk," and patients in "high" and "very high" categories according to IPSS-R were classified as "higher risk". Afterward, we made another risk stratification according to MDS subtype at the time of sample collection. Patients that had refractory anemia with excess blasts-1 (RAEB-1) and refractory anemia with excess blasts-2 (RAEB-2) were classified as "higher risk" and all other MDS subtypes were classified as "lower risk".

Our study protocol received the approval of the institutional research committee. All procedures that we performed in this study were in accordance with the ethical standards of the institutional research committee and with 1964 Helsinki declaration and its later amendments.

2.2. Determination of AID mRNA expression levels in peripheral blood samples

We collected the peripheral blood samples from patients and healthy controls in sterile tubes containing etylenediaminetetraacetic acid (EDTA). We isolated total RNA using High Pure RNA Isolation Kit (Roche), in accordance with the instructions of the manufacturer. We measured the density of RNAs that were obtained from the samples spectrophotometrically using NanoDrop 2000c (Thermo Scientific, USA). We transcribed complementary DNA (cDNA) from 100 ng of total RNA using Fermentas, RevertAid First Strand cDNA Synthesis Kit (Roche). We performed TaqMan-based quantitative real-time PCR (QRT-PCR) using a LightCycler®TaqMan Master Kit (Roche Diagnostics) and we used a LightCycler® 480II instrument (Roche Diagnostics, Mannheim, Germany) to analyze the target gene AID and the reference gene HPRT1 (hypoxanthine phosphoribosyl transferease-1). Primers and probes were designed at the Universal Probe Library website of Roche. Primers specific to the target gene AID were as follows: forward: 5′-TGGACACCACTATGGACAGC-3' and reverse: 5'-GCGGACATTTTTGAATTGGT-3'. Primers specific to the reference gene HPRT1 were as follows: forward: 5'-GACCAGTCAACAGGGGACAT-3' and reverse: 5′-GTGTCAATTATATCTTCCACAATCAAG-3′.

To calculate the relative expression, we obtained C_T values of AID and HPRT for all samples. We obtained the normalized expression for each sample by subtracting the C_T of HPRT1 from the C_T of AID of the same sample. This was designated as ΔC_T. Afterward, we transformed this value using $2^{-(\Delta CT)}$ formula [24].

2.3. Statistical analysis

Firstly, we reported AID mRNA expression levels of MDS patients and healthy controls using mean and standard deviation. Next, we checked these expression levels for normal distribution. Due to their nonnormal distribution, we then reported AID mRNA expression levels of MDS patients and healthy controls using median, first, and third quartile values. Next, we used the nonparametric Mann–Whitney U test to compare AID mRNA expression levels in both groups. We then checked AID mRNA expression levels of "lower risk" and "higher risk" MDS subgroups according to IPSS and IPSS-R scores at the time

of diagnosis and according to MDS subtypes at the time of sample collection for normal distribution. None of the subgroups demonstrated a normal distribution. We then reported AID mRNA expression levels of "lower risk" and "higher risk" subgroups using median, first, and third quartile values. Next, we used the nonparametric Mann–Whitney U test to compare AID mRNA expression levels in "lower risk" and "higher risk" subgroups. After that, we checked AID mRNA expression levels of patients that received hypomethylating agents and those that did not receive hypomethylating agents for normal distribution. Due to absence of normal distribution, we reported AID mRNA expression levels of "hypomethylating agent" and "no hypomethylating agent" subgroups using median, first, and third quartile values. After that, we used nonparametric Mann–Whitney U test to compare AID mRNA expression levels in these two subgroups. Finally, we separately compared the AID expressions of "hypomethylating agent" subgroup and "no hypomethylating agent" subgroup with the healthy control group.

Hypotheses were two tailed with $p < 0.05$ accepted as the cutoff for statistical significance. We performed all statistical analyses using SPSS 17.0.

3. Results

3.1. General characteristics of patients and healthy controls

We enrolled thirty patients and thirty healthy controls in our study. In the patient group, there were 15 male and 15 female patients. Mean age of the patients was 63.03 ± 9.67. In the healthy control group, there were 15 males and 15 females. Mean age of the healthy control group was 59.10 ± 9.39. Characteristics of patients and healthy controls are demonstrated in Tables 1 and 2, respectively. Conventional cytogenetic analysis was performed in 27 patients (90%), cytogenetic analysis was not performed in 3 patients (10%) because a sufficient number of metaphases was not obtained. Twenty-two patients (73.33%) had normal karyotype. 1 patient (3.33%) had a karyotype of 46, XX, del(5q), 1 patient (3.33%) had a karyotype of 46, XX, del(20q), 1 patient had a karyotype of 45, X-Y (3.33%), 1 patient had a karyotype of 45, XX, -7,der(14) (3.33%), 1 patient had a karyotype of 46, XY, der(1),der(2),der(20) (3.33%).

We classified thirty MDS patients into subtypes according to 2008 classification of World Health Organization. Their MDS subtypes at the time of diagnosis: 9 patients (30%) had RCUD (refractory cytopenia of unilineage dysplasia), 1 patient (3.33%) had RARS (refractory anemia with ring sideroblasts), 8 patients (26.67%) had RCMD (refractory cytopenia of multilineage dysplasia), 1 patient (3.33%) had MDS with 5q deletion, 4 patients (13.33%) had RAEB-1 (refractory anemia with excess blasts-1), and 7 patients (23.33%) had RAEB-2 (refractory anemia with excess blasts-2). MDS subtypes of patients at the time of sample collection: 7 patients (23.33%) had RCUD, 5 patients (16.67%) had RCMD, 1 patient (3.33%) had MDS with 5 q deletion, 7 patients (23.33%) had RAEB-1, and 10 patients (33.33%) had RAEB-2.

Nineteen (63.33%) MDS patients received hypomethylating agents as treatment. Eighteen (60%) of these patients received azacitidine, 5 (16.67%) of them received decitabine. Four of these patients received both treatments. The remaining 11 patients received only supportive treatment such as erythropoietin and transfusions.

We classified IPSS and IPSS-R scores at the time of diagnosis for 27 patients, in 3 patients, these scores were not calculated due to the lack of cytogenetic analysis. Mean IPSS was 0.72 ± 0.79, with a minimum score of 0 and a maximum score of 3. Twenty-two patients were in the "lower risk" subgroup and 5 patients were in the "higher risk" subgroup. Mean IPSS-R was 3.57 ± 1.88 with a minimum score of 1 and a maximum score of 8.5. Nineteen patients were in the "lower risk" subgroup and 8 patients were in the "higher risk" subgroup. Next, we classified the patients into "higher risk" and "lower risk" subgroups according to their MDS subtypes at the time of sample collection. Thirteen patients were in the "lower risk" subgroup and 17 patients were in the "higher risk" subgroup.

3.2. Comparison of AID expression in MDS patients and healthy controls

Mean AID mRNA level in the peripheral blood of the 30 MDS patients was 0.034410 ± 0.026487 and the mean AID mRNA level of 30 healthy controls was 0.006060 ± 0.003260 (Figure 1). The distribution of AID expression of both MDS patients and healthy controls was nonnormal. Since both groups demonstrated a nonnormal distribution, we compared AID expression of MDS patients and healthy controls using the nonparametric Mann–Whitney U test. AID mRNA levels in MDS patients (median: 0.021906; Q1: 0.015775–Q3: 0.057967) was higher compared to healthy controls (median: 0.004792; Q1:0.003569–Q3: 0.009088). The Mann–Whitney U test indicated that this difference was statistically significant ($U = 47$, $p < 0.001$).

3.3. Comparison of AID expression in "lower risk" and "higher risk" subgroups according to IPSS, IPSS-R and MDS subtypes

According to IPSS, the mean AID mRNA level was 0.039728 ± 0.028614 in the "lower risk" subgroup, and was 0.022977 ± 0.010285 in the "higher risk" subgroup (Figure 2). Neither the "lower risk" subgroup nor the "higher risk" subgroup demonstrated a normal distribution. Therefore, we used the nonparametric Mann–Whitney U test to compare these subgroups. AID mRNA expression in the "lower risk" subgroup (median: 0.027776; Q1: 0.016477–Q3: 0.067757) and the "higher risk" subgroup (median: 0.017579; Q1: 0.014563–Q3: 0.034091) were compared and the Mann–Whitney U test indicated that this difference was not statistically significant. ($U = 39.5$, $p = 0.333$).

Table 1. Clinical characteristics of the myelodysplastic syndrome patients.

Patient	Age and sex	MDS subtype at the time of diagnosis	Cytogenetic analysis at the time of diagnosis	IPSS score at the time of diagnosis (risk group)	IPSS-R score at the time of diagnosis (risk group)	MDS subtype at the time of study	Treatment received
1	58, female	RCUD	46, XX	0 (lower)	2 (lower)	RAEB-2	Azacitidine
2	73, female	RAEB-2	46, XX	1 (lower)	3.5 (lower)	RAEB-2	Azacitidine
3	69, male	RAEB-2	46, XY	2 (higher)	5.5 (higher)	RAEB-2	Azacitidine and decitabine
4	57, male	RCMD	46, XY, der(1), der(2), der(20)	1 (lower)	3.5 (lower)	RAEB-1	Azacitidine
5	42, male	RAEB-1	46, XY	1 (lower)	5.5 (higher)	RAEB-1	Azacitidine
6	72, male	RAEB-2	-	-	-	RAEB-2	Azacitidine
7	78, female	RCMD	46,XX/47,XX+8,der21 (p11),del(20q)	1.5 (higher)	4 (lower)	RCMD	Supportive
8	69, male	RAEB-2	46, XY	2 (higher)	5 (higher)	RAEB-2	Azacitidine
9	56, male	RAEB-1	-	-	-	RAEB-1	Decitabine
10	73, female	RAEB-1	46, XX	1 (lower)	5.5 (higher)	RAEB-1	Azacitidine and decitabine
11	61, male	RCUD	-	-	-	RCUD	Supportive
12	62, male	RCMD	46, XY	0.5 (lower)	4 (lower)	RCMD	Supportive
13	58, male	RAEB-1	45X,-Y	0.5 (lower)	3.5 (lower)	RAEB-1	Azacitidine and decitabine
14	63, male	RCMD	46, XY	0.5 (lower)	2.5 (lower)	RCMD	Supportive
15	85, male	RCUD	46, XY/46, XY, t(5;21) (q33;q22)	0.5 (lower)	2.5 (lower)	RCUD	Supportive
16	62, female	5q deletion	46, XX, del(5q)	0.5 (lower)	2.5 (lower)	5q deletion	Supportive
17	57, female	RARS	46, XX	0 (lower)	2.5 (lower)	RAEB-1	Azacitidine
18	63, male	RCMD	46, XY	0 (lower)	2.5 (lower)	RAEB-1	Azacitidine
19	53, female	RCMD	46, XX	0 (lower)	1.5 (lower)	RCMD	Azacitidine
20	78, female	RCMD	46, XX, del(20q)	0 (lower)	1.5 (lower)	RCMD	Supportive
21	39, female	RAEB-2	45, XX,-7 der(14)	3 (higher)	8.5 (higher)	RAEB-2	Supportive
22	79, male	RAEB-2	46, XY	1 (lower)	5.5 (higher)	RAEB-2	Azacitidine and decitabine
23	70, female	RCMD	46, XX	0.5 (lower)	2.5 (lower)	RAEB-2	Azacitidine
24	71, female	RCUD	46, XX	0 (lower)	1 (lower)	RCUD	Supportive
25	69, female	RCUD	46, XX	0 (lower)	2 (lower)	RCUD	Azacitidine
26	64, female	RCUD	46, XX	0 (lower)	1 (lower)	RCUD	Azacitidine
27	70, female	RCUD	46, XX	0.5 (lower)	2 (lower)	RCUD	Supportive
28	69, female	RCUD	46, XX, del(20q)	0 (lower)	6.5 (higher)	RAEB-2	Azacitidine
29	44, male	RCUD	46, XY	0 (lower)	4 (lower)	RCUD	Supportive
30	73, male	RAEB-2	46, XY	2 (higher)	6 (higher)	RAEB-2	Azacitidine

Abbreviations: RAEB-1: Refractory anemia with excess blasts-1, RAEB-2: Refractory anemia with excess blasts-2, RARS: Refractory anemia with ring sideroblasts, RCMD: Refractory cytopenia of multilineage dysplasia, RCUD: Refractory cytopenia of unilineage dysplasia

According to IPSS-R, mean AID mRNA level was 0.040604 ± 0.028518 in the "lower risk" subgroup, and was 0.027178 ± 0.021065 in the "higher risk" subgroup (Figure 3). Neither the "lower risk" subgroup nor the "higher risk" subgroup demonstrated a normal distribution. Therefore, we used the nonparametric Mann–Whitney U test to compare these subgroups. AID mRNA expression in the "lower risk" subgroup (median: 0.030606; Q1: 0.016688–Q3: 0.066985) and the "higher risk" subgroup (median: 0.017162; Q1: 0.015570–Q3: 0.034736) were compared and the Mann–Whitney U test indicated that this difference was not statistically significant. (U = 56; p = 0.288).

According to MDS subtypes of the patients at the time of sample collection, mean AID mRNA level was 0.043139 ± 0.030846 in the "lower risk" subgroup and 0.027735 ±

Table 2. Characteristics of the healthy controls.

Healthy control	Age and sex
1	55, male
2	57, female
3	57, female
4	71, female
5	55, female
6	54, female
7	47, female
8	56, female
9	56, female
10	76, female
11	49, female
12	47, male
13	67, male
14	68, female
15	46, female
16	45, female
17	62, male
18	62, male
19	43, male
20	65, male
21	62, male
22	66, female
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27	70, male
28	75, male
29	41, female
30	57, male

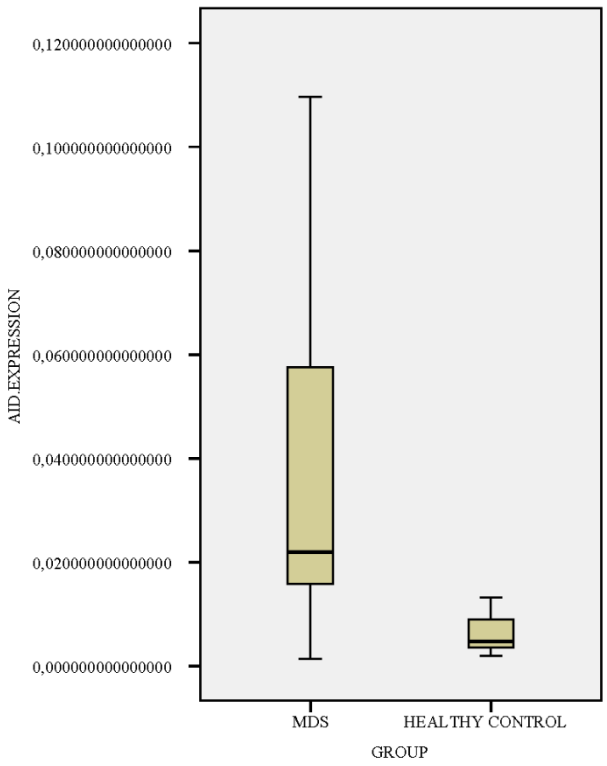

Figure 1. AID mRNA expression levels of MDS patients and healthy controls.

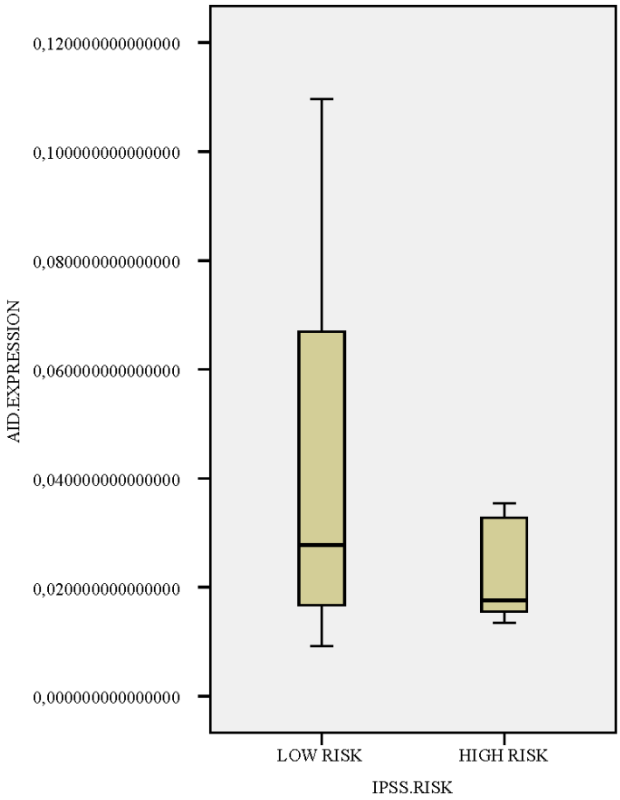

Figure 2. AID mRNA expression levels of "lower risk" and "higher risk" MDS patients according to their initial IPSS score.

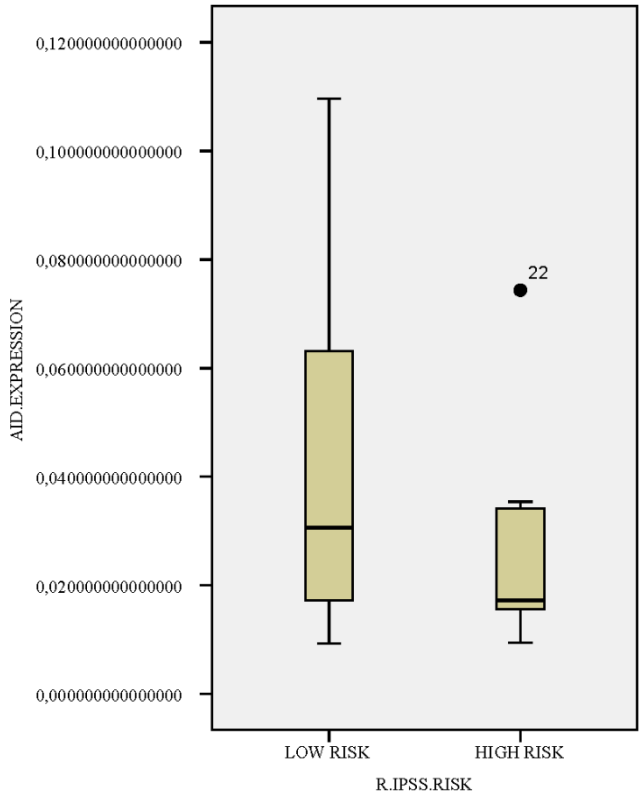

Figure 3. AID mRNA expression levels of "lower risk" and "higher risk" MDS patients according to their initial IPSS-R score.

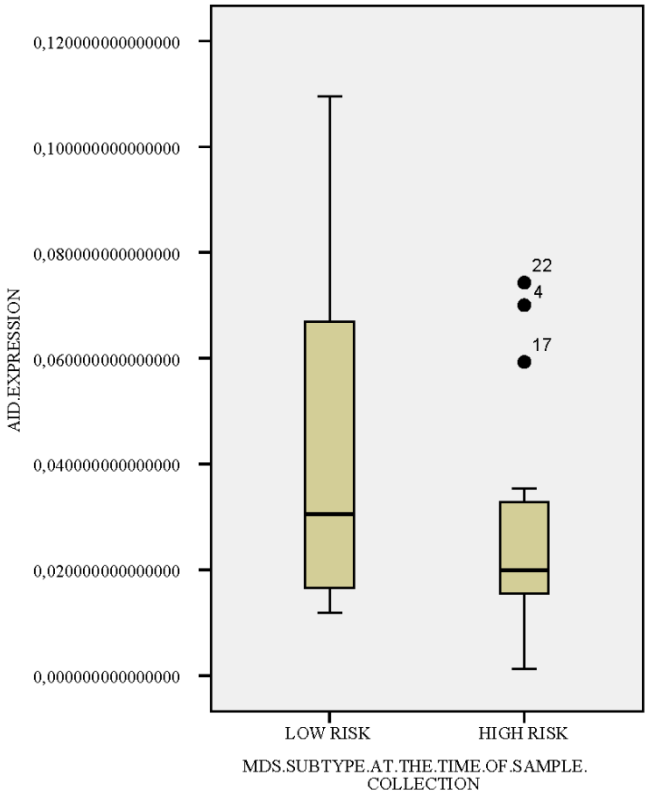

Figure 4. AID mRNA expression levels of "lower risk" and "higher risk" MDS patients according to their MDS subtype at the time of the study.

0.021183 in the "higher risk" subgroup (Figure 4). Neither the "lower risk" subgroup nor the "higher risk" subgroup demonstrated a normal distribution. Therefore, we used the nonparametric Mann–Whitney U test to compare these subgroups. AID mRNA expression in the "lower risk" subgroup (median: 0.030606; Q1: 0.016265–Q3: 0.069514) and the "higher risk" subgroup (median: 0.019915; Q1:0.015570–Q3:0.034091) were compared, and the Mann–Whitney U test indicated that this difference was not statistically significant (U = 81; p = 0.217).

3.4. Comparison of AID expression in patients that received hypomethylating agents and patients that did not receive hypomethylating agents

Mean AID mRNA level of the patients that received hypomethylating agents (azacitidine and/or decitabine) was 0.032146 ± 0.027421. Mean AID mRNA level of patients that did not receive hypomethylating agents was 0.038319 ± 0.025584 (Figure 5). Neither group demonstrated a normal distribution; therefore, we used the nonparametric Mann–Whitney U test to compare them. AID mRNA expression in the "hypomethylating agent" group (median: 0.020263; Q1: 0.015570–Q3: 0.035380) and the "no hypomethylating agent" (median: 0.022020; Q1: 0.015843–Q3: 0.066985) group were compared and the Mann–Whitney U test indicated that this difference was not statistically significant (U = 90; p = 0.533).

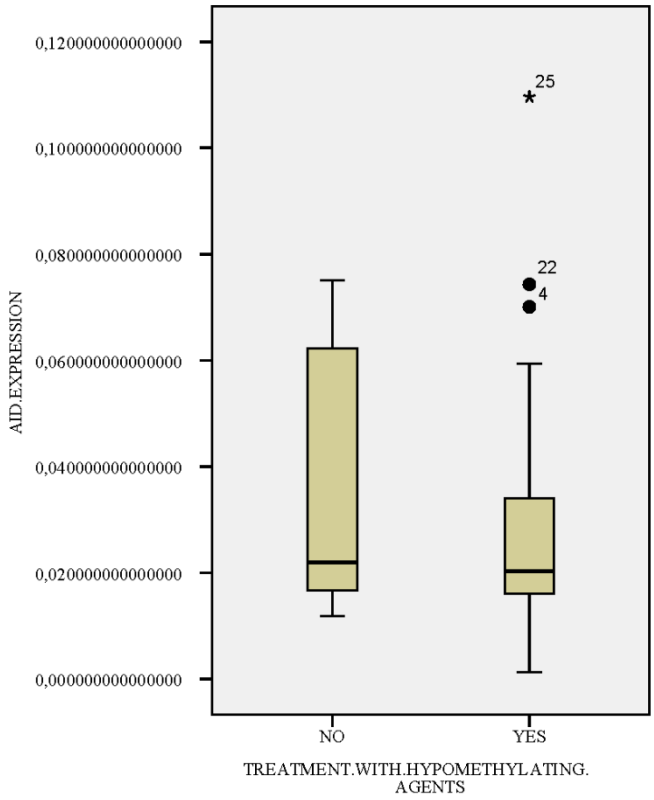

Figure 5. AID mRNA expression levels of patients that received and that did not receive hypomethylating agents.

When we compared AID expression of each of these subgroups separately with the healthy control group, we found that both subgroups had a significantly higher AID expression ($U = 44$; $p < 0.001$ for patients that received hypomethylating agents and $U = 3$; $p < 0.001$ for patients that did not receive hypomethylating agents) compared to the healthy control group.

4. Discussion

Increased AID mRNA expression may have a role in the pathogenesis of MDS, a premalignant disease with an increased risk of leukemic transformation, by inducing mutations and cytogenetic abnormalities.

AID is known to cause DNA mutations and double strand breaks. This enables antibody diversity in B lymphocytes, but in other cell lines it can lead to tumor formation by inducing chromosome translocations and protooncogene mutations [17].

In a study by Marusawa et al., constitutive and excessive expression of AID in transgenic mice was demonstrated to cause lymphoma by inducing mutations in T cell receptors and in genes such as myc. In the same study, there was also an increase in dysgenetic lesions in lungs and in epithelial neoplasia of liver and stomach [25]. AID expression was present in gastric tissue infected with *Helicobacter pylori*, and a relationship between epithelial neoplasia and increased AID expression was proposed [26]. AID expression was associated with many different types of hematological malignancies such as Burkitt leukemia/lymphoma, diffuse large B cell lymphoma, Philadelphia chromosome positive acute lymphoblastic leukemia, chronic lymphocytic leukemia, and chronic myeloid leukemia in blastic crisis [27–32].

AID mRNA expression can also play a role in MDS pathogenesis through its possible role in the epigenetic mechanisms of DNA methylation and demethylation.

Mutations in genes which regulate DNA methylation, such as DNMT and TET 2, were demonstrated in MDS patients [10,11,33,34]. An animal study by Arioka et al. suggested the possible role of AID in regulating intracellular localization of TET proteins. This study also proposed the possible significance of the coordinated function of AID and TET in regulating epigenetic changes [35]. In another study by Tsai et al., a possible role of AID in stabilization of DNMT was hypothesized [36]. According to these studies, AID may have a functional interaction with TET and DNMT, two genes whose roles in epigenetic changes in MDS pathogenesis have already been demonstrated.

DNMT1 and DNMT3A are upregulated and overexpressed in MDS patients, causing increased methylation of cytosine residues [37]. TET2, whose loss of function mutations are demonstrated in MDS, enables demethylation by catalyzing conversion of 5-methylcytosine to 5-hydroxymethylcytosine [8,9]. AID contributes to DNA demethylation by catalyzing deamination of 5-hydroxymethylcytosine to 5-hydroxymethyluracil. This activates base excision repair mechanisms that convert 5-hydroxymethyluracil to unmethylated cytosine [38]. According to the study by Morgan et al., AID may play an additional role in DNA demethylation by also deaminating 5- methylcytosine to 5-methyluracil (thymine) [39].

Figure 6 demonstrates the role of DNMT and TET in DNA methylation-demethylation reactions and the possible role of AID in these reactions [38].

In MDS patients, AID mRNA expression may be increased in order to compensate the increased DNA methylation (due to mutations causing DNMT overexpression) and decreased DNA demethylation (due to TET-2 mutations causing loss of function). Due to overexpression of DNMT, 5-methylcytosine production is increased. Since TET2 function is lost, 5-methylcytosine cannot be converted to 5-hydroxymethylcytosine. AID expression may increase as a compensating mechanism, enabling DNA demethylation by converting increased 5-methylcytosine is to 5-methyluracil (thymine). Therefore, increased AID expression can be secondary to mutations in DNMT and/or TET2 rather than a primary factor in the disease pathogenesis.

However, not all studies support this hypothesis. The review of DNA demethylation pathways by Bochtler et al. demonstrated that recent studies investigating the expression of AID on embryonic stem cells and the involvement of AID in DNA demethylation in these cells have yielded conflicting results. Some studies supported AID's role, whereas some studies argued that it does not play a role in DNA demethylation [40]. In this review, the authors also stated that according to some studies, AID's catalytic effect on 5-methylcytosine as a substrate was much less efficient than its effect on cytosine, which undermined the direct role of AID on DNA demethylation [40]. The authors then proposed that AID acted indirectly on DNA demethylation by acting on cytosine, triggering

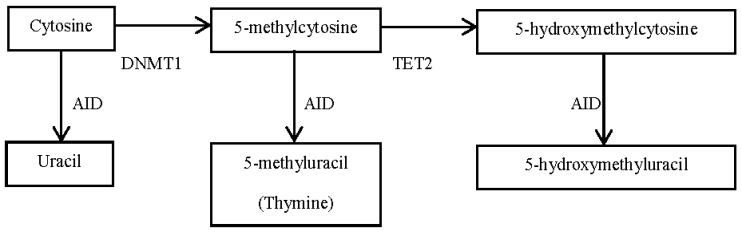

Figure 6. Role of DNMT, TET, and AID in DNA methylation-demethylation reactions. AID: Activation induced cytidine deaminase, DNMT1: DNA methyltransferase 1, TET2: Ten-eleven translocation family protein 2.

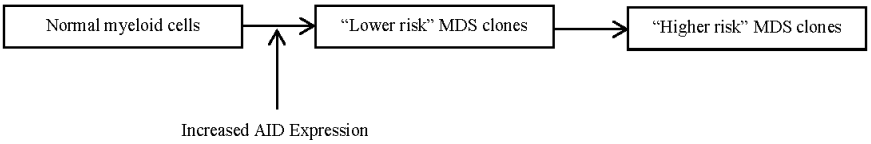

Figure 7. Increased AID expression may occur in the earlier steps of MDS pathogenesis. AID: Activation induced cytidine deaminase, MDS: Myelodysplastic syndrome.

repair mechanisms which also replaced the 5-methylcytosine molecules in the vicinity [40].

Although the AID expression is significantly increased in MDS patients compared to healthy controls, there was no statistically significant difference in AID mRNA levels of "lower risk" and "higher risk" subgroups according to IPSS, IPSS-R, and MDS subtypes. Considering our findings, we hypothesize that increased AID mRNA expression may occur in the earlier steps of MDS pathogenesis. As normal myeloid cells transform into "lower risk" MDS clones, AID expression increases. This increase may account for the statistically significant increase in AID expression of MDS patients compared to healthy controls. However, as "lower risk" MDS clones transform to "higher risk" clones, there is no further increase in AID expression because this change has already occurred in earlier steps. This hypothesis is summarized in Figure 7.

The low number of patients is a limitation of our study. There are 4 risk groups in the original IPSS classification and 5 risk groups in the original IPSS-R classification. However, due to the low number of our patients, they were classified into "lower risk" and "higher risk" subgroups according to these scoring systems and then a comparison of AID expression of these subgroups was performed. Further studies which include more patients will enable AID expression of each risk group to be directly compared with each other. Due to the low number of patients in our study, instead of comparing AID expression of each MDS subtype with each other, we divided the MDS subtypes into 2 categories and compared the AID expression of these 2 categories. Due to the heterogeneous nature of MDS, AID expression of MDS subtypes could differ from each other. Therefore, larger studies that compare AID expression in each different MDS subtype are also necessary.

Our study compared the AID expression of patients that received hypomethylating agents with those that did not receive this treatment. Although both subgroups had a significantly higher AID expression compared to healthy controls, there was no significant difference in the AID expression of these 2 subgroups. Due to the cross-sectional nature of our study, we were unable to compare the AID expression before and after hypomethylating agent treatment. We believe that such a comparison could have yielded a statistically significant difference. In the literature, there is evidence that links AID with hypomethylating agents. For example, in the study by Tsai et al., hypomethylating agent decitabine was shown to bind the active region of AID and inhibit its expression by proteosomal degradation [36]. Future studies are necessary to determine whether AID expression is affected by hypomethylating agents in MDS patients.

In this cross-sectional study, we determined AID mRNA levels of 30 MDS patients and 30 healthy controls. AID expression was significantly higher in the peripheral blood of MDS patients compared to healthy controls. However, there was no significant difference in AID expression of MDS patients that were classified as "lower risk" and "higher risk" subgroups according to IPSS, IPSS-R scores and MDS subtypes. There was also no significant difference in the AID expression of patients that received hypomethylating agents, compared to the patients that did not receive this treatment. Future studies which include a larger number of MDS patients and a larger number of age-matched healthy controls will improve our understanding of the possible role of AID in MDS pathogenesis, risk stratification and response to treatment with hypomethylating agents.

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Conflict of interest
The authors declare that they have no conflicts of interest.

Informed consent
This study protocol (file number 2016/999) received the approval of institutional review board (İstanbul Tıp Fakültesi Klinik Araştırmalar Etik Kurulu). All participants in both the patient group and the healthy control group provided informed consent in the format required by the institutional review board.

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