Activation Induced Cytidine Deaminase Expression in Patients with Myelodysplastic Syndrome and its Relationship with Prognosis and Treatment

**Background and 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 analysed 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 expression of different groups were compared using 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.
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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 could 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, was originally described as a B lymphocyte specific factor [14,15]. AID enables generation of antibody diversity in B lymphocytes by the mechanisms of somatic hypermutation, isotype switching and gene conversion [16,17]. However, AID may also
facilitate tumorigenesis by inducing proto-oncogene mutations, chromosome breaks and translocations in other cell lineages [18].

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 [19]. 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 [20]. 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 [21].

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

Thirty 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 were enrolled in this study. Their blood samples were taken. Blood samples of an age-matched healthy control group of thirty people were also obtained. All participants in both the patient group and healthy control group provided informed consent in the format required by the institutional research committee.
The history, physical examination findings, complete blood count, bone marrow biopsy and cytogenetic findings of MDS patients at the time of diagnosis were recorded. MDS subtypes both at the time of diagnosis and at the time of sample collection were analysed separately because transformation to other subtypes occurred in some patients. MDS subtypes were determined according to the 2008 classification of World Health Organization (WHO) [22]. The International Prognostic Scoring System (IPSS) [23] and Revised International Prognostic Scoring System (IPSS-R) [24] scores of the patients at the time of diagnosis were also recorded. Then, patients that were treated with hypomethylating agents (azacitidine and decitabine) were determined.

Patients were then classified 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”. Then, another risk stratification was made 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”.

Study protocol received the approval of the institutional research committee. All procedures that were 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

Peripheral blood samples from patients and healthy controls were collected in sterile tubes containing etylenediaminetetraacetic acid (EDTA). Total RNA was isolated using High Pure RNA Isolation Kit (Roche), in accordance with the instructions of the manufacturer. The density of RNAs that were obtained from the samples was measured spectrophotometrically using NanoDrop 2000c (Thermo Scientific, USA). Complementary DNA (cDNA) was transcribed from 100 ng of total RNA using Fermentas, RevertAid First Strand cDNA Synthesis Kit (Roche). TaqMan based Quantitative Real-time PCR (QRT-PCR) was performed using a LightCycler® TaqMan Master Kit (Roche Diagnostics) and a LightCycler® 480II instrument (Roche Diagnostics, Mannheim, Germany) was used to analyse 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′-TGGACACCACCTATGGACAGC-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, C_T values of AID and HPRT1 were obtained for all samples. The normalized expression for each sample was obtained by subtracting the C_T of HPRT1 from the C_T of AID of the same sample. This was designated as ΔC_T. This value was then transformed using $2^{-\Delta C_T}$ formula [25].

2.3 Statistical analysis

AID mRNA expression levels of MDS patients and healthy controls were first reported using mean and standard deviation. Then, these expression levels were checked for normal
distribution. Due to their non-normal distribution, AID mRNA expression levels of MDS patients and healthy controls were then reported using median, first and third quartile values. Then, non-parametric Mann Whitney U test was used to compare AID mRNA expression levels in both groups.

Then, 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 were checked for normal distribution. None of the subgroups demonstrated a normal distribution. AID mRNA expression levels of “lower risk” and “higher risk” subgroups were reported using median, first and third quartile values. Then, non-parametric Mann Whitney U test was used to compare AID mRNA expression levels in “lower risk” and “higher risk” subgroups.

After that AID mRNA expression levels of patients that received hypomethylating agents and those that did not receive hypomethylating agents were checked for normal distribution. Due to absence of normal distribution AID mRNA expression levels of “hypomethylating agent” and “no hypomethylating agent” subgroups were reported using median, first and third quartile values. Then, non-parametric Mann Whitney U test was used to compare AID mRNA expression levels in these two subgroups. Finally, the AID expressions of “hypomethylating agent” subgroup and “no hypomethylating agent” subgroup were separately compared with the healthy control group.

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

3. Results

3.1 General Characteristics of Patients and Healthy Controls

Thirty patients and thirty healthy controls were enrolled 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 Table 1 and Table 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. 22 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 45X,-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%).

Thirty MDS patients were classified 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 erythropoetin and transfusions.

IPSS and IPSS-R scores at the time of diagnosis were calculated 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. 19 patients were in the “lower risk” subgroup and 8 patients were in the “higher risk” subgroup.

Then, the patients were classified into “higher risk” and “lower risk” subgroups according to their MDS subtypes at the time of sample collection. 13 patients were in “lower risk” subgroup and 17 patients were in “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 non-normal. Since both groups demonstrated a non-normal distribution, AID expression of MDS patients and healthy controls was compared using the non parametric 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). 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, 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 the non parametric Mann-Whitney U test was used 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 Mann-Whitney U test indicated that this difference was not statistically
significant. (U= 39.5, p =0.333).

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, the non parametric Mann-Whitney U test was used 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 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 was 0.027735
±0.021183 in the “higher risk” subgroup (Figure 4). Neither the “lower risk” subgroup nor the
“higher risk” subgroup demonstrated a normal distribution. Therefore, the non parametric
Mann-Whitney U test was used 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 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 $0.038319 \pm 0.025584$ (Figure 5). Neither
group demonstrated a normal distribution, therefore, the non parametric Mann-Whitney U test
was used 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”
group (median:0.022020; Q1:0.015843-Q3:0.066985) were compared and Mann-Whitney U
test indicated that this difference was not statistically significant (U=90; p=0.533). When AID
expression of each of these subgroups were compared separately with the healthy control
group, 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 pre-
malignant 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 [18].

In a study by Marusawa et al., constitutive and excessive expression of AID in
trangenic 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 [26]. AID
expression was present in gastric tissue infected with *Helicobacter pylori* and a relationship
between epithelial neoplasia and increased AID expression was proposed [27]. AID
expression was associated with many different types of hematological malignancies such as
Burkitt leukemia/lymphoma [28,29], diffuse large B cell lymphoma [30], Philadelphia chromosome positive acute lymphoblastic leukemia [31], chronic lymphocytic leukemia [32], chronic myeloid leukemia in blastic crisis [33].

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 [10,11,34,35]. 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 [36]. In another study by Tsai et al, a possible role of AID in stabilization of DNMT was hypothesized [37]. 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 over-expressed in MDS patients, causing increased methylation of cytosine residues [38]. 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 [39]. 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) [40].

Figure 6 demonstrates the role of DNMT and TET in DNA methylation-demethylation reactions and the possible role of AID in these reactions [39].
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 over-expression of DNMT, 5-methylcytosine production is increased. Since TET2 function is lost, 5-methylcytosine can not 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 [41]. 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 [41]. The authors then proposed that AID acted indirectly on DNA demethylation by acting on cytosine, triggering repair mechanisms which also replaced the 5-methylcytosine molecules in the vicinity [41].

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 are some evidences that link 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 [37]. Future studies are necessary to determine whether AID
expression is affected by hypomethylating agents in MDS patients.

5. Conclusion

In this cross-sectional study, AID mRNA levels of 30 MDS patients and 30 healthy
controls were determined. 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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Table 1: Clinical characteristics of the myelodysplastic syndrome patients that were enrolled in our study. 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
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Table 2: Characteristics of the healthy controls that were enrolled in our study
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
Figure 5: AID mRNA expression levels of patients that received and that did not receive hypomethylating agents.
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