Original Article

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Aberrant Activation-Induced Cytidine Deaminase Gene Expression Links BCR/ABL1-Negative Classical Myeloproliferative Neoplasms

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Abstract

Aim: Activation-induced cytidine deaminase (AID) has been associated with tumor initiation and development because of its ability to generate DNA damage and somatic mutations that cause genomic instability. This study aimed to investigate the relationship between AID expression levels and the risk of developing BCR/ABL1-negative myeloproliferative neoplasms (MPNs) by comparing the AID expression levels of the patients and controls.

Methods: This case-control study was conducted on 117 cases (64 essential thrombocythemia, 23 primary myelofibrosis, and 30 polycythemia vera) with MPNs and 69 healthy controls. The *JAK2* V617F somatic mutation analysis was performed using a real-time polymerase chain reaction (RT-PCR). The relative expression levels of *AID* in the patient and the control groups were analyzed using quantitative RT-PCR and the $2^{-\Delta\Delta CT}$ method.

Results: *AID* expression levels were significantly higher in the patient group compared to the control group ($p<0.001$). *AID* expression levels were higher in patients with the *JAK2* V617F mutation compared to patients without the mutation, but the difference was not statistically significant.

Conclusion: The results of our study suggest that although overexpression of *AID* does not seem to support the *JAK2* driver gene, it may contribute to the development of MPNs through other mechanisms.

Keywords: Cytidine deaminase, mutations, genomic instability

Introduction

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders in which erythroid, granulocytic, and megakaryocytic cells are overproduced. The major diseases within BCR/ABL1-negative classical MPNs are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). In MPN, there is a deterioration in the JAK2 kinase signaling pathway. Mutations in the genes encoding *JAK2, CALR,* and *MPL* are driver mutations in MPN (1). Additional genetic alterations in genes involved in epigenetic mechanisms, such as *TET2, IDH1/2, ASXL1, EZH2, SF3B1, SRSF2,* and *U2AF1*, are also common in MPNs (2,3). The single point mutation *JAK2* V617F is present in approximately

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95% of PV patients and 50-60% of ET and PMF patients (4). Additionally, some MPN patients may have a "mutator" phenotype (5,6). Neoplastic clones may arise in neoplastic cells gradually because of the accumulation of mutations that occur mainly in DNA repair pathways (7).

Activation induced cytidine deaminase (AID), a member of the AID/apolipoprotein B editing complex (APOBEC) catalytic protein family, is highly expressed in germinal center B lymphocytes (8). By deaminating the cytosine residues in the immunoglobulin (Ig) variable region, the AID enzyme normally generates somatic hypermutation (SHM) and class-switch recombination (CSR) processes in *Ig* genes (7). SHMs create point mutations and insertions/deletions in the DNA sequence with a very high frequency of 10^{-2} to 10^{-3} base pairs per generation (9). The conversion of cytosine to uracil by AID is attempted and repaired through base excision repair or mismatch repair mechanisms. These processes are prone to errors as they can create mutations that cause dU: dG mismatches. Moreover, AID can alter gene expression by DNA demethylation, which can induce tumor initiation or progression because of genomic instability, so its tight control is important (10). AID-induced demethylation has also been shown to play a role in the expression of tumor progression factors (8). Several research groups have reported the aberrant expression of Igs in non-lymphoid cancer cells, suggesting a complex mechanism emphasizing Ig expression in cancer cells (10,11). Furthermore, there is evidence that AID is expressed not only in lymphoid cells but also in non-lymphoid cells, suggesting that AID also mutates genes other than Igs (8). Therefore, many studies have focused on *AID* for its potential role in the generation of both point mutations and chromosomal rearrangements in different types of cancer. It has been reported that many genes, such as *c-MYC*, *CARD11*, *EZH2,* and *MMP14* are affected by AID activity in various types of cancers (12).

Various researchers are trying to elucidate the role of *AID* in different types of cancer, but there is no study that has been carried out on MPN patients. In this study, we hypothesized that somatic mutations in the *JAK2* driver gene could be induced by both the SHM and the methylation-demethylation activity of AID. Thus, we investigated whether *AID* expression was involved in the etiopathogenesis of BCR-ABL1-negative classical MPNs by comparing patients and controls in terms of *AID* gene expression levels and by investigating the relationship between *AID gene* expression levels and the presence of the *JAK2* V617F mutation.

Materials and Methods

Ethical Approval and Study Design

Approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Ethics Committee (approval number: 2010/1025-325 date: 04.01.2011) for our project, which we carried out in accordance with the Declaration of Helsinki. This study comprised 117 patients with BCR/ABL1-negative classic MPNs who were followed up in the Hematology out-patient Clinic of Istanbul University, Istanbul Faculty of Medicine between January 2009 and January 2011. The patients' diagnoses were reviewed according to the recommendations of the World Health Organization, revealed in 2008 (13). Clinical and laboratory data collected during diagnosis and at study entry were collected from patients' medical files and electronic medical records. The control group consisted of 69 healthy volunteers who were hospital staff and had no history of MPN in themselves or their relatives (as age-appropriate with patients). Peripheral blood samples were drawn in 2 mL of sterile tubes containing EDTA. A complete blood count was also performed during sampling.

DNA Extraction and *JAK2* V617F Mutation Analysis

Automated DNA extraction was carried out with the MagNA Pure Compact Instrument (Roche Diagnostic, Germany). The *JAK2* V617F mutation assay with RT-PCR was performed by the JAK2 MutaScreen Kit using the manufacturer's recommended protocol (Ipsogen, Luminy Biotech, France).

RNA Extraction and cDNA Synthesis

Total RNA was isolated from whole blood with a High Pure RNA Isolation Kit (Roche Diagnostics, Germany) following the manufacturer's instructions. The quantity and purity of RNAs were measured using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). Then, cDNA was synthesized from 1 µg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany).

Quantitative Real-time PCR

Quantitative Real-time PCR (qRT-PCR) was performed with 50 ng total RNA in 20 µL total volume using the Real-Time Ready Universal ProbeLibrary Assay (Roche Diagnostics, Germany). The RT reactions were carried out on a LightCycler® 480II system. Each sample was studied in duplicate. Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used for the normalization of relative qRT-PCR studies. Relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method.

The primers used for the amplification of AID were as follows: forward: 5′-TGGACACCACTATGGACAGC-3′ and reverse: 5′-GCGGACATTTTTGAATTGGT-3′)

(ENST00000229335). The primers used for amplification of the reference gene *HPRT1* were as follows: forward: 5′-GACCAGTCAACAGGGGACAT-3′ and reverse: 5′-GTGTCAATTATATCTTCCACAATCAAG-3′).

Statistical Analysis

The statistical software program SPSS (ver. 21.0) was used for the analysis. The Kolmogorov-Smirnov test was used to check whether the distribution was normal. The Student's t-test and the Mann-Whitney U test were used for the analysis of the data with and without normal distribution, respectively. Chi-square tests were used to analyze categorical data. Continuous variables were defined as mean ± standard deviation. Statistical significance was set as a p-value less than 0.05.

Results

The clinical characteristics and laboratory analysis of the patients in the MPN patient group are presented in Table 1. The patient group consisted of 117 cases with MPN (30 PV, 64 ET, and 23 PMF). Two of the patients with PMF had transformed from acute myeloid leukemia. The mean age was 53.95±13.86. The mean ages between the two groups and between females and males were similar. The female to male ratios of the groups were not different. Leukocyte counts at diagnosis and during sampling were similar between ET, PV, and PMF patients. The platelet count at the time of diagnosis and during sampling was found to be significantly higher in ET patients than in PMF patients. (p=0.001, p=0.005, respectively). The LDH count detected at the time of diagnosis in PMF patients was significantly higher than in ET and PV patients (p=0.001, p=0.047, respectively). LDH count during sampling was also significantly higher in PMF patients than in ET and PV patients (p<0.001, p=0.02, respectively). The disease duration and lymphocyte count at diagnosis/during sampling were similar between the three subgroups. The *JAK2* mutation was present in 38 of 64 ET (59.38%), 15 of 23 PMF (65.22%), and 28 of 30 PV (93.33%) patients. The frequency of *JAK2* V617F was found to be significantly higher in PV patients than in patients with ET and PMF (p=0.004).

AID expression levels in the study groups are shown in Table 2 and Figure 1a. *AID* expression levels were significantly higher in the patient group than

Table 1. Clinical features and laboratory findings of the patient group

	ET	PMF	PV	All patients
Number of patients	64	23	30	117
Female/Male	30/34	14/9	14/16	58/59
Mean age (mean ± SD)	51.33±13.99	60.3±14.32	54.67±11.82	53.95±13.86
Leukocyte at diagnosis ($X10^9$/L) (mean ± SD)	10±4.11	13.39±10.93	12.65±4.51	11.27±6.23
Leukocyte during sampling ($X10^9$/L) (mean ± SD)	7.82±3.28	8.74±5.16	10.83±4.98	8.79±4.33
Lymphocyte at diagnosis ($X10^9$/L) (mean ± SD)	2.32±1.01	2.37±1.68	2.23±8.03	2.31±1.11
Lymphocyte during sampling ($X10^9$/L) (mean ± SD)	2.08±0.92	2.16±2.3	2.03±0.8	2.08±1.26
Platelet count at diagnosis ($X10^9$/L) (mean ± SD)	982.33±405.63	472.35±400.09	415.93±202.32	747.71±452.72
Platelet count during sampling ($X10^9$/L) (mean ± SD)	617.55±396.84	353.35±272.66	366.53±232.41	500.51±359.42
LDH at diagnosis (U/L) (mean ± SD)	430.41±182.04	727.47±335.94	547.12±197.11	513.53±246.37
LDH during sampling (U/L) (mean ± SD)	442.78±208.24	953.52±387.77	667.78±422.77	607.49±372.71
JAK2 V617F (+) n (%)	38 (59.38%)	15 (65.22%)	28 (93.3%)	81 (69.23%)
Bone marrow reticulin fibrosis degree n (%)				
0	14 (21.88%)	0	2 (6.67%)	16 (13.68%)
1	32 (50%)	0	22 (73.33%)	54 (46.15%)
2	17 (26.56%)	3 (13.04%)	4 (13.33%)	24 (20.51%)
3	1 (1.56%)	17 (73.92%)	2 (6.67%)	20 (17.1%)
4	0	3 (13.04%)	0	3 (2.56%)
Splenomegaly (+) n (%)	23 (35.9%)	22 (95.7%)	19 (63.3%)	64 (54.7%)
Thrombosis risk n (%)	13 (20.31%)	6 (26.09%)	13 (43.33%)	32 (27.35%)
Use of hydroxyurea n (%)	43 (67.18%)	15 (65.22%)	22 (73.33%)	80 (68.38%)
Hydroxyurea usage time; years (mean ± SD)	3.68±4.72	2.8±3.43	4.2±5.14	3.64±4.60
Disease duration, years (mean ± SD)	4.89±5.1	7±5.78	5.2±5.54	5.38±5.36

Data are presented as mean ± SD and n (%).
ET: Essential thrombocythemia, PMF: Primary myelofibrosis, PV: Polycythemia vera, LDH: Lactate dehydrogenase, n: Number of samples, SD: Standard deviation

Table 2. AID expression levels in the study groups		
Study groups	***AID* expression levels**	**p-value**
All patients (n=117)	0.033±0.084	0.001[a]
Controls (n=69)	0.020±0.018	
ET (n=64)	0.035±0.698	NS
PMF (n=23)	0.046±0.151	
PV (n=30)	0.020±0.018	
JAK2 V617F positivity		
JAK2 V617F (+) (n=81)	0.036±0.096	0.467
JAK2 V617F (-) (n=36)	0.024±0.044	

Data are presented as mean ± SD: Standard deviation
n: Number of samples, ET: Essential thrombocythemia, PMF: Primary myelofibrosis, PV: Polycythemia vera, NS: Not significant
[a]: Students' t-test, p<0.001 (*AID* expression levels were significantly higher in the patient group than in the control group)

in the control group. (p<0.001). But *AID* expression levels were similar within the three subgroups of MPN (Figure 1b). Although *AID* expression was higher in patients harboring *the JAK2* V617F mutation than in patients without this mutation, there was no statistically significant difference. *AID* expression levels in terms of the *JAK2* V617F mutation situation are shown in Table 2. *AID* expression levels were found to be higher in hydroxyurea users than in non-users, but the difference was not significant. There was no statistical difference between the *AID* levels of the patients with regard to age, gender, splenomegaly, risk of thrombosis, and degree of reticulin degree.

Discussion

Recently, it has been understood that the AID/APOBEC DNA deaminase family generates mutations/mutation showers by recognizing certain motifs in the DNA chain (14). Since AID, a member of this family, causes point mutations or chromosomal rearrangements, was identified as the first mutator enzyme (15).

In this study, our aim was to explore the gene expression levels of *AID*, which causes SHM and CSR in both Ig and non-Ig genes that may contribute to the etiology of MPN, in the Turkish population. Although there are publications investigating the relationship of the *AID* gene with various cancers, there is no study investigating its role in the development of MPNs.

Several studies have discovered a link between increased AID expression and various types of cancer, including hematologic cancers (8,16-25). First, aberrant *AID* expression was reported in transgenic mice with lymphoma and diffuse large B cell lymphoma (26,27). A study on breast cancer cells demonstrated the importance of AID on the epithelial to mesenchymal transition, which is essential in normal morphogenesis and tumor metastasis (8). Then, AID was shown to play a crucial role in regulating myeloid and erythroid lineage differentiation but not in self-renewal or myeloid transformation of hematopoietic stem/progenitor cells (28). AID takes part in the hypermutation of tumor suppressor genes as well as DNA repair genes in CML (21) and *AID* expression levels were found to be higher in BCR-ABL1 (+) acute lymphoblastic leukemia (ALL) cells (secondary to CML or *de novo*) compared with BCR-ABL1 (-) ALL cells (18). AID expression levels have also been found to be elevated in cases of myelodysplastic syndrome, chronic lymphocytic leukemia (CLL), and T-cell leukemia/lymphoma (19,16,25). A study on multiple myeloma patients emphasized the function of AID in early mutagenesis, implying that it causes mutations in driver genes that are the targets of AID (22). A recent study in mice reported a close

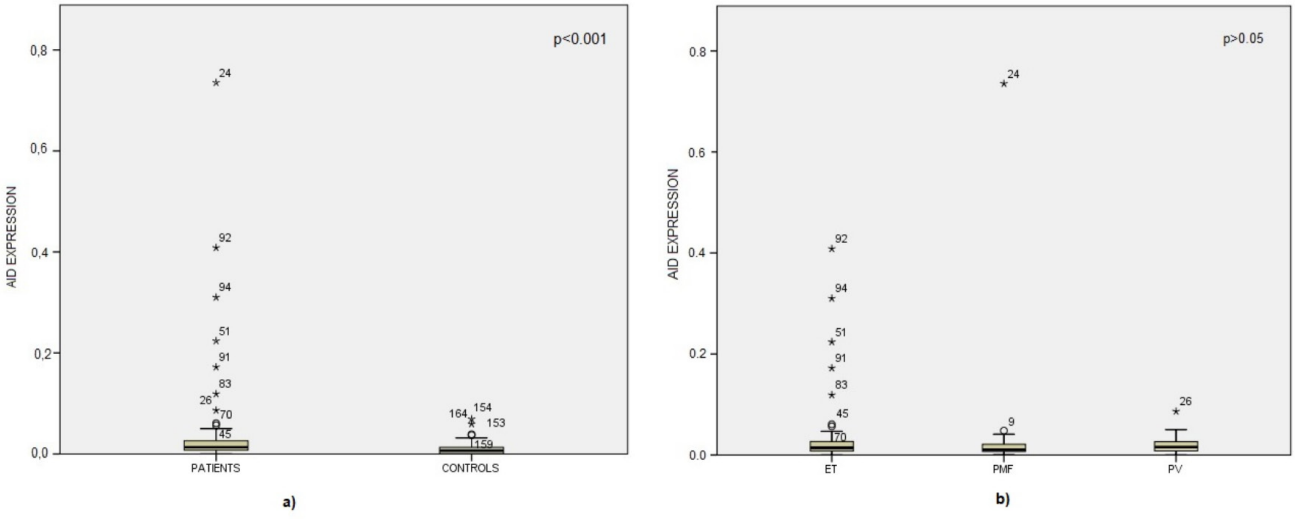

Figure 1. a) *AID* expression levels in patient and control groups b) *AID* expression levels in patients with ET, PMF and PV
ET: Essential thrombocythemia, PMF: Primary myelofibrosis, PV: Polycythemia vera

association between CLL driver mutations and increased AID activity, suggesting that AID encourages aggression in CLL (23). *AID* was also suggested to be an oncogene that triggers tumorigenesis and a treatment that suppresses AID can also suppress cell proliferation, migration and invasion (14,24,28,29). Moreover, tumor cells escaped from therapy through various genetic and epigenetic mechanisms due to genetic instability (30).

Our results were compatible with the previous studies conducted in patients with multiple types of cancer, as we found elevated *AID* expression levels in our patient group. Because of the mutagenic role of AID, we examined the possible relationship between *AID* expression and *JAK2* V617F, a common driver mutation in MPNs. Although *AID* expression was higher in patients with *JAK2* V617F than in those without *JAK2* V617F, the difference did not reach statistical significance, suggesting the contribution of other mechanisms in the generation of this mutation. Interestingly, *AID* expression levels were higher in patients who were not using hydroxyurea compared with those who were using it, but the difference was not statistically significant, suggesting that hydroxyurea treatment increases *AID* expression in MPNs. However, hydroxyurea treatment may also lead to mutations through chemotoxicity and DNA damage (31). Owing to its ability to generate mutations, AID appears to play a crucial role in the development of MPNs as well as in various other types of cancer.

Study Limitations

There were some limitations in our study. The first limitation was the relatively small number of study groups enrolled in the study. The second limitation was the lack of data regarding patients' status of *CALR* and *MPL* gene mutations, which are also driver mutations in MPNs. Therefore, these driver mutations could not be compared with *AID* expression. Despite all these limitations, we believe that our study will make important contributions to the literature.

Conclusion

Our data demonstrate the overexpression of *AID* in patients with MPN compared with controls, suggesting an important role in the etiogenesis of MPNs. To our knowledge, this is the first study investigating the role of *AID* in the development of MPNs. Prospective, randomized genetic and functional studies with larger groups are needed to elucidate the function and significance of overexpression of the *AID* gene in MPN patients.

Ethics

Ethics Committee Approval: Approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Ethics Committee (approval number: 2010/1025-325 date: 04.01.2011) for our project.

Informed Consent: Written informed consent was taken from all patients and healthy controls.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.D., A.S.Y., Design: H.D., A.S.Y., Data Collection and/or Processing: H.D., M.N., A.S.Y., Analysis and/or Interpretation: A.D.A., A.B.A.T., V.S.H., M.Y.G., Literature Research: H.D., A.S.Y., Writing: A.D.A., H.D., A.S.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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