*Original Article*

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# Effect of the Epigenetic Regulators (JAK2 and IDH-2) on the Methylation Status of Tumor Suppressor Genes (p15, FHIT, Calcitonin and SOCS-1) and Prognosis of MDS Patients: A Study from India

**Rekha Chaubey[1], Sudha Sazawal[2], Manoranjan Mahapatra[3], Sunita Chikkara[4], Renu Saxena[5]**

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**Abstract**

The Myelodysplastic Syndromes (MDS) are a group of clonal disorders of the hematopoietic system characterized by the presence of ineffective hematopoiesis, peripheral cytopenias and an increased risk of transformation to acute myeloid leukemia (AML). The pathogenesis of MDS involves a complex pattern of genetic, epigenetic and immune mediated mechanisms. However, recent advances in the understanding of the disease have identified altered epigenetic mechanisms, particularly aberrant DNA methylation as a key pathogenic factor in MDS. Since presently very sparse data is available on the epigenetic regulators and their effect on the methylation machinery, we studied the mutations in the epigenetic regulators i.e. JAK2V617F and IDH-2 ina series of 45 MDS patients and analyzed their effect on the methylation of the four tumor suppressor genes (p15, FHIT, calcitonin and SOCS-1) and clinical profile of MDS patients. We have found JAK2 mutation in 29% and IDH2 in 7% of MDS cases. JAK2 mutation was significantly correlated with the SOCS-1 gene methylation ($p<0.05$). No effect of IDH2 mutation on methylation was observed. Also, we did not find any correlation of co-occurrence of JAK2 /IDH2 mutation and SOCS -1 methylation on the prognosis and treatment outcome. Our study suggests the importance of epigenetic regulation of SOCS -1 methylation through JAK/STAT pathway in the leukemogenesis.

**Keywords:** Epigenetic Regulators; JAK2; IDH-2; FHIT; Calcitonin and SOCS-1

## Introduction

The Myelodysplastic Syndromes (MDS) are a group of clonal disorders of the hematopoietic system characterized by the presence of ineffective hematopoiesis, peripheral cytopenias, and an increased risk of transformation to acute myeloid leukemia (AML). Although MDS has been recognized as an important disease for more than 50 years, its molecular pathogenesis and the molecular basis for progression to AML remains largely undefined. The pathogenesis of MDS involves a pattern of genetic, epigenetic, and immune mediated mechanisms. However, recent

**Author's Affiliation:** [1]Research Scientist [2]Scientist [3]Professor [4]Research Associate [5]Professor and Head, Department of Hematology, All India Institute of Medical Sciences, New Delhi 110029, India.

**Corresponding Author: Rekha Chaubey,** Research Scientist, Department of Hematology, All India Institute of Medical Sciences, New Delhi 110029, India.

**E-mail:** drrekhacgupta@gmail.com

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advances in the understanding of the disease have identified altered epigenetic mechanisms, particularly aberrant methylation of DNA, as key pathogenic factors in MDS [1,2]. Numerous single locus and recent genome-wide studies of DNA methylation in MDS have revealed the complexity of the disease at an epigenetic level. An incredible amount of progress has been made in the past few years towards the elucidation of the epigenetic mechanisms involved in development of MDS. Novel mutations have been described as the target of epigenetic machinery and may explain the profoundly aberrant epigenetic profiles in MDS patients [3]. Recent genomic studies have identified novel recurrent somatic mutations in patients with myeloid malignancies, including myeloproliferative neoplasms (MPNs), MDS and AML. These newer mutations are known to play an important role in regulating chromatin and/or methylation states in hematopoietic progenitors. Few genetic and functional studies have elucidated a role for specific mutations in altering epigenetic patterning in myeloidmalignancies [4]. Past studies have demonstrated that the role of JAK2 in hematopoiesis and leukemogenesis is mediated through cytoplasmic phosphorylation of the various signal transducer and activator of transcription (STATs), which are subsequently translocated into the nucleus to activate STAT target genes [5]. A recently published study in Journal 'Nature' reported the activation of a leukemogenic gene, lmo2 by JAK2 through phosphorylation of histone H3 in nuclei. This study revealed a novel mechanism by which JAK2 contributes to the leukemogenesis process [6]. IDH mutations also plays an important role in metabolic and epigenetic deregulation. Recent studies in AML have elucidated the possible different mechanisms responsible for the metabolic chaos and epigenetic disruption [7]. Since presently very sparse data available on the mechanism of epigenetic regulators and their effect on the methylation machinery in MDS, we studied the mutations in the epigenetic regulators i.e. JAK2 and IDH-2 in MDS patients and tried to analyze their effect on the methylation of the four tumor suppressor genes (p15, FHIT, Calcitonin and SOCS-1) andclinical profile of MDS patients.

## Materials and Methods

A total of 45MDS patients presenting randomly at the Department of Hematology, All India Institute of Medical Sciences (AIIMS), New Delhi between September 2014 and December 2016, were the study subjects. The selection of patients was based on the availability of the sample. Ethical approval for the study was obtained from the Ethics Committee of the AIIMS, New Delhi. Morphologic subtypes of MDS were classified according to the WHO 2008 criteria [8].

Mononuclear cells (MNC) were isolated from by density gradient centrifugation on Ficoll-Hypaque. DNA was isolated by commercially available kit (Qiagen, Germany). Mutation analysis of JAK2 V617F (Exon 12) was initially performed using allele-specific PCR based on Amplification Refractory Mutation System (ARMS) technology. Genomic DNA was extracted from each PB and BM aspiration sample, using a QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA). After adding 3 μL of primer mixture to 20 μL of PCR master mixture, we added 2 μL of sample DNA (10–20 ng/μL) to bring the total volume to 25 μL. The wild-type allele was amplified using a forward primer with the sequence 5′-TCC TCA GAA CGT TGA TGG CAG-3′ and a reverse primer with the sequence 5′-ATT GCT TTC CTT TTT CAC AAG AT-3′. The mutant allele was amplified using a forward primer with the sequence 5′-GCA TTT GGT TTT AAA TTA TGG AGT ATA TG-3′ and a reverse primer with the sequence 5′-GTT TTA CTT ACT CTC GTC TCC ACA AAA-3′. The PCR conditions used were as follows: heating at 94$^{o}$C for 15 min (initial denaturation), then 34 cycles of 94$^{o}$C for 30 s (denaturation), 58$^{o}$C for 45s (annealing), and 72$^{o}$C for 45s (extension), followed by a final extension at 72$^{o}$C for 4 min. The amplified products were electrophoresed on 3% agarose gel and visualized using ethidium bromide staining. The wild-type phenotype was scored by observation of a single 229-bp fragment only, homozygous mutations were scored by observation of a single 279-bp fragment only, and heterozygosity was identified by the presence of both a 229-bp fragment and a 279-bp fragment, irrespective of band signal strength.

For IDH2 R140Q Mutation, ARMS analysis was performed by using two control primers flanking exon 23 and two allele specific primers; IDH2 –R1 and IDH2- F1 that were complementary to the wild type (wt) and two mutated alleles respectively. The composition of reaction mixture was as follows : 10x PCR buffer -5 ul, $MgCl_2$ (25 mM) – 6ul, dNTPs (2 mM)-1.25 ul, Control Primers (Fo and Ro ) – 1ul each, allele specific Primers (F1 and R1) – 2ul each, Ampli Taq Gold (5U/ul) – 0.5 ul and Genomic DNA – 10-20 ng/ul. PCR conditions were as follows : Initial Denaturation - 95°C for 5min, denaturation - 95°C for 60 sec, Annealing – 65°C for 60 sec, Extension - 72°C for 45 sec and final extension for 10 min. The amplified products were electrophoresed on

1.5% agarose gel and visualized using ethidium bromide staining. In the presence of the mutation the PCR the PCR reaction generated 3 different size of fragments with size 613bp (control band), 446bp (mutation band), and 233bp (Wild type band).

Methylation status of the promoter regions of the p15, SOCS-1, Calcitonin, and FHIT gene was assessed by methylation specific PCR. Bisulphate-modified DNA was amplified by PCR using primer sets as described previously [9]. Patient's baseline parameters like hemogram, TLC, platelet, transfusion requirements (U/months) were recorded at the time of initial diagnosis. All statistical analysis was performed by using STATA 11 (Texas, USA). In case of categorical parameters chi square, Fisher exact test was used to compare the groups.

## Results

Of the total 54 patients studied, 32 (59%) were male and 22 (41%) were females (M:F::1.5:1) diagnosed as having primary MDS according to the WHO criteria. Mean age at presentation was 46 (SD ± 16.1) and median age was 45 years (Range: 14-75).

The frequency of different morphological subtypes (WHO 2008) in this study was as follows: refractory anemia with unilineage dysplasia (RCUD): 64%, refractory anemia with excessive blasts-2 (RAEB-2): 9%, refractory anemia with excessive blasts-1 (RAEB-1): 7%, refractory cytopenia with multilineage dysplasia (RCMD): 6%, refractory anemia with ringed sideroblasts (RARS): 2%, refractorycytopenia with multilineage dysplasia –ringed sideroblasts (RCMD): 6%. A total of five patients transformed during the follow up. Two patients transformed to AML-M2, one from RCUD and other from RAEB-1. One patient initially diagnosed as RAEB-2 and later transformed to ALL. 1 patient transformed from RAEB-2 to AML -M1 and 1 patient progress to RCMD from RA.

The different hematological parameters were as follows: Mean hemoglobin (Hb) was 6.7 g/L (SD ± 2.5), median total leukocyte count (TLC) was $3.9x10^9/L$ (range $0.8–116x10^9/L$) and median platelet count was 88 $x10^9/L$ (range 0.1–381 $x10^9/L$).

Out of 45 cases, JAK2 mutation was present in 13 (29%) of patients. 2 out of these 13 cases (15%) were homozygous mutant and 11 (24%) were heterozygous mutant (Fig. 1) (Table 1). 3 out of 45 cases (7%) were positive for IDH-2 mutation and all these patients were heterozygous mutant (Fig. 2) (Table 1).

**Table 1:** Frequency of JAK2 mutation in MDS cases

| JAK2 Mutation (n=45) | Frequency | Percent | Cum. Percent | 95% CI Lower | 95% CI Upper |
|---|---|---|---|---|---|
| Homozygous Mutant | 2 | 4.44% | 4.44% | 0.54% | 15.15% |
| Heterozygous Mutant | 11 | 24.44% | 28.89% | 12.88% | 39.54% |
| Wild Type | 32 | 71.11% | 100.00% | 55.69% | 83.63% |

A total of 45 MDS patients were screened for IDH-2 mutation. (Fig. 2) Out of 45 cases IDH-2 mutation was present in 3 (7%) of patients. All these patients were heterozygous mutant. (Table 1)

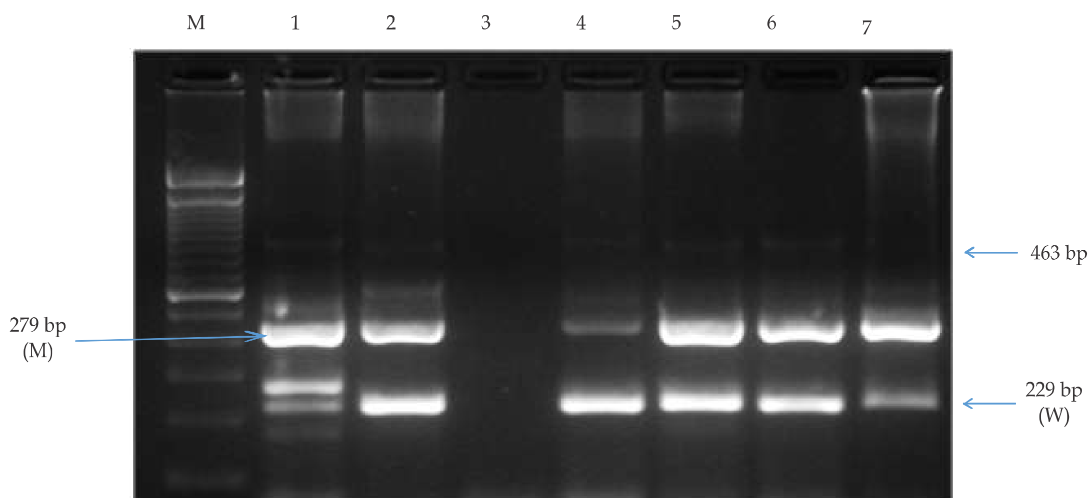


**Fig. 1:** JAK2 mutation detection on 3% Agarose gel. Lane M is 100 bp molecular marker. Lane 1 is patient positive for JAK2 mutation. Lane 2, 4, 5, 6, 7 is patients sample negative for JAK2 mutation. Lane 3 is negative control.

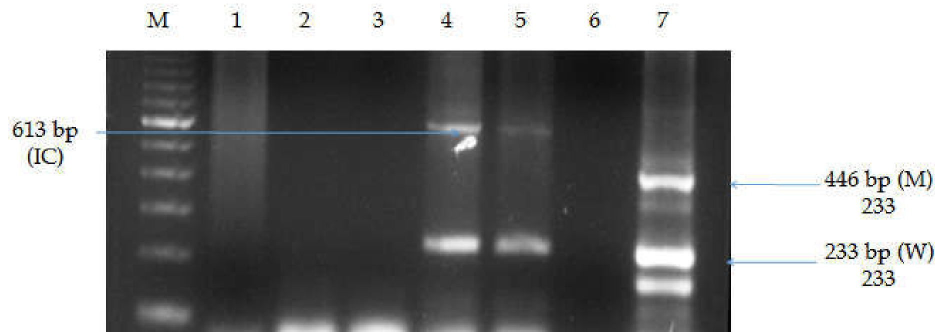


Fig. 2: IDH2 mutation detection on 3% agarose gel. Lane M is 100 bp molecular marker. Lane 1,4,5 is negative for IDH2 mutation. Lane 6 is negative control.

**Table 2:** Frequency of IDH2 mutation in MDS cases

| IDH2 (n=45) | Frequency | Percent | Cum. Percent | 95% CI Lower | 95% CI Upper |
|---|---|---|---|---|---|
| Heterozygous Mutant | 3 | 6.67% | 6.67% | 1.40% | 18.27% |
| Wild Type | 42 | 93.33% | 100.00% | 81.73% | 98.60% |

*Combined frequency of both JAK2 and IDH2 mutations*

A total of 38 were available with both JAK2 and IDH2 mutation studies. The results has been shown in the Table 3. There were 15 (39%) cases who were either positive for JAK2 mutation or IDH2 mutation or positive for both. Only one patient was positive for both JAK2 and IDH2 mutation.

**Table 3:** Frequency of JAK2+ IDH2 mutation in MDS cases

| JAK2 +IDH2 mutation (n=38) | Frequency | Percent | Cum. Percent | 95% CI Lower | 95% CI Upper |
|---|---|---|---|---|---|
| Mutant | 15 | 39.47% | 39.47% | 24.04% | 56.61% |
| Wild Type | 23 | 60.53% | 100.00% | 43.39% | 75.96% |

*JAK-2 Mutation and its relation of methylation status of four genes*

All the patients samples studied for JAK2 mutation were also screened for the methylation status of four tumor suppressor genes – SOCS-1, FHIT, p15 INK4b and Calcitonin. It was found that the SOCS-1 gene methylation was significantly associated with the JAK2 positivity ($p < 0.07$). (Table 4)

**Table 4:** Association of gene methylation and JAK2 mutation status

| TSGs | JAK2 + (N=13) (%) | JAK2 (-) (N=32) (%) | p-Value |
|---|---|---|---|
| SOCS-1 | 9 (69) | 13 (41) | 0.07 |
| Calcitonin | 5 (38) | 11(34) | 0.52 |
| p15 | 4(30) | 9 (28) | 0.56 |
| FHIT | 4(30) | 11(34) | 0.55 |

*JAK-2 Mutation and its relation of methylation status of four genes*

When we analysed the combined frequency of JAK2 and IDH2 mutation and then compared it with the methylation of TSGs, we found that it was associated with the SOCS-1 gene methylation. (Table 5)

**Table 5:** Association of gene methylation and mutation status of JAK2 and IDH2.

| Gene Methylated | JAK2 +/IDH2 + (N=15) (%) | JAK2 (-)/IDH2 (-) (N=32) (%) | p-Value |
|---|---|---|---|
| SOCS-1 | 11 (73) | 14 (43) | 0.055 |
| CALCA | 6 (46) | 11 (34) | 0.34 |
| p15 | 6 (46) | 11 (34) | 0.34 |
| FHIT | 5 (38) | 14 (43) | 0.5 |

In order to study the effect of epigenetic regulators and gene methylation on clinical profile MDS patients clinical parameters were compared for the group of patients positive for JAK2, IDH2 mutations and SOCS-1 methylation with the group of patients negative for JAK2, IDH2 mutations and SOCS-1 methylation. No significant difference was observed when it was compared for age, sex, TLC, Hb, Platelet, disease progression, transfusion dependency and response to treatment (Table 6).

**Table 6:** Comparison of clinical parameters of patients positive for JAK2, IDH2 mutations and SOCS-1 methylation with the patients negativefor JAK2, IDH2 mutations and SOCS-1 methylation

| Variable | JAK2 (+) IDH2 (+) SOCS-1 (M) (n=11)) | JAK2 (-) IDH2 (-) SOCS-1 (U) (n=18) | p-value |
|---|---|---|---|
| Age (years) | 41 (20-72) | 45 (14-70) | 0.5 |
| Sex (M:F) | 3:8 | 7:2 | |
| TLC (median) (X109 /L) | 4.7 (1.7-116) | 3.8 (0.95-63.3) | 0.2 |
| Hb (Mean) (gm/dl) | 5.2 (SD ± 1.4) | 7.3 (SD ± 2.8) | 0.3 |
| Plt (Median) (X109 /L) | 98 (28-116) | 60.5 (10-381) | 0.25 |
| Progression | 2 (18) | 1 (6) | 0.5 |
| Transfusion Dependent | 11 (100) | 16 (88) | 0.5 |
| Non Responder | 9 (82) | 6 (78) | 0.6 |

## Discussion

An incredible amount of progress has been made in the past few years towards the elucidation of the molecular mechanisms involved in the development of MDS. These genetic aberrations include mutations that alter the structure of the gene product (single point mutations, or larger genetic deletions or amplifications) or mutations that modify gene expression (mutations in the promoters and enhancers, or genes involved in epigenetic alterations) [3]. Epigenetics plays a central role in the pathophysiology of MDS; even small changes in the expression of epigenetic modifying proteins can have profound consequences on the epigenetic profile, resulting in aberrant expression or repression of potentially thousands of genes. Based on the studies on methylomes, there are evidences indicating the crucial role of hypomethylating agents in reprogramming the epigenetic status of MDS cells. Activation of JAK2 plays animportant role in normal hematopoiesis and leukemogenesis. Dawson et al. reported that JAK2 performs this functionby displacing the heterochromatin protein HP1a from chromatin through phosphorylation of histone H3 [6]. Similarly IDH-2 is also plays an important role in epigenetic regulation and alteration in its function results in oncogenesis.

Presently, apart from our previous research work published from AIIMS New Delhi, on the molecular studies in MDS patients, no molecular data of MDS patients from India is presently available. Moreover, the epigenetic studies have also not been studied from this region. In the present study frequency of JAK2 mutation in our patients was 29% (n=45). Its frequency in other Asian countries is reported to be from 0% -14% [10,11]. The frequency of JAK2 mutation was higher in the Western countries i.e., 5%-23% [12-13]. In our study, we have found higher frequency of JAK2 mutation as compared to other Asian countries although our results. Frequency of IDH2 was found to be 7% in our study which is similar to reported by other parts of the world [14].

We tried to study the effect of mutation in epigenetic regulators i.e, JAK2 and IDH2 on one of the most important epigenetic change i.e, gene methylation. For the same we analysed the methylation status of tumor suppressor genes on the same samples analysed for mutations. We have found that out of the four gene studied (SOCS-1, p15, Calcitonin and FHIT), only SOCS-1 gene was found associated with the JAK2 mutations.SOCS-1 switching cytokine signaling by means of its direct interaction with JAK. In our previous study we have shown the high frequency of SOCS 1 (53%) in MDS patients. We identified the association of JAK2 constitutive activation with SOCS-1 methylation, thus supporting the role of JAK/ STAT pathway in leukemogenesis. Whereas, we were unable to find the role IDH2 as it was found in only small number of cases. More studies with large sample size are needed to elucidate the exact role of JAK2 and IDH2 in the development of myelodysplastic syndromes.

Further, in order to study the role of epigenetic regulators and methylation on the prognosis of MDS patients we divided the patients into two groups, group 1-JAK2, IDH2 mutated and SOCS-1 methylated and group 2- JAK2, IDH2 unmutated and SOCS-1 unmethylated. We have not found any significant difference between the two groups in terms of age, sex, TLC, Hb, Platelet, disease progression, transfusion dependency and response to treatment. All these patients were either receiving supportive care or cyclosporine and thalidomide. It may be possible that we can better understand the difference in response in these patients if we administers hypomethylating agents. Bejar et al. published an analysis of 18 genes with respect to mutations (TET2, ASXL1, RUNX1, TP53, EZH2, NRAS, JAK2, ETV6, CBL, IDH2, NPM1, IDH1, KRAS, GNAS, PTPN11, BRAF, PTEN, CDKN2A) using different techniques on 439 patients [15-16]. They found high frequencies of mutations in TP53, TET2 and ASXL1 as well as in RUNX1 which demonstrated that addition of molecular information has significant impact on prognostication of patients with MDS and potentially in selecting therapy. At the moment, it remains uncertain whether mutations in genes coding for epigenetic machinery can predict response to hypomethylating agents. For example, one study reported that TET2 mutations predict response to azacitidine, but do not predict response duration or overall survival [17]. In contrast, other studies suggest that TET2 mutations predict decreased responsiveness to hypomethylating agents [18]. Such studies are limited by small patient samples.

## Conclusion

JAK2 mutation affect increase methylation of tumor suppressor gene SOCS-1 which is well described poor prognostic marker of prognosis. IDH2 mutation has no effect on the methylation of tumor suppressor genes. Although present study is a very small attempt to study the mechanism of epigenetics in vivo. Further research is required

into the subtle interplays between epigenetic regulators and DNA methylation, and how they are influenced by genetic and cytogenetic changes, in order to improve the future outcome of MDS patients.

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