
DNMT3A Co-Mutation with SF3B1, ASXL1 and TET2 in Indian Patients with Myelodysplastic Syndrome (MDS)

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Abstract: Myelodysplastic syndrome (MDS) encompasses a diverse group of closely related clonal hematopoietic disorders. Several genes are often mutated in MDS, of which only five (DNMT3A, SF3B1, ASXL1, TET2 and SRSF2) are known to be mutated in >10% of cases. In this perspective, we studied the frequency of somatic mutations in DNMT3A, SF3B1, ASXL1 and TET2 genes and also the impact of DNMT3A co-mutation with SF3B1, ASXL1 and TET2 genes in a series of 21 Indian patients primarily diagnosed with MDS. Patients with de novo MDS were examined for mutations in DNMT3A, SF3B1, ASXL1 and TET2 genes by Sanger's sequencing. The prognostic impact of DNMT3A mutations was evaluated in juxtaposition with SF3B1, ASXL1 and TET2 gene. Around 62% patients (13 out of 21) were found to have a somatic mutation in at least one of the genes studied herein. Of the 13 patients, 2 patients had single mutation of DNMT3A, 5 carried SF3B1 mutation and one patient each had ASXL1 and TET2 mutation. Likewise, 2 patients carried double mutation of DNMT3A/TET2 and one each carried DNMT3A/SF3B1 and DNMT3A/ASXL1 co-mutation. Our study identified novel missense, nonsense and frameshift mutations in DNMT3A, SF3B1, ASXL1 and TET2 genes for the first time in Indian MDS patients. Distinct mutations of DNMT3A in juxtaposition with SF3B1, ASXL1 and TET2 gene were predictive of clinical status.

Keywords: Sangers' Sequencing, Co-mutations, Stop-gained Frameshift Mutation, IPSS Risk Score

1. Introduction

Myelodysplastic syndrome (MDS) encompasses a diverse group of closely related clonal hematopoietic disorders [1]. Of late, genetic analysis have identified a set of somatic genes, that are persistently mutated in MDS [2, 3]. Such genes are essentially involved in pivotal cellular process such as RNA splicing, signal transduction, epigenetic and traditional transcriptional regulation [4]. Aberrant DNA methylation is a major epigenetic change observed in MDS and plays an important role in their pathogenesis and transformation to AML [5, 6]. DNMT3A, belonging to DNA

methyltransferase (DNMT) family is one of the most commonly mutated genes found in MDS [5] and commonly co-occur with SF3B1, NPM1, FLT3 and IDH1 gene mutation [6]. By and large, patients carrying DNMT3A mutations have a worse overall prognosis [7, 8], however this varies significantly on which collaborating mutation co-exist in that patient [9].

Several studies reported the manifestation of DNMT3A and SF3B1 co-mutation, albeit with varied findings. A study by Martin et al [10] showed worse overall survival (OS) with increased tendency for disease progression in patients who carried double mutation of DNMT3A and SF3B1. However,

Song *et al* [11] ascertained that DNMT3A/SF3B1 double mutation showed a better prognosis than patients with DNMT3A single mutation. However, data on the impact of DNMT3A/ASXL1 and DNMT3A/TET2 co-mutation on the patients' prognosis in MDS is scarce. Yozhizato *et al* [12] found strong evidence of poorer OS and higher rates of transformation to AML in aplastic anemia patients with DNMT3A/ASXL1 co-mutation.

Even though, complete registry of driver mutations recurrently found in a distinguishable fraction of MDS patients have been revealed, prevalence of DNMT3A co-mutation with SF3B1, TET2 and ASXL1 mutations in Indian population has not been explored. Under this milieu, we intended to evaluate the frequency of somatic mutations in DNMT3A, SF3B1, ASXL1 and TET2 genes and the impact of DNMT3A co-mutation in a series of 21 Indian patients primarily diagnosed with MDS.

2. Patients & Methods

Twenty-One Indian subjects with a first diagnosis of myelodysplastic syndrome (MDS) who attended Department of Hematology, Krishna Institute of Medical Sciences, Telangana, between December 2018 and July 2019, were recruited for this study. The Institutional Research Advisory Board and Ethics Committee approved this study. All the individuals participated in the study had given their written informed consent. The study was conducted by following accepted guidelines for human material use in the 1964 Helsinki Declaration as well as its later amendments. The morphological subtype of MDS was classified according to the French-American-British (FAB) criteria. Patients with blast count ranged between 5 – 30% were included in the study. Exclusion criteria include patients with MDS-AML and Chronic myelomonocytic leukemia (CMML).

As a part of diagnostic workup, Patients' history, laboratory test reports and treatment regime were obtained for each participant. An inquiry form was used to collect data about demography, family history of any hematological disorder and other information relevant to the study. Approximately 5mL of bone marrow was collected from each participant for consequent mutational analysis.

3. Mutational Analysis

Bone marrow mononuclear cells were enriched using the Histopaque®-1077 density gradient centrifugation method and total cellular DNA was extracted using FlexiGene DNA kit (Qiagen), according to the manufacturers' instruction. DNAs were screened for mutations in the genes such as DNMT3A (exon 23), SF3B1 (exon 13 – 15), ASXL1 (exon 12) and TET2 (exon 3 and 11). Each gene-specific exon was amplified using primers (Table 1) designed using Primer 3 Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

PCR amplification was performed in 25µl volume containing 100ng cellular DNA; 1X reaction buffer; 1.5–3

mM magnesium chloride; 200 µM dNTP; 20–50 pm each primer; and 0.25 U Taq Polymerase. PCR products were then purified and sequenced by Sangers' sequencing. If a mutation was identified, a new PCR product from the same participant was sequenced on sanger's platform to confirmed the result.

Evaluation of clinical characteristics and disease-free/overall survival pertaining to mutation of DNMT3A, SF3B1, TET2 and ASXL1 genes as single and co-mutation

Clinical characteristics including FAB classification, disease recurrence and death during follow-up were assessed pertaining to mutation so as to ascertain the clinical significance of the mutations identified in our study. All the patients were reviewed at regular intervals till the end of study period (24 months). Disease-free survival (DFS) was measured from the date of initial diagnosis to disease progression. Overall survival (OS) was measured from the date of admission into the study to the date of demise or last follow-up.

4. Results

Following FAB classification, 21 patients with de novo MDS were recruited in this study for mutational analysis. Comprehensive clinical features of the MDS subjects are presented in Table 1. Of these 21 subjects, 10 (55.63%) had refractory anemia (RA), 5 (15.79%) had RA with ring sideroblasts (RARS), 4 (21.05%) had RA with excess blasts (RAEB) and 2 (10.53%) had RAEB in transformation (RAEB-T). According to the Revised IPSS (IPSS-R), 3 subjects (15.79%) were classified as No risk, 4 (15.79%) as very low risk, 7 (31.58%) as low risk, 5 (26.32%) as intermediate risk and 2 subjects (10.53) as high risk. The median follow-up was 19 months (17 – 24 months).

4.1. Mutations Identified in DNMT3A, SF3B1, ASXL1 and TET2 Genes

We amplified and sequenced exon 23 of DNMT3A, exon 13, 14 and 15 of SF3B1, exon 12 of ASXL1 and exon 3 and 11 of TET2 for all the 21 samples. Patient-wise detection of genetic mutations in the DNMT3A, SF3B1, ASXL1 and TET2 genes is shown as Table 2.

Two non-synonymous missense mutations (Q886H and K906N) were detected in the DNMT3A genes, with none of these affecting amino acid at hotspot position 882. Similarly, we found two frameshift mutation (I598LfsX3 and K700KfsX28) in SF3B1, single stop gained frameshift mutation (Y634X) and a missense mutation (D1464N) in ASXL1 and two nonsense mutation (C834X and G93X) in TET2 genes. One mutation (Q886H) was already documented in other hematological diseases [13] but not in MDS. All other mutations were reported first time herein in this study.

Around 62% patients (13 out of 21) were found to have a somatic mutation in at least one of the genes studied. Of the 13 patients, 2 had single mutation of DNMT3A, 5 patients carried SF3B1 mutation alone and one patient each had ASXL1 and TET2 mutation. Likewise, 2 patients carried double mutation of DNMT3A/TET2 and one each carried DNMT3A/SF3B1 and DNMT3A/ASXL1 co-mutation.

Table 1. Clinical Characteristics of MDS Subjects.

Distribution of FAB Group (%)	Sample ID	Age (yrs)	Hemoglobin (g/dL)	WBC (10 ⁹ /L)	ANC (10 ⁹ /L)	Platelets (10 ⁹ /L)	BM Blasts (%)	Risk Factor
RA (55.63)	1	59/M	14.5	4.71	2.6	114	1	0
	2	61/M	5.7	10.47	7.7	408	1	1.12
	3	71/M	7.7	6.5	3.8	276	1	1.54
	4	60/M	9.0	3.1	1.5	25	2	1.6
	5	69/M	6.1	3.32	1.5	43	3	3.47
	6	47/F	10.3	4.59	2.3	90	2	0
	7	56/F	10.8	4.1	2.6	104	1	0
	8	58/F	8.9	4.3	2.5	79	1	0.99
	9	66/F	6.0	2.66	1.1	70	2	1.84
	10	75/F	6.8	3.4	1.3	304	3	2.69
	11	65/F	6.5	7.3	4.9	196	3	1.29
RARS (15.79)	12	73/M	6.1	5	3.6	110	1	1.63
	13	64/M	6.4	2.4	0.8	63	3	2.79
	14	52/F	6.9	3.2	1.4	125	1	1.32
	15	58/M	6.1	4.1	2.3	98	2	2.14
RAEB (21.05)	16	60/F	8.9	3.56	2.1	86	9	3.18
	17	50/F	6.5	4.82	2.6	15	8	3.95
	18	60/F	9.3	0.9	0.2	112	19	4.23
RAEB-T (10.53)	19	46/F	7.7	1.5	0.6	59	17	4.96
	20	73/M	8.7	2.29	1.4	137	25	4.09
	21	31/M	5.3	0.6	0.1	39	28	5.22

FAB – French American and British group classification, RA – Refractory anemia; RARS – Refractory anemia with ring sideroblast; RAEB – Refractory anemia with excess of blast; RAEB-T – Refractory anemia with excess of blast transformation.

Risk factor – (IPSS-RA – Age adjusted calculation of Risk) None (0); Very low (<1.5); Low (1.5 – 3.0); Intermediate (3.0 – 4.5); High (4.5 – 6.0) and very high (>6.0).

Table 2. Patient-wise detection of genetic mutations in the DNMT3A, SF3B1, ASXL1 and TET2 genes.

Sample ID	DNMT3A (NM_022552.5)		SF3B1 (NM_012433.4)		ASXL1 (NM_001363734.1)		TET2 (NM_001127208.3)	
	Sequence Change	Amino acid Change	Sequence Change	Amino acid Change	Sequence Change	Amino acid Change	Sequence Change	Amino acid Change
1	ND	ND	c.2127delA	K700KfsX28	ND	ND	ND	ND
2	c.2935G>T	Q886H	ND	ND	c.4633G>A	D1464N	ND	ND
3	c.2995G>C	K906N	ND	ND	ND	ND	c.2798T>A	C834X
4	ND	ND	c.2127delA	K700KfsX28	ND	ND	ND	ND
5	c.2935G>T	Q886H	c.1761delA	I578LfsX3	ND	ND	ND	ND
6	ND	ND	c.2127delA	K700KfsX28	ND	ND	ND	ND
7	ND	ND	c.2127delA	K700KfsX28	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND	ND
10	c.2995G>C	K906N	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND	ND
12	c.2935G>T	Q886H	ND	ND	ND	ND	ND	ND
13	ND	ND	ND	ND	ND	ND	c.573G>T	G93X
14	ND	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	ND	ND	ND	ND
17	c.2995G>C	K906N	ND	ND	ND	ND	c.2798T>A	C834X
18	ND	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	c.2127delA	K700KfsX28	ND	ND	ND	ND
20	ND	ND	ND	ND	c.2198C>A	Y634X	ND	ND
21	ND	ND	ND	ND	ND	ND	ND	ND

ND – Not Detected.

Table 3. Association of clinical characteristics and disease-free/overall survival with respect to mutation status of DNMT3A, SF3B1, TET2 and ASXL1 genes.

Sample ID	FAB	DNMT3A	SF3B1	ASXL1	TET2	Risk Factor	DFS (Month)	OS (Months)
Patients with event free survival								
1	RA	ND	K700KfsX28	ND	ND	0	24	24
2	RA	Q886H	ND	D1464N	ND	1.12	24	24
4	RA	ND	K700KfsX28	ND	ND	1.6	24	24
5	RA	Q886H	I578LfsX3	ND	ND	3.47	24	24
6	RA	ND	K700KfsX28	ND	ND	0	24	24

Sample ID	FAB	DNMT3A	SF3B1	ASXL1	TET2	Risk Factor	DFS (Month)	OS (Months)
7	RA	ND	K700KfsX28	ND	ND	0	24	24
9	RA	ND	ND	ND	ND	1.84	24	24
12	RARS	Q886H	ND	ND	ND	1.63	24	24
14	RARS	ND	ND	ND	ND	1.32	24	24
15	RARS	ND	ND	ND	ND	2.14	24	24
16	RAEB	ND	ND	ND	ND	3.18	24	24
19	RAEB	ND	K700KfsX28	ND	ND	4.96	24	24
Patients with disease relapse								
3	RA	K906N	ND	ND	C834X	1.54	12	21
8	RA	ND	ND	ND	ND	0.99	22	24
10	RA	K906N	ND	ND	ND	2.69	13	19
11	RARS	ND	ND	ND	ND	1.29	15	21
13	RARS	ND	ND	ND	G93X	2.79	20	24
17	RAEB	K906N	ND	ND	C834X	3.95	19	24
18	RAEB	ND	ND	ND	ND	4.23	6	8
20	RAEB-T	ND	ND	Y634X	ND	4.09	8	9
21	RAEB-T	ND	ND	ND	ND	5.22	20	24

ND – Not Detected.

4.2. Association of Clinical Characteristics and Disease-free/Overall Survival Pertaining to Mutational Status of DNMT3A, SF3B1, TET2 and ASXL1 Genes

We determined disease-free (DFS) and overall survival (OS) of all 21 patients with respect to the presence or absence of mutations in the studied gene and presented as Table 3. Nine out of 21 patients had disease relapse during the follow-up period and five of them died owing to AML or complications raised out of MDS. Patients with specific mutant of DNMT3A gene (K906N) either singularly or as co-mutation with C834X of TET2 gene had shorter median disease-free survival (DFS) of 13 months. Similarly, patient (No.20) with distinct stop gained frameshift mutation of ASXL1 had worse prognosis and shortest OS of approximately 9 months, as compared to other mutations detected. Another patient (No.13) with nonsense mutation of TET2 (G93X) were also associated with disease relapse. However, four patients with no detectable mutation in the studied genes were also found to have disease relapse and two of succumbed to the disease during the follow-up period. Conversely, distinct mutations of DNMT3A (Q886H), SF3B1 (I598LfsX3 and K700KfsX28) and ASXL1 (D1464N) seem to have a favorable prognosis as most of the patients carrying these mutations had event-free survival.

5. Discussion

Several genes are often mutated in MDS and among them, only five (DNMT3A, SF3B1, ASXL1, TET2 and SRSF2) are established to be mutated in more than 10% of cases [1]. In this perspective, we evaluated the frequency of DNMT3A, SF3B1, ASXL1 and TET2 mutations and the impact of DNMT3A co-mutation in a series of Indian patients.

We found that almost 62% of our MDS patients had at least one mutation in the studied genes, which is comparable to certain reports [14]. In this study, we identified missense, nonsense and frameshift mutations in DNMT3A, SF3B1, ASXL1 and TET2 gene reported herein for the first time in

the Indian population.

We detected two novel missense mutations in DNMT3A gene with none affecting amino acid at hotspot position R882. Patients with mutation at 886th position showed an improved event-free survival, irrespective of whether mutation occurred singularly or as co-mutation with SF3B1 and ASXL1. We presume that Q886H variant might not have impacted the catalytic domain of DNMT3A protein and its enzymatic activity, as hotspot R882 mutation [15]. On the other hand, patients with K906N variant singularly or in juxtaposition with TET2 mutation was found to have a poor prognosis regardless of favourable clinical status and low IPSS score, reinforcing the fact that mutations' location as well as co-operating mutation could be crucial components in assessing the extrapolative significance of the mutated gene. SF3B1 emerges to be the only gene for which somatic mutations are associated with a good prognosis in MDS [16]. Our results intently aligned with published reports [17, 11] as our patients harbouring the frameshift mutations of SF3B1 showed improved clinical outcome with event-free survival. Furthermore, one patient with a distinct stop-gained frameshift mutation (Y634X) showed worse prognosis and succumbed to the disease during the follow-up period. Another patient with nonsense mutation (G93X) of TET2 gene also had disease relapse. We presume that frameshift and nonsense mutation of ASXL1 and TET2 gene could have caused truncation of protein leading to the loss of function. Nevertheless, clinical significance of such truncated protein has not been reported earlier. However, ASXL1 mutations in myeloid malignancies have been shown to be loss-of-function mutations resulting in myeloid transformation via loss of polycomb repressive complex 2 (PRC2)-mediated H3K27 tri-methylation [18].

6. Conclusion

Our study identified missense, nonsense and frameshift mutations in DNMT3A, SF3B1, ASXL1 and TET2 genes for the first time in Indian MDS patients. Besides, we presented that distinct mutations of DNMT3A in juxtaposition with

SF3B1, ASXL1 and TET2 gene were predictive of clinical status. Construction of mutation profile by combining several gene mutations could assist in the initial assessment of disease process and also aid in risk stratification of MDS patients for more assertive treatment strategy. However, our findings should be regarded as a preliminary data and need to be validated in a larger clinical setting to effectively develop a mutation-based treatment system for MDS.

Author's Contribution

All authors contributed to drafting and revising the article and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Declaration of Interest

The authors declare that they have no competing interests.

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