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Role of Molecular Mutations, Telomerase and Promoter Methylation Status of the TSGs ($p15^{INK4b}$, SOCS-1, Calcitonin and FHIT) in Disease Severity, Progression and Survival in a Series of 100 MDS Patients: A Study from India

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Abstract

In contrast to the Western countries, Myelodysplastic syndromes (MDS) in India is being increasingly seen in young adults. The severity of the disease is more in Indian patients at time of presentation; moreover their response to treatment is poor. In the present study molecular markers which may affect the biology of MDS patients were studied.Conventional cytogenetics, molecular mutations of RAS and FLT3 genes, hTERT gene expression, telomerase activity (TA) and promoter methylation status of four tumor suppressor genes (TSGs) ($p15^{INK4b}$, SOCS-1, calcitonin and FHIT) were analyzed in a series of 100 MDS patients and correlated with disease severity, progression and survival. It was found that the prevalence of MDS was higher in patients with age<60 years as compared to the patients with age ≥ 60 years (75% vs. 25%). Normal cytogenetics was present in 26/51 (51%) patients and 25/51 (49%) patients had chromosomal abnormalities. The frequency of N-RAS mutation was 3% and K-RAS mutation was 9%. FLT3-ITD and FLT3-TKD mutations were absent. TA was increased in 17/100 (17%) cases. hTERT expression was present in 17/100 (17%) cases. 40 patients (40%) had $p15^{INK4b}$ gene methylation. $p15^{INK4b}$, SOCS-1, FHIT and calcitonin gene methylation was observed in 40 (40%), 53 (53%), 43 (43%) and 58 (58%) patients respectively. After multivariate analysis, only $p15^{INK4b}$ gene methylation was found as an independent predictor for progression of disease in MDS patients. In conclusion, $p15^{INK4b}$ gene methylation may play pivotal role in the diagnosis and disease progression in younger Indian MDS patients.

Keywords: Myelodysplastic Syndromes; $p15^{INK4b}$; SOCS1; Calcitonin; FHIT; hTERT.

Introduction

Myelodysplastic syndromes (MDS) are a group of clonal hematologic disorders characterized by inefficient hematopoiesis, hypercellular bone marrow, dysplasia of hematopoietic cells and cytopenias. In the Western and European countries, most patients are diagnosed in their late 60s to early 70s. But this disease is being increasingly reported in young adults in India and other Asian countries (Silvia et al. 2013). As reports on these younger MDS patients are very limited hence, both clinical and molecular data relevant for the prognostication is lacking.

Presently very few studies on the pathophysiology and molecular biology of MDS have been reported from the Asian countries and the results are contradictory (Matsuda et al. 2005; Lee et al. 1999;

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Chen et al. 2005). Also, it has been observed that there is increased severity of MDS in Indian patients at time of presentation and treatment response in these patients is poorer than reported from the West and Europe (Chaubey et al. 2015). Moreover studies on cytogenetics, molecular genetics and epigenetics (gene methylation) from India have either not been reported or are contradictory (Mansoor et al. 1993; Korgaonkar et al. 2008; Kannan et al. 1999). Molecular abnormalities in young MDS patients and their impacton clinical features and survivalmay provide better the understanding of disease pathology.

Materials and Methods

A total of 100 MDS patients presenting consecutively at the Department of Hematology, All India Institute of Medical Sciences (AIIMS), New Delhi between July 2010 and June 2014, 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 criteria 2008 (Vardiman JW et al. 2009). The IPSS scoring system was applied for the risk stratification of the patients. Also the patients were stratified into good, intermediate and poor groups according the cytogenetic abnormalities as described in IPSS. Chromosome analysis was carried out as we described in our previous study (Chaubey et al. 2011).

Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque. DNAwas isolated by proteinase K/ phenol-chloroform – isoamyl alcohol method. RNA was extracted from the commercially available kit Qiagen, Germany. N-RAS and K-RAS gene mutations (codon 12) were analysed by PCR-RFLP (Constantinidou et al.1997; Zhou et al. 2004). FLT3 gene mutations (ITD and TKD) were analysed by RT-PCR(Noguera et al. 2002; Yamamoto 2001). Semi quantitative analysis of expression of hTERT, component of telomerase was assessed by RT-PCR (Gil et al. 2004). PCR-ELISA TRAP assay was used for the detection and quantification of telomerase activity. Methylation status of the promoter regions of the p15, SOCS-1, Calcitonin, and fragile histidine triad (FHIT) gene was assessedby methylation specific PCR. Bisulphate-modified DNA was amplified by PCR using primer sets as described previously (Hermanet al. 1996; Lin et al. 2008; Fukushima et al. 2003; Ot´avia et al. 2007).

Patient's baseline parameters like hemogram, TLC, platelet, transfusion requirements (U/months) were recorded at the time of initial diagnosis. The criteria for anemia, and thrombocytopenia were Hb <10 g/dL and platelets $<100\times10^9$/L, respectively. All statistical analysis was performed by using STATA 11 (Texas, USA). The values for IPSS variables were based on data obtained at the time of initial diagnosis. Survival curves were estimated according to the method of Kaplan and Meier and statistical differences between curves were assessed by the log-rank test. In case of categorical parameters chi square, Fisher exact test was used to compare the groups. For multivariate analysis, a Cox proportional hazards model was constructed for survival and time to AML transformation and adjusted for potential confounding covariates.

Results

Out of 100 MDS patients, 67 (67%) were male and 33 (33%) were female (M: F:: 2:1) with median age 48 years (range 17 - 84 years). The frequency of different morphological subtypes (WHO 2008) in this study was as follows: refractory anemia with unilineage dysplasia (RCUD) (49%), refractory anemia with excessive blasts-2 (RAEB-2) (16%), refractory anemia with excessive blasts -1 (RAEB-1) (10%), refractory cytopenia with multilineage dysplasia (RCMD) (10%), refractory anemia with ringed sideroblasts (RARS) (6%), del (5q) (6%), refractory anemia with excessive blasts 1 – ringed sideroblasts (RAEB1-RS) (1%), refractorycytopenia with multilineage dysplasia –ringed sideroblasts (RCMD-RS) (1%) and MDS unclassified (MDS-U) (1%).

The different hematological parameters were as follows:Median hemoglobin (Hb) was 6.7 g/L (range of 2.4–14.5 g/L), median total leukocyte count (TLC) was 4.4×10^9/L (range $0.8–38.2\times10^9$/L) and median platelet count was 122 × 109/L (range $0.1–800\times10^9$/L). Bone marrow was normocellular in 64 patients, hypercellular in 16 patients andin hypocellular in 20 patients.

21 (21%) patients progressed during the follow up. 2 patients progressed from RA to AML, 10 patients from RAEB2 to AML, 5 patients with RAEB-1 to AML, 1 patient with RAEB-2 to ALL, 2 patients with RA to RCMD and 1 patient with RAEB2 to MF. The median time of progression was 15 months (range 1-16 months). 32 patients died during the follow up. The median follow-up time of all the patients was 32 months (range 5-121 months).

Table 1: Distribution of mutations in different subtypes of MDS.

Molecular abnormality	RCUD (n=49)	RARS (n=6)	RCMD (n=11)	del (5q) (n=6)	RAEB-1 (n=11)	RAEB-2 (n=16)	MDS-U (n=1)	Total MDS (n=100)	MDS progressed (n=21)	Average duration of progression (months)
(n=) (%)	23	0	4	0	8	11	0	46	18	17.5
	(46.9)	(0)	(36.4)	(0)	(72.7)	(68.8)	(0)	(46)	(85.7)	
N-RAS (%)	0	0	0	0	1	2	0	3	2	8
	(0)	(0)	(0)	(0)	(9)	(12.5)	(0)	(3)	(10)	
K-RAS (%)	5	0	0	0	1	2	0	9	4	21
	(10.2)	(0)	(0)	(0)	(9)	(18.7)	(0)	(9)	(19)	
FLT3-LM (%)	0	0	0	0	0	0	0	0	0	–
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	
FLT3-TKD (%)	0	0	0	0	0	0	0	0	0	–
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	
hTERT expression (%)	10	0	2	0	3	2	0	17	5	21
	(20.4)	(0)	(18.2)	(0)	(27.3)	(12.5)	(0)	(17)	(24)	
Telomerase activity	8	0	2	0	3	4	0	17	7	20
	(16.3)	(0)	(18.2)	(0)	(27.3)	(25)	(0)	(17)	(30)	

Table 2: p15^{ink4b} Methylation and its correlation with hematological parameters.

Characteristics	Methylated p15^{INK4b} (n=40) (%)	Unmethylated p15 ^{INK4b} (n=60) (%)	p value
Age (years) (Range)	50 (14-84)	47.5 (9-80)	0.56
≥ 60	11 (28)	14 (23)	0.819
<60	29 (72)	46 (77)	
Hemoglobin (g/dl) (Range)	6.4 (3-14.5)	6.3 (2.4-14.5)	0.5
≥ 10	38 (95)	09 (15)	0.192
<10	02 (5)	51 (85)	
WBC (109/L) (Range)	4 (0.8-35)	4.4 (1.5-38.2)	0.8
Platelet (109/L) (Range)	42 (0.1-381)	112.5 (4.71-800)	0.03
≥ 100	12 (30)	33 (55)	0.015
<100	28 (70)	27 (45)	
RCUD (49)	14 (35)	35 (58.3)	0.02
RARS (6)	5 (12.5)	1 (1.6)	0.03
RCMD (11)	6 (15)	5 (8.3)	0.32
del 5q (6)	1 (2.5)	5 (8.3)	0.39
MDS-U (1)	0	1 (1.66)	1
RAEB-1 (11)	4 (10)	7 (11.6)	1
RAEB-2 (16)	10 (25)	6 (0.1)	0.05
Karyotype (n=51)	21	30	0.56
Good (n)	13 (62)	21 (70)	
Poor +Intermediate (n)	08 (38)	09 (30)	
IPSS score (n=51)	21	30	0.581
Low (n)	08 (39)	17 (57)	
Intermediate (n)	10 (25)	09 (15)	
High (n)	03 (14)	04 (13)	

Table 3: Calcitonin gene methylation and its correlation with hematological parameters.

Characteristics	Methylated calcitonin gene (n=58) (%)	Unmethylated calcitonin gene (n=42) (%)	p value
Age (years) (Range)	50 (9-84)	45.5 (11-80)	0.3
≥ 60	20(34)	05(12)	
< 60	38(66)	37(88)	0.011
Hemoglobin(g/dl) (Range)	6.2 (2.4-13.3)	6.5 (3.5-14.5)	0.68
≥ 10	08 (14)	03 (7)	
< 10	50 (86)	39 (93)	0.35
WBC (10^9/L) (Range)	5 (1.5-38.2)	3.9 (0.8-14.2)	0.4
Platelet (10^9/L) (Range)	89.5 (2.5-746)	82.5 (0.1-800)	0.5
≥ 100	28(48)	17(40)	
< 100	30(52)	25(60)	0.54
RCUD (49)	22 (37.9)	27 (64.2)	0.015
RARS (06)	5 (8.6)	1 (2.3)	0.39
RCMD (11)	9 (15.5)	2 (4.7)	0.11
del 5q (06)	2 (3.4)	4 (9.5)	0.23
MDS-U (01)	1 (1.7)	0 (0)	1
RAEB-1 (11)	9 (15.5)	2 (4.7)	0.11
RAEB-2 (16)	10 (17.2)	6 (14.2)	0.78
Karyotype (51)	38	13	0.03
Good (n)	22(58)	12(92)	
Poor +Intermediate (n)	16(42)	01(8)	
IPSS score (n=51)	38	13	0.001
Low	15(39.4)	10 (76.9)	
Intermediate	16 (10.6)	03(7.6)	
High	07 (18.4)	00 (0)	

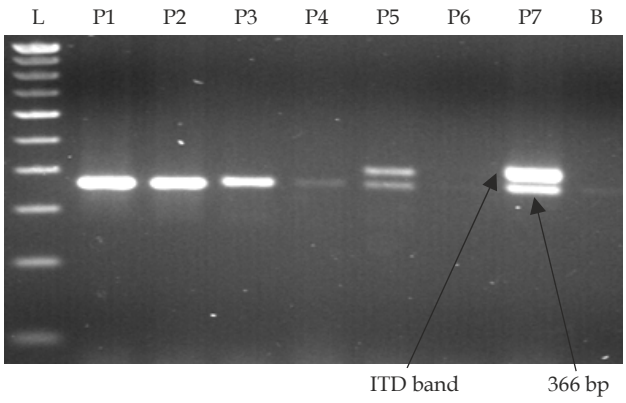
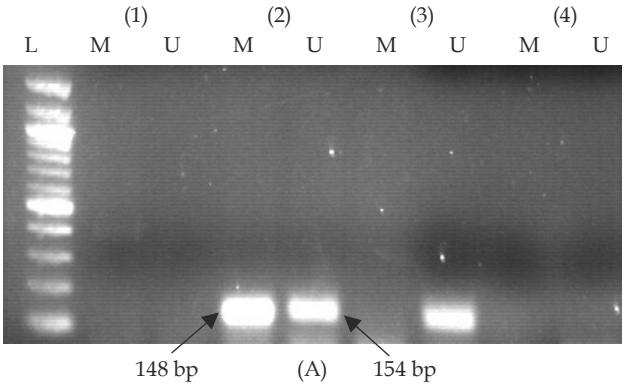
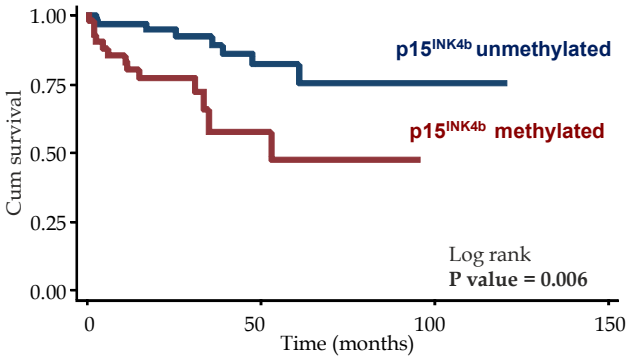
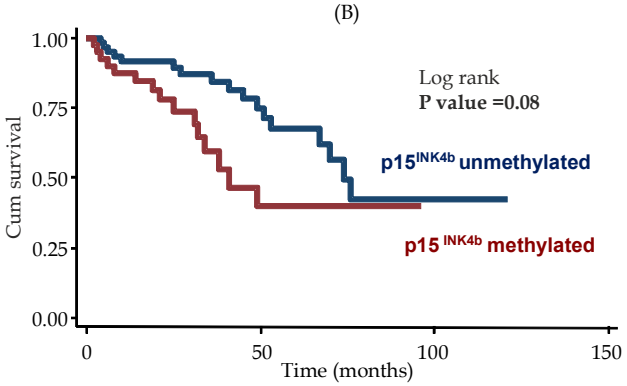


Fig. 1: FLT3 - ITD mutation.





(B)



(C)

Fig. 2: (A) p15 gene methylation B) Comparison of PFS and OS of patients with methylated and unmethylated p15

Table 4: FHIT methylation and its correlation with hematological parameters.

Characteristics	Methylated FHIT gene (n=43) (%)	Unmethylated FHIT gene (n=57) (%)	p value
Mean Age (years) (Range)	48 (9-77)	48 (11-84)	0.8
≥ 60	10 (23)	15 (26)	
<60	33 (77)	42 (74)	0.818
Hemoglobin(g/dl) (Range)	6.7 (3-13.3)	6.3 (2.4-14.5)	0.8
≥ 10	04 (9)	07 (12)	
<10	39 (91)	50 (88)	0.753
WBC (10^9/L) (Range)	4.3 (0.8-38.2)	4.4 (1.57-35)	0.65
Platelet (10^9/L) (Range)	83 (0.1-381)	85 (2.95-800)	0.8
≥ 100	20 (47)	25 (44)	
< 100	23 (53)	32 (56)	0.841
RCUD (49)	19 (44.1)	30 (52.6)	0.42
RARS (6)	1 (2.3)	5 (8.7)	0.23
RCMD (11)	5 (11.6)	6 (10.5)	1
del 5q (6)	0 (0)	6(10.5)	1
MDS-U (1)	0 (0)	1 (1.7)	1
RAEB-1 (11)	7 (16.2)	4 (0.7)	0.19
RAEB-2 (16)	11 (25.5)	5 (8.7)	0.02
Karyotype (51)	23	28	
Good (n)	12 (37)	22 (79)	0.07
Poor +Intermediate	11 (33)	6 (21)	
IPSS score (51)	23	28	0.07
Low	12 (37)	22 (79)	
Intermediate + High	11 (33)	06 (21)	

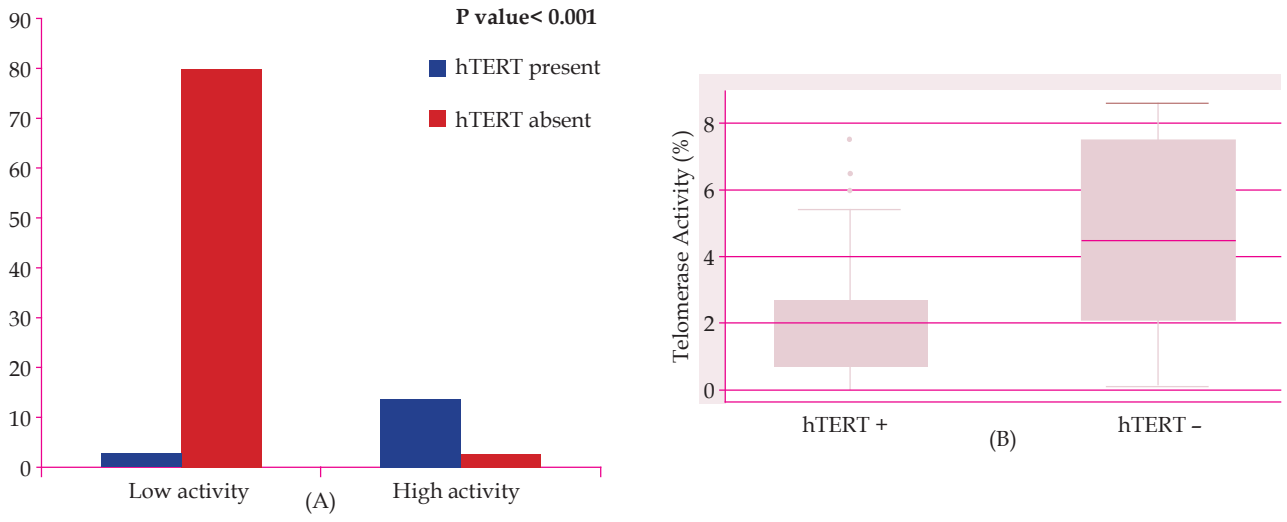


Fig. 3: (A) Comparison of telomerase activity and hTERT Expression (B) Box and whiskers plot showing the comparison of telomerase activity and hTERT expressionunmethylated p15.

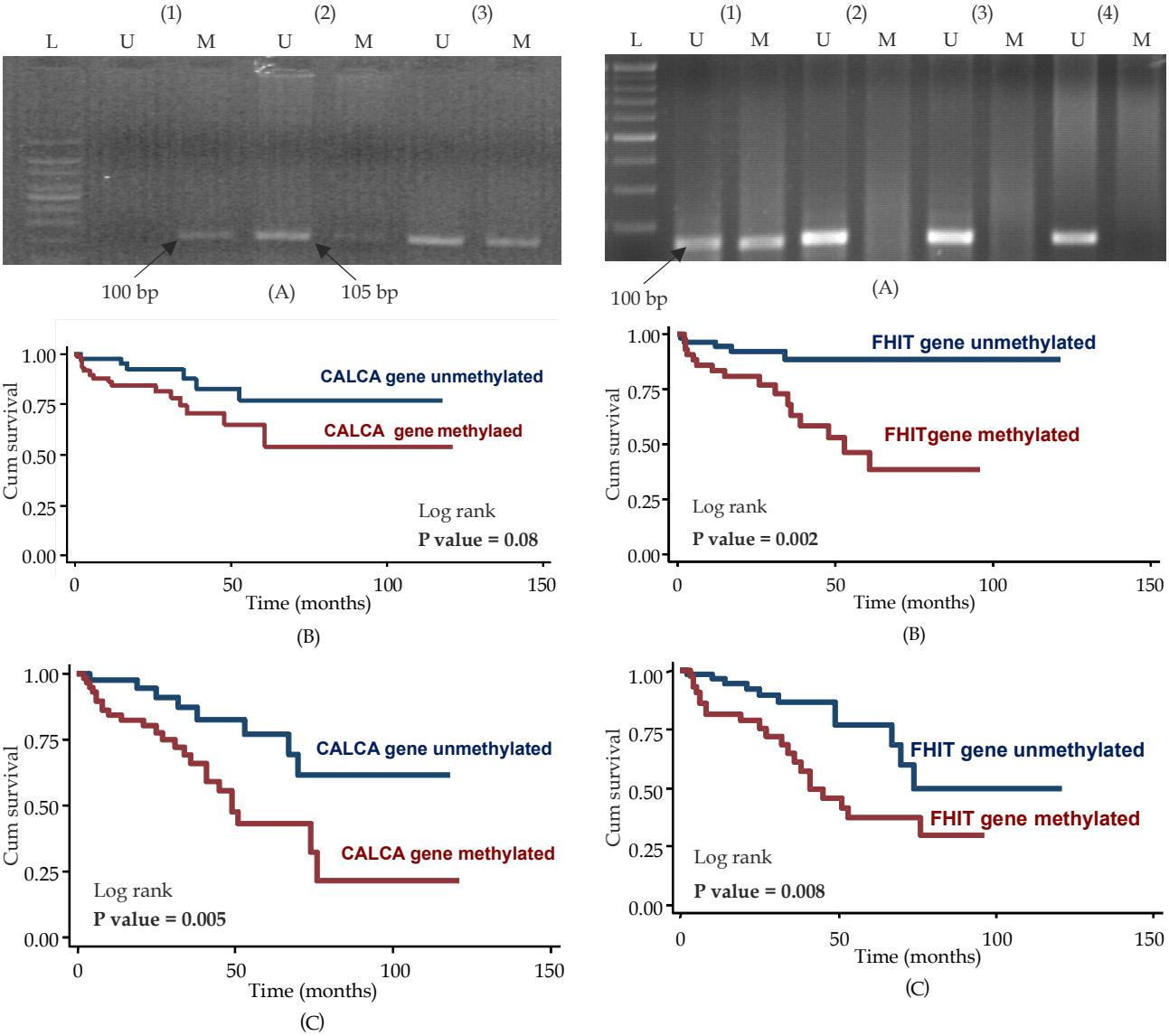


Fig. 4: (A) Methylation specific PCR for FHIT gene (B) and (C) PFS and OS of patients with methylated and unmethylated FHIT gene.

Of the 100 patients, karyotyping was successfully carried out in 51 patients. 26 (51%) patients had normal karyotype, while 25 (49%) patients had abnormal karyotype. Results which included the details of the cytogenetic findings were already published in our previous paper (Chaubey et al. 2011).

The results of the molecular abnormalities of the 100 patients within the different subgroups have been shown in Table 1. RAS mutations (codon 12) were detected in 12/100 patients (12%) of which N-RAS mutation was detected in 3 patients; 2 patients in RAEB-2 and 1 was in RAEB-1 group.2 out these 3 patients progressed to acute leukemia.K-RAS mutation was detected in 9/12 RAS positive patients; 4 patients progressed to acute leukemia 3 were in RAEB-2, 1 patient with RAEB-1 group (Table 1).

FLT3-ITD and FLT3-TKD mutations were absent in all the 100 MDS patients (figure 1).The TA was ranging from 0 to 0.86 units (mean 0.24±0.21 U). TA was high in 17/100 cases (17%). 14 out of these 17 cases (82%) co-expressed hTERT. The remaining 83 patients had low TA and hTERT expression was present in only 3 cases. This strongly suggests the association of increasedTA with hTERT expression in MDS patients ($p<0.001$)(Figure 2-A and 2-B).

40/100 (40%) patients showed methylated p15 INK4b(Figure 3-A). On comparing the methylation status with the hematological parameters, it was observed that the patients with p15^{INK4b} gene methylation had significantly lower platelet counts ($p<0.03$). The frequency of methylated allele in different WHO subtypes, clinico-hematological parameters and prognostication based on IPSS scoring is as in (Table 2).

The disease progression was observed in 13 (32.5%), 8 (13.3%) patients with methylated $p15^{INK4b}$ and unmethylated $p15^{INK4b}$ gene respectively ($p<0.02$).A significant difference was observed between PFS of patients in methylated $p15^{INK4b}$ and unmethylated $p15^{INK4b}$ gene (p=0.006) (Figure 3-B). The median overall survival (OS) (4 years) was significantly shorter in patients with methylated $p15^{INK4b}$ gene as compared to the patients with unmethylated $p15^{INK4b}$ gene (41 months vs. 74 months, p=0.04) (Figure 3-C).

53/100 (53%) casesshowed SOCS-1 methylation. No difference was noted in the hematological parameters in the patients with methylated SOCS-1 and the patients with unmethylated SOCS-1. Results which included frequency of methylated allele in different WHO subtypes, clinico-hematological profile and prognostication based on IPSS scoring were already published in our previous paper (Chaubey et al. 2015).

58/100 (58%) casesshowed calcitonin methylation (Figure 4-A). There was no difference in the hematological parameters in the patients with methylated calcitonin and the patients with unmethylated calcitonin. The frequency of methylated allele in different WHO subtypes, clinico-hematological profile and prognostication based on IPSS scoring is as in Table 3.

There was a no significant difference observed between the PFS of patients in methylated calcitonin group and unmethylated calcitonin group (p = 0.08) (Figure 4-B). The median OS (4 years) was significantly shorter in patients with methylated calcitoningene when compared to the patients with unmethylated calcitoningene (49 months vs. – months (not reached, p=0.005) (Figure 4-C).

43/100 (43%) cases showed FHIT gene methylation(Figure 5-A). No difference was found in hematological parameters between the patients with methylated and the patients with unmethylated FHIT gene.The frequency of methylated allele in different WHO subtypes, clinico-hematological profile and prognostication based on IPSS scoring is as in Table 4.

A significant difference was observed between the PFS of patients in methylated FHIT group and unmethylated FHIT group (p=0.002) (Figure 5-B). The median OS (4 years) was significantly shorter in patients with methylated FHITgene when compared to the patients with unmethylated FHITgene (41 months vs. 74 months, p=0.008) (Figure 5-C).

Multivariate Analysis for Prognostic Factors

Disease Progression

Univariate analysis for the molecular factors such as gene methylation of $P15^{INK4b}$, SOCS-1, FHIT, Calcitonin, telomerase activity, hTERT expression and RAS gene mutations was performed for the prediction of progression. Out of these factors, gene methylation of SOCS-1 (HR 4.68 , 95% CI, 1.57-13.9, P -0.006), FHIT (HR 4.85 , 95% CI, 1.77-13.26, P -0.002), $p15^{INK4b}$ gene,(HR 3.55 , 95% CI, 1.45-8.71, P - 0.006) mutations of RAS gene (HR 2.76 , 95% CI, 1.07-7.1, P 0.03) and level of telomerase activity (HR 2.5, 95% CI, 1.03-6.3, P = 0.04) were found significant.

After adjusting WHO subtypes in multivariate analysis which was the only independent predictors for the progression of disease in univariate analysis $p15^{INK4b}$ gene methylation was found as an important predictor for progression of disease (HR 5.15, 95% CI, 1.64-16.1 P=0.005).

Overall Survival

Univariate analysis for the molecular factors such as gene methylation of SOCS-1, FHIT, calcitonin, TA, hTERT expression and RAS gene mutations was performed for the prediction of OS. Out of these factors only methylation of SOCS-1 (HR 5.73, 95% CI, 2.20-14.9, P -0.00), FHIT (HR 2.68, 95% CI, 1.29-5.57, P -0.008), calcitonin (HR 2.99, 95% CI, 1.33-6.72, P -0.008) gene and TA were identified as factor predicting OS.

After adjusting age, WHO risk groups, IPSS risk groups and disease progression in multivariate analysis which were the independent predictors for the progression of disease in univariate analysis, none of the molecular factors was found as a predictor for OS.

Discussion

MDS are a heterogeneous group of myeloid neoplasms that develop primarily in elderly patients. The median age at diagnosis in most of the reported case series from the USA and Europe is 60 and 75 years (Haase et al. 2007; Germing et al. 2008). In our study the median age of patients at diagnosis was 48 years which signifies the prevalence of MDS in younger population in Indian subcontinent. It has been reported that the patients from China, Southeast Asia, Turkey and Central

Africa are also generally younger than those from Western countries, and the reason for this difference is unclear (Yoshida 1987; Demirkan 2007; Mukiibi 1994; Wang 2010). Presently, the impact of race on the biology of MDS is poorly understood (Silvia et al. 2013). We have already reported a comparative study in year 2016, depicting the difference in molecular biology of Indian MDS patients from the Western patients (Chaubey et al. 2016). However, it is speculated that younger age of Indian patients may be due to differential action of various environmental factors, including an increase in exposure to etiological relevant risk factors such as organic solvents, pesticides, radiation and environmental pollution (Solé et al. 2005). Although reports are available on the racial and geographic differences in the morphology and prognosis of MDS, very sparse data exists which compares the clinical , hematological and molecular characteristics of Western and Asian patients with MDS.

Several studies conducted with large number of MDS patients have shown that the incidence of mutation at codon 12 of the N-RAS gene ranged from 0% to 33% (Constantinidou et al. 1997), which is similar to our observation of the present study in which it was found in 12% of MDS patients. In contrast to few studies which reported more frequency of mutated N-RAS than K-RAS in MDS patients, Lyon et al reported higher frequency of K-RAS than N-RAS (5.8% vs. 3%) (Browett et al. 1989; Yunis et al. 1989). In our study, we found K-RAS mutated more frequent than N-RAS (9% vs. 3%) which is line with study by Lyon et al (Lyonset al.1988). The difference in the reported frequencies may be because of the use of different techniques having different sensitivity by different authors.

In the present study 2/3 patients with N-RAS mutations and 4/9 patients with K-RAS progressed to acute leukemia. Of the these 2 patients with N-RAS mutated, one patient belonged to RAEB-1 and other belonged to RAEB-2. Of the 4 patients with K-RAS three belonged to RAEB-2 group and one to RAEB-1 group. Our findings suggest a RAS point mutation may be one of the factor involved in the progression of disease.

Internal tandem duplication (ITD) within JM/ TK-1 domains as a somatic mutation of the FLT3 gene has been reported in 20 – 25% of adult patients with acute myeloid leukemia (AML) and 3% of adult MDS (Yokota et al. 1997; Georgiou et al. 2006). In the present study, none of patients had FLT3 genetic aberrations. This may be due to the very low frequency of these mutations were reported in MDS. Therefore, large sample size may be needed for confirmation of this observation.

Telomerase is thought to play important role in cell immortalization and carcinogenesis. The most widely used strategy for detecting TA is the TRAP assay. By this method, TA can be detected in as few as 10 immortal cancer cells (Wright et al. 1995). In present study, TA was increased in 17% patients in contrast to other studies who have reported the frequency of increased telomerase activity 5.4% (Engelhardt et al. 2000; Li et al. 2000). In the present study, the frequency of hTERT mRNA expression in MDS patients is 17%, similarly reported by Ohshima K et al. (23%) (Ohshima et al. 2003). We have selected the catalytic subunit of telomerase (hTERTmRNA) because it is believed to be critical in producing and regulating TA.

We have also found that the increased TA was highly associated with hTERT expression ($p< 0.001$). Similar study by Briatore et al.also reported that higher TA and hTERT expression in MDS patients (Briatore et al. 2009). There are some studies which reported the variable TA in MDS patients (Engelhardt et al. 2000; Ohyashiki et al. 2001).

The $p15^{INK4b}$ gene plays an important role in the negative regulation of the proliferation of hematopoietic cells and in the prevention of malignant transformation. The observed frequency of 15^{INK4b} gene methylation in this study is 40% which more or less similar to the findings of others (Rigolin et al. 2004; Uchida et al. 1997). This is the first study from India reporting the methylation status of $p15^{INK4b}$ gene by using highly sensitive methylation specific PCR in MDS patients. Solomon et al. also reported the $p15^{INK4b}$ genemethylation in 61% of Indian patients but used another technique i.e. restriction digestion followed by semi-nested multiplex PCR (Kim et al. 2010). No significant association was found between the $p15^{INK4b}$ gene methylation and age, sex, baseline hemoglobin and, WBC counts. However, it was observed that patients with methylated $p15^{INK4b}$ gene were more thrombocytopenic as compared to the patients without $p15^{INK4b}$ methylation. In this study patients with $p15^{INK4b}$ gene methylation had a shorter progression free survival than those without. Progression was found in half of the patients (50%) with $p15^{INK4b}$ gene methylation which indicates the role of $p15^{INK4b}$ gene hypermethylation as an important event in the progression of MDS.

In this study a substantial portion of patients (53%) with MDS had SOCS-1 gene methylation. In present study, SOCS-1 genemethylation occurred more frequently in high-risk subtypes of MDS.

Also, the disease progression was observed more frequent in these patients (p=0.006). Hence, the patients with SOCS1 methylated gene had both poor PFS (p=0.006) as well as OS (p=0.000). This is the first time from India we have demonstrated the associations of SOCS1 gene methylation with clinical and biological features of MDS and disease progression (Chaubey et al. 2015).

In the present study calcitonin gene was methylated in 58 % of patients which is slightly lower than the reported frequency (68–92%). Dhodapkar M et al. found calcitonin gene methylation in 65% of patients and in nine patients with MDS and normal cytogenetics (Dhodapkar et al. 1995; Solomon et al. 2008) but they didn't found any correlation between calcitonin gene methylation and subtype of MDS. Presently, its role in disease progression is not clearly understood. In the present study no statistical correlation was found between calcitonin gene methylation and disease progression. However, the median OS (4 years) was significantly shorter in patients with methylated calcitonin genethan those without (p=0.005). We speculate that calcitonin promoter methylation may play an important role in the disease progression.

FHIT gene plays an important role in anti-cancer and the abnormal methylation of FHIT gene is found in many carcinomas. In this study, the frequency of FHIT gene methylation was 43%, which is line with the studies in Japanese (55%) and Chinese population (47.2%) (Germing et al. 2008; Dhodapkar et al. 1995). Both investigators also indicated that the frequency and density of FHIT methylation was increased in the advanced-stages of MDS and the relapsed AML cases.

Of all the molecular factors studied, only p15INK4b gene methylation is an independent factor for predicting PFS (p=0.005). The patients with methylated $p15^{INK4b}$ gene had 5 fold increased risk of leukemic transformation than the patients with unmethylated $p15^{INK4b}$ gene [HR = 5.15 (95% CI: 1.64-16.1)]. This suggests the important role of hypomethylating agents in the treatment of MDS patients. Presently, very few centres in India areusing the hypomethylating agents for management of MDS. Furthermore, the usage of hypomethylating agents and monitoring of $p15^{ink4b}$ gene methylation in more health care facilities may provide better insight in understanding the pathophysiology and prognosis of MDS.

In the present study, we analyzed and depicted the involvement of molecular markers withthe prognosis for the first time in Indian MDS patients. Although there are many similarities between Indian patients with the Western data in terms of molecular biology, some striking differences also exits.These differences may be due the aetiology, ethnicity or environmental factors. To better explore the pathogenesis of MDS in Indian patients who are much younger than rest of the world, comparative studies which includes samples from India as well as rest of the world is needed. This will provide the crucial information about the exact development of MDS and will be helpful in excluding the age related dysplastic changes. Also more studies in larger subsets of the patients is suggested to confirm our findings.

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