RESEARCH ARTICLE

Genetic landscape of myelodysplastic syndrome and its prognostic relevance: a study from Pakistan

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Abstract

Objective: To determine the genetic landscape of myelodysplastic syndrome patients, and to evaluate the impact of gene mutations on disease prognosis and overall survival.

Method: This descriptive study was conducted from April 2019 to April 2021 at the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, and comprised myelodysplastic syndrome patients of either gender. Targetted gene panel sequencing and Sanger sequencing were performed on blood and bone marrow samples. Different variant analysis was performed on sequencing data to identify the frequency of various mutations in genes related to myelodysplastic syndrome. Survival analysis and other tools were employed to analyse the impact of prognostic factors and gene mutations. Data was analysed using SPSS 24 and GraphPad Prism 8.

Results: Of the 47 patients, 32(68.1%) were males and 15(31.9%) were females. The overall median age was 66 years (interquartile range: 20 years). Targetted gene panel sequencing was done in 09(19.14%) cases, and Sanger sequencing in 38(80.85%). Mutation was present in 15(32%) cases, while it was absent in 32(68%). The most commonly mutated genes were DDX10 (9%), TET2 (6%), RUNX1(6%), and ASXL1(6%). In general, presence of any gene mutation reflected a poor prognosis (HR=1.54, p=0.24) and shorter median overall survival (median OS=7.5 months, p-trend=0.07) in MDS patients. In individual patients harbouring these mutations, the DDX10 and TET2 mutations suggested a low-risk and favourable prognosis, while RUNX1 mutations had an adverse prognosis, translating into high-risk myelodysplastic syndrome. Whereas, the ASXL1 gene exhibited both low-risk and high-risk MDS disease. In addition, SF3B1 gene mutation in MDS-MLD-RS presented with a favourable outcome. Median overall survival was 11 months (ranging from 3-38 months with an IQR of 11 months), with 36(76.6%) patients succumbing to the disease.

Conclusion: Involvement of genetic variants in the initiation, diagnosis and prognosis of myelodysplastic syndrome was noted.

Key Words: Myelodysplastic syndromes, Exome sequencing, High-throughput nucleotide sequencing, Mutation, Survival rate.

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Introduction

Myelodysplastic syndrome (MDS) is a group of clonal haematopoietic stem cell disorders characterised by cytopenias, dysplasia in one or more of the major myeloid lineages, ineffective haematopoiesis, recurring genetic abnormalities, and an increased chance of progressing acute myeloid leukaemia (AML).[1] Pallor is the most common presenting clinical feature, followed by symptoms of fatigue, repeated infections, and bleeding/bruising. Diagnosis of MDS primarily depends on morphological proof of dysplasia upon visual inspection of a peripheral blood film and bone marrow aspirate.[2] Studies such as karyotype, flow cytometry or molecular genetic testing supplement and refine the diagnosis further.[2] MDS may be primary (de novo) in nature, arising from unknown causes, or may be acquired due to secondary causes, like vitamin B12 or folate deficiency, infections, chemotherapy and radiation. A substantial number of studies regarding the mutational landscape of MDS have persistently authenticated the presence of specified genetic mutations in >90% cases.[3]

A large number of genes have been established to be responsible for causing recurrent mutations in MDS that can be driver or passenger mutations. Mostly involving genes responsible for ribonucleic acid (RNA) splicing (including splicing factor 3b subunit 1 [SF3B1], Serine and arginine-rich splicing factor 2 [SRSF2], U2 small nuclear RNA auxiliary factor 1 [U2AF1] and Zinc Finger CCCH-

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Type, RNA Binding Motif And Serine/Arginine Rich 2 [ZRSR2]), deoxyribonucleic acid (DNA) methylation (including DNA methyltransferase 3A [DNMT3A], tet methylcytosine dioxygenase 2 [TET2], and Isocitrate dehydrogenase 1 and 2 [IDH1/2]) and chromatin modification (including Enhancer of zeste homolog 2 [EZH2], ASXL transcriptional regulator 1 [ASXL1], and BCL6 corepressor [BCOR]).[4] It has been reported that mutations in genes involved in RNA splicing, DNA methylation, histone modification, transcription factors, signal transduction proteins and the components of the cohesion complex participate in the pathogenesis and development of MDS. MDS patients with SF3B1 mutations have good prognoses, while tumour protein p53 (TP53), ASXL1 and DNMT3 mutations are well known to be affiliated with bad results.[5] Current studies using next-generation sequencing (NGS) provide substantial progress in recognising the relationship between commonly found gene mutations and clinical phenotypes of MDS.[5] In Pakistan, to our knowledge, no such study has been carried out. The current study was planned to fill the gap in literature by defining the genetic landscape of MDS in the Pakistani population, and to determine the relevance of these gene mutations to its prognosis.

Materials and Methods

The descriptive study was conducted from April 2019 to April 2021 at the Department of Haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, and comprised MDS patients of either gender. Approval was obtained from the institutional ethics review committee, and written informed consent was obtained from all the subjects before enrolment. The mutational analysis did not need sample size determination, but to increase generalisability of the findings, sample size was calculated using the World Health Organisation (WHO) sample size calculator[6] with a mutation detection rate of 88% in MDS patients[7] and absolute precision of 10%. The sample size was inflated by 15%.

Diagnosis of MDS was based on the morphological assessment of peripheral blood film and bone marrow slides, in accordance with the WHO classification and criteria of MDS diagnosis (2016).[8] Only treatment-naive primary MDS patients were considered. Those with secondary or therapy-related MDS were excluded.

Genomic DNA was extracted from bone marrow aspirate or peripheral blood mononuclear cells using a pure link genomic DNA kit (Qiagen, Germany). Quantitative and qualitative assessments of extracted DNA samples were made using Qubit 4 fluorometer (Thermo Fisher Scientific, USA) as per the manufacturer's recommendations. In the first stage, clinical whole exome sequencing (WES) was performed on a subset of nine MDS samples. Extracted DNA samples were amplified in a multiplex polymerase chain reaction (PCR), followed by tagging with sample-specific indexes and sequencing adapters using the HiSeq® platform (Illumina, USA). With a mean read length of 240 base pairs (bps) and an average sequencing depth of >600x, coverage of more than 100x was achieved for >95% target bases.

Supplementary Table-S1: The 142 genes analysed in the clinical exome sequencing of myelodysplastic syndrome (MDS) patients.

ACD	CUX1	XRCC2	IRF1	PDS5B	RPS15	SMC1A	ZRSR2
ANKRD26	DDX10						
DDX41	FANCL	JAK2	PHF6	RPS17	SMC3		
ARID2	DKC1	FANCM	JARID2	POT1	RPS19	SRSF2	
ASXL1	DNAJC21	FANCN	KDM6A	PRPF8	RPS24	STAG2	
ATM	DNMT3A	FANCO	KIT	PTEN	RPS26	SUZ12	
ATRX	EED	FANCP	KMT2	PTPN11	RPS27	TCAB1	
BCOR	EFL1	FANCQ	KRAS	RAD21	RPS27A	TERT	
BCORL1	ERCC4	FANCR	LUC7L2	RAD51	RPS28	TET2	
BRAF	ESCO2	FANCS	MPL	RAD51C	RPS29	TINF2	
BRCA1	ETV6	FANCT	MPO	RIT1	RPS7	TP53	
BRCA2	EZH2	FANCU	NAF1	RPL11	RTEL1	TPP1	
BRCC3	FANCA	FANCV	NCOR2	RPL15	RUNX1	TR	
BRIP1	FANCB	FBWX7	NF1	RPL23	SAMD9	TSR2	
CBL	FANCC	FLT3	NHP2	RPL26	SAMD9L	U2AF1	
CDKN2A	FANCD1	GATA1	NIPBL	RPL27	SBDS	U2AF2	
CEBPA	FANCD2	GATA2	NOP10	RPL31	SETBP1	UBE2T	
CEBPA	FANCE	GNAS	NPM1	RPL35A	SF1	WRAP53	
CSF3R	FANCF	GNB1	NRAS	RPL36	SF3B1	WT1	
CTC1	FANCG	IDH1	PALB2	RPL5	SF3B1	XRCC2	
CTCF	FANCI	IDH2	PARN	RPS10	SLX4	ZCCHC8	

The NGS reads thus generated were aligned with the human reference genome assembly [Genome Reference Consortium Human Build 37 (GRCh37)/hg19] and the variants were called using DRAGEN somatic v3.2.8 and germline v3.0.2 pipelines (Illumina, USA) for somatic and germline variants, respectively. Standard filters were applied to meet the expected quality control (QC) metrics where low confidence variants (likely mapping or sequencing artifacts) were removed based on a somatic/germline quality score threshold. The variant detection was restricted to the targetted regions covered by the clinical exome library preparation kit that was used. A list of 142 genes was generated (Table S1).

Given 100x coverage (the median for clinical WES performed), probabilities of detecting a somatic variant with at least 3 mutant reads, which is the minimum required for de novo variant calling, given a true variant allele fraction (VAF) of 15%, 10% and 5% were 99.9%,

Supplementary Table-S2: Primer sequences for Sanger sequencing-based targetted mutational analysis of the selected variants identified from clinical exome sequencing data.

Sr. No	Target genes	Forward primer	Primer sequence	Reverse primer	Primer sequence	Tm (C°)	Product size (bp)
1	*ASXL1*	ASXL1F	TTAAAGGTCAGCCCACTTAC	ASXL1R	CATCATCACTTTCCCAGGAA	55	648
2	*RUNX1*	RUNX1F	TCAGTCACCATCCTTTCTTG	RUNX1R	CATATTTGAACAAGGGCCAC	55	523
3	*SF3B1*	SF3B1F	AGGAGTTGCTGCTTCAG	SF3B1R	TGGTCTGGCTACTATGATCT	55	768
4	*RTEL1*	RTEL1F	GAAGGTGAATGGCATCCTG	RTEL1R	GTCCTGGAGGCGTAAATAAT	56	593
5	*DDX10*	DDX10bF	TCTGAAAGTCACATAAGGGC	DDX10bR	GCAACAAACATCTCTTGGAG	55	399
6	*BCOR*	BCORF	GCTAACTGTCATGTATGGCT	BCORR	CTCGACTCGCCAAATAAGTA	55	522
7	*BCORL1*	BCORL1F	GAAGCATCTGTAGCTCCTTT	BCORL1R	CTTCTTGCTCTGTCAGGTAC	55	363
8	*CBLB*	CBLBF	TCATAAGCACTCCAACTTCC	CBLBR	AGTTTTTGTCTGAGGTAGGC	55	576
9	*CEBPA*	CEPBAF	TACTCGTTGCTGTTCTTGTC	CEPBAR	GACATCAGCGCCTACATC	55	668
10	*DDX10*	DDX10F	GTCTCTTGGTCTTGCTGTG	DDX10R	CGTCTTTAAGGTCCAATCCA	55	452
11	*DNMT3A*	DNMT3AF	TGTGGCTGAATCTTCCTTAC	DNMT3AR	CTCCAGATGTTCTTCGCTAA	55	552
12	*FANCC*	FANCCF	GGAAGTTGAGGAGAAGGTG	FANCCR	TGATTTGTGGCTGTGATAGG	55	516
13	*FOXP1*	FOXP1F	AATGGTGGTCTAACTTCTGC	FOXP1R	CAAGTGTCACTCTCTCCAAG	56	350
14	*JAK2*	JAK2F	TTCAGGTGTTATGGGTCAAG	JAK2R	TGGGCATTGTAACCTTCTAC	55	590
15	*KANSL1*	KANSL1F	GAGGAGCTTTGTGTTTTCTG	KANSL1R	CTCTGATGCTGAGGAACAAT	55	360
16	*SAMD9*	SAMD9F	TTCCCAGAGAATGTGCATAG	SAMD9R	CCCACACATATTTCAGGGAT	55	523
17	*SAMD9L*	SAMD9LF	GATGGTGTATTGTTCTGCAG	SAMD9LR	TGATCCCAGAGAGATCAGAG	55	537
18	*SETBP1*	SETBP1aF	CCTTAAAACCAAAGCACCAG	SETBP1aR	GTAATCCGGACATGACTTGA	55	595
19	*SETBP1*	SETBP1bF	CAACTACACCAAGATCCTGT	SETBP1bR	CTTTTGGCTTTCACCTCCT	55	504
20	*TET2*	TET2F	GCTGATGATGCTGATAATGC	TET2R	GTTGTGTTACTTTGGTTGGG	55	439

BP: base pair, C: Celsius, F: forward, R: Reverse, and Tm: melting temperature.

97.9% and 70.5%, respectively. For germline variant calling, mutant VAF of at least 0.2 was considered. The detected somatic/germline single nucleotide variant (SNV) and indel calls were processed for variant filtration and annotation to determine high probability oncogenic mutations.

Initially, synonymous and intronic variants (except essential splice site variants) were filtered out. The selected variants were further shortlisted based on their minor allele frequency in the South Asian population from gnomAD v2.1.1 database and 1000 genomes project (Ensembl genome database), and those with MAF<0.01 or unknown allele frequency were retained. All the identified variants were evaluated with respect to their pathogenicity and causality using common pathogenicity prediction and tertiary annotation tools, including PolyPhen-2, Sorting Intolerant From Tolerant (SIFT), Mutation Taster and others.[9] To support pathogenicity assessments, the detected variants were corroborated with mutation records, if available, from the general or cancer-specific variant databases, including ClinVar[10], dbSNP[11] and COSMIC[12]. Variant reporting was done in accordance with American College of Medical Genetics and Genomics (ACMG) guidelines[9], where all variants related to the phenotype of the patients, except benign or likely benign variants, were considered. As per standard protocols to ensure quality in tertiary reporting, the resultant variants of relevance were individually validated in-house, but those variants that met the internal QC criteria, based on extensive validation processes, did not require validation by Sanger sequencing. Targetted sequencing of specific variants of 20 mutated genes identified in the initial NGS analysis was performed by using custom designed primers (Table S2). Further, 25-50ng genomic DNA was amplified in a standard PCR reaction for each target region, and the purified amplicons were subjected to bidirectional sequencing using BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Alignment of resultant chromatograms to wild-type (WT) target gene sequences from the human reference genome assembly GRCh37 and variant detection was performed using

SeqMan Ultra 17 packages of DNAStar Lasergene (DNAStar Inc., USA). Pathogenicity predictions, tertiary annotations, and reporting of thus identified variants were same as described above in clinical exome analysis sub-section.

Data was analysed using SPSS 24 and GraphPad Prism.[8] Categorical variables were presented as frequencies and percentages or as mean ± standard deviation for normally distributed data, while median with interquartile range (IQR) was used for skewed, continuous data. Statistical comparisons were made using chi-square (or Fisher's exact test for qualitative variables, and unpaired student's t-test or Mann-Whitney U test for quantitative variables. The patients were followed up from the time of diagnosis till the conclusion of the study, and 2-year overall survival (OS) was documented. The prognostic significance of demographic, clinical and genetic variables was explored with respect to OS using Cox proportional hazard model. Survival curves were plotted using Kaplan-Meier estimates, and log-rank statistics were used to evaluate differences between prognostic factors and OS curves. Two-sided $p<0.05$ was considered statistically significant.

Results

Of the 47 patients, 32(68.1%) were males and 15(31.9%) were females. The overall median age was 66 years (IQR: 20 years), with 30(63.8%) patients aged >60 years. Anaemia was present in 26(59.1%), cases, thrombocytopenia in 21(44.6%) and neutropenia in 14(29.8%).

Mutation was absent in 32(68%) cases, while it was present in 15(32%). Of these, 7(46.7%) patients had single gene mutations and 8(53.3%) had multiple (Figure 1, Table S3).

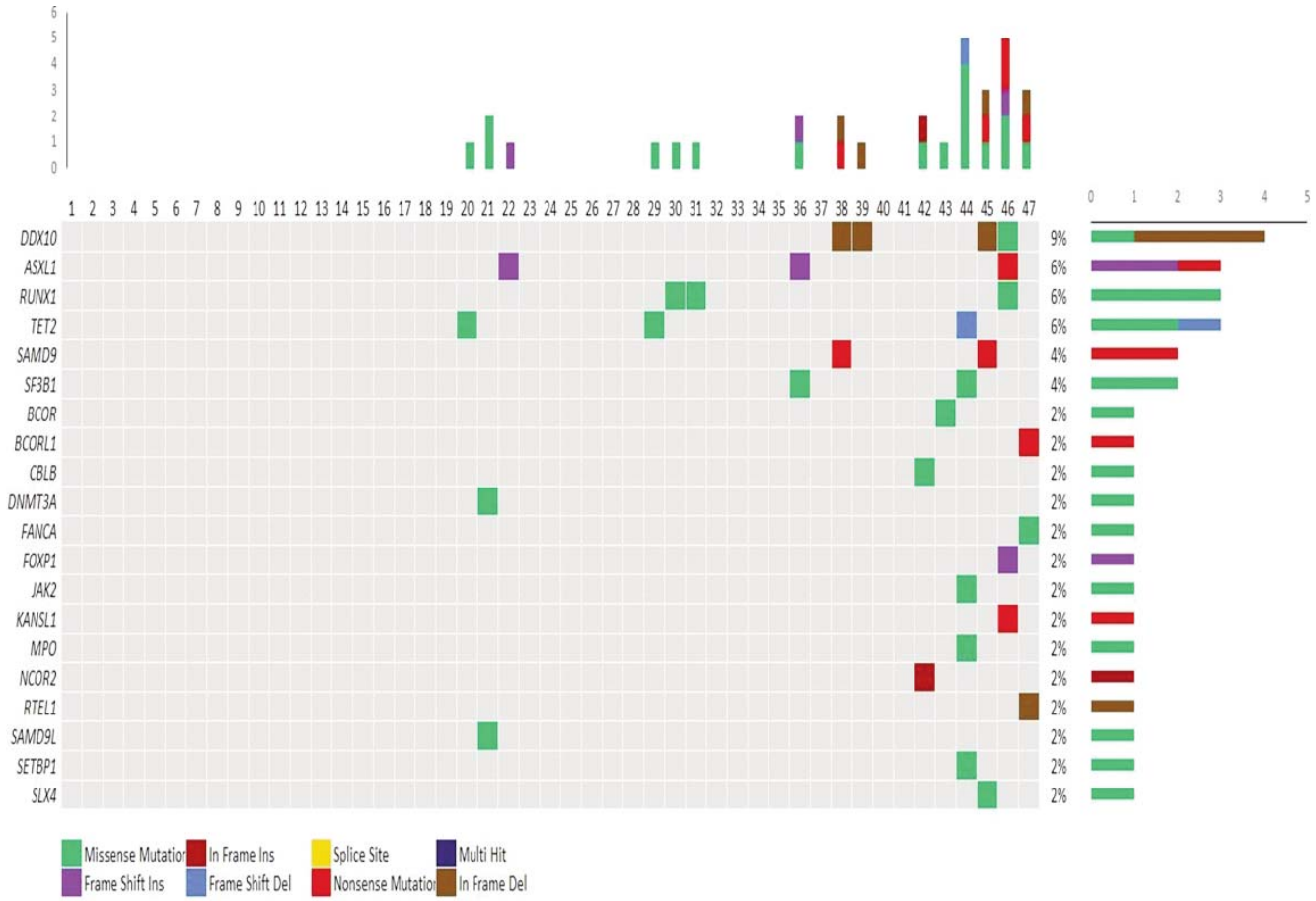

Figure-1: Oncoplot displaying the spectrum of mutations in 20 MDS-associated candidate genes.

Each column represents an individual MDS patient and each row represents an individual mutated gene, while each coloured cell represents a mutation of the gene pertaining to that row.

BCORL1: BCL6 corepressor-like 1, CBLB: Casitas B lymphoma-b, FANCA: Fanconi anemia complementation group A, FOXP1: Forkhead box protein P1, JAK2: Janus kinase 2, KANSL1: KAT8 regulatory NSL complex subunit 1, MDS: Myelodysplastic syndrome, MPO: myeloperoxidase, NCOR2: Nuclear receptor corepressor 2, RTEL1: regulator of telomere length 1, SAMD9: sterile alpha motif domain-containing protein 9, SAMD9L: sterile alpha motif domain-containing protein 9 like, SETBP1: SET binding protein 1, and SLX4: SLX4 structure-specific endonuclease subunit.

Supplementary Table-S3: Candidate gene mutations identified.

Patient ID	WES/ Sanger	Gene	Transcript	cDNA	Protein	Mutation Type	Exon/ Intron	Zygosity	Variant classification	COSMIC	ClinVar	dbSNP	Reference
MDS20	Sanger	TET2	NM_001127208.3	c.1088C>T	p.Pro363Leu	Missense	Exon 3	Het	VUS	COSM5020141	VCV000135309.1	rs17253672	Novel in MDS
MDS21	WES	DNMT3A	NM_175629.2	c.1934C>T	p.Thr645Ile	Missense	Exon 16	Het	Likely pathogenic	NR	NR	rs1387749234	Novel in MDS
MDS21	WES	SAMD9L	NM_152703.3	c.697A>G	p.Thr233Ala	Missense	Exon 5	Het	Likely pathogenic	NR	NR	rs780573740	Novel gene & variant in MDS
MDS22	Sanger	ASXL1	NM_015338.6	c.1927_1928insA	p.Gly643GlufsTer15	Frameshift insertion	Exon 13	Het	Pathogenic	COSM142310	NR	NR	Pubmed: 23018865
MDS29	Sanger	TET2	NM_001127208.3	c.1088C>T	p.Pro363Leu	Missense	Exon 3	Het	VUS	COSM5020141	VCV000135309.1	rs17253672	Novel in MDS
MDS30	Sanger	RUNX1	NM_001754.5	c.814C>G	p.Gln272Glu	Missense	Exon 8	Het	Likely pathogenic	NR	NR	NR	Novel in MDS
MDS31	Sanger	RUNX1	NM_001754.5	c.814C>G	p.Gln272Glu	Missense	Exon 8	Het	Likely pathogenic	NR	NR	NR	Novel in MDS
MDS36	WES	SF3B1	NM_012433.2	c.1873C>T	p.Arg625Cys	Missense	Exon 14	Het	Likely pathogenic	COSM110696	VCV000376535.1	rs775623976	Pubmed: 21886174
MDS36	WES	ASXL1	NM_015338.5	c.1994_1995insTGGTGATGGTGGTGAGGCC	p.Cys672rpfsTer1	Frameshift insertion	Exon 13	Het	Likely pathogenic	NR	NR	NR	Novel in MDS
MDS38	WES	DDX10	NM_004398	c.2340_2342del	p.Asp788del	Nonframeshift deletion	Exon 17	Het	Likely pathogenic	COSM1721800	NR	rs756833840	Novel in MDS
MDS38	WES	SAMD9	NM_001193307	c.1792C>T	p.Gln598Ter	stopgain	Exon 2	Het	Pathogenic	NR	NR	rs767325816	Novel in MDS
MDS39	Sanger	DDX10	NM_004398	c.2340_2342del	p.Asp788del	Nonframeshift deletion	Exon 17	Het	Likely pathogenic	COSM1721800	NR	rs756833840	Novel in MDS
MDS42	WES	CBLB	NM_001321796	c.743T>C	p.Ile248Thr	Missense	Exon 6	Het	Likely pathogenic	NR	NR	NR	Novel in MDS
MDS42	WES	NCOR2	NM_001077261	c.5487_5488insAGCAGCGGCGGGGGGTGGGGGCAGCAGCGGC	p.Gly1829_Gly1830insSerSerGlyGlyGlyGlySerSerGly	Nonframeshift insertion	Exon 38	Het	Pathogenic	NR	NR	rs1555301110	Novel gene & variant in MDS
MDS43	WES	BCOR	NM_017745.5	c.965C>T	p.Thr322Ile	Missense	Exon 4	Hemi	VUS	NR	NR	NR	Novel in MDS
MDS44	WES	JAK2	NM_004972.3	c.1849G>T	p.Val617Phe	Missense	Exon 14	Het	Pathogenic	COSM12600	VCV000014662.58	rs77375493	Pubmed: 21034166
MDS44	WES	SF3B1	NM_012433.2	c.2098A>G	p.Lys700Glu	Missense	Exon 15	Het	Likely pathogenic	COSM84677	VCV000376004.7	NR	Pubmed: 21886174

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MDS44	WES	SETBP1	NM_015559	c.860G>A	p.Gly287Glu	Missense	Exon 4	Het	VUS	NR	NR	NR	Novel in MDS
MDS44	WES	TET2	NM_001127208	c.1256del	p.Pro419LeufsTer8	Frameshift deletion	Exon 3	Het	Pathogenic	COSM4383791	NR	rs12400 75151	Pubmed: 24030381
MDS44	WES	MPO	NM_000250.1	c.752T>C	p.Met251Thr	Missense	Exon 6	Het	VUS	COSM3755703	VCV000003628.7	rs5637 8716	Novel gene & variant in MDS
MDS45	WES	SLX4	NM_032444.2	c.5212G>T	p.Gly1738Trp	Missense	Exon 15	Het	VUS	NR	VCV000848629.5	rs5562 17576	Novel gene & variant in MDS
MDS45	WES	DDX10	NM_004398	c.2340_2342del	p.Asp788del	Nonframeshift deletion	Exon 17	Het	Likely pathogenic	COSM1721800	NR	rs7568 33840	Novel in MDS
MDS45	WES	SAMD9	NM_001193307	c.1792C>T	p.Gln598Ter	stopgain	Exon 2	Het	Pathogenic	NR	NR	rs7673258 16	Novel in MDS
MDS46	WES	ASXL1	NM_015338.5	c.2077C>T	p.Arg693Ter	stopgain	Exon 13	Het	Pathogenic	COSM4169684	VCV000620281.3	rs3732210 34	Pubmed: 21576631
MDS46	WES	RUNX1	NM_001754.4	c.964_965del	p.Ser322AsnfsTer277	Frameshift deletion	Exon 8	Het	Likely pathogenic	COSM26017	NR	NR	Pubmed: 17910630
MDS46	WES	DDX10	NM_004398	c.1525C>T	p.Arg509Cys	Missense	Exon 13	Het	Pathogenic	COSM6641509	NR	rs7550867 24	Novel in MDS
MDS46	WES	FOXP1	NM_001244813.1	c.940dup	p.Leu314ProfsTer47	Frameshift insertion	Exon 9	Het	Pathogenic	COSM6666485	VCV000194567.7	rs7970446 52	Novel in MDS
MDS46	WES	KANSL1	NM_001193466.1	c.1816C>T	p.Arg606Ter	stopgain	Exon 6	Het	Pathogenic	COSM980250	VCV000031693.11	rs2818654 69	Novel in MDS
MDS47	WES	BCORL1	NM_001184772	c.4012C>T	p.Arg1338Ter	stopgain	Exon 6	Hemi	Pathogenic	NR	NR	NR	Novel in MDS
MDS47	WES	RTEL1	NM_001283009.1	c.287_289del	p.Ala96del	Nonframeshift deletion	Exon 3	Het	Likely pathogenic	COSM5278937	NR	rs7699090 59	Novel gene & variant in MDS
MDS47	WES	FANCA	NM_000135.2	c.2236G>T	p.Ala746Ser	Missense	Exon 25	Het	VUS	NR	VCV000321349.13	rs5751084 46	Novel gene & variant in MDS

Het: Heterozygous, Hom: Homozygous, NR: not reported, and VUS: variant of unknown significance.

Targetted gene panel sequencing was done in 9(19.14%) cases, and Sanger sequencing was done in 38(80.85%) Figure 2), and different representative mutations were detected in different oncogenes, including DDX10 gene (deletion of amino acid Aspartate [Asp] at position 788), ASXL1 gene (insertion of nucleotide A that resulted in replacement of Glycine [Gly] by Glutamic acid [Glu] amino acid at position 643 and subsequent shifting of reading frame), RUNX1 gene (substitution of nucleotide C by G that resulted in amino acid Glu instead of Glutamine [Gln] at position 272), and TET2 gene (substitution of nucleotide T that resulted in amino acid Leucine [Leu] instead of Proline [Pro] at position 363). The most commonly mutated genes included DDX10 (2 different mutations in 4[9%] patients), ASXL1 (3 different mutations in 3[6%] patients), RUNX1 (2 different mutations in 3[6%] patients), and TET2 (2 different mutations in 3[6%] patients) (Figure 3). The most commonly detected oncogenic variants were missense mutations 14(53.8%) followed by stop gain 04(15.4%), frameshift insertions 3(11.5%), frameshift deletions 2(7.7%), non-frameshift deletions 2(7.7%), and non-frameshift insertions 1(3.8%). Most patients harboured mutations in genes related to epigenetics 10(66.7%), followed by transcription and

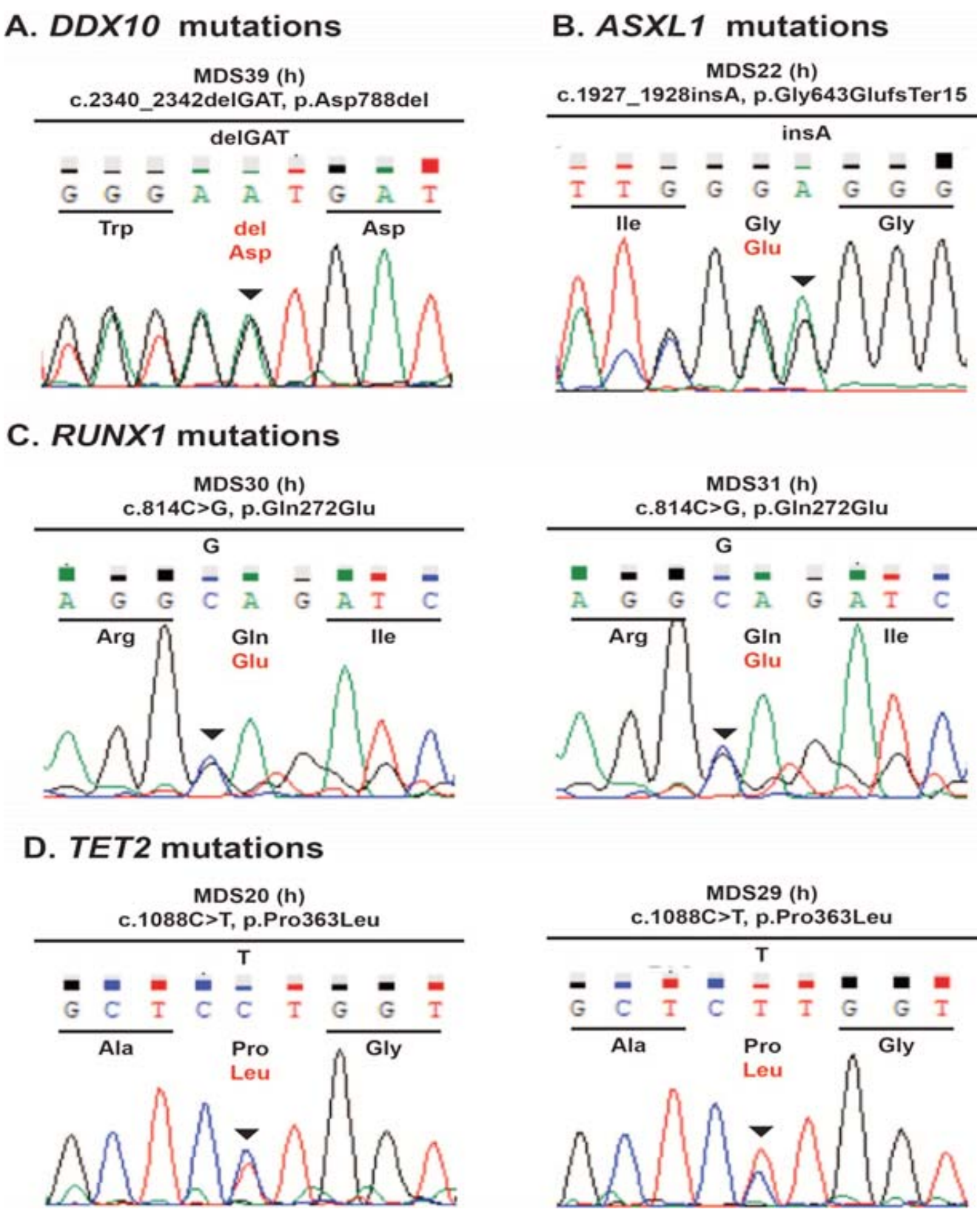


Figure-2 Sequencing electropherograms of representative mutations identified by Sanger deoxyribonucleic acid (DNA) sequencing analysis.

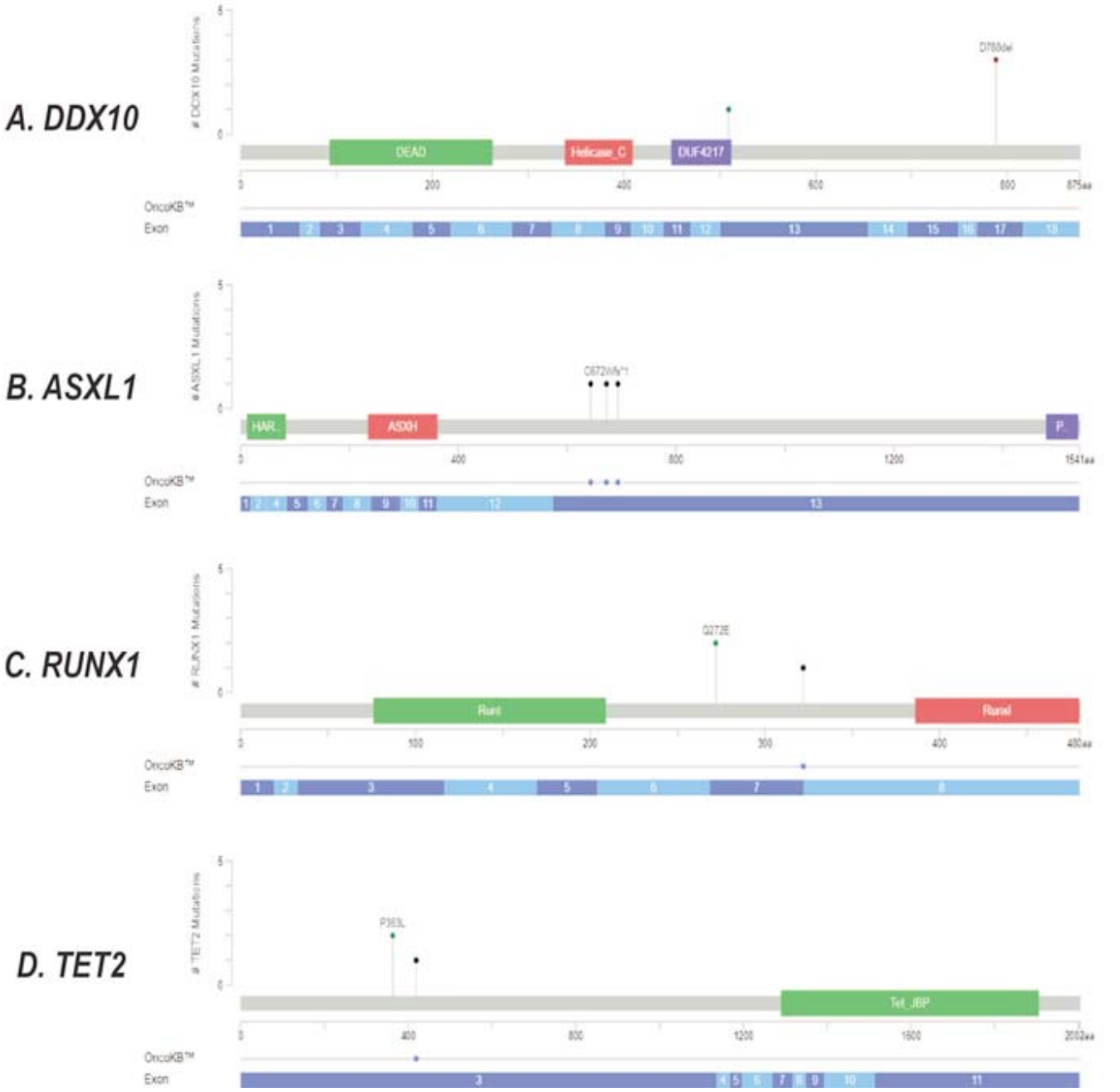


Figure-3: Lollipop plots for mutation distribution in top mutated genes in myelodysplastic syndrome (MDS) showing each gene with domain organisation of resultant protein having amino acid positions and corresponding coding exons on the X-axis, and a number of mutations on the Y-axis with the most common mutation in each gene labelled.

translation factors 6(40%), cell proliferation and apoptosis 3(20%), DNA binding, replication and repair 3(20%), cell signalling 2(13.3%) and splicing 2(13.3%) (Figure 4).

Most of the demographic and clinic-pathological factors were comparable among MDS patients with or without mutation (p>0.05) (Table 1, Figure S1).

In univariate analysis, thrombocytopenia of <50x109/L was associated with a poor 2-year OS (p=0.002). No significant association was observed for the presence of any gene mutation on the prognosis of MDS patients (Table 2).

Median OS was 11 months (ranging from 3-38 months with an IQR of 11 months), with 36(76.6%) patients

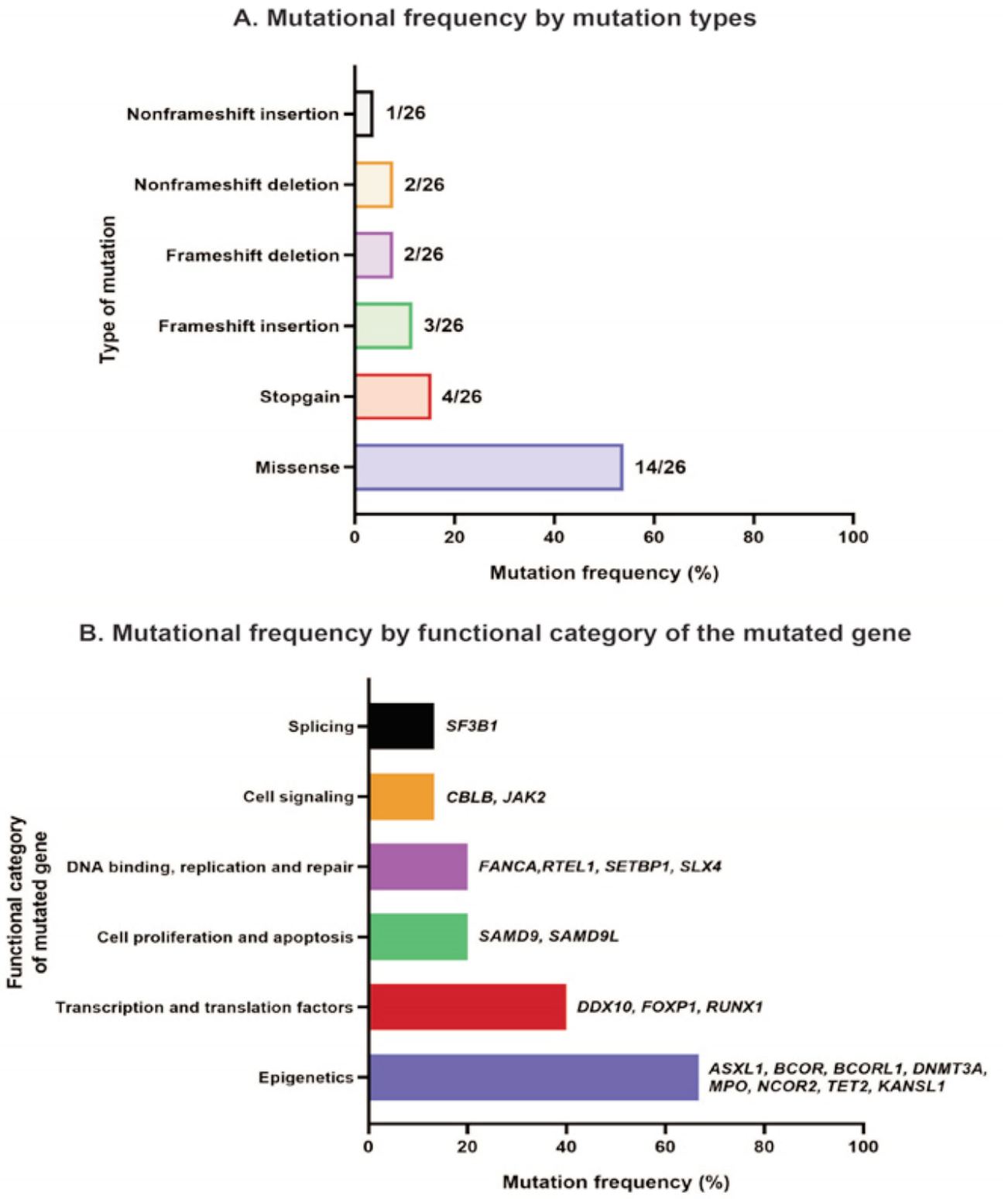
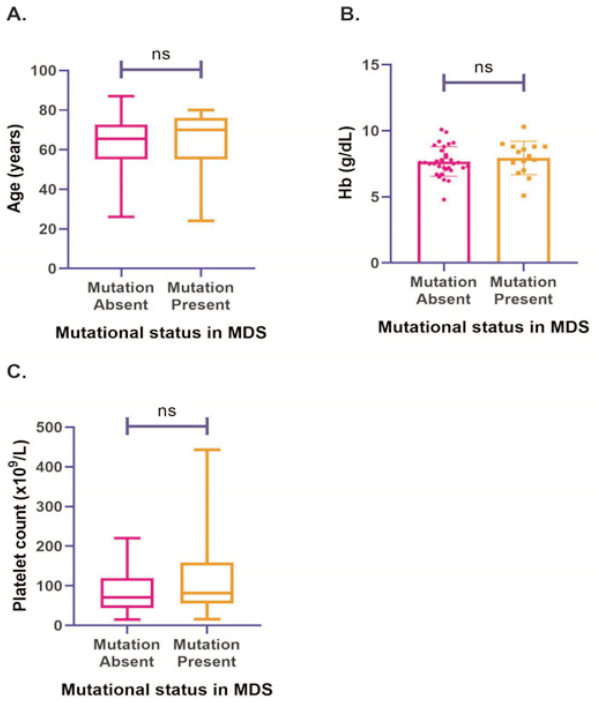


Figure-4: Bar plots displaying (A) distribution of different mutation types detected in myelodysplastic syndrome (MDS) patients, and (B) mutational frequency by functional category of the mutated gene.

Table-1: Intergroup comparison of baseline demographic and clinical characteristics.

Patient characteristics	Frequency (n, %)/median (IQR)/mean (±SD) Mutation absent (n = 32)	Mutation present (n = 15)	p-value for comparison
Median Age with IQR (years)	65.5 (17.75)	70 (21)	0.479*
Age >60 years	21 (65.6%)	09 (60%)	0.961†
Age ≤60 years	11 (34.4%)	06 (40%)	
Males	23 (71.9%)	09 (60%)	0.627†
Females	09 (28.1%)	06 (40%)	
Mean haemoglobin levels with SD (g/dL)	7.7 ± 1.1	8.0 ± 1.3	0.469‡
Haemoglobin <8g/dL	22 (68.8%)	04 (33.3%)	0.040†
Haemoglobin ≥8g/dL	10 (31.2%)	11 (66.7%)	
Median platelet counts with IQR (x109/L)	71 (75.8)	82 (104)	0.190*
Platelets <50 x109/L	11 (34.4%)	03 (20%)	0.504†

*p-values computed from Mann-Whitney U test
†p-values computed from Chi-square test
‡p-values computed from unpaired student's t test
dL: desi Litre, IQR: Interquartile range, L: litre, n: number, SD: Standard deviation.



Supplementary Figure-S1: Baseline quantitative variables, including (A) Age, (B) Hb, and (C) platelet count, stratified by mutational status in 47 myelodysplastic syndrome (MDS) patients.
ns: not significant.

succumbing to the disease. Survival curve with platelets count <50x109/L was associated with decreased OS with a median OS of 05 months (IQR: 7 months) (p=0.0007). Mutation status also showed a trend of dismal OS with single mutation (median OS: 10 months, IQR: 10 months)

Table-2: Prognostic factors for overall survival in MDS patients of present study.

Variables	Univariate HR (95% CI)	p-value	Multivariate HR (95% CI)	p-Value
Age	0.99 (0.97-1.01)	0.472	-	-
Gender (Female)	0.84 (0.40-1.76)	0.647	-	-
Haemoglobin (<10 g/dL)	1.69 (0.82-3.47)	0.153	-	-
Platelets (<50 x109/L)	2.97 (1.49-5.90)	0.002	2.27 (0.99-5.18)	0.052
Gene mutation (Present)	1.54 (0.77-3.06)	0.224	-	-

HR: Hazard ratio, CI: Confidence interval.

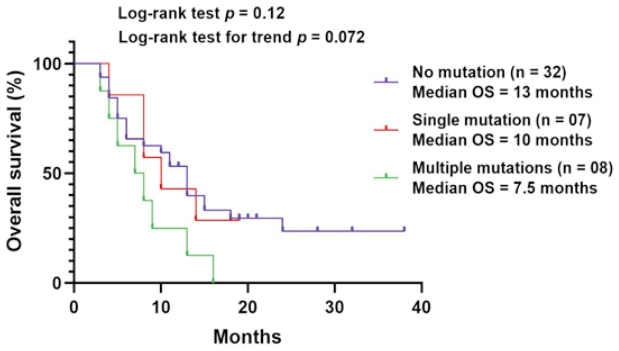


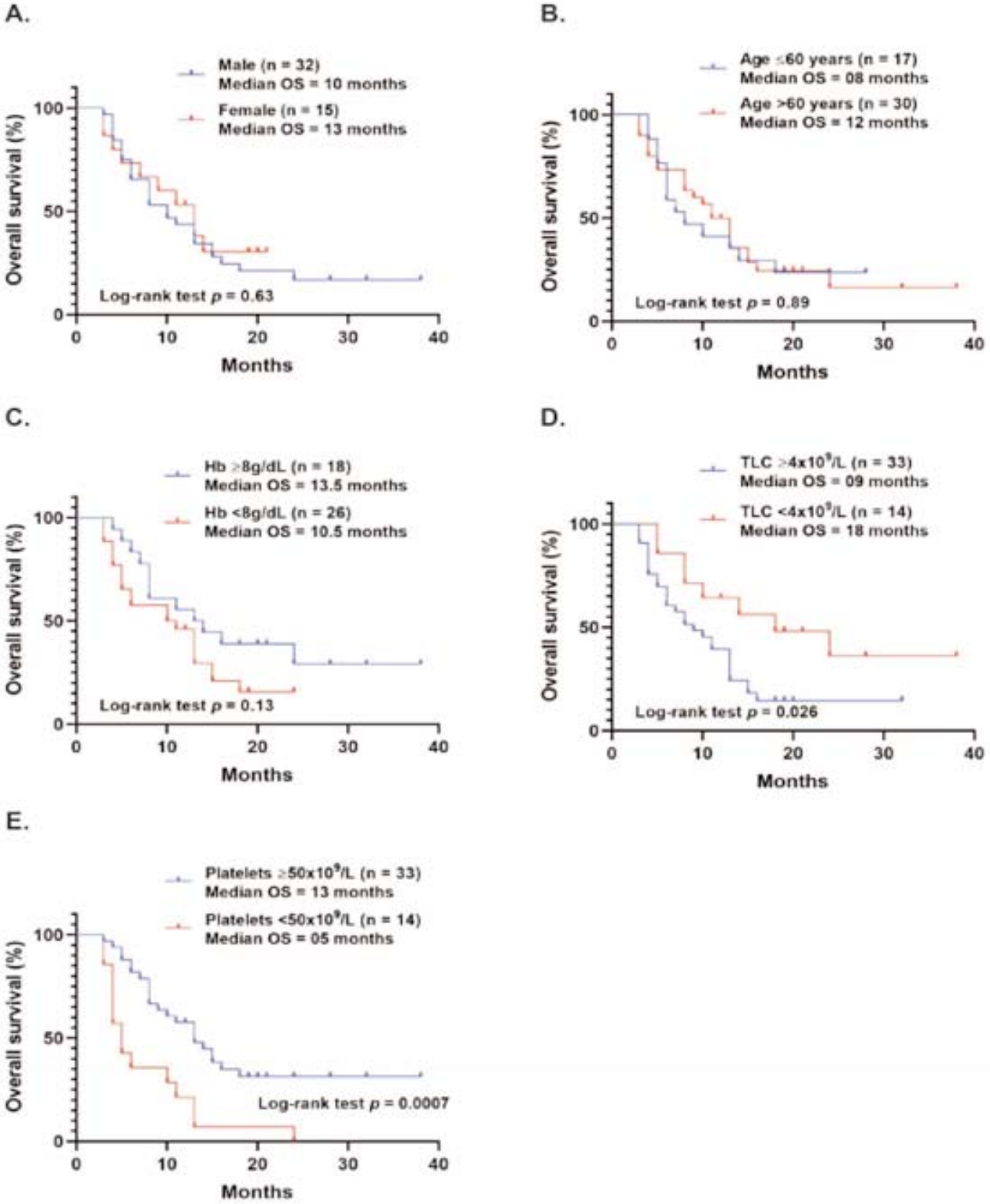
Figure-5: Overall survival (OS) with respect to mutational status.

and multiple mutations (median OS: 7.5 months, IQR: 6.5 months), although this association was not statistically significant (p=0.072) (Figures 5, S2-3).

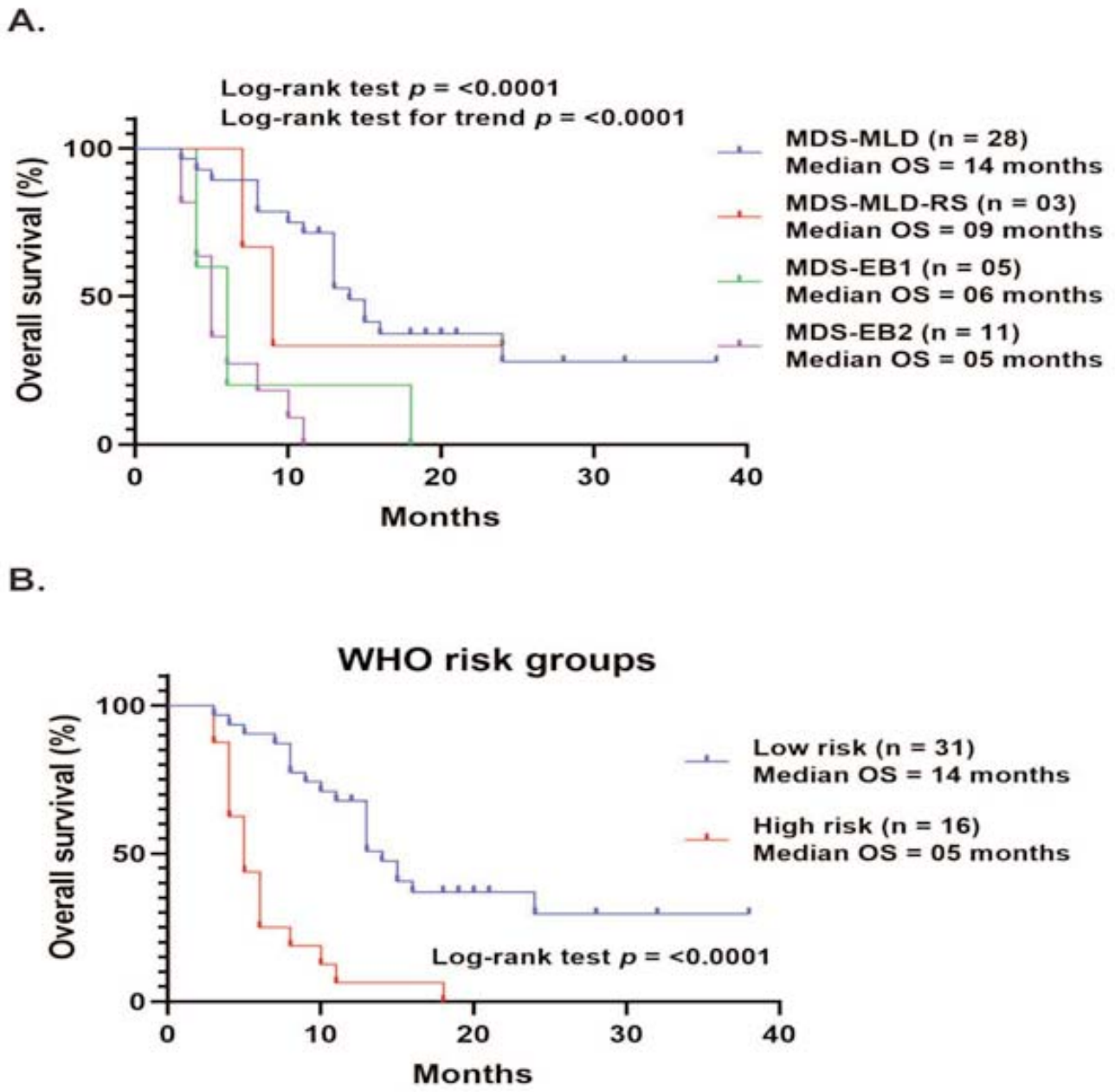
Discussion

MDS is characterised by ineffective haematopoiesis with morphological dysplasia and cytopenias. MDS in general is considered to be more common in elderly individuals with a predisposition to AML. In the current study, the median age of the patients was 66 years Compared to other studies reporting 60 years[13], 64 years[14], 67 years[15], and 71 years.[16,17] The current study showed a male-to-female ratio of 1.6:1 which is consistent with earlier reports from other Asian and European regions.[13, 15, 17]

The most common presenting clinical findings was pallor (mean haemoglobin [Hb] 7.82g/dl), followed by fatigue. This was compatible with an earlier study[18] in the Indian population, while a local study[13] reported mean Hb of 7.7gm/dl which was inconsistent with the current findings. Another study[16] reported Hb 9.9g/dl which was higher than the current study. This may be because Pakistan is an underdeveloped country, and patients do not have early access to tertiary care medical facilities. The mean platelet count in the current study was 77x10^9/L. A platelet count of 20x10^9/L was reported by an Indian study,[19] while another study[20] reported 158x10^9/L. A study in China[7] reported 60x10^9/L.



Supplementary Figure-S2: Overall survival (OS) with respect to baseline demographic and laboratory parameters including (A) gender, (B) age groups, (C) Hb, (D) TLC, and (E) Platelets.



Supplementary Figure- S3: Overall survival (OS) in MDS patients with respect to (A) WHO MDS classification and (B) WHO risk categories.

The most commonly mutated genes in the current study were DDX10 (26.7%), ASXL1(20%), RUNX1 (20%) and TET2 (20%). However, the findings were not in accordance with an India study[19] which reported SF3B1(25.2%), SRSF2(19%) and U2AF1(14.4%) as the predominant genetic alterations. In China[7] as study reported U2AF1 (22%), ASXL1 (18%) and RUNX1 (18%), while another study[21] showed TET2 (37.6%), SF3B1(36.8%) and ASXL1(26.6%) as the most common mutated genes. A German study[22] reported SF3B1 (24%), ASXL1 (15%) and RUNX1 (8%). Mutations in RUNX1 and ASXL1 were the common denominators in these studies which was in line with the current study, while DDX10, which was most commonly seen in the current study was not reported in the other studies.[7, 19, 21, 22] DDX10 is a protein coding gene and is associated with de novo MDS and is more common in the Asian population.[23]

Among the functional category of mutated genes, those involved in epigenetic regulation were most common (66%) in the current study, while studies conducted in

India[19] and China[7] reported RNA splicing pathway. One study[24] showed epigenetic functional category of mutated genes in 48.6% of Taiwanese patients, which was similar to the current sample. The mutation status showed a trend of dismal OS for MDS patients with single mutation (median: 10 months) and multiple mutations (median: 7.5 months), although this association was not statistically significant (p =0.072). Similar findings were documented by earlier studies.[5, 25]

Several sequencing studies indicated that mutations determined in DDX10 and TET2 are associated with low risk and good prognosis, while RUNX1 has a negative impact on the prognosis and was associated with high-risk MDS.[5] ASXL1 gene is associated with both low-risk and high-risk MDS.[21] ASXL1 mutation is also associated with the mutation in other genes, like SF3B1, RUNX1 and others.[26] SF3B1 gene was identified in[2] patients having MDS-MLD-RS in the current study, which was similar to earlier findings.[5] The[2] patients in the current study had a good prognosis, which was in line with literature.[25]

The current study provided a comprehensive analysis of common mutations in MDS patients by using both next-generation and Sanger sequencing showing that numerous genetic variants were involved in the initiation, development and survival related to MDS patients. The study will likely be helpful in identifying mutations essential for the diagnosis and prognosis of MDS.

The current study has its limitations related to a small sample size from a single medical centre. A large cohort from multiple centres is recommended to enhance the robustness and generalisability of the current findings.

Conclusion

Multiple sequencing showed that numerous genetic variants were involved in the initiation, development and survival related to MDS patients. Mutations were identified in DDX10, TET2, RUNX1 and ASXL1 genes that were essential for the diagnosis and prognosis of MDS.

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AUTHOR'S CONTRIBUTION:

AW: Concept, formal analysis, methodology, validation, investigation, writing, original draft, final approval and agreement to be accountable for all aspects of the work.

SAK: Concept, writing, review, editing, final approval and agreement to be accountable for all aspects of the work.

RM: Formal analysis, data curation, investigation and agreement to be accountable for all aspects of the work.

HSM: Formal analysis, writing, review, editing and agreement to be accountable for all aspects of the work.

HSS: Methodology, Software, writing, original draft and agreement to be accountable for all aspects of the work.

FFK: Methodology, Software and agreement to be accountable for all aspects of the work.