Screening of mutations in the additional sex combs like 1, transcriptional regulator, tumor protein p53, and *KRAS* proto-oncogene, *GTPase/NRAS* proto-oncogene, *GTPase* genes of patients with myelodysplastic syndrome

CAROLINA LEITE[1], LUCAS DELMONICO[2], GILDA ALVES[2], ROMARIO JOSÉ GOMES[2], MARIANA RODRIGUES MARTINO[2], ALINE RODRIGUES DA SILVA[2], ALINE DOS SANTOS MOREIRA[3], MARIA CHRISTINA MAIOLI[1], LUCIANO RIOS SCHERRER[4], ELENICE FERREIRA BASTOS[5], ROBERTO IRINEU[6] and MARIA HELENA ORNELLAS[2]

[1]Haematology Service, Pedro Ernesto University Hospital; [2]Circulating Biomarkers Laboratory, Faculty of Medical Sciences, State University of Rio de Janeiro, Rio de Janeiro 20550-170; [3]Bioinformatics and Functional Genomic Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro 21040-900; [4]Department of Engineering and Production, Kennedy Faculty, Belo Horizonte 31535-040; [5]Department of Medical Genetic, Fernandes Figueira Institute, Oswaldo Cruz Foundation, Rio de Janeiro 22250-020; [6]Pedro II School, Realengo II Campus, Rio de Janeiro 21710-261, Brazil

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Abstract. Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal bone marrow disorders characterized by ineffective hematopoiesis, different degrees of cellular dysplasia, and increased risk of progression to acute myeloid leukemia. International Prognostic Scoring System is the gold standard for MDS classification; however, patients exhibiting different clinical behaviors often coexist in the same group, indicating that the currently available scoring systems are insufficient. The genes that have recently been identified as mutated in MDS, including additional sex combs like 1, transcriptional regulator (*ASXL1*), tumor protein p53 (*TP53*), and *KRAS* proto-oncogene and *GTPase* (*KRAS*)/*NRAS* proto-oncogene, *GTPase* (*NRAS*), may contribute to a more comprehensive classification, as well as to the prognosis and progression of the disease. In the present study, the mutations in the *ASXL1*, *TP53* and *NRAS*/*KRAS* genes in 50 patients were evaluated by sequencing genomic bone marrow DNA. Nine patients (18%) presented with at least one type of mutation. Mutations in *TP53* were the most frequent in six patients (12%), followed by *ASXL1* in two patients (4%) and *NRAS* in one patient (2%). The nine mutations were detected in patients with low- and high-risk MDS. The screening of mutations in MDS cases contributes to the application of personalized medicine.

Correspondence to: Mr. Lucas Delmonico, Circulating Biomarkers Laboratory, Faculty of Medical Sciences, State University of Rio de Janeiro, Av. Prof Manuel de Abreu, 444, 4º andar, Vila Isabel, Rio de Janeiro 20550-170, Brazil
E-mail: lucasdelmonico@gmail.com

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Introduction

Myelodysplastic syndrome (MDS) is characterized by multiple cellular events, including cell clonality, ineffective hematopoiesis, apoptosis, and increased risk of progression to acute myeloid leukemia (AML) (1). The pathogenesis remains unclear, although previous studies have investigated the genetic signatures that may explain its origin (2-5).

Diagnosis continues to be based on morphological (cytopenias in blood count associated with the degree of dysplasia and blast percentage in myelogram) and cytogenetic findings (6). Overall, diagnostic criteria enable the stratification of patients into risk groups according to the International Prognostic Scoring System (IPSS) (7). Other scoring systems emerged, such as the WHO classification-based Prognostic Scoring System in 2005 (8), which added transfusion support as a novel criterion to those that were already established by the IPSS. IPSS-R (9) was created in 2012, adding further variables, such as lactate dehydrogenase (LDH), ferritin, β_2 microglobulin, marrow fibrosis, comorbidities and performance status. Even after the introduction of these novel parameters, it remains clear that patients with different clinical behaviors coexist in the same IPSS and IPSS-R group, indicating that improvement of the prognostic scoring system is still required.

The evaluation of molecular changes in the pathogenesis of MDS has been the subject of a number of studies (10,11). In such

studies, the gene, additional sex combs like 1, transcriptional regulator (*ASXL1*), located in the chromosome 20q11 region has been described as the most frequently mutated gene in advanced MDS, with rates ranging from 10 to 23% (4,5,12-14).

Similarly, located mutations in the gene *NRAS* proto-oncogene, *GTPase* (NRAS; located on chromosome 1p13.2) were identified in ~8-35% of patients with MDS, and were associated with the worst prognosis and a higher rate of leukemic transformation (15,16).

Another gene, tumor protein p53 (*TP53*) located in chromosomal region 17p13, is the most frequently mutated gene among neoplastic diseases, as it is important in the integrity of the genome. In MDS, these mutations are observed in ~10-15% of cases, although this frequency may be higher in patients exposed to alkylating agents or radiation (17). These mutations that inactivate the *TP53* gene are associated with advanced disease, complex karyotype, and resistance to treatment, all of which lead to a poor prognosis (17,18).

The aim of the present study was to investigate the presence of mutations in *ASXL1* (exon 12), *TP53* (exon 4-8) and *NRAS/KRAS* proto-oncogene and *GTPase* (*KRAS*; exon 1,2) genes from 50 patients with MDS, through the extraction and sequencing of genomic bone marrow DNA. In addition, the clinical consequences of the mutations that were revealed in the cohort are discussed.

Materials and methods

Patients. A total of 50 patients [18/50 (36%) men and 32/50 (64%) women] diagnosed with MDS and AML secondary to MDS, treated at the Pedro Ernesto University Hospital (HUPE; Rio de Janeiro, Brazil) from 2012 to 2014 were included in the present study. The mean age was 56-years-old (standard deviation, 19 years) and 57-years-old (standard deviation, 13 years), for men and women, respectively.

All patients underwent concomitant bone marrow studies for morphological analysis, according to the WHO criteria (1), and cytogenetic (19) and molecular analyses. Patients provided informed consent in accordance with the Declaration of Helsinki, and the scientific analysis of the samples was approved by the Ethics Committee of the Pedro Ernesto University Hospital (code no. CAAE 08084712.4.00 00.5259). To validate possible mutations and polymorphisms (germline changes) of the evaluated genes, the peripheral blood of 129 eligible healthy control subjects [38/129 (29%) men and 91/129 (71%) women] were collected in HUPE (Rio de Janeiro, Brazil). The mean age was 57-years-old (standard deviation, 13 years) and 51-years-old (standard deviation, 10 years), for men and women, respectively.

DNA sequencing. Isolation of bone marrow cells was performed with erythrocyte lysis solution (10 mM Tris-HCl, 5 mM $MgCl_2$ and 10 mM NaCl) at 4˚C. The extraction of genomic DNA was performed according to standard protocol (20).

For the amplification of exon 12 of the *ASXL1* and *KRAS/NRAS* genes, the basic solution contained ultra-pure and sterile water (36.5 μl), 5 μl 10X STR buffer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA); 2 μl dNTPs (10 mM; Invitrogen; Thermo Fisher Scientific, Inc.), 2.5 μl $MgCl_2$ (50 mM; Invitrogen; Thermo Fisher Scientific, Inc.), 2.5 μl of primer set at a concentration of 10 pmol/μl; 0.5 μl Taq DNA polymerase (1 U/μl; Invitrogen; Thermo Fisher Scientific, Inc.) and 50-100 ng/μl genomic DNA with a final volume of 50 μl. The primers were described previously by Rocquain *et al* (5).

In the Axygen-1000 thermal cycler, the amplifications of the fragments were initiated with a pre-denaturation of 5 min at 94˚C followed by 35 cycles of denaturation at 94˚C for 45 sec, annealing of the primers at 58˚C for 40 sec, and extension at 72˚C for 45 sec; and terminated with an extension step at≈72˚C for 10 min. For the amplifications of fragments 4, 5 and 6 of the *ASXL1* gene, the same reactions and cycling conditions were used, differing by the use of the enzyme, Platinum Taq DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.) and the increase of the annealing temperature to 61˚C. For amplification of the *KRAS1* gene, fragment 1, and the *NRAS2* gene, fragment 2, the same reactions and cycling conditions were used; however, the annealing temperature was increased to 55˚C.

To amplify exons 4-8 of the *TP53* gene, the basic solution contained 35 μl of ultra-pure, sterile water, 5 μl 10X STR buffer, 2 μl dNTPs (10 mM), 3 μl of $MgCl_2$ (50 mM), 3 μl of the pair of primers for each region to be amplified (concentration, 10 pmol/μl), 0.5 μl of the enzyme DNA polymerase (1 U/μl) and 50-100 ng/μl genomic DNA. The primers were designed using GenBank (https://www.ncbi.nlm.nih.gov/nuccore/NC_000017.9/, access no. NC_000017-9; Table I).

The polymerase chain reaction (PCR) products were purified using the PCR kit, GFX™ DNA and Gel Band Purification kit (cat. no. 28903470, GE Healthcare Life Sciences, Chalfont, UK). Subsequent to purification, sequences were loaded onto an ABI 3730XL DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Comparisons were made between the reference sequences of genes *ASXL1*, *NRAS*, *KRAS* and *TP53* (accession nos. NC_000001405.26, NG_007572.1, NG_007524.1 and NC_000017-9, respectively; GenBank) using the sample sequences obtained by sequencing. All samples were sequenced in duplicate and the mutations were confirmed. This comparison was performed using Sequencher version 5.1 program (Gene Codes, Ann Arbor, MI, USA).

Statistical analysis. Contingency tables were used to associate mutation with sex, age, mortality, bone marrow fibrosis, hematologic alterations, and need for transfusion. The χ^2 and Fisher's exact test was adopted to evaluate the statistical significance of the association between these variables.

A contingency table was used to assign the case and control groups with genotypes CC, CG, and GG, in exon 4 of the *TP53* gene (R72P polymorphism). The Pearson χ^2 test was adopted to analyze the statistical significance of the association between these variables.

The survey data were processed in the statistical program Predictive Analytics Software (PASW Statistics 18; Quarry Bay, Hong Kong). In all statistical tests used, the significance level was set at 5%. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients. Of the patients analyzed, 18/50 (36%) were men and 32/50 (64%) were women. The mean age was 57-years-old

Table I. Primer sequences of the *TP53* gene.

Exon *TP53*	Forward	Reverse	Product size, bp
4	5'-TGAGGACCTGGTCCTCTGAC-3'	5'-AGAGGAATCCCAAAGTTCCA-3'	413
5	5'-TGTTCACTTGTGCCCTGACT-3'	5'-AGCAATCAGTGAGGAATCAG-3'	310
6	5'-TGGTTGCCCAGGGTCCCCAG-3'	5'-CGGAGGGCCACTGACAACCA-3'	224
7	5'-CTTGCCACAGGTCTCCCCAA-3'	5'-AGGGGTCAGAGGCAAGCAGA-3'	237
8	5'-TTGGGAGTAGATGGAGCCCT-3'	5'-AGAGGCAAGGAAAGGTGATA-3'	337

TP53, tumor protein p53.

Table II. Clinical and hematologic characteristics of patients [n=50; age, 57 years (range, 19-90 years)] in the present study.

Variables	Total, n
Sex	
Male	18
Female	32
Hb (g/dl)	
<10	10
≥10	25
No follow up	3
Platelet (x1,000/μl)	
<100,000	24
≥100,000	23
No follow up	3
Neutrophil count	
<1,000	35
≥1,000	11
Missing	4
WHO classification 2008	
AML	9
RCUD (refractory anemia)	11
RCUD (refractory neutropenia)	3
RCUD (refractory thrombocytopenia)	7
Refractory cytopenia with multilineage dysplasia	14
RAEB1	1
RAEB2	3
Unclassified	2
International Prognostic Scoring System	
Low/Intermediate grade 1	18
Intermediate grade 2/high	3
Transformation to AML	9
Missing	20
Succumbed	
Yes	16
No	29
No follow up	6

AML, acute myeloid leukemia; RCUD, refractory cytopenia with unilineage dysplasia; RAEB, refractory anemia with excess blast.

(range, 19-90 years) with a median of 61 years (standard deviation, 15 years). The clinical and hematologic characteristics of patients are presented in Table II.

DNA sequencing. Of the 50 patients evaluated, 9/50 (18%) harbored mutations in one of the target genes. Mutation in the *TP53* gene was the most frequent in 6/50 patients (12%), followed by *ASXL1* in 2/50 patients (4%), and *NRAS* in 1/50 patients (2%). Table III summarizes the mutations that were detected. The R72P polymorphism in exon 4 of the *TP53* gene was analyzed, but no statistical significance was identified (Table IV).

Treatment. Regarding treatment, 9/50 (18%) of the patients with AML received chemotherapy. Of these patients, 5/9 (55%) succumbed and one is in disease remission following conventional chemotherapy. The three remaining patients underwent allogeneic bone marrow transplantation; two of these patients are alive and in disease remission; however, the third patient succumbed due to complications of the transplant.

For the 5/50 (10%) cases receiving treatment with hypomethylating agents, one patient was referred for allogeneic bone marrow transplantation and currently has graft versus host disease. The other four patients succumbed; three due to sepsis and one due to disease progression. Of the remaining group, 10/50 patients (24%) were in transfusion support, 1/50 (2%) were using erythropoietin, and 25/50 patients (50%) were not undergoing a specific type of therapy, but were treated with a more conservative approach. Table III presents the treatment information of the mutated cases.

Furthermore, no statistical associations between hematological, and survival of the cases with mutation and those without mutation.

Discussion

ASXL1, *TP53* and *KRAS*/*NRAS* were investigated in the present study, as they are considered to be significant in the evolution of MDS. *TP53* is an imperative tumor suppressor gene, *ASXL1* is involved in the regulation of DNA methylation, and *KRAS*/*NRAS* are involved in important proliferation pathways. These are also some of the most analyzed genes, with the highest frequency of mutations described in MDS (2-5). In the present study, 9/50 (18%) cases exhibited mutations in these genes. The most frequent mutations were observed in the *TP53* gene

Table III. Summary of the mutations.

Gene	Case no.	Classification (WHO 2008)	Nucleotide change/ amino acid change	Sex	Age (years)	Survival (months)	Treatment
Additional sex combs like 1, transcriptional regulator	5	AML	c.1846G>A, D615N	F	44	16 (succumbed)	Decitabine
	29	RCUD (RT)	c.1923C>T, I641I	F	56	110 (alive)	No specific therapy with conservative approach
Tumor protein p53	20	RCMD	c.384T>A, P128P	F	65	No follow-up	No specific therapy with conservative approach
	24	Refractory anemia with excess blast 2	c.204InsT, Q68V	F	NI	40 (alive)	Azacitidine + Exjade® + allogeneic bone marrow transplantation
	28	RCUD (RT)	c.216InsA, A72T	M	70	44 (alive)	No specific therapy with conservative approach
	37	Unknown	c.669C>T, P223L	M	NI	0 (succumbed)	No specific therapy with conservative approach
	40	AML	c.669C>T, P223L	M	31	4 (succumbed)	7+3 + FLAG-IDA + mitoxantrone, etoposide and intermediate-dose Ara-C
	49	RCMD	c.384T>A, P128P	M	67	32 (alive)	No specific therapy with conservative approach
NRAS proto-oncogene, *GTPase*	38	RCUD (RT)	c.Ins279C, R97T	F	68	46 (alive)	No specific therapy with conservative approach

AML, acute myeloid leukemia; RCUD, refractory cytopenia with unilineage dysplasia; RT, refractory thrombocytopenia; RCMD, refractory cytopenia with multilineage dysplasia; F, female; M, male.

Table IV. Summary of R72P polymorphism in exon 4 of the tumor protein p53 gene, in the cases (n=50) and control subjects (n=129).

	Group		
Genotype	Cases, n (%)	Controls, n (%)	P-value[a]
CC	7 (14)	25 (19)	
CG	20 (40)	60 (47)	0.318
GG	23 (46)	44 (34)	

[a]χ^2 test.

[6/50 patients (12%)], followed by in *ASXL1* [2/50 patients (4%)] and *NRAS* [1/50 patients (2%)].

The frequency of *TP53* mutation ranges from 5 to 10% (4,12,21,22-24). However, certain studies demonstrated that those with poor prognosis, high cytopenias, complex karyotype, chromosome 17 abnormalities and del(5q) have a higher frequency of mutation in *TP53* when high-risk groups are stratified (International Prognostic Scoring System) (25). A recent study by Devillier *et al* (24) examined 125 cases of AML with myelodysplasia changes and reported that 28 (22%) exhibited mutations in *TP53*, with the majority of cases being from the high-risk group. Conversely, the present study observed the *TP53* mutations in just 2/50 (4%) high-risk patients (the other four patients were low risk; Table III). Furthermore, in the present study, the majority of mutations identified in the *TP53* gene were missense. Regarding the mutations of the *TP53* gene, none have been described in the Catalogue of Somatic Mutations in Cancer database, except for the substitution, c.384T>A, P128P, observed in two of the present study cases. This mutation was first described by Andersen *et al* (26) in metastatic breast cancer.

Further to the polymorphism of exon 4 of the *TP53* gene (Arg72Pro), the study by Machado-Neto *et al* (27), evaluating 77 cases of MDS and 103 control subjects, indicated that this polymorphism was not associated with clinical and laboratory parameters, disease progression, and overall survival. Similarly, in the present study, no association between the Arg72Pro polymorphism and the increasing risk for MDS was identified in this population (Table IV; P=0.318) nor with clinical and laboratory parameters. By contrast, McGraw *et al* (28), investigating 705 MDS patients from different populations (United States, Canada, Spain, Germany and England), demonstrated that the C-allele homozygosity in del(5q) MDS was associated with prolonged overall and progression-free survival, as well as non-terminal interstitial deletions that excluded 5q34. Conversely, G-allele homozygosity was associated with inferior outcomes and terminal deletions involving 5q34 (P=0.05). In addition, McGraw *et al* (29) described the correlation of the *TP53* single nucleotide polymorphism with survival in cases of non-del(5q) MDSs. Utilizing a novel functional SNP scoring system ranging from +2 to -2 based upon predicted p53 activity, the authors demonstrated the difference between the role of *TP53* gene polymorphism R72P in del(5q) MDS patients and MDS patients with normal chromosome 5 (29).

The frequency of mutation in *ASXL1* ranges from 10 to 23% (4,5,12-14,22) and is associated with a worse prognosis. *ASXL1* gene mutations affect histone modifications via their interaction with polycomb complex 2, together with enhancer of zeste 2 polycomb repressive complex 2 subunit gene mutations and are involved in epigenetic dysregulation, which alters gene expression and acts as a major driver in the pathogenesis of MDS. *ASXL1* gene mutations are also frequent in cases of Clonal Hematopoiesis of Indeterminate Potential (CHIP) and therefore prevalent in disorders other than MDS, thus, an isolated mutation in this gene cannot be considered diagnostically helpful. *ASXL1* mutation is more common in chronic myelomonocytic leukemia (CMML; 40% of cases) and is associated with leukemic transformation (30).

Thol *et al* (13) followed 193 patients diagnosed with MDS and concluded that the presence of mutations in *ASXL1* was associated with the increased risk of progression to AML, and reduced the rates of overall survival. In another study, Boultwood *et al* (12) demonstrated an association between the increased risk group and the frequency of *ASXL1* mutation. Furthermore, the authors observed 6% of mutated cases in the refractory anemia group, 31% in the refractory anemia with excess blasts 1 and 2 groups, and 25% in the AML group (12). Although the frequency of mutation in the gene *ASXL1* in the present study was lower, this fact is justified due to the number of cases studied herein. However, consistent with the previous study (12), the presence of mutation in the *ASXL1* gene, and the risk of AML and poor prognosis were observed in one of two cases evaluated in the present study (Table III).

Concerning the *NRAS*/*KRAS* genes, previous reviews demonstrated mutations at a frequency of 5 to 10% of patients with MDS, and are more common in CMML (15%) and correlate strongly with the percentage of bone marrow blasts (4,22,30). In the present study, only one case was identified as having a mutation in *NRAS*. This particular patient was diagnosed with refractory cytopenia with unilineage dysplasia and is clinically stable, with no transfusion required and receiving no specific therapy. These data were consistent with that of Al-Kali *et al* (31), where the prognostic impact of the *NRAS* mutation was investigated in 1,067 patients with MDS. The authors indicated that the mutation in the *NRAS* gene was an infrequent event in MDS and there was no association between the presence of the mutation and overall survival or response to treatment. However, in studies conducted by Bejar *et al* (2,21), patients exhibiting mutations in the *NRAS* gene exhibited a worse prognosis, particularly in groups of low risk where the presence of the mutation led to a reduced overall survival, indicating that the mutational status of *NRAS* may be an isolated risk factor.

With regard to the additional clinical hematologic data, bone marrow fibrosis has been described as a poor prognostic factor in previous studies (32,33) and contributes to the risk stratification that is proposed by the IPSS-R (34). In the present study, no association was identified between fibrosis and mutational status (data not shown). Furthermore, transfusion dependency has been demonstrated as an independent prognostic factor in MDS patients and may be considered a reliable index of disease severity (8,30,33). Malcovati *et al* (8) evaluated 467 patients between 1992 and 2002, and identified that patients who were transfusion-dependent had a lower overall survival when compared with transfusion-independent patients [odds ratio (OR)=2.16; P<0.001). Survival without progression to AML was significantly worse in transfusion-dependent patients (OR=2.02; P<0.001). In the present study, nine cases of transfusion-dependency were identified (data not shown), although no association was observed with severity of the disease or with the mutational data.

In conclusion, the results indicate that alterations in the *ASXL1*, *TP53* and *NRAS* genes may occur in early stages of the MDS disease. Furthermore, genetic testing at the time of initial evaluation aids with establishing a diagnosis and provides additional, clinically relevant information. The present study hypothesizes that the combination of mutations in these genes may contribute to the progression of the disease; however, a study with a larger number of cases from the Brazilian population is required to validate the current findings.

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