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New polymorphisms of *Xeroderma Pigmentosum* DNA repair genes in myelodysplastic syndrome

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ABSTRACT

The association between *Xeroderma Pigmentosum* DNA repair genes (*XPA* rs1800975, *XPC* rs2228000, *XPD* rs1799793 and *XPF* rs1800067) polymorphisms and myelodysplastic syndrome (MDS) have not been reported. To assess the functional role between these polymorphisms and MDS, we evaluated 189 samples stratified in two groups: 95 bone marrow samples from MDS patients and 94 from healthy elderly volunteers used as controls. Genotypes for all polymorphisms were identified in DNA samples in an allelic discrimination experiment by real-time polymerase chain reaction (qPCR). We also studied the mRNA expression of *XPA* and *XPC* genes to evaluate if its polymorphisms were functional in 53 RNAm MDS patients by qPCR methodologies. To the rs2228000 polymorphism, the CT and TT polymorphic genotype were associated with increased odds ratio (OR) of more profound cytopenia (hemoglobin and neutrophils count). To the rs1799793 polymorphism, we found that the GG homozygous wild-type genotype was associated with a decreased chance of developing MDS. We observed low expression of *XPA* in younger patients, in hypoplastic MDS and patients with abnormal karyotype when presented AG or AA polymorphic genotypes. We also found that there was a statistically significant interaction between the presence of micromegakaryocyte on down regulation of *XPC* regarding the CT heterozygous genotype of the rs1800975 polymorphism. Our results suggest that new functional polymorphisms of *Xeroderma Pigmentosum* DNA repair genes in MDS are related to its pathogenesis and prognosis.

1. Introduction

Myelodysplastic Syndrome (MDS) is a clonal disorder of hematopoietic stem cell (HSC) characterized by cytopenias, bone marrow dysplasia, ineffective hematopoiesis and increased risk of progression to acute myeloid leukemia (AML) [1]. Chromosomal abnormalities are detected in up to 50% of MDS patients and are considered the most significant marker of prognosis [1]. Papaemmanuil et al., using a painel of 111 genes, reported that mutations are detected in up to 80% of patients when using Cytogenetic and Next Generation Sequencing [2], improving the prognostic markers.

The maintenance of genome is commonly executed by genes related to single and double strand breaks of DNA. The estimated numbers of single-strand breaks and spontaneous base losses in nuclear DNA may be up to 10^5 lesions per cell per day [3]. If not properly corrected, these lesions may evolve into double strand breaks which may predispose to chromosomal abnormality [3]. DNA damage repair pathways are important for removing different types of DNA damage in HSCs. Our group has endeavored to assess the importance and the frequency of specific polymorphisms in DNA double strand-breaks (DSB) genes of MDS patients [4,5]. Ribeiro et al. [4,5] demonstrated that polymorphisms in genes related to homologous recombination (i.e. *ATM*, *BRCA1*, *BRCA2* and *RAD51*) and non-homologous end-joining (i.e. *XRCC5*, *XRCC6*

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and *LIG4*) repair mechanisms were associated with susceptibility, pathogenesis and prognosis of MDS [4,5]. Recently, of utmost importance, we showed that many of these polymorphisms are functional influencing its level of expression [6].

Nucleotide excision repair (NER) is an important mechanism that repairs long nucleotide segments that cause distortion of single-strand breaks (SSB) [7]. It acts mainly in damage caused by UV radiation and a variety of exogenous chemicals including environmental and chemotherapeutic agents such as benzopyrenes and cisplatin [8]. NER acts by two different ways based on the recognition of the damage in eukaryotes: *Transcription-Coupled Repair – TCR* and the *Global Genome Repair – GGR*. The GGR mechanism removes lesions in any part of the genome, while the TCR specifically removes tape transcribed genes active lesions [9]. The main participants of NER repair pathway include seven proteins that make up the *Xeroderma pigmentosum complementation group*, especially the protein complexes XPA, XPC, XPD and XPF [9].

Functional defects in genes which encode these NER proteins are related to certain diseases such as Xeroderma Pigmentosum, Cockayne's syndrome, and several types of cancers [10]. Several studies have associated certain polymorphisms of Xeroderma pigmentosum genes with solid tumors [11–13]. Recently, Joshi et al. [13] have studied one important single-nucleotide polymorphisms of XPD DNA repair gene (Lys751Gln or rs13181) in clinical laboratory variables of MDS patients (ie. haemoglobin, bone marrow cellularity, blast count and WHO 2008 classification subtypes). However, the role of these genes (XPA rs1800975, XPC rs2228000, XPD rs1799793 and XPF rs1800067) in MDS is unclear. Thus, we aimed to study if these polymorphisms would be associated with any clinical or laboratory characteristics of MDS patients.

2. Patients, materials and methods

2.1. Patients

We evaluated 189 samples stratified in two groups: 95 bone marrow samples from MDS patients and 94 from healthy elderly volunteers used as controls (sex and age-matched). Ninety-five MDS patients (7 RA, 12 RARS, 51 RCDM, 14 RAEB, 2 CMML and 9 secondary MDS) (Table 1) were diagnosed at Federal University of Ceara according to WHO 2008 classification [14] and International Prognosis Score System revised (R-IPSS) [15].

We evaluated the possible associations between the *XPA* rs1800975, *XPC* rs2228000, *XPD* rs1799793 and *XPF* rs1800067 polymorphisms with the variables of the gender (i.e. male and female), age (i.e. patients younger than 60 years and patients older than 60 years, according to IPSS-R [15]), number of cytopenias (i.e. 0–1 and 2–3 cytopenias, according to IPSS-R [15]), cytopenias analysis (i.e. hemoglobin, neutrophil and platelets count), karyotype (i.e. normal and abnormal), bone marrow cellularity (i.e. hypocellular and normocellular + hypercellular) [16], presence of micromegacaryocyte (i.e presence or abscense) [14], WHO 2008 classification [14] (refractory anemia (RA), refractory anemia with ringer sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts (RAEB) and secondary MDS), IPSS-R score [15] (very-low + low and intermediate + high + very high), and death (i.e. yes or no) (Table 1).

This study was approved by the research ethics committee of the Federal University of Ceara (CEP/HUWC/UFC) according to the protocol #129.25.12.

2.2. Cytogenetic analysis

Conventional G-Band karyotype analysis was performed on bone marrow cells of fifty one *de novo* MDS patients according to the protocol already established in our laboratory. Briefly, cultures were established in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 30% fetal calf serum. For the 24-h culture, colcemid was added at a final concentration of 0.05 μg/ml for the final 30 min of culture. After harvesting, the cells were exposed to a hypotonic KCl solution (0.068 mol/L) and fixed with Carnoy buffer (acetic acid/methanol in a 1:3 proportion). The slides were prepared and stained using Giemsa solution. A minimum of 20 metaphases were analyzed whenever possible. The karyotype was prepared using a CytoVision Automated Karyotyping System (Applied Imaging, San Jose, CA, USA) and described according to the International System for Human Cytogenetic Nomenclature (2013).

2.3. DNA extraction

Genomic DNA was extracted from 95 bone marrow mononuclear samples of MDS patients and of 94 peripheral blood samples obtained from sex and age-matched healthy elderly volunteers, using TRizol Reagent™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

2.4. Allelic discrimination by real time-PCR (RT-PCR)

Genotypes were identified in DNA samples by real-time polymerase chain reaction (RT-PCR), using TaqMan SNP Genotyping Assay (ThermoFisher Scientific, Foster City, CA, USA). We performed an allelic discrimination assay for rs1800975 (C_482935_1_), rs2228000 (C_16018061_10), rs1799793 (C_3145050_10) and rs1800067 (C_3285104_10) polymorphisms using the TaqMan Genotyping Master Mix kit® (catalog #4371355, ThermoFisher Scientific, Foster City, CA, USA), according to the manufacturer's protocol.

Each 10 μl PCR reaction contained ~30 ng of genomic DNA in final volume of 4.5 μl, 5.0 μl of 2X TaqMan Genotyping Master Mix and 0.5 μl of 20X TaqMan Genotyping assay mix. The following thermal cycling conditions were: an initial activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s of denaturation step at 95 °C and annealing/enzyme extension at 60 °C for 1 min. PCR was performed using 7500 Fast System® (Applied Biosystems, Carlsbad, CA, USA).

2.5. Total RNA extraction

Total RNA extractions from isolated mononuclear cells (bone marrow), obtained from 53 MDS patients, were performed with TRizol Reagent™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

2.6. cDNA synthesis

cDNA was generated from total RNA using the High Capacity cDNA Reverse Transcription kit® (Applied Biosystems, San Jose, CA, USA), according to the manufacturer's protocol. cDNA synthesis was generated using Mastercycler Pro Vapor Protect Technology® machine (Eppendorf, Hamburg, Germany). cDNA samples were stored at −20 °C until further use.

2.7. Genotyping analysis by quantitative PCR (qRT-PCR)

We also studied the mRNA expression of *XPA* and *XPC* genes to evaluate if its polymorphisms were functional in 53 MDS patients. Quantitative PCR (qRT-PCR) reactions were based on TaqMan methodology® (Applied Biosystems, Carlsbad, CA, USA) and ran on a 7500 Fast System® (Applied Biosystems, Carlsbad, CA, USA). Predeveloped TaqMan gene expression assays (Assays-on-Demand, Applied Biosystems, Carlsbad, CA, USA) for *XPA* (Hs00166045_m1)

Table 1
Clinical Characteristics, Cytogenetic, IPSS and WHO classification of the MDS patients.

Patients	Cytogenetic	Gender	Age	IPSS	WHO
1	46,XX[20]	F	83	Very low	RA
2	46,XX[10]	F	57	Low	RCDM
3	No metaphase	F	83	–	RARS
4	46,XX[20]	F	56	Very low	RCDM
5	46,XX[6]	F	71	Very low	RA
6	No metaphase	F	66	–	RARS
7	46,XX[8]	F	46	Low	RA
8	No metaphase	F	55	–	RCDM
9	No metaphase	M	66	–	RAEB I
10	46,XY[20]	M	61	Low	RCDM
11	46,XY[20]	M	81	Intermediate	CMML II
12	46,XY[5]	M	33	High	RCDM
13	47,XX,t(4;11)(q27;q32),+mar[4]/46,XX[16]	F	80	Low	RCDM
14	37,X,−2,−3,−9,−11,−12,−15,−16,−18,-Y[8]/46,XY,del(5)(q15q33)[5]/46,XY[6]	M	63	Very high	RAEB II
15	No metaphase	F	56	–	RCDM
16	No metaphase	M	85	–	RCDM
17	46,XY[11]	M	73	High	RAEB I
18	No metaphase	F	77	–	RCDM
19	46,XX[12]	F	72	Low	RARS
20	46,XY[6]	M	74	Low	RARS
21	No metaphase	F	74	–	sec. MDS
22	46,XX[5]	F	80	Low	RARS
23	46,XY,del(5)(q31q35)[6]/46,XY [17]	M	88	Low	RCDM
24	46,XX[14]	F	78	Very low	RCDM
25	46,XX[25]	F	66	Low	RCDM
26	46,XX,del(5)(q15q33)[4]/46,XX [18]	F	22	Very low	RCDM
27	No metaphase	F	72	–	RAEB II
28	46,XX[5]	F	86	Low	RCDM
29	46,XY[5]	M	35	Low	RCDM
30	46,XX[20]	F	63	Low	RCDM
31	47,XY,+mar[5]/46,XY,del(5)(q31)[5]/46,XY[15]	M	47	Low	RCDM
32	No metaphase	M	56	–	RCDM
33	71 ∼ 99<4N>,XY···[4]/46,XY, del(17)(p11.2)[9]/46,XY[7]	M	75	Low	RCDM
34	40–45,XX,−5,−6,−7,−9,−17[7]/46,XX,add(5)(p15)[3]/46,XX[14]	F	75	Intermediate	RCDM
35	47,XY,+mar[5],46,XY[11]	M	65	Intermediate	RCDM
36	No metaphase	F	70	–	RARS
37	No metaphase	F	64	–	RCDM
38	No metaphase	F	74	–	RCDM
39	No metaphase	F	44	–	RCDM
40	46,XY[20]	M	48	Low	RCDM
41	46,XY[20]	M	66	Intermediate	RAEB I
42	47,XY,+mar[3]/46,XY[17]	M	45	High	RCDM
43	90,XXXX,−6,−7,−8,−11,+21,+22[5]/46,XX,del(7)(q23),del(20)(q13.1)[3]/45,XX,−7[5]/45 ∼ 46,XX,−7,del(7)(q32),del(11)(q32),−17,del(17)(p11.2),del(20)(q13.1)[cp11]	F	30	Very high	RAEB II
44	No metaphase	M	53	–	RCDM
45	No metaphase	F	86	–	RARS
46	47,XY,+mar[6]/48,XY,+8,del(16)(?q22),+mar[4]/47 ∼ 50,XY,del(4)(?q35),+8,+10,+11, del(16)(?q22),+21,+mar[cp8]	M	49	Very high	RCDM
47	46,XX[11]	F	50	Very low	RARS
48	46,XY[17]	M	70	Low	RARS
49	46,XY,del(5)(q15q33)[7]/46,XY[11]	M	68	Low	RCDM
50	46,XY,del(5)(q33),del(11)(q23)[20]	M	69	Low	RCDM
51	No metaphase	F	81	–	sec. MDS
52	No metaphase	M	67	–	AREB II
53	46,XY[4]/46,XY[16]	M	91	Very low	RARS
54	46,XX,del(11)(q23)[9]/46,XX[5]	F	70	Very low	RCDM
55	46,XY,del(5)(q22q33)[5]/46,XY[7]	M	15	Very low	RCDM
56	46,XX[20]	F	28	Low	RA
57	46,XY[24]	M	30	Low	RCDM
58	46,XX[20]	F	81	Low	RARS
59	No metaphase	F	41	–	RARS
60	No metaphase	F	79	–	RA
61	46,XY[8]	M	45	Low	RCDM
62	46,XX[20]	F	88	Intermediate	RAEB I
63	46,XX,del(5)(q31)[5]/46,XX[19]	F	64	Low	RCDM
64	46,XX[20]	F	80	Low	RCDM
65	No metaphase	M	79	–	RCDM
66	46,XY[10]	M	41	Low	RCDM
67	46,XY[20]	M	53	Intermediate	RCDM
68	46,XY[17]	M	88	Very low	RAEB II

Table 1 (*Continued*)

Patients	Cytogenetic	Gender	Age	IPSS	WHO
69	46,XY[9]	M	24	Low	RCDM
70	46,XX,del(5)(q15q33)[9]/46,XX,del(5)(q15q33),del(11)(?q25)[7]/46,XX[4]	F	40	Low	RCDM
71	No metaphase	M	62	–	RCDM
72	46,X,i(x)(q10),del(17)(q22q23.3)[7]/46,XX[13]	F	78	Low	RA
73	47,XX,+mar[4]/46,XX[14]	F	73	Intermediate	CMML I
74	No metaphase	F	77	–	sec. MDS
75	89,XXY,−20,−22,−Y[4]/46,XY,del(16)(?q22)[5]/46,XY[11]	M	82	Low	RCDM
76	46,XY,add(12)(9q24.33)[2]/46,XY,del(17)(q23)[3]/46,XY[10]	M	67	Intermediate	RCDM
77	44,XX,−13,−17[4]/46,XX[5]	F	41	High	RCDM
78	No metaphase	M	66	–	RAEB I
79	42,XY,−4,−5,−16,−22[10]	M	68	Intermediate	RCDM
80	47,XY,+8[6]/47,XY,del(7)(q32),+8[7]/46,XY[2]	M	62	Very high	RAEB II
81	80∼90<3n>,[3]/46,XY,del(5)(q32),del(11)(q32),del(17)(p11.2)[7]/46,XY[11]	M	70	High	RAEB I
82	46,XY,add(13)(p11)[12]/46,XY,del(7)(q32),add(13)(p11)[4]/48,XY,add(13)(p11),+22,+mar[9]/48,XY,del(7)(q32),add(13)(p11),+22,+mar[3]/46,XY[2]	M	77	High	RCDM
83	No metaphase	M	79	–	RCDM
84	46,XX[20]	F	81	–	sec. MDS
85	No metaphase	F	62	–	RCDM
86	175,XXXXXXXX,−5,−6,−7,−8,−9,−11,−13,−14[4]/46,XX,del(5)(q15q33)[8]/46,XX[19]	F	71	–	sec. MDS
87	No metaphase	F	65	–	sec. MDS
88	46,XX[20]	F	87	–	sec. MDS
89	No metaphase	F	71	–	RA
90	No metaphase	F	81	–	RAEB II
91	46,XY[9]	M	64	–	sec. MDS
92	39,XX,−1,−5,−11,−12,−15,−20,−21[5]/46,XX,del(5)(?q14q33)[5]/46,XX[4]	F	37	–	sec. MDS
93	No metaphase	M	59	–	RCDM
94	No metaphase	M	81	–	RCDM
95	No metaphase	M	71	–	RAEB II

and *XPC* (Hs01104213_m1) as well as the TaqMan Universal Master Mix II, with UNG® (Applied Biosystems, Carlsbad, CA, USA) were used to quantify mRNA expression.

The expression level of the beta-2-microglobulin gene (*B2 M*, Hs99999907_m1) and ubiquitin (*UBC*, Hs00824723_m1) were used to normalize differences in input cDNA. Each sample was performed in duplicate and the expression ratios were calculated using the $2^{-\Delta Cq}$ method [17].

2.8. Statistical analysis

The Pearson chi-square test (x^2) was used to determine the Hardy–Weinberg (H-W) equilibrium and the differences in genotypes distribution. All genotypes were divided and analyzed in three distinct genetic models, according to the study of Clarke et al. [18]: 1. Genotype distribution model (wild-type *versus* heterozygous *versus* mutated); 2. Dominant genetic model (wild-type *versus* heterozygous + mutated) and 3. Recessive genetic model (mutated *versus* wild-type *versus* heterozygous + wild-type). Differences in allele and genotype frequencies and comparisons of variables between the control and MDS groups were evaluated using the Pearson's chi-square test or Fisher's exact test. Multinomial logistic regression analysis was used to measure the association between an exposure and an outcome (odds ratio) with 95% confidence intervals (C.I.).

The *one-way* ANOVA and *two-way* ANOVA (both with Tukey *pos-hoc* test) were used to analyze the influence of relative expression regarding polymorphisms and clinical variables. All tests were two-sided and considered to be statistically significant if $p \leq 0.05$. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for analysis.

3. Results

3.1. Characteristics of MDS patients

The features of MDS patients are presented in Table 1. Approximately 52% of the patients were female with a median age of 64.4 years (Table 1). According to WHO 2008 classification [14] and IPSS-R scores [15], most of the cases were considered as RCMD (51/95/53.68%), and with predominance of low risk prognosis (27/55/49.09%) (Table 1). Cytogenetic analyses were performed for all MDS patients. Of those patients, 35 (36.8%) exhibited a normal karyotype, 28 (29.5%) exhibited an abnormal karyotype and 32 patients (33.7%) presented no metaphases (Table 1).

3.2. Genotype frequencies and allele distributions of NER repair polymorphisms

The distributions of allele and genotype frequencies of the *XPA* rs1800975, *XPC* rs2228000, *XPD* rs1799793 and *XPF* rs1800067 polymorphisms of 94 MDS patients and 95 elderly volunteers are presented in Table 2.

3.2.1. XPC *rs2228000 polymorphism*

For the *XPC* rs2228000 polymorphism, the CT heterozygous genotype was associated with increased odds ratio (OR) of presenting hemoglobin (Hb) < 8.0 g/dL ($p = 0.010$; OR = 3.385; 95% CI 1,343 – 8,529) (Table 2). However, the CC wild genotype was associated with increased OR for Hb >8 g/dL in the dominant model ($p = 0.027$; OR = 2.75; 95%CI 1.125 – 6.722) (Table 2).

Table 2
Distribution of genotypes in MDS patients and health controls and associations with clinical variables.

Polymorphism / Genotype	Groups		x^2	Multinomial Logistic Regression*				Variables	Polymorphism / Genotype	Multinomial Logistic Regression*				
	Control	MDS												
*XPA*rs1800975			*p-value*	*p-value*	OR	95% CI			*XPC*rs2228000	Wald	*p-value*	OR	95% CI	
GG	53 (56.4)	49 (57.0)	0.243	–	–	–	–	**Hb**						
GA	37 (39.4)	28 (32.5)		–	–	–	–	<8g/dL	CC	–	–	–	–	–
AA	4 (4.2)	9 (10.5)		–	–	–	–		CT	**6.685**	**0.01**	**3.385**	**1.343**	**8.529**
*XPC*rs2228000									TT	0.062	0.803	0.8	0.139	4.618
CC	54 (57.4)	46 (52.9)	0.922	–	–	–	–	≥8g/dL	CC	**4.921**	**0.027**	**2.75**	**1.125**	**6.722**
CT	33 (35.1)	34 (39.1)		–	–	–	–		CT	–	–	–	–	–
TT	7 (7.4)	7 (8.0)		–	–	–	–		TT	–	–	–	–	–
*XPD*rs1799793														
GG	61 (64.9)	41 (47.7)	**0.045**	0.009	0.445	0.242	0.816	**ANC**						
GA	29 (30.9)	39 (45.3)		–	–	–	–	<800/mm^3	CC	–	–	–	–	–
AA	4 (4.2)	6 (7.0)		–	–	–	–		CT	1.42	0.233	1.826	0.678	4.917
*XPF*rs1800067									TT	**5.678**	**0.017**	**8.75**	**1.47**	**52.098**
GG	79 (84.0)	70 (79.5)	0.233	–	–	–	–	≥800/mm^3	CC	**4.615**	**0.032**	**2.833**	**1.096**	**7.327**
GA	15 (16.0)	15 (17.0)		–	–	–	–		CT	–	–	–	–	–
AA	0 (0.0)	3 (3.5)		–	–	–	–		TT	–	–	–	–	–

Hb.: Hemoblogin. **ANC.**: Neutrophil count. **MDS.**: Myelodysplastic syndrome. **OR.**: Odds ratio. **CI.**: Confidence interval.
Bold text indicates significant differences ($p < 0.05$) between groups analyzed by Chi-square test or Multinomial Logistic Regression.

The CC wild genotype was also associated with an increased odds ratio of ≥800 neutrophils/mm^3 in the dominant model ($p = 0.032$; OR = 2.833; 95% CI 1.096 – 7.327) and in the homozygous model ($p = 0.013$; OR = 17.500; 95% CI 1.828 – 167.558) while the TT polymorphic genotype showed an increased OR of neutrophils <800/mm^3 ($p = 0.017$; OR = 8.750; 95%CI 1.470 – 52.098) (Table 2).

We found no significant associations between the *XPC* rs2228000 polymorphism, and the variables of the gender, age, origin, number of cytopenias, karyotype, bone marrow cellularity, presence of micromegacaryocyte, WHO 2008 classification, IPSS-R prognosis, and death ($p > 0.05$).

3.2.2. XPD *rs1799793 polymorphism*

For the *XPD* rs1799793 polymorphism, we found that the GG homozygous wild-type genotype was associated with a decreased chance of developing MDS when compared to control group in the dominant model ($p = 0.009$, OR = 0.445, 95%CI 0.242 – 0.816) and in the heterozygous dominant model ($p = 0.010$, OR = 0.440, 95% CI 0.235 – 0.825) (Table 2).

We found no significant associations between the *XPD* rs1799793 polymorphism and the variables of the gender, age, origin, number of cytopenias, cytopenias analysis, karyotype, bone marrow cellularity, presence of micromegacaryocyte, WHO 2008 classification, IPSS-R prognosis, and death ($p > 0.05$).

3.2.3. XPA *rs1800975 polymorphisms*

We found no significant associations between the *XPA* rs18000975 polymorphism and the variables of the gender, age, origin, number of cytopenias, cytopenias analysis, karyotype, bone marrow cellularity, presence of micromegacaryocyte, WHO 2008 classification, IPSS-R prognosis, and death ($p > 0.05$) (data not present).

3.2.4. XPF *rs1800067 polymorphisms*

We found no significant associations between the *XPF* rs1800067 polymorphism and the variables of the gender, age, origin, number of cytopenias, cytopenias analysis, karyotype, bone marrow cellularity, presence of micromegacaryocyte, WHO 2008 classification, IPSS-R prognosis, and death ($p > 0.05$) (data not present).

3.3. *Functional polymorphisms analysis of NER repair genes*

In a second step, we did a two-way ANOVA to evaluate the effect of rs1800975 and rs2228000 polymorphisms on *XPA* and *XPC* mRNA expression in MDS patients. The analysis of associations between the level of expression of repair genes and their respective polymorphisms were performed for all patients using the clinical variables presented in Section 2.1.

3.3.1. *Effect of rs2228000 polymorphism on* XPA *gene expression*

We detected strong evidence that the rs2228000 polymorphism modifies the *XPA* gene expression while associated with age, bone marrow cellularity, karyotype, and death. We observed low expression of *XPA* in younger patients (F test = 5.947; $p = 0.0190$) (Fig. 1, Table 3), patients with abnormal karyotype (F test = 4.891; $p = 0.016$) (Fig. 2, Table 4), and who died (F test = 4.591; $p = 0.038$) (Fig. 3, Table 5) when presented AG or AA genotypes (Fig. 1). We also detected a decrease of *XPA* expression in hypoplastic MDS patients with the AG polymorphic genotype when analyzed for dominant and recessive genetic models (F test = 6.780; $p = 0.015$) (Fig. 4, Table 6).

We found no significant associations between the *XPA* expression, rs2228000 polymorphism and the variables of the gender, origin, number of cytopenias, cytopenias analysis, presence of micromegacaryocyte, WHO 2008 classification, and IPSS-R prognosis ($p > 0.05$).

3.3.2. *Effect of rs1800975 polymorphism on* XPC *gene expression*

Regarding *XPC* gene, we found that there was a statistically significant interaction between the presence of micromegakaryocyte (F test = 5.325; $p = 0.029$) on down regulation of *XPC* regarding the CT heterozygous genotype of the rs1800975 polymorphism (Fig. 5, Table 7).

We found no significant associations between the *XPC* expression, rs1800975 polymorphism and the variables of the gender, age, origin, number of cytopenias, cytopenias analysis, karyotype, bone marrow cellularity, WHO 2008 classification, IPSS-R prognosis, and death ($p > 0.05$).

4. Discussion

To the best of our knowledge, we evaluated for the first time the association of NER repair gene polymorphisms (*XPA* rs1800975,

Table 3
Data of the comparisons of *XPA* gene expression levels within MDS patients, which were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism in clinical variable analysis of the age.

	Genotypes*	Variable**	N	Mean	Std. Deviation	Tests of Between-Subjects Effects***			
Gene						Source	Mean square	*F* test	*p*-value
XPA	GG	≤60 years old	8	0.0025177	0.0010984	Genotypes	3.813E-006	5.947	**0.019**
		>60 years old	14	0.0015832	0.0006636	Age	2.399E-006	3.742	0.060
	GA	≤60 years old	7	0.0015278	0.0008734				
		>60 years old	11	0.0014663	0.0007778	Genotypes*Age	2.279E-006	3.555	0.066
	AA	≤60 years old	3	0.0013014	0.0008211				
		>60 years old	2	0.0013468	0.0005827				

*Genotypes of the rs2228000 polymorphism. **Age variable.
***Two-way ANOVA analysis with significant difference in $p < 0.05$.

Table 4
Data of the comparisons of *XPA* gene expression levels within MDS patients, which were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism in clinical variable analysis of the karyotype.

	Genotypes*	Variable**	N	Mean	Std. Deviation	Tests of Between-Subjects Effects***			
Gene						Source	Mean square	*F* test	*p*-value
XPA	GG	Normal	9	0.0012505	0.0004236	Genotypes	4.921E-007	1.535	0.234
		Abnormal	6	0.0019556	0.0007672	Karyotype	1.355E-007	0.423	0.521
	GA	Normal	5	0.0015242	0.0007160				
		Abnormal	7	0.0009533	0.0003977	Genotypes*Karyotype	21.568E-006	4.891	**0.016**
	AA	Normal	3	0.0015508	0.0005382				
		Abnormal	2	0.0009727	0.0006146				

*Genotypes of the rs2228000 polymorphism. **Karyotype.
***Two-way ANOVA analysis with significant difference in $p < 0.05$.

Table 5
Data of the comparisons of *XPA* gene expression levels within MDS patients, which were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism in clinical variable analysis of the death.

	Genotypes*	Variable**	N	Mean	Std. Deviation	Tests of Between-Subjects Effects***			
Gene						Source	Mean square	*F* test	*p*-value
XPA	GG	Yes	7	0.0005381	0.0006540	Genotypes	1.002E-006	3.249	0.079
		No	14	0.0013610	0.0004956	Death	7.325E-010	0.002	0.961
	GA	Yes	7	0.0011219	0.0004936				
		No	10	0.0013842	0.0006548	Genotypes*Death	1.416E-006	4.591	**0.038**
	AA	Yes	1	0.0005381	–				
		No	4	0.0015149	0.0004453				

*Genotypes of the rs2228000 polymorphism. **Death.
***Two-way ANOVA analysis with significant difference in $p < 0.05$.

Table 6
Data of the comparisons of *XPA* gene expression levels within MDS patients, which were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism in clinical variable analysis of the bone marrow cellularity.

	Genotypes*	Variable**	N	Mean	Std. Deviation	Tests of Between-Subjects Effects***			
Gene						Source	Mean square	*F* test	*p*-value
XPA	GG	Hypocellular	2	0.0024196	0.0005515	Genotypes	1.101E-006	4.842	**0.037**
		Norm + Hyper	13	0.0013336	0.0005076	Bone Marrow Cellularity	1.542E-006	6.780	**0.015**
	GA	Hypocellular	1	0.0016776	–				
		Norm + Hyper	12	0.0011663	0.0004829	Genotypes*Bone Marrow Cellularity	6.164E-007	2.710	0.111
	AA	Hypocellular	1	0.0011959	–				
		Norm + Hyper	2	0.0013468	0.0000855				

*Genotypes of the rs2228000 polymorphism. **Bone marrow cellularity variable.
***Two-way ANOVA analysis with significant difference in $p < 0.05$.

Table 7
Data of the comparisons of *XPC* gene expression levels within MDS patients, which were stratified according to genotypic distribution genetic model for the rs1800975 polymorphism in clinical variable analysis of the presence of micromegacaryocyte.

	Genotypes*	Variable**	N	Mean	Std. Deviation	Tests of Between-Subjects Effects***			
Gene						Source	Mean square	*F* test	*p*-value
XPC	CC	Absence	11	0.0120801	0.0037920	Genotypes	7.873E-005	1.617	0.218
		Presence	5	0.0217984	0.0120440	Micromegacaryocyte	1.890E-007	0.004	0.951
	CT	Absence	11	0.0142808	0.0060030				
		Presence	1	0.0050735	–	Genotypes*Micromegacaryocyte	0.000	5.325	**0.029**
	TT	Absence	3	0.0118329	0.0095185				
		Presence	–	–	–				

*Genotypes of the rs1800975 polymorphism. **Presence of Micromegacaryocyte.
***Two-way ANOVA analysis with significant difference in $p < 0.05$.

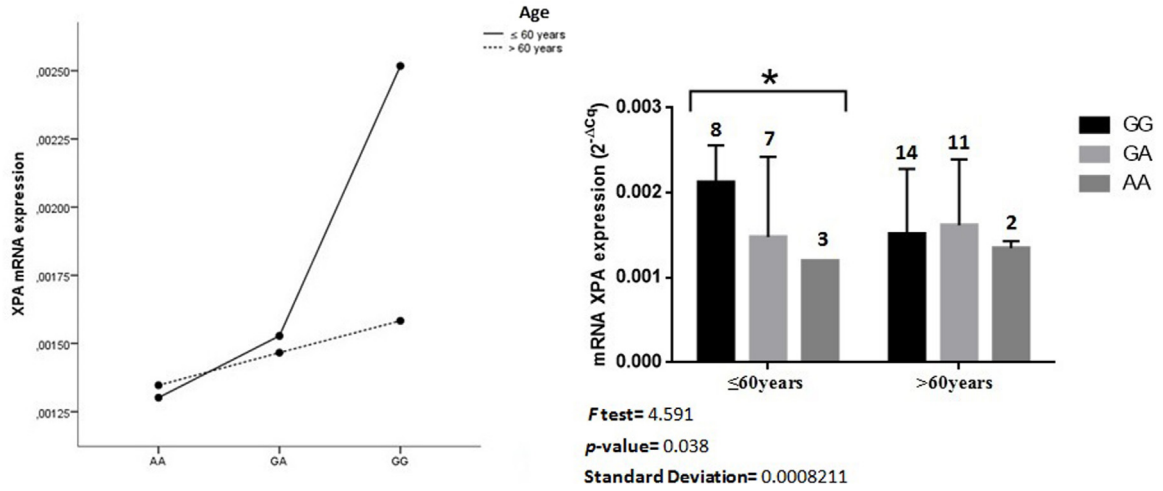

Fig. 1. A. Profile plots and **B.** bar graph of the comparisons of *XPA* gene expression within MDS patients that were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism regarding age variable. Two-way ANOVA test. Results are shown as the median ±SD. Significant difference in $p < 0.05$.

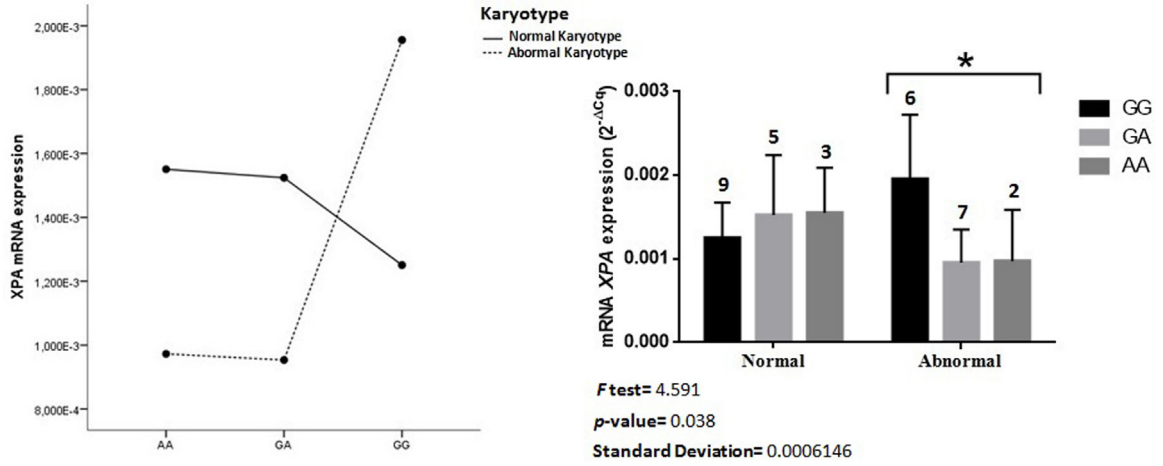

Fig. 2. A. Profile plots and **B.** bar graph of the comparisons of *XPA* gene expression within MDS patients that were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism regarding karyotype variable. Two-way ANOVA test. Results are shown as the median ±SD. Significant difference in $p < 0.05$.

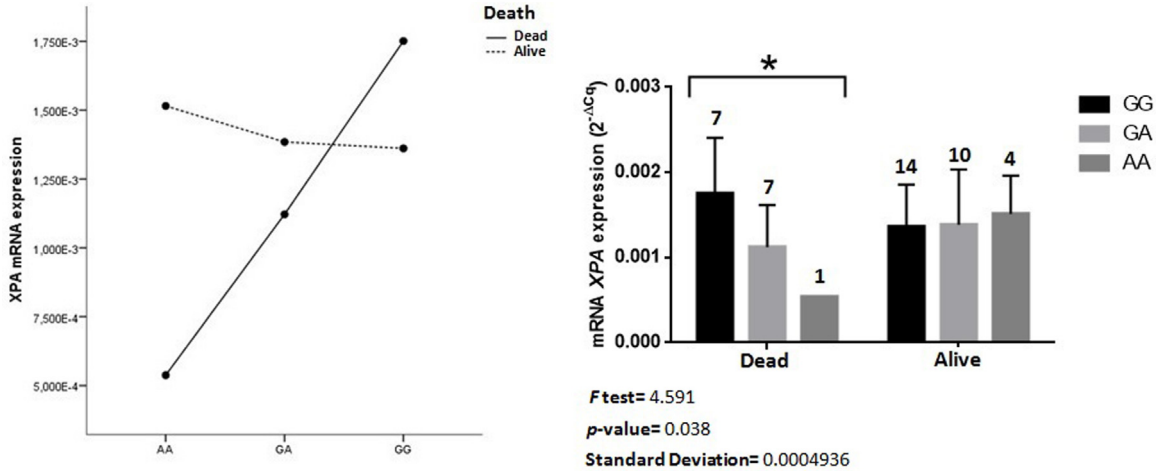

Fig. 3. A. Profile plots and **B.** bar graph of the comparisons of *XPA* gene expression within MDS patients that were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism regarding death variable. Two-way ANOVA test. Results are shown as the median ±SD. Significant difference in $p < 0.05$.

XPC rs2228000, *XPD* rs1799793 and *XPF* rs1800067) and expression levels of those genes in MDS patients.

Our results demonstrated that the C/C wild genotype of the *XPC* rs2228000 polymorphism was associated with favorable prognostic variables such as the presence of Hb ≥8.0 g/dL and ≥800

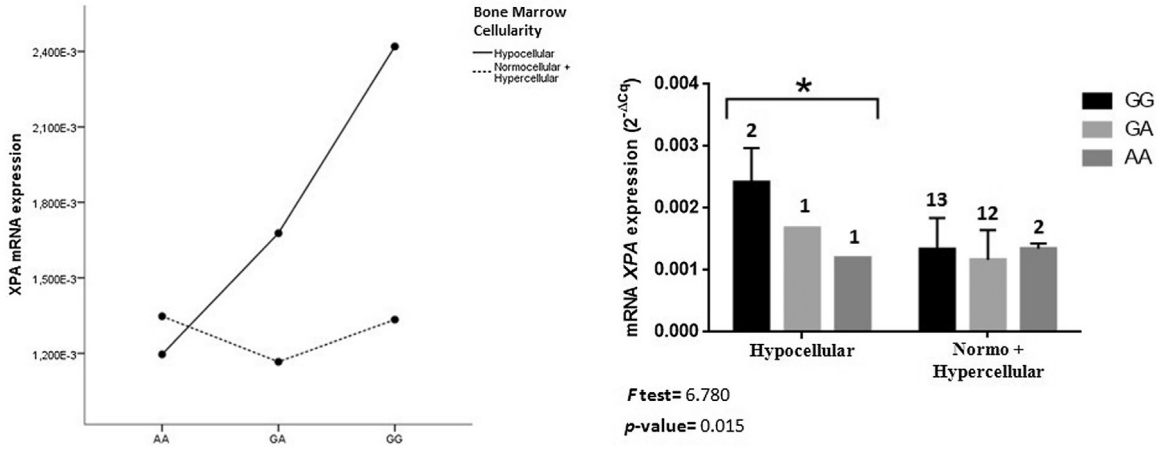

Fig. 4. A. Profile plots and **B.** bar graph of the comparisons of *XPA* gene expression within MDS patients that were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism regarding bone marrow cellularity variable. Two-way ANOVA test. Results are shown as the median ±SD. Significant difference in $p < 0.05$.

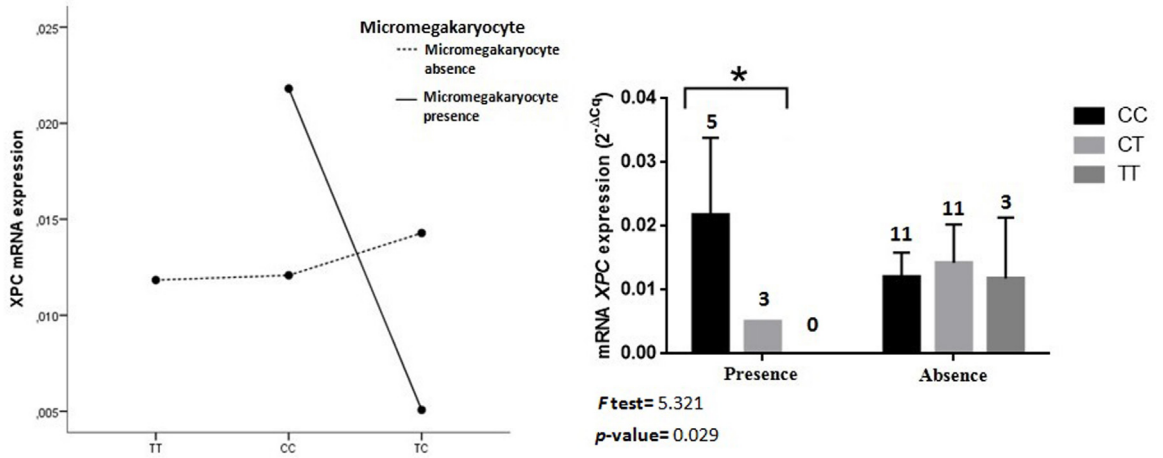

Fig. 5. A. Profile plots and **B.** bar graph of the comparisons of *XPA* gene expression within MDS patients that were stratified according to genotypic distribution genetic model for the rs2228000 polymorphism regarding presence of micromegacaryicyte variable. Two-way ANOVA test. Results are shown as the median ±SD. Significant difference in $p < 0.05$.

neutrophils/mm^3. Such findings indicate that the C wild-type allele homozygous seems to have a protective effect regarding severity of anemia and neutropenia in MDS patients. On the other hand, the presence of the TT polymorphic variant in rs2228000 polymorphism was associated with the presence of profound neutropenia (<800 neutrophils/mm^3), a marker of grim prognosis for MDS. In the revised IPSS, Greenberg et al. [15], evaluated data from 7012 patients from multiple institutional databases and detected, using multivariate analysis, that depth of cytopenias (hemoglobin, platelet, and neutrophils) presented a grim impact on prognostic risk. Profound anemia (Hb < 8 g/dL) in MDS patients may contribute to the high no- leukemic mortality regarding cardiac disease. De Castro et al., demonstrated a strong correlation between low hemoglobin levels and high LVEDV (left ventricular end-diastolic volume), high LVESV (left ventricular end-systolic volume) and high LAV (left atrial volume). The reduction of blood viscosity in severe anemia increases blood return and ventricular preload which lead to atrial and ventricular enlargement observed in MDS patients with low hemoglobin (< 8 g/dL) [19]. Besides that, patients with neutrophils under 800/mm^3 present high risk of infectious which increases the mortality of MDS.

We detected only two reports of *XPC* rs2228000 polymorphism in hematological neoplasm. Guillem and researchers [20] showed that wild genotypes (CC) of the *XPC* rs2228000 polymorphism are related to better response to treatment and better disease-free survival in patients with chronic myeloid leukemia (CML) [20] while Strom and colleagues [21] showed that CT and TT polymorphic genotype are associated with a lower overall survival in AML patients. Thus, we hypothesized that the T allelic polymorphic variant in the *XPC* rs2228000 polymorphism may be considered an important candidate of unfavorable prognosis in MDS.

When we evaluated the *XPD* rs1799793 polymorphism, we found that the GG homozygous wild-type genotype was associated with a decreased chance of developing MDS when compared to control group in the dominant model and in the heterozygous dominant model. We can speculate that XPD helicase is related to genomic stability in MDS. The XPD helicase is an essential component of NER repair pathway of SSB lesion and its function is critical for the correction of damages caused by tobacco and other carcinogens [22]. According to several epidemiological studies, *XPD* polymorphisms are associated with increased risk of several cancer types, such as breast [12,23], lung [24–26], colon [27] and prostate [28]. However, studies relating *XPD* polymorphisms with hematologic diseases are scarce and have conflicting results [29–32], focusing mainly on rs13181 polymorphism. To the best of our knowledge, our report is the first evaluation of the polymorphism rs1799793 in MDS which demonstrates association with a protective effect. New reports are necessary to confirm our result.

We found an association of the effects of rs2228000 polymorphism on *XPA* mRNA expression in clinical and laboratory variables of MDS patients. We detected that the presence of AG or AA polymorphic genotypes of rs2228000 SNP are associated with a down-regulation of *XPA* gene in clinical variables of MDS patients, such as younger patients, hypocellular bone marrow, abnormal karyotype, and death. Several studies correlate the presence of this rs2228000 SNP with increased risk of solid tumors such as lung [25,33,34], kidney [29], breast [35] and colon [36]. Some authors [37,38] explain that the *XPA* gene encodes a zinc-zinc DNA binding protein, playing a central role in the NER pathway in modulating the recognition of lesions, participating in DNA repair for maintain genomic integrity. Also, It is known that any variations that may occur in the *XPA* gene may have the potential of affect protein function and subsequently reduce DNA repair capacity, which may play a role in the development of cancer [37,38]. Thus, based on our molecular findings, we speculate that the A allelic polymorphic variant of rs1800975 SNP may be a possible new marker of unfavorable prognosis for MDS.

This study presents limitations. We found very significant associations and correlations, but the number of cases evaluated is low and we can't exclude that the absence of associations related to other genes may be explained by this factor. We didn't evaluate how these genes are truly correlated in specific cells because we studied the whole bone marrow when performing RT-PCR. Another important point was the use of peripheral blood samples from elderly volunteer donors for genotyping experiments. Because MDS is a bone marrow disease, it is necessary to use bone marrow samples to identify possible genotypic and gene expression changes associated with MDS pathophysiology. When we evaluated the controls, we used peripheral blood instead to study the genotype. However, it is known that the polymorphic changes are somatic and are present in the genome of all cells of the organism which normally don't change the results of controls.

Functional polymorphisms (FPs) are defined as alterations in DNA that have an effect on the gene function (expression of mRNA or its protein) [39]. Joshi and colleagues [13] have indicated correlation of DNA SSB polymorphisms with inter-individual differences in susceptibility to MDS and clinical variations among MDS patients [13]. The authors identified that the *XPD* rs13181 ($p = 0.011$) was significantly associated with MDS, but did not find significant associations with the clinical laboratory variables MDS [13] against we detected. Thus, in our study, we demonstrated new functional polymorphisms of *Xeroderma Pigmentosum* DNA genes (*XPA* rs1800975, *XPC* rs2228000, and *XPD* rs1799793) in MDS, corroborating the idea that polymorphic variant of these genes are probably related to the MDS pathogenesis prognosis.

Author contributions

SPS, HLRJ, JCS, DPB, RTGO, IRF, MBC, ARSM, MNI, SMMM and RFP designed the study, were responsible for collection and assembly of data, carried out the genotyping by Real-time PCR, analyzed the data and wrote the manuscript. SMMM and RFP provided medical care to the patients. All drafted and edited the manuscript. All authors have approved the final version of manuscript before publication.

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