

DNA repair gene expressions are related to bone marrow cellularity in myelodysplastic syndrome

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ABSTRACT

Objective To evaluate the expression of genes related to nuclear excision (*ERCC8*, *XPA* and *XPC*), homologous recombination and non-homologous end-joining (*ATM*, *BRCA1*, *BRCA2* and *LIG4*) repair mechanisms, using quantitative PCR methodologies, and its relation with bone marrow cellularity in myelodysplastic syndrome (MDS).

Methods and results A total of 51 adult de novo patients with MDS (3 refractory anaemia (RA), 11 refractory anaemia with ringed sideroblasts (RARS), 28 refractory cytopenia with multilineage dysplasia (RCMD), 3 refractory cytopenia with excess blasts type I (RAEB-I), 5 refractory anaemia with excess blasts type II (RAEB-II), and 1 chronic myelomonocytic leukaemia (CMML)) were evaluated. For karyotype, 16.2% patients were defined as very low prognosis, 59.5% low risk, 8.1% intermediate risk, 5.4% high risk and 10.8% very high risk. For bone marrow cellularity, 17.6%, 17.6% and 64.7% presented as hypocellular, normocellular and hypercellular, respectively. Patients with hypocellular MDS had significantly decreased expression of *ATM* ($p=0.000$), *BRCA1* ($p=0.014$), *BRCA2* ($p=0.003$), *LIG4* ($p=0.004$) and *ERCC8* ($p=0.000$) than those with normocellular/hypercellular bone marrow, whereas *XPA* ($p=0.049$) and *XPC* ($p=0.000$) genes were increased. In patients with hypoplastic MDS, a low expression of *ATM* ($p=0.0268$), *LIG4* ($p=0.0199$) and *ERCC8* ($p=0.0493$) was significantly associated with the presence of chromosomal abnormalities. We detected positive correlations between *BRCA1* and *BRCA2* ($r=0.416$; $p=0.007$), *ATM* and *LIG4* ($r=0.472$; $p=0.001$), *LIG4* and *BRCA1* ($r=0.333$; $p=0.026$), *LIG4* and *BRCA2* ($r=0.334$; $p=0.025$), *ATM* and *XPA* ($r=0.377$; $p=0.008$), *ATM* and *XPC* ($r=0.287$; $p=0.046$), *LIG4* and *XPC* ($r=0.371$; $p=0.007$) and *XPA* and *XPC* genes ($r=0.895$; $p=0.0000$). We also found among all patients evaluated that correlation with *LIG4* occurred most often.

Conclusions These correlations demonstrate the important intrinsic relations between single and double DNA strand breaks genes in MDS, emphasising that these genes are related to MDS pathogenesis.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal haematopoietic stem cell disorders characterised by peripheral cytopenias due to ineffective haematopoiesis.^{1–3} Bone marrow cellularity in patients with MDS is usually hypercellular or normocellular.⁴ According to WHO,⁵

10–15% of patients with MDS can present hypocellular bone marrow, which has prognostic significance for hypoplastic MDS (hMDS).^{5 6–8}

Schemenau *et al*⁹ evaluated the prognostic importance of cellularity among 1270 patients with MDS and showed that for cases with hypercellular, normocellular and hypocellular bone marrow there was a significant difference in median survival of 25 months, 42 months and 38 months, respectively.⁹ Acute myeloid leukaemia progression rates were 33% after 2 years (hypercellular), whereas normocellular and hypocellular cases showed a progression of only 19%. These results suggest that hypercellular bone marrow in MDS is related to a poor prognosis.⁹

Patients with hypocellular bone marrow are usually younger, have immune abnormalities such as vasculitis, pyoderma and respond to antithymocyte globulin (Atgam) treatment.^{10–12} In a study of the pathogenesis of hMDS, Young *et al*,¹³ reported that T cells affect bone marrow destruction as in aplastic anaemia, reinforcing hMDS as a distinct entity with a different prognosis.

DNA repair is an important mechanism for maintaining the integrity of multicellular organisms and for ensuring genomic stability.¹⁴ DNA damage constantly arises from DNA replication, spontaneous chemical reactions and assaults by external or metabolism-derived agents.^{15 16} At least four main, partially overlapping damage repair pathways operate in mammals: nucleotide-excision repair (NER), base excision repair (BER), homologous recombination (HR) and non-homologous end-joining (NHEJ). NER and BER are related to single-strand lesion and HR and NHEJ, to double-strand breaks (DSBs). DSBs are mutagenic DNA lesions^{3 14 17} and are considered to exert important biological effects on the formation of chromosomal aberrations, cell death and transformation,^{3 14 17} which are common features of MDS. NER eliminates helix-distorting DNA damage, a broad category of damage that affects one of the two DNA strands.¹⁵ Problems in NER are also related to cancer and senescence.¹⁵

We previously demonstrated that polymorphisms of DNA repair genes related to HR are associated with the pathogenesis of MDS.^{18 19} We found that the polymorphisms of *ATM*, *RAD51*, *XRCC5* and *XRCC6* are associated with the genesis and aetiology of MDS.^{18 19} Importantly, we recently showed that these polymorphisms are functional, influencing the level of expression of *ATM*, *XRCC6* and *LIG4*.²⁰

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The aim of this study was to evaluate the relation between expression of genes related to NER (*ERCC8*, *XPA* and *XPC*), HR and NHEJ (*ATM*, *BRCA1*, *BRCA2* and *LIG4*) repair mechanisms and bone marrow cellularity in MDS.

PATIENTS AND METHODS

Patients

Fifty-one adult de novo patients with MDS were diagnosed at Federal University of Ceara according to WHO.¹⁰ The patients were evaluated according to WHO classification¹⁰ and the International Prognosis Score System revised (IPSS-R).²¹

Bone marrow cellularity was assessed on biopsy specimens and was stratified as hypocellular, normocellular and hypercellular, in relation to the patient's age. Hypocellularity in patients with MDS was defined as <30% in those aged <70 years, and 20% in patients >70 years, according to the established criteria of the European consensus on grading bone marrow fibrosis and assessment of cellularity.²²

The study was approved by the ethics committee of the Federal University of Ceara (No. 1.292.509) and conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants included in this study.

Cytogenetic analysis

Conventional G-band karyotype analysis was performed on bone marrow cells of 51 de novo patients with MDS according to the protocol already established in our laboratory.²³ Briefly, cultures were established in RPMI 1640 medium (Gibco, Grand Island, New York, USA) containing 30% fetal calf serum. For the 24-hour culture, colcemid was added at a final concentration of 0.05 µg/mL for the final 30 min. 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 was analysed whenever possible. The karyotype was prepared using a CytoVision Automated Karyotyping System (Applied Imaging, San Jose, California, USA) and described according to the International System for Human Cytogenetic Nomenclature (2013).

Total RNA extraction

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

Table 1 Characteristics of patients with MDS according to bone marrow cellularity

| Variables | Hypocellular MDS (n=9) | Normal/hypercellular MDS (n=9/n=33) | p Value |
|--|--------------------------|-------------------------------------|--------------|
| Age (years), mean (range) | 43 (22–58) | 67 (41–88) | 0.000 |
| Gender | | | |
| Male | 4 (44.4) | 20 (47.6) | 0.863 |
| Female | 5 (55.6) | 22 (52.4) | |
| Haemoglobin (g/dL), mean (range) | 8.87 (6.2–13.1) | 8.46 (4.9–12.9) | 0.620 |
| ANC ($\times 10^9 L^{-1}$), mean (range) | 527.11 (217–1075) | 2185.55 (144–7429) | 0.000 |
| Platelets (mm^3), mean (range) | 827 900 (12 100–300 000) | 7 857 110 (10 500–676 000) | 0.140 |
| Ring sideroblasts (%), mean (range) | 8 (0–70) | 19 (0–80) | 0.290 |
| Blasts (%), mean (range) | 2 (0–11) | 3.12 (0–37) | 0.634 |
| Transfusal dependence | | | |
| Dependence | 4 (44.4) | 23 (56.1) | 0.525 |
| Non-dependence | 5 (55.6) | 18 (43.9) | |
| WHO classification | | | |
| RA | 0 (0.0) | 3 (7.1) | |
| RARS | 1 (11.1) | 10 (23.8) | |
| RCMD | 7 (77.8) | 21 (50.0) | |
| RAEB | 1 (11.1) | 7 (16.7) | |
| CMML-1 | 0 (0.0) | 1 (2.4) | |
| High-grade (RA, RARS, RCMD and CMML) | 8 (88.9) | 35 (83.3) | |
| Low-grade (RAEB) | 1 (11.1) | 7 (16.7) | 0.173 |
| Karyotype | | | |
| Normal | 2 (28.6) | 19 (63.3) | 0.095 |
| Abnormal | 5 (71.4) | 11 (36.7) | |
| No metaphase | 2 (14.3) | 12 (85.7) | |
| Cytogenetics prognosis | | | |
| Very low+low+intermediate | 6 (85.7) | 29 (96.7) | 0.249 |
| High+very high | 1 (14.3) | 1 (3.3) | |
| IPSS-R score | | | |
| Very low+low+intermediate | 5 (71.4) | 26 (86.7) | 0.325 |
| High+very high | 2 (28.6) | 4 (13.3) | |
| AML transformation | 2 (25.0) | 5 (12.8) | 0.378 |

Results are shown as number (%) unless stated otherwise.

Bold text indicates significant differences ($p < 0.05$) between groups.

AML, acute myeloid leukaemia; ANC, absolute neutrophil count; CMML, chronic myelomonocytic leukaemia; IPSS-R, International Prognosis Score System revised; MDS, myelodysplastic syndrome; RA, refractory anaemia; RAEB, refractory anaemia with excess blasts type I or type II; RARS, refractory anaemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia.

cDNA synthesis

cDNA was generated from total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, San Jose, California, 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 .

Quantitative real-time PCR

Quantitative real-time PCR reactions were based on TaqMan methodology (Applied Biosystems, Carlsbad, California, USA) and run on a 7500 Fast System (Applied Biosystems, Carlsbad, California, USA). Predeveloped TaqMan gene expression assays for *ATM* (Hs01112344_m1), *BRCA1* (Hs01556191_m1), *BRCA2* (Hs01037423), *LIG4* (Hs00934061_m1), *ERCC8* (Hs01122124_m1), *XPA* (Hs00166045_m1) and *XPC* (Hs01104213_m1) as well as the TaqMan Universal Master Mix II, with uracil N-glycosylase (Applied Biosystems, Carlsbad, California, USA) were used to quantify mRNA expression. The expression level of the β 2-microglobulin gene (*B2M*) and ubiquitin (*UBC*) were used to normalise differences in input cDNA. Each sample was performed in duplicate and the expression ratios were calculated using the $2^{-\Delta\Delta C_q}$ method.²⁴

Statistical analysis

Data on relative mRNA expression (ΔC_q values—quantitative cycle) were expressed as mean and range (maximum and minimum) in order to determine the possible association between relative gene expressions and the analysed variables. Normality was evaluated by Shapiro–Wilk test. Outliers were removed.

Student's t-test or one-way analysis of variance with Tukey/Games–Howell post hoc test were used to analyse the associations between gene expression and the variables. The homogeneity of variances for all variables was tested by Levene's test. Multinomial logistic regression (MLR) analysis was used to estimate the ORs for each gene that might contribute to cellularity.

The best DNA repair expression cutoff point predicting MDS overall survival was calculated using the web-based Cutoff Finder software⁹ (Cutoff Finder—<http://molpath.charite.de/cutoff/index.jsp>) by Kaplan–Meier curve analysis based on the log-rank test.

Pearson's correlation test was used to obtain r and r^2 values. Statistical analyses were performed using the GraphPad Prism 5 (GraphPad Prism software, La Jolla, California, USA) and SPSS 20.0 (SPSS Inc, Chicago, Illinois, USA) computer software programs. Probability level (p value) <0.05 was assumed.

RESULTS

Patients

Characteristics of the patients with MDS are shown in [table 1](#). A total of 51 adult de novo patients with MDS (3 refractory anaemia (RA), 11 refractory anaemia with ringed sideroblasts (RARS), 28 refractory cytopenia with multilineage dysplasia (RCMD), 3 refractory anaemia with excess blasts type I (RAEB-I), 5 refractory anaemia with excess blasts type II (RAEB-II), and 1 chronic myelomonocytic leukaemia (CMML) were analysed according to WHO classification.¹⁰ Cytogenetic analyses were performed in 37/51 (72.5%) patients. Of those, 21 (56.8%) showed normal karyotype, 16 (43.2%) showed abnormal karyotype; 14 (27.5%) presented no metaphases ([table 1](#)). For karyotype, 6 (16.2%) patients were defined as having a very low prognosis, 22 (59.5%) as low risk, 3 (8.1%) as intermediate risk, 2 (5.4%) as high risk and 4 (10.8%) as very high risk, according to IPSS-R²¹ ([table 1](#)).

Table 2 Gene expression of patients with MDS according to bone marrow cellularity

| BM cellularity | ATM gene expression ($2^{-\Delta C_q}$) | | BRCA1 gene expression ($2^{-\Delta C_q}$) | | BRCA2 gene expression ($2^{-\Delta C_q}$) | | LIG4 gene expression ($2^{-\Delta C_q}$) | | p Value |
|----------------|---|---------|---|----------|---|----------|--|---------|---------|
| | Mean (range) | p Value | Mean (range) | p Value* | Mean (range) | p Value* | Mean (range) | p Value | |
| DSB | | | | | | | | | |
| Hypocellular | 0.001849 (0.0033379–0.000663) | 0.000† | 0.003351 (0.007095–0.001553) | 0.014‡ | 0.001423 (0.0035092–0.0000601) | 0.003‡ | 0.000767 (0.001238–0.000335) | 0.004† | |
| Normocellular | 0.00474 (0.008631–0.00107) | – | 0.005962 (0.011155–0.001788) | – | 0.002212 (0.0033876–0.000504) | – | 0.001443 (0.002379–0.000692) | – | |
| Hypercellular | 0.004471 (0.010709–0.001067) | 0.000† | 0.006369 (0.012149–0.002896) | 0.014‡ | 0.003845 (0.0091746–0.0004713) | 0.003‡ | 0.001297 (0.002429–0.000464) | 0.004† | |
| | | | | | | | | | |
| BM cellularity | ERCC8 gene expression ($2^{-\Delta C_q}$) | | XPA gene expression ($2^{-\Delta C_q}$) | | XPC gene expression ($2^{-\Delta C_q}$)* | | | | |
| | Mean (Range) | p value | Mean (Range) | p value* | Mean (Range) | p value* | | | |
| SSB | | | | | | | | | |
| Hypocellular | 0.0001183 (0.000048–0.000187) | 0.000† | 0.001730 (0.0005042–0.002810) | 0.049† | 0.01657 (0.01438–0.01834) | 0.000§ | | | |
| Normocellular | 0.0002028 (0.000118–0.000307) | – | 0.001383 (0.0005689–0.002637) | – | 0.01212 (0.003582–0.02825) | 0.000§ | | | |
| Hypercellular | 0.0002028 (0.000118–0.000307) | 0.000† | 0.001198 (0.0005661–0.002416) | 0.049† | | | | | |

Asterisk indicates significant differences ($p < 0.05$) between groups.

*These genes were analysed for the hypocellular versus normocellular+hypercellular groups.

†These data were analysed by one-way analysis of variance (ANOVA) with the Games–Howell post hoc test.

‡These data were analysed by one way ANOVA with the Tukey post hoc test.

§These data were analysed by a two-sided Student t test.

BM, bone marrow; DSB, double-strand break genes related to double DNA strand break repair pathway; MDS, myelodysplastic syndrome; SSB, single-strand break genes related to single DNA strand break repair pathway.

For bone marrow cellularity, 9 (17.6%) patients presented hypocellular bone marrow, 9 (17.6%) normocellular and 33 (64.7%) hypercellular (table 1). For patients with hypocellular MDS, 5/7 (71.4%) had chromosomal abnormalities (table 1), whereas for patients with normocellular/hypercellular hMDS 11/30 (36.7%) were found to have abnormalities (table 1). We determined that hMDS was associated with a lower count of neutrophils ($p=0.000$) and younger patients ($p=0.000$) (table 1).

mRNA expression of DNA repair genes in relation to bone marrow cellularity

We evaluated mRNA expression of *ATM*, *BRCA1*, *BRCA2*, *LIG4*, *ERCC8*, *XPA* and *XPC* genes related to the bone marrow cellularity analysis. The mRNA expressions of patients with MDS are shown in table 2.

Patients with hypocellular MDS presented significantly lower *ATM* ($p=0.000$; 95% CI=0.001330 to 0.003914) mRNA expression levels than patients with hypercellular bone marrow (figure 1A). We also detected an important decrease of *BRCA1* expression ($p=0.014$; 95% CI 0.000514 to 0.005521), *BRCA2* ($p=0.003$; 95% CI 0.000709 to 0.004136), *LIG4* ($p=0.004$; 95% CI 0.000162 to 0.000899) and *ERCC8* ($p=0.000$; 95% CI 0.000070 to 0.000245) genes (figure 1B–E) in hMDS.

On the other hand, we found that there is an increase of *XPA* ($p=0.049$; 95% CI 0.00000182 to 0.001064) and *XPC* ($p=0.000$; 95% CI 0.002192 to 0.006704) expression in hMDS (figure 1F, G).

Analysis of karyotype in patients with hypoplastic MDS in relation to DNA repair genes

Patients with hMDS who showed low expression of *ATM* ($p=0.0268$; 95% CI 0.005891 to 0.0006105), *LIG4* ($p=0.0199$; 95% CI 0.001239 to 0.0001663) and *ERCC8* ($p=0.0493$; 95% CI=0.000303 to 0.0000007) genes were significantly associated with the presence of chromosomal abnormalities (see figure 2A–C).

Low expression was considered when the mean of values of *ATM*, *LIG4* and *ERCC8* gene expressions were significantly lower than the mean observed for patients with hMDS with or without presence of chromosomal abnormalities (table 3).

Overall survival by Cutoff Finder of DNA repair genes in patients with MDS

We determined the cutoff value using the Cutoff Finder biostatistical tool to discriminate survival by Kaplan–Meier with log-rank post-test⁹ (table 4). This method correlates the dichotomised biomarker with a binary outcome using logistic regression.⁹

First, we selected 0.001638 as the optimal cutoff value for all subsequent analyses to differentiate between lower *LIG4* (<0.001638) and higher *LIG4* (>0.001638) gene expression (figure 3A). Overall, 36 (70.6%) patients with MDS were considered to be lower *LIG4* and 15 (29.4%) considered higher *LIG4* (figure 3A, B). Lower *LIG4* patients with MDS were associated with a higher overall survival ($p=0.017$; HR=2.42; 95% CI 1.14 to 5.13) (figure 3C).

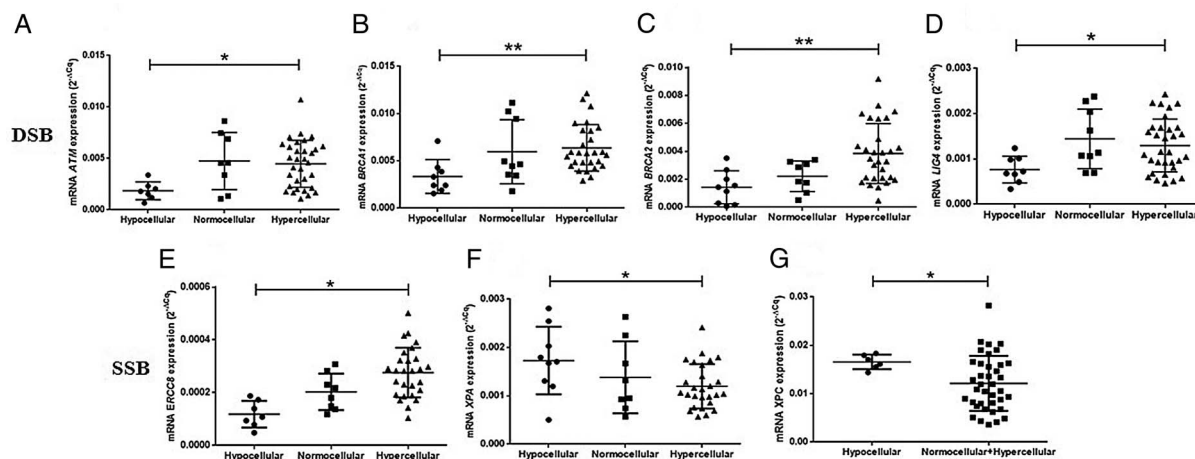


Figure 1 DNA repair genes analysis according to bone marrow cellularity. (A) mRNA *ATM* expression. (B) mRNA *BRCA1* expression. (C) mRNA *BRCA2* expression. (D) mRNA *LIG4* expression. (E) mRNA *ERCC8* expression. (F) mRNA *XPA* expression. (G) mRNA *XPC* expression. Asterisk indicates significant differences ($p<0.05$) between groups by one-way analysis of variance test. DSB, double-strand break genes related to double DNA strand break repair pathway; SSB, single-strand break genes related to single DNA strand break repair pathway.

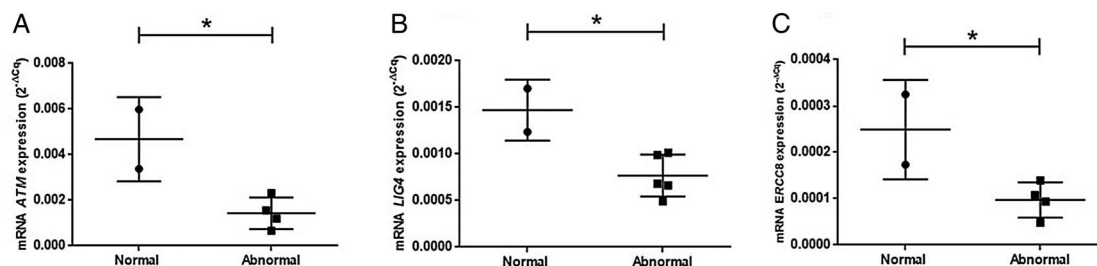


Figure 2 Plots of *ATM*, *LIG4* and *ERCC8* gene amplification analysis compared with the presence of chromosomal abnormalities in patients with hypocellular myelodysplastic syndrome. Asterisk indicates significant differences ($p<0.05$).

Table 3 Comparison of DNA repair gene expression according to presence of chromosomal abnormalities in subgroups of bone marrow cellularity

| BM cellularity | <i>ATM</i> gene expression ($2^{-\Delta Cq}$) | | <i>BRCA1</i> gene expression ($2^{-\Delta Cq}$) | | <i>BRCA2</i> gene expression ($2^{-\Delta Cq}$) | | <i>LIG4</i> gene expression ($2^{-\Delta Cq}$) | |
|----------------|---|---------------|---|---------|---|---------|--|---------------|
| | Mean (range) | p Value | Mean (range) | p Value | Mean (range) | p Value | Mean (range) | p Value |
| <i>DSB</i> | | | | | | | | |
| Hypocellular | | | | | | | | |
| Normal | 0.004681 (0.003379–0.005983) | 0.026* | 0.00335 (0.002408–0.004292) | 0.583 | 0.0017 (0.001551–0.002004) | 0.442 | 0.00147 (0.001238–0.001702) | 0.019* |
| Abnormal | 0.001430 (0.000663–0.002309) | | 0.00278 (0.001553–0.003859) | | 0.0011 (0.000160–0.002669) | | 0.00076 (0.000492–0.001014) | |
| Normocellular | | | | | | | | |
| Normal | 0.006017 (0.004554–0.007479) | 0.324 | 0.00387 (0.003453–0.00464) | 0.543 | 0.0026 (0.001743–0.003388) | 0.803 | 0.00138 (0.000697–0.002379) | 0.969 |
| Abnormal | 0.003973 (0.003383–0.004563) | | 0.00312 (0.001788–0.004451) | | 0.0024 (0.001836–0.003073) | | 0.00135 (0.001074–0.001628) | |
| Hypercellular | | | | | | | | |
| Normal | 0.005026 (0.001408–0.01071) | 0.775 | 0.00680 (0.003871–0.01215) | 0.546 | 0.0043 (0.001413–0.009175) | 0.177 | 0.00135 (0.000464–0.002429) | 0.864 |
| Abnormal | 0.004750 (0.00167–0.007398) | | 0.00611 (0.004197–0.008506) | | 0.0031 (0.001572–0.006852) | | 0.00131 (0.000512–0.002215) | |
| BM cellularity | <i>ERCC8</i> gene expression ($2^{-\Delta Cq}$) | | <i>XPA</i> gene expression ($2^{-\Delta Cq}$) | | <i>XPC</i> gene expression ($2^{-\Delta Cq}$) | | | |
| | Mean (Range) | p Value | Mean (Range) | p Value | Mean (Range) | p Value | | |
| <i>SSB</i> | | | | | | | | |
| Hypocellular | | | | | | | | |
| Normal | 0.00024 (0.0001733–0.000324) | 0.049* | 0.00085 (0.000504–0.001196) | 0.070 | 0.00979 (0.005201–0.01438) | 0.242 | | |
| Abnormal | 0.000097 (0.000048–0.000139) | | 0.00190 (0.001308–0.002810) | | 0.01829 (0.01089–0.03180) | | | |
| Normocellular | | | | | | | | |
| Normal | 0.000422 (0.000230–0.000731) | 0.418 | 0.00137 (0.000568–0.002637) | 0.564 | 0.00755 (0.00411–0.01369) | 0.942 | | |
| Abnormal | 0.000231 (0.000180–0.000282) | | 0.00084 (0.000737–0.00095) | | 0.00787 (0.00679–0.00895) | | | |
| Hypercellular | | | | | | | | |
| Normal | 0.000396 (0.000103–0.000501) | 0.234 | 0.00025 (0.000191–0.00036) | 0.471 | 0.01631 (0.00434–0.05181) | 0.336 | | |
| Abnormal | 0.000255 (0.000191–0.000369) | | 0.00168 (0.000597–0.00133) | | 0.01221 (0.00622–0.02023) | | | |

Asterisk in bold text indicates significant differences ($p < 0.05$) between groups analysed by Student's t-test two sided.

BMC, bone marrow cellularity; DSB, double-strand break genes related to double DNA strand break repair pathway; SSB, single-strand break genes related to single DNA strand break repair pathway.

Table 4 Multinomial logistic regression for the DNA repair gene expressions concerning bone marrow cellularity of patients with MDS

| Gene | BMC | Cases (n) | Cut-off point* | χ^2 | Multinomial logistic regression† | | | |
|-------------------------|---------------|-----------|----------------|--------------|----------------------------------|--------------|---------------|-------------------------|
| | | | | | Wald | p Value | OR | 95% CI |
| <i>ATM</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 4 (36.4) | 0.001821 | 0.052 | – | – | – | – |
| | Normocellular | 2 (18.2) | | | – | – | – | – |
| | Hypercellular | 5 (45.4) | | | – | – | – | – |
| Higher§ | Hypocellular | 3(8.3) | | | – | – | – | – |
| | Normocellular | 6 (16.7) | | | – | – | – | – |
| | Hypercellular | 27 (75.0) | | | – | – | – | – |
| <i>BRCA1</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 8 (19.5) | 0.0105 | 0.817 | – | – | – | – |
| | Normocellular | 8 (19.5) | | | – | – | – | – |
| | Hypercellular | 25 (61.0) | | | – | – | – | – |
| Higher§ | Hypocellular | 0 (0.0) | | | – | – | – | – |
| | Normocellular | 1 (25.0) | | | – | – | – | – |
| | Hypercellular | 3 (75.0) | | | – | – | – | – |
| <i>BRCA2</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 6 (50.0) | 0.001741 | 0.007 | 8.075 | 0.004 | 12.500 | 2.190 to 71.363 |
| | Normocellular | 2 (16.7) | | | 0.563 | 0.453 | 2.083 | 0.306 to 14.168 |
| | Hypercellular | 4 (33.3) | | | – | – | – | – |
| Higher§ | Hypocellular | 3 (8.8) | | | 8.075 | 0.004 | 0.080 | 0.014 to 0.457 |
| | Normocellular | 6 (17.7) | | | 0.563 | 0.453 | 0.480 | 0.071 to 3.264 |
| | Hypercellular | 25 (73.5) | | | – | – | – | – |
| <i>LIG4</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 8 (22.2) | 0.001638 | 0.196 | – | – | – | – |
| | Normocellular | 6 (16.7) | | | – | – | – | – |
| | Hypercellular | 22 (61.1) | | | – | – | – | – |
| Higher§ | Hypocellular | 0 (0.0) | | | – | – | – | – |
| | Normocellular | 3 (23.1) | | | – | – | – | – |
| | Hypercellular | 10 (76.9) | | | – | – | – | – |
| <i>ERCC8</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 7 (30.4) | 0.0002351 | 0.019 | 4.712 | 0.030 | 11.900 | 1.272 to 111.351 |
| | Normocellular | 6 (26.1) | | | 3.216 | 0.073 | 5.100 | 0.859 to 30.265 |
| | Hypercellular | 10 (43.5) | | | – | – | – | – |
| Higher§ | Hypocellular | 1 (5.0) | | | 4.712 | 0.030 | 0.084 | 0.009 to 0.786 |
| | Normocellular | 2 (10.0) | | | 3.216 | 0.073 | 0.196 | 0.033 to 1.164 |
| | Hypercellular | 17 (85.0) | | | – | – | – | – |
| <i>XPA</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 3 (9.7) | 0.001674 | 0.172 | – | – | – | – |
| | Normocellular | 7 (22.6) | | | – | – | – | – |
| | Hypercellular | 21 (67.7) | | | – | – | – | – |
| Higher§ | Hypocellular | 6 (30.0) | | | – | – | – | – |
| | Normocellular | 9 (17.6) | | | – | – | – | – |
| | Hypercellular | 12 (60.0) | | | – | – | – | – |
| <i>XPC</i> expression | | | | | | | | |
| Lower‡ | Hypocellular | 1 (6.2) | 0.009534 | 0.345 | – | – | – | – |
| | Normocellular | 4 (25.0) | | | – | – | – | – |
| | Hypercellular | 11 (68.8) | | | – | – | – | – |
| Higher§ | Hypocellular | 8 (22.9) | | | – | – | – | – |
| | Normocellular | 5 (14.3) | | | – | – | – | – |
| | Hypercellular | 22 (62.9) | | | – | – | – | – |

Bold text indicates significant differences ($p < 0.05$) between groups analysed by one-way analysis of variance.

*Cut-off points established by the Cutoff Finder web tool.

†Multinomial logistic regression analysis with significant differences in $p < 0.05$.

‡Patients were included when the gene expression was lower than or equal to the cutoff point according to Cutoff Finder software. The reference category is higher for MLR analysis.

§Patients were included when the gene expression was higher than the cutoff point according to Cutoff Finder software. The reference category is lower for MLR analysis.

χ^2 , Pearson's χ^2 ; BMC, bone marrow cellularity; MDS, myelodysplastic syndrome; MLR, multinomial logistic regression.

Based on 0.001674 cutoff point value for *XPA* gene analysis, we found that there were 21 (41.2%) patients with a higher *XPA* and 30 (58.8%) patients with a lower *XPA* (figure 3A, B). We observed that higher *XPA* (> 0.001674) patients with MDS were

associated with a lower overall survival ($p = 0.019$; HR=3.23; 95% CI 1.15 to 9.06) (figure 3E, F).

No difference in overall survival was detected for other genes related to HR (*ATM*, *BRCA1*, *BRCA2*) and NER (*ERCC8* and

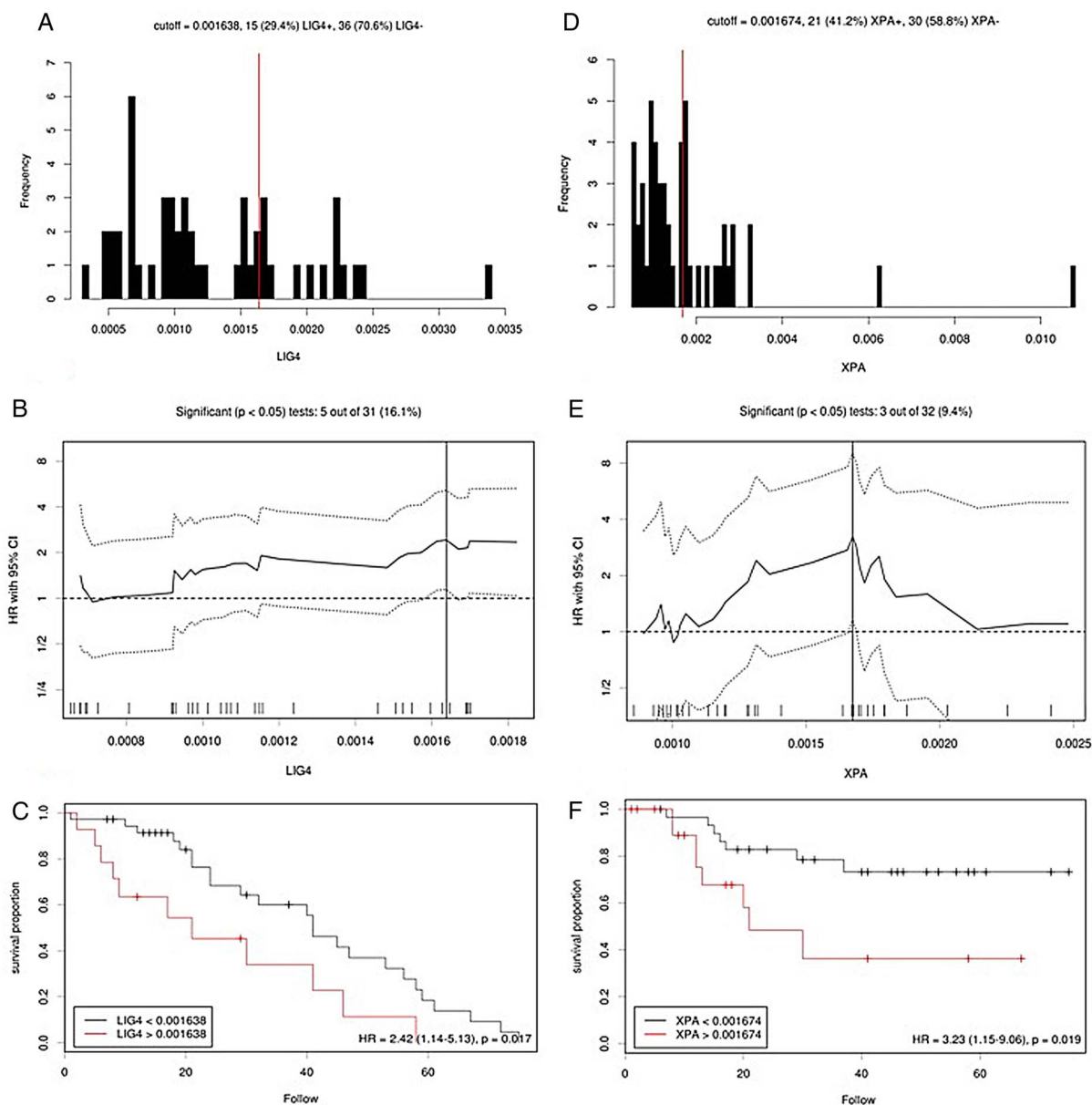


Figure 3 Analysis of cutoff points for *LIG4* and *XPA* genes as predictive marker for higher survival of patients with myelodysplastic syndrome. Red lines mark the optimal cutoff point for *LIG4* gene expression. (A, D) Histogram of *LIG4* and *XPA* gene expressions in 51 patients with myelodysplastic syndrome (MDS). (B, E) HR for disease-free survival depending on the cutoff point. (C, F). HR for overall survival depending on the cutoff point. The plots were generated using the biostatistical tool Cutoff Finder software.

XPC) when their values were dichotomised as biomarkers with a binary outcome using logistic regression (data not presented).

MLR analysis of expressions of DNA repair genes

The expression of genes related to DSBs (*ATM*, *BRCA1*, *BRCA2*, *LIG4*) and single-strand breaks (SSBs) (*ERCC8*, *XPA*, *XPC*) was divided into two groups: low and high expression, according to cutoff point established by Cutoff Finder biostatistical web application.⁹

We identified two subgroups of patients according to gene expression: lower group (when expression was lower than or equal to the cutoff value) and higher group (when expression was higher than the cutoff value).⁹ We detected that the decreased expression of *BRCA2* ($p=0.004$, OR=12.500, 95% CI 2.190 to 71.363) and *ERCC8* ($p=0.030$, OR=11.900, 95% CI 1.272 to 111.3512) genes were associated with diagnosis of hMDS (table 4).

No significant associations were found between the *ATM*, *BRCA1*, *LIG4*, *XPA* and *XPC* expressions and the bone marrow cellularity by MLR analysis ($p>0.05$) (table 3).

Correlations of the mRNA expressions of DNA repair genes in patients with MDS

By Pearson's correlation analysis, we observed significant correlations between the DSB (*ATM*, *BRCA1*, *BRCA2*, *LIG4*) and SSB (*ERCC8*, *XPA* and *XPC*) genes in MDS, demonstrating that these genes work in a dependent manner as a cascade of events (table 5).

We observed positive correlations between *BRCA1* and *BRCA2* ($r=0.416$; $r^2=0.173$; $p=0.007$) (figure 4A), *ATM* and *LIG4* ($r=0.472$; $r^2=0.222$; $p=0.001$) (figure 4B); *LIG4* and *BRCA1* ($r=0.333$; $r^2=0.110$; $p=0.026$) (figure 4C), *LIG4* and *BRCA2* ($r=0.334$; $r^2=0.111$; $p=0.025$) (figure 4D), *ATM* and *XPA* ($r=0.377$; $r^2=0.142$; $p=0.008$) (figure 4E), *ATM* and

Table 5 Pearson's correlation analysis of DNA repair genes in patients with MDS

| Pearson's correlation analysis | <i>ATM</i> | <i>BRCA1</i> | <i>BRCA2</i> | <i>LIG4</i> | <i>ERCC8</i> | <i>XPA</i> | <i>XPC</i> |
|--------------------------------|--------------|--------------|--------------|--------------|--------------|---------------|---------------|
| <i>ATM</i> | | | | | | | |
| Pearson correlation (r) | 1 | 0.255 | 0.232 | 0.472 | 0.267 | 0.377 | 0.287 |
| r ² | – | 0.065 | 0.053 | 0.222 | 0.071 | 0.142 | 0.082 |
| p Value (2-tailed) | – | 0.099 | 0.135 | 0.001 | 0.088 | 0.008 | 0.046 |
| N | 49 | 43 | 43 | 49 | 42 | 49 | 49 |
| <i>BRCA1</i> | | | | | | | |
| Pearson correlation (r) | 0.255 | 1 | 0.416 | 0.333 | 0.303 | 0.153 | 0.257 |
| r ² | 0.065 | – | 0.173 | 0.110 | 0.091 | 0.023 | 0.066 |
| p Value (2-tailed) | 0.099 | – | 0.007 | 0.026 | 0.061 | 0.315 | 0.088 |
| N | 43 | 45 | 41 | 45 | 39 | 45 | 45 |
| <i>BRCA2</i> | | | | | | | |
| Pearson correlation (r) | 0.232 | 0.416 | 1 | 0.334 | 0.272 | –0.195 | 0.003 |
| r ² | 0.053 | 0.173 | – | 0.111 | 0.073 | 0.038 | 0.000009 |
| p Value (2-tailed) | 0.135 | 0.007 | – | 0.025 | 0.090 | 0.199 | 0.987 |
| N | 43 | 41 | 45 | 45 | 40 | 45 | 45 |
| <i>LIG4</i> | | | | | | | |
| Pearson correlation (r) | 0.472 | 0.333 | 0.334 | 1 | 0.243 | 0.219 | 0.371 |
| r ² | 0.222 | 0.110 | 0.111 | – | 0.059 | 0.047 | 0.137 |
| p Value (2-tailed) | 0.001 | 0.026 | 0.025 | – | 0.116 | 0.123 | 0.007 |
| N | 49 | 45 | 45 | 51 | 43 | 51 | 51 |
| <i>ERCC8</i> | | | | | | | |
| Pearson correlation (r) | 0.267 | 0.303 | 0.272 | 0.243 | 1 | –0.173 | –0.173 |
| r ² | 0.071 | 0.091 | 0.073 | 0.059 | – | 0.0299 | 0.0299 |
| p Value (2-tailed) | 0.088 | 0.061 | 0.090 | 0.116 | – | 0.267 | 0.275 |
| N | 42 | 39 | 40 | 43 | 43 | 43 | 43 |
| <i>XPA</i> | | | | | | | |
| Pearson correlation (r) | 0.377 | 0.153 | –0.195 | 0.219 | –0.173 | 1 | 0.895 |
| r ² | 0.142 | 0.023 | 0.038 | 0.047 | 0.0299 | – | 0.801 |
| p Value (2-tailed) | 0.008 | 0.315 | 0.199 | 0.123 | 0.267 | – | 0.0000 |
| N | 49 | 45 | 45 | 51 | 43 | 51 | 51 |
| <i>XPC</i> | | | | | | | |
| Pearson correlation (r) | 0.287 | 0.257 | 0.003 | 0.371 | –0.173 | 0.895 | 1 |
| r ² | 0.082 | 0.066 | 0.000009 | 0.137 | 0.0299 | 0.801 | – |
| p Value (2-tailed) | 0.046 | 0.088 | 0.987 | 0.007 | 0.275 | 0.0000 | – |
| N | 49 | 45 | 45 | 51 | 43 | 51 | 51 |

Bold text indicates significant differences ($p < 0.05$) between groups analysed by Pearson's correlation test. MDS, myelodysplastic syndrome.

XPC ($r = 0.287$; $r^2 = 0.082$; $p = 0.046$) (figure 4F), *LIG4* and *XPC* ($r = 0.371$; $r^2 = 0.137$; $p = 0.007$) (figure 4G) and *XPA* and *XPC* genes ($r = 0.895$; $r^2 = 0.801$; $p = 0.0000$) (figure 4H).

DISCUSSION

We evaluated genes related to double and single DNA strand breaks in patients with MDS and detected that these genes are positively correlated and work in a dependent manner as a cascade of events and may be related to pathogenesis and prognosis of MDS. We found that genes recruited and activated by DNA double (*ATM*) and single (*ERCC8*) strand breaks are downregulated in hMDS. Ataxia telangiectasia mutated (*ATM*) is a serine/threonine protein kinase that, once activated, phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis.^{19–25} *ERCC8* (CSA), together with *ERCC6* (CSB), is the cell signalling that begins the transcription-coupled repair subpathway of NER.²⁶ As both genes act as primary sensors of DNA lesions, we suggest that DNA repair is deficient in hMDS and DNA damage tends to accumulate.

Importantly, we also found that for patients with hMDS with low expression of *ATM* and *ERCC8* there was a significant association with the presence of chromosomal abnormalities, reinforcing the intrinsic link between single- and double-strand breaks in MDS, principally in hypoplastic MDS. SSBs are not usually associated with a major DNA lesion and,¹⁹ consequently, chromosomal breaks, but may be the first step to a DSB if not properly corrected.²⁷ The estimated numbers of SSBs and spontaneous base losses in nuclear DNA are as high as 10^4 per cell per day.¹⁹ If the cell signalling of this type of lesion shows low expression, it may help to increase the chance of DSBs and consequent chromosomal abnormalities, as presented here.

Among several protein complexes involved in HR repair pathways, *BRCA1/2*, which are members of the BRCA family, were initially identified as biomarkers of breast cancer.^{28–30} *BRCA1/2* work with proteins of the RAD complex to control of genomic stability.^{28–30} To the best of our knowledge, this is the first report to demonstrate that both genes show low expression in hMDS and are intrinsically and positively correlated, reinforcing the suggestion that *BRCA1/2* work in a similar way. This result

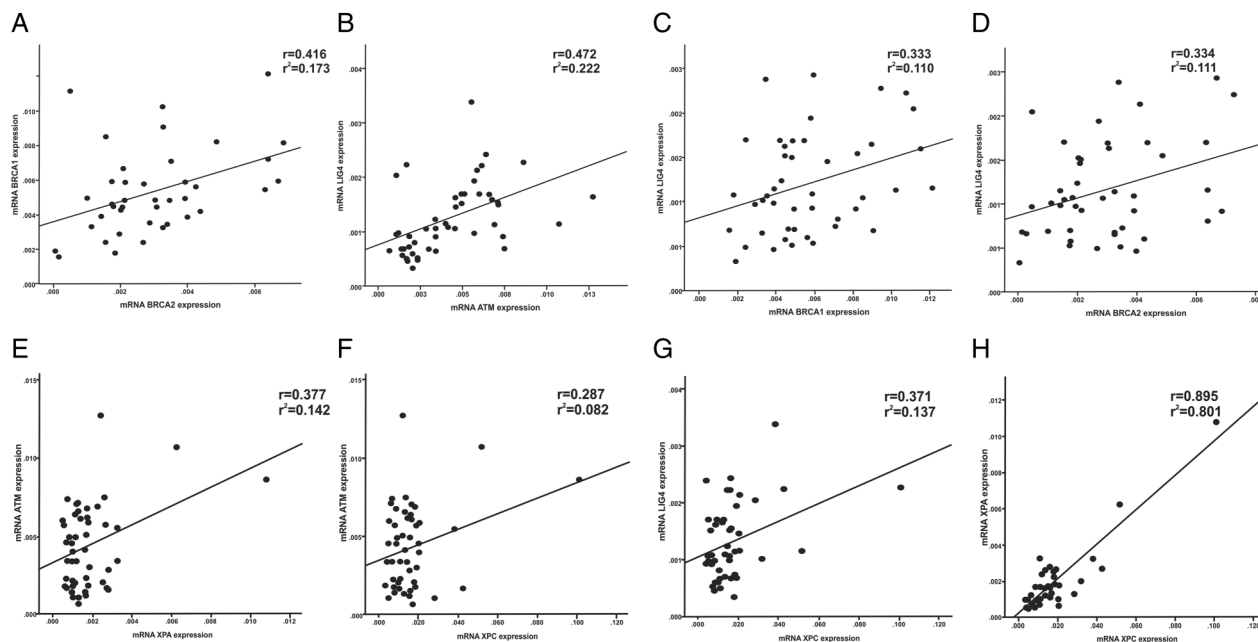


Figure 4 Plots of Pearson's correlation analysis of DNA repair gene expressions of patients with myelodysplastic syndrome.

also corroborates the importance of both genes in maintaining genomic stability because their low expression among patients reported here was associated with an increase of chromosomal abnormalities.

The low expressions of *BRCA1* and *BRCA2* genes have also been reported in solid tumours, such as epithelial ovarian carcinoma, prostate cancer and breast cancer.^{31 32} Using MLR analysis, we detected that decreased expression of *BRCA2* was associated with an increased chance of developing hMDS with an OR of 12. We were initially surprised with this result because *BRCA2* is associated with DNA repair, but Jeong *et al*³³ showed that *BRCA2* deficiency leads to T cell loss and immune dysfunction. In a very elegant murine model, the researchers demonstrated that mice with deficiency of *BRCA2* show reduction in splenic T cell number and less interferon γ and interleukin 4, suggesting a functional defect of *BRCA2*-deficient T cells.³³ *BRCA2* deficiency mice showed a greatly reduced production of IgM (ninefold reduction) compared with IgG1 (threefold reduction) or IgG2a (less than threefold reduction) isotypes.³² All these results suggest that *BRCA2* is also linked to immune abnormalities.³² As hMDS is considered an immune disorder,^{34 35} our result, showing decreased expression of *BRCA2* with increased chance of developing hMDS, was probably not obtained by chance. New reports and in vitro models are necessary to confirm our associations.

LIG4 (DNA ligase 4) encodes a protein that is a DNA ligase which joins SSBs in a double-stranded polydeoxynucleotide in an ATP-dependent reaction.³⁶ This protein is essential for V(D)J recombination, generation of the T cell receptor, immunoglobulin molecules and DNA DSB repair through NHEJ.³⁶ We were interested to detect low expression of *LIG4* among patients with hMDS because deficiency of *LIG4* has been associated with pancytopenia, combined immunodeficiency, cellular radiosensitivity, and recently, its mutation was associated with alterations of T and B cell counts with an increased percentage of memory cells and hypogammaglobulinaemia,³⁷ all markers of immune dysregulation. Hypoplastic MDS is considered an 'autoimmune disorder' owing to the concept that T cells

contribute to bone marrow failure and that T cell depleting agents, such as rabbit and equine antithymocyte globulin, are important options of treatment.^{32 33}

Importantly, we also detected among all patients evaluated (not only patients with hMDS) that correlation with the *LIG4* gene occurred most often. It was positively correlated with *ATM*, *BRCA1*, *BRCA2* and *XPC* genes, which are linked to HR and NER. These correlations demonstrate the important intrinsic relations between single and double DNA strand breaks in MDS, reinforcing the suggestion that these genes are all related to MDS pathogenesis.

We detected an increased expression of *XPC* and *XPA* in hMDS. Damage to DNA single strand that occurs in the genome is recognised by the XPC and XPE protein complexes, which are specific components of the global genome NER system.^{26 38} The DNA helix is opened by the XPB and XPD helixes of the repair, allowing damage verification by the XPA protein.^{26 38} So, one important cell signalling (*XPC*) and the 'check' (*XPA*) of NER are hyperactivated in hMDS. As expected, we also found that these two genes were also positively correlated. Using the Cutoff Finder biostatistical tool to discriminate the survival by Kaplan–Meier with log-rank post-test, we also found that patients with higher *XPA* (>0.001674) expression had a shorter overall survival ($p=0.019$; HR=3.23; 95% CI 1.15 to 9.06). This method correlates the dichotomised biomarker with a binary outcome using logistic regression.⁹ This is the first report to demonstrate the importance of the single-strand DNA repair system in MDS and these modifications may be the first step in genomic instability which, if not properly corrected, would predispose to DSBs and, consequently, chromosomal abnormalities.

When evaluating patients with hMDS, most researchers have focused on evaluation of immunological abnormalities.^{34–35 39} We show, for the first time, that genes related to DNA repair may also be associated with the pathogenesis of hMDS. Recently, Danjou *et al*,³⁹ evaluated a large cohort of patients with MDS exploring 147 954 single nucleotide polymorphisms localised in regions which display some degree of association

with immunological disorders. One of the most significant results was related to the gene *RTEL1*, an ATP helicase implicated in telomere-length regulation, DNA repair and genomic instability.³⁹ Of utmost importance, mutations in *RTEL1* are responsible for nail dystrophy, mucosal leucoplasia and progressive bone marrow failure, the hallmark of MDS.³⁹ The authors demonstrate the intrinsic association of immunological disorders and genes related to DNA repair, reinforcing our proposal that DNA repair genes may be intrinsically related to the pathogenesis of hMDS.

Our study has some limitations. Limitations due to sample size are common in scientific publications, as stated by Danjou *et al*³⁹ and Gonçalves *et al*.⁴⁰ We found significant associations and correlations, but the number of cases evaluated was low and we cannot exclude the possibility that the absence of associations related to other genes might be explained by this factor. We must also emphasise that the number of cases with hMDS was low, only nine cases. Hypolastic MDS is a rare entity among cases of MDS—<10% of cases—which makes it difficult to study large numbers.

We believe that the pathogenesis of hMDS is associated with an immune-mediated process,³⁴ and also with the repair mechanism of DSB and SSB lesions. One important question arises: might the expression of these genes be new biomarkers of MDS? New reports are necessary to confirm or not our results.

Take home messages

- ▶ Previously, our group has shown that double and single DNA strand breaks are related to the pathogenesis of myelodysplastic syndrome (MDS).
- ▶ Our study determined that DNA repair gene expressions are positively correlated and work in a dependent manner as a cascade of events and may be related to the pathogenesis and prognosis of MDS.
- ▶ To the best of our knowledge, our study demonstrates for the first time that hypolastic MDS is associated with an immune-mediated process, and also with the repair mechanism of double-and single-strand breaks.

Handling editor Mary Frances McMullin.

Contributors HLRJ, ARSM, RTGdO, MBC, IRF, DdPB, JcDs, SMMM and RFP designed the study, provided patient materials and were responsible for the collection and assembly of data. HLRJ, ARSM, RTGdO, MBC, IRF, DdPB, JcDs, SMMM and RFP performed the molecular procedures and analysed the data. All authors drafted and edited the manuscript and approved the final version of manuscript before publication.

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Competing interests None declared.

Ethics approval The study was approved by the ethics committee of the Federal University of Ceara (No. 1.292.509) and conducted according to the Declaration of Helsinki.

Patient consent Obtained.

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DNA repair gene expressions are related to bone marrow cellularity in myelodysplastic syndrome

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