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ORIGINAL ARTICLE

Bone marrow PARP1 mRNA levels predict response to treatment with 5-azacytidine in patients with myelodysplastic syndrome

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Abstract

Poly (ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme that participates in the DNA repair of malignant cells, with various consequences on their survival. We have recently shown that PARP1 mRNA levels in the bone marrow of patients with myelodysplastic syndrome (MDS) are correlated to prognosis. To evaluate PARP1 as a biomarker of response to 5-azacytidine in patients with MDS, we measured PARP1 mRNA levels by a quantitative real-time PCR in diagnostic bone marrow samples of 77 patients with MDS treated with 5-azacytidine. Patients with higher PARP1 mRNA levels had a better response to 5-azacytidine per the IWG criteria ($p = 0.006$) and a longer median survival after 5-azacytidine initiation ($p = 0.033$). Multivariate analysis revealed that PARP1 mRNA level was the only factor affecting response to treatment and survival after treatment with 5-azacytidine. A next-generation sequencing for 40 genes of interest in MDS and quantification of the methylation levels of the PARP1 promoter were also carried out in a subset of samples (16 and 18 samples respectively). It is the first time that a single, easily measurable biomarker shows a clear correlation with response to treatment and survival in a patient population consisting of previously untreated patients with MDS homogeneously treated with 5-azacytidine. The fact that PARP1 is also a treatment target in several malignancies underscores the importance of our finding for the potential use of PARP1 inhibitors in MDS.

Keywords Poly (ADP-ribose) polymerase 1 (PARP1) · Myelodysplastic syndrome · Prognosis · 5-Azacytidine · Response to treatment

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Introduction

Poly (ADP-ribose) polymerase 1 (PARP1) is the major member of a family of nuclear enzymes that play important roles in DNA repair. Upon binding to DNA breaks, PARP1 polymerizes and, by poly (ADP-ribosylation), participates in DNA repair and gene transcription [1, 2] by recruiting various proteins involved in DNA repair to sites of DNA damage [3]. Low levels of DNA damage trigger its detection and repair whereas high levels of DNA damage may lead to cell death by necrosis through PARP1 overactivation [3], which causes depletion of the cellular NAD+ and ATP pool [4, 5], or by apoptosis through caspase activation and PARP1 cleavage. It has been shown that PARP1 is not a necessary molecule under normal conditions. However, when DNA damage exceeds the levels at which the repair enzymes can function, PARP1 becomes important for the cell response [6].

PARP1 inhibition leads to the "preservation" of DNA damage that would have otherwise been repaired, and to the dysfunction of the malignant cell. The pharmaceutical research focuses on the development of potent competitive inhibitors of PARP1/NAD+ [7, 8]. The use of PARP1 inhibitors mainly aims at the sensitization of malignant cells to cytotoxic agents, thus leading to treatment potentiation.

Due to the multiple roles of PARP1, studies about its role in hematologic malignancies have conflicting results. However, PARP1 overexpression has been correlated with poor treatment response in children with acute lymphoblastic leukemia [9], while PARP1-driven apoptosis has been shown to be important in patients with chronic lymphocytic leukemia [10]. Moreover, PARP1 inhibitors have been tested in vitro in hematologic malignancies, mostly lymphoid malignancies [11, 12], but also in acute myelogenous leukemia (AML), MDS, and acute promyelocytic leukemia (APL). It has been shown that, in AML cell lines, combining 5-azacytidine with the PARP1 inhibitor olaparib causes synergistic cell lethality [13]. Moreover, in vitro PARP inhibitors have been found to potentially exploit defects in dsDNA break repair in leukemic cells, thus inducing cell cycle arrest and apoptosis; however, the addition of decitabine failed to increase cytotoxicity of PARP inhibitors [14].

We have recently shown that PARP1 mRNA levels in the bone marrow of patients with MDS are correlated to prognosis; high levels of PARP1 mRNA at diagnosis are an independent risk factor for lower overall survival (OS) in lower risk MDS patients per both the International Prognostic Scoring System (IPSS) and the revised IPSS (IPSS-R) [15]. It has also been shown that PARP1 is correlated with hypermethylation since it upregulates DNA methylotransferase 1 (*DNMT1*) gene and activates DNMT1 protein [16].

In the present study, based on the prognostic significance of PARP1 levels in MDS, the correlation of PARP1 with DNA hypermethylation, which is a central event in the pathogenesis of MDS, and the fact that hypomethylating agents (HMAs) are the most effective available choice for patients with higher risk MDS, we investigated the correlation of PARP1 mRNA levels in the bone marrow of patients with MDS with response to treatment with 5-azacytidine and survival.

Patients and methods

Patients

Adult patients with MDS per the 2008 World Health Organization (WHO) classification were included in the study. Patients that would have been classified as having MDS based on the French–American–British (FAB) classification (i.e. chronic myelomonocytic leukemia (CMML) and refractory anemia with excess blasts in transformation (RAEB-t)) were censored. The demographic, clinical, and hematologic characteristics of the patients were recorded retrospectively. Only data from patients that were eventually treated with 5-azacytidine monotherapy per common clinical practice were finally analyzed. All patients at diagnosis had MDS while patients progressing to AML before 5-azacytidine administration were excluded from the analysis, so that the final population consisted only of patients with MDS per the 2008/2016 WHO classification [17, 18]. The patients were stratified according to the IPSS [19], IPSS-R [20] and the WHO classification-based Prognosis Scoring System (WPSS) [21] for MDS. Informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Ethics Committee.

We also performed next-generation sequencing (NGS) for 40 genes of interest in MDS and identified the methylation level of PARP1 gene promoter in a small subset of patients.

5-Azacytidine administration

5-Azacytidine was administered subcutaneously (and in a minority of cases intravenously) at the approved dosing schedule by the US Food and Drug Administration and the European Medicines Agency of 75 mg/m^2/day for 7 days every 28 days. Patients having received at least one complete cycle of treatment and evaluated by a bone marrow aspiration after at least two cycles of treatment, or patients who died or progressed at an earlier time point were considered evaluable. Patients previously treated with cytotoxic agents or other disease-modifying treatment were excluded from the analysis. Finally, supportive treatment with erythropoietin-stimulating agents (ESAs), granulocyte colony-stimulating factor (G-CSF), and/or red blood cell transfusions before 5-azacytidine administration was not considered an exclusion criterion.

Evaluation of response and survival

Evaluation of response was based on the International Working Group (IWG) criteria for MDS [22]. Responses were independently reviewed, and responders were defined as patients achieving complete remission (CR), partial remission (PR), or hematological improvement (HI), while non-responders were defined as patients with stable disease (SD) or failure (F) to respond. OS rate was defined as the time interval from diagnosis to death from any cause, and survival after treatment initiation with 5-azacytidine (OS_T) was defined as the time interval from treatment initiation with 5-azacytidine to death from any cause.

Sample collection and processing

Bone marrow samples were collected in ethylenediaminetetraacetic acid (EDTA) during a routine bone marrow aspiration at diagnosis and processed within 6 h from collection. RNA extraction and cDNA synthesis followed and the samples were kept at − 80 °C. PARP1 mRNA levels were measured using a quantitative real-time polymerase chain reaction (qRT-PCR).

RNA extraction and reverse transcription

The Trizol protocol (Invitrogen, Carlsbad, CA, USA) was used to extract and purify total RNA from bone marrow samples. An MMLV-derived reverse transcriptase enzyme (M-MLV RT, Invitrogen, Carlsbad, CA, USA) was used for reverse transcription according to standard protocols.

Primer design for real-time PCR

Primers for PARP1 and β-actin were designed with the use of the primer3 software (University of Massachusetts, USA), using the relevant annotated cDNA sequences from NCBI BLAST (NM_001618.3 for PARP1 and NM_001101.3 for β-actin), and have already been described in a previous paper [15]. Primer sequences are as follows: for PARP1 forward, CCTGATCCCCCACGACTTT; reverse, GCAGGTTG TCAAGCATTTC and for β-actin forward, AGGATGCA GAAGGAGATCACT; reverse GGGTGTAACGCAAC TAAGTCATAG.

Real-time PCR

An RT-PCR was performed with the use of 2× iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions for both PARP1 and β-actin: 5 s at 95 °C, 15 s at 59 °C, and 5 s at 72 °C. All steps were repeated for 40 cycles. The standard curve method was used for the relative quantitation of PARP1 and β-actin transcripts, and PARP1 mRNA levels were expressed as a ratio of PARP1 to actin transcript levels.

DNA extraction

DNA was extracted from bone marrow leukocytes using the Purelink DNA mini kit (Invitrogen, Carlsbad, CA, USA) following standard protocols.

Next-generation sequencing

A multiple-gene panel (Ion Torrent Oncomine Myeloid Research Assay (Thermo Fisher Scientific, Waltham, MA, USA)) was used to interrogate relevant DNA mutations and fusion transcripts associated with MDS. The panel comprises 40 key DNA target genes: *ABL1*, *BRAF*, *CBL*, *CSF3R*, *DNMT3A*, *FLT3*, *GATA2*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *MYD88*, *NPM1*, *NRAS*, *PTPN11*, *SETBP1*, *SF3B1*, *SRSF2*, *U2AF1*, *WT1*, *ASXL1*, *BCOR*, *CALR*, *CEBPA*, *ETV6*, *EZH2*, *IKZF1*, *NF1*, *PHF6*, *PRPF8*, *RB1*, *RUNX1*, *SH2B3*, *STAG2*, *TET2*, *TP53*, *ZRSR2*. DNA was amplified by PCR using standard techniques. NGS was performed on an Ion S5 System per the manufacturer's instructions. Deep sequencing analysis (depth per variant; 500×/average depth; 1500×) was conducted to detect mutations at a rate as low as 5%. Variants were identified with Oncomine Knowledgebase reporting software (Thermo Fisher Scientific, Waltham, MA, USA).

PARP1 promoter methylation

The EpiTect Methyl qPCR Array (SA Bioscience, Qiagen, Hilden, Germany) was used according to manufacturer's instructions to identify gene promoter methylation. Four digestion reactions were carried out (no enzyme (Mo), methylation-sensitive enzyme (Ms), methylation-dependent enzyme (Md), and methylation-sensitive and dependent enzymes (Msd)), and a SYBR green-based RT-PCR was performed on a CFX96 RT-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using pre-designed primers that flank the loci of interest (CpG island 101327) (EPHS101327-1A)—CpG island location Chr1: 226595333–226595939 (606 bp)). The level of the promoter methylation of *PARP1* was measured and displayed as a percentage of unmethylated (F_{UM}) and methylated (F_M) fraction. To test the cutting efficiencies of the restriction enzymes, two control DNA molecules were used (one healthy and one completely methylated > 99.9%). The Ct value difference of Ms and Mo (ΔCt[Ms-Mo] > 4) as well as the difference between the Ct values of Md and Mo were greater than four (ΔCt[Md-Mo] > 4). The significance of the level of methylated DNA (% of total input DNA) was defined after parallel analysis of measuring the corresponding

expression levels of *PARP1* in experimental DNA samples and comparing them with control samples [23].

Statistical analysis

IBM SPSS statistics, version 23.0 (IBM Corporation, North Castle, NY, USA) was used for the statistical analysis of the results. The individual tests used are cited in the "Results" section, separately for each correlation.

Results

Seventy-seven (77) patients were included in the study. The baseline demographic and hematologic characteristics of the patients are shown in Table 1.

Response to 5-azacytidine

Response per the IWG criteria is presented in Table 1. The median PARP1 mRNA levels, expressed as a ratio of PARP1/beta actin transcript level, were 0.0712. Using this value as a cut-off point, we found that patients with higher PARP1 mRNA levels responded to treatment with 5-azacytidine (CR, PR, HI) at a significantly higher rate than patients with lower PARP1 mRNA levels (64.1% versus 34.2% respectively, Pearson chi-square, two-sided $p = 0.012$). This difference was more pronounced in lower (low and intermediate-1) risk patients (52.6% of patients with higher PARP1 mRNA levels were responders versus 16.7% of patients with lower PARP1 mRNA levels, Pearson chi-square, two-sided $p = 0.022$) than in higher (intermediate-2 and high) risk patients (75.0% versus 47.1% respectively, Pearson chi-square, two-sided $p = 0.081$). Moreover, the median PARP1 mRNA levels of responders were 3.3 times higher than those of non-responders (independent samples Mann-Whitney *U* test, two-sided $p = 0.006$). None of the remaining factors that were tested (MDS type, categories of IPSS, IPSS-R, and WPSS, karyotype risk per the IPSS/IPSS-R, cytopenias, bone marrow blast percentage (< 5% versus 5–10% versus 11–19%), hemoglobin levels, neutrophil and platelet count, reduction in the dose of 5-azacytidine during treatment, previous use of G-CSF or ESAs, and transfusion dependence) were correlated to response to treatment. On the other hand, PARP1 levels were not correlated to any other tested prognostic factor for MDS (IPSS, IPSS-R, and WPSS risk groups, karyotype risk per IPSS/IPSS-R, bone marrow blast percentage, hemoglobin levels, platelet and neutrophil count, number of cytopenias); thus, factors such as the risk per IPSS/IPSS-R or the cytogenetic risk were not correlated to lower or higher PARP1 mRNA levels. In multivariate analysis, PARP1 retained its statistical significance in several tested models being the only independent factor predicting response to 5-azacytidine (Table 2, models A, B, and C).

The median duration of response was higher in patients with high PARP1 mRNA levels (24.0 versus 19.3 months),

Table 1 Patient characteristics and response to treatment

Number of patients, *N* (%)	77 (100)
Median age (at diagnosis), years (range)	74.4 (51–89)
Median age (at treatment initiation), years (range)	75.2 (52–89)
Sex (male:female)	3.14
WHO 2008/2016 (at diagnosis), *N* (%)	
RA/MDS-SLD	2 (2.6)
RARS/MDS-RS-SLD	2 (2.6)
RCMD/MDS-MLD	22 (28.6)
RCMD-RS/MDS-RS-MLD	1 (1.3)
RAEB1/MDS-EB-1	25 (32.5)
RAEB2/MDS-EB-2	25 (32.5)
IPSS category, *N* (%)	
Low	11 (14.3)
Intermediate-1	26 (33.8)
Intermediate-2	25 (32.5)
High	15 (19.5)
IPSS-R category, *N* (%)	
Very low	3 (3.9)
Low	17 (22.1)
Intermediate	14 (18.2)
High	26 (33.8)
Very High	17 (22.1)
WPSS category, *N* (%)	
Very low	0 (0)
Low	3 (3.9)
Intermediate	13 (16.9)
High	46 (59.7)
Very high	15 (19.4)
Starting dose of 5-azacytidine, mg/m^2/day (range)	75 (75–75)
Median cycles of 5-azacytidine, *N* (%)	7 (2–45)
AML transformation, *N* (%)	19 (24.7)
Time to AML transformation (since diagnosis), *N* (%)	19.2 (3.8–42)
Response (IWG criteria), *N* (%)	
CR/TF	17 (22.1)
PR	12 (15.6)
HI	9 (11.7)
SD	21 (27.3)
F	18 (23.4)

WHO, World Health Organization; *RA*, refractory anemia; *MDS*, myelodysplastic syndrome; *SLD*, single lineage dysplasia; *RS*, ring sideroblast; *RCMD*, refractory cytopenia with multilineage dysplasia; *MLD*, multilineage dysplasia; *EB*, excess blasts; *IPSS*, International Prognostic Scoring System; *IPSS-R*, revised IPSS; *WPSS*, WHO Classification-Based Prognostic Scoring System; *AML*, acute myeloid leukemia; *CR*, complete remission; *TF*, treatment failure; *PR*, partial remission; *HI*, hematologic improvement; *SD*, stable disease; *F*, failure.

Table 2 Multivariate analysis

Response to 5-azacytidine	Multivariate regression analysis		
	HR	95% CI for HR	*p* value
Model A			
PARP1 mRNA level	2.555	1.229–5.310	0.012
IPSS category	1.451	0.855–2.461	0.167
Model B			
PARP1 mRNA level	2.603	1.246–5.437	0.011
IPSS-R category	1.204	0.794–1.825	0.382
Model C			
PARP1 mRNA level	2.529	1.220–5.243	0.013
Cytopenias (IPSS)	1.269	0.564–1.929	0.658
Karyotype risk (IPSS)	1.474	0.442–3.643	0.227
Bone marrow blast percentage (IPSS)	1.043	0.786–2.765	0.892
Survival after 5-azacytidine	Multivariate regression analysis		
	HR	95%CI	p value
Model D			
PARP1 mRNA level	2.001	1.038–3.865	2.001
IPSS category	0.728	0.383–1.383	0.332
Model E			
PARP1 mRNA level	1.984	1.029–3.823	0.041
IPSS-R category	1.365	0.703–2.648	0.358
Model F			
PARP1 mRNA level	1.948	0.987–3.847	0.055
Cytopenias (IPSS)	0.546	0.241–1.238	0.148
Bone marrow blast percentage (IPSS)	1.488	0.936–2.366	0.093
Karyotype risk (IPSS)	1.281	0.808–2.030	0.293

Models A, B, and C assessed response to 5-azacytidine. Model A concomitantly assessed PARP1 mRNA levels and IPSS categories. Model B concomitantly assessed PARP1 mRNA levels and IPSS-R categories. Model C concomitantly assessed PARP1 with the individual components of IPSS (bone marrow blasts—< 5%, 5–10%, 11–20%, cytogenetic risk—low, intermediate, high, and number of cytopenias—< 1 and > 1). In all tested models, PARP1 mRNA level was the single independent factor predicting response to 5-azacytidine, always with a HR above 2.5.

Models D, E, and F assessed survival after 5-azacytidine initiation. The same parameters were tested as with models A, B, and C. PARP1 mRNA levels retained their significance as a prognostic factor of survival after treatment with 5-azacytidine in the first two models, while in the third one, there was a borderline statistical significance.

HR, hazard ratio; *CI*, confidence intervals; *PARP1*, poly (ADP-ribose) polymerase 1; *IPSS*, International Prognostic Scoring System; *R*, revised

but this result was not statistically significant (log rank, Mantel-Cox, $p = 0.864$, Fig. 1a). Moreover, PARP1 levels did not differ between patients that developed AML transformation and patients that did not, during treatment with 5-azacytidine (independent samples Mann-Whitney *U* test, two-sided $p = 0.451$, Table 1).

Survival analysis

Patients with higher PARP1 mRNA levels had a median OS_T of 27.0 months, while patients with lower levels had a median OS_T of 19.7 months (Kaplan-Meier, log rank 0.033, Fig. 1c). As shown in the figure, survival of patients with higher PARP1 mRNA levels tends to plateau after a certain amount of time (i.e., more patients with survival longer than 22 months, which was the median survival for the whole group, belonged to the higher PARP1 mRNA level group). If we isolate these long-term survivors (18 patients, 12 with high and 6 with low PARP1 mRNA levels), we notice that there is a clear difference of their median OS_T. The median OS_T of patients with higher PARP1 mRNA levels was not reached, while the median OS_T of patients with lower PARP1 mRNA levels was 30.6 months (log rank 0.003, Fig. 1d). The median PARP1 mRNA levels of responders were 7.1

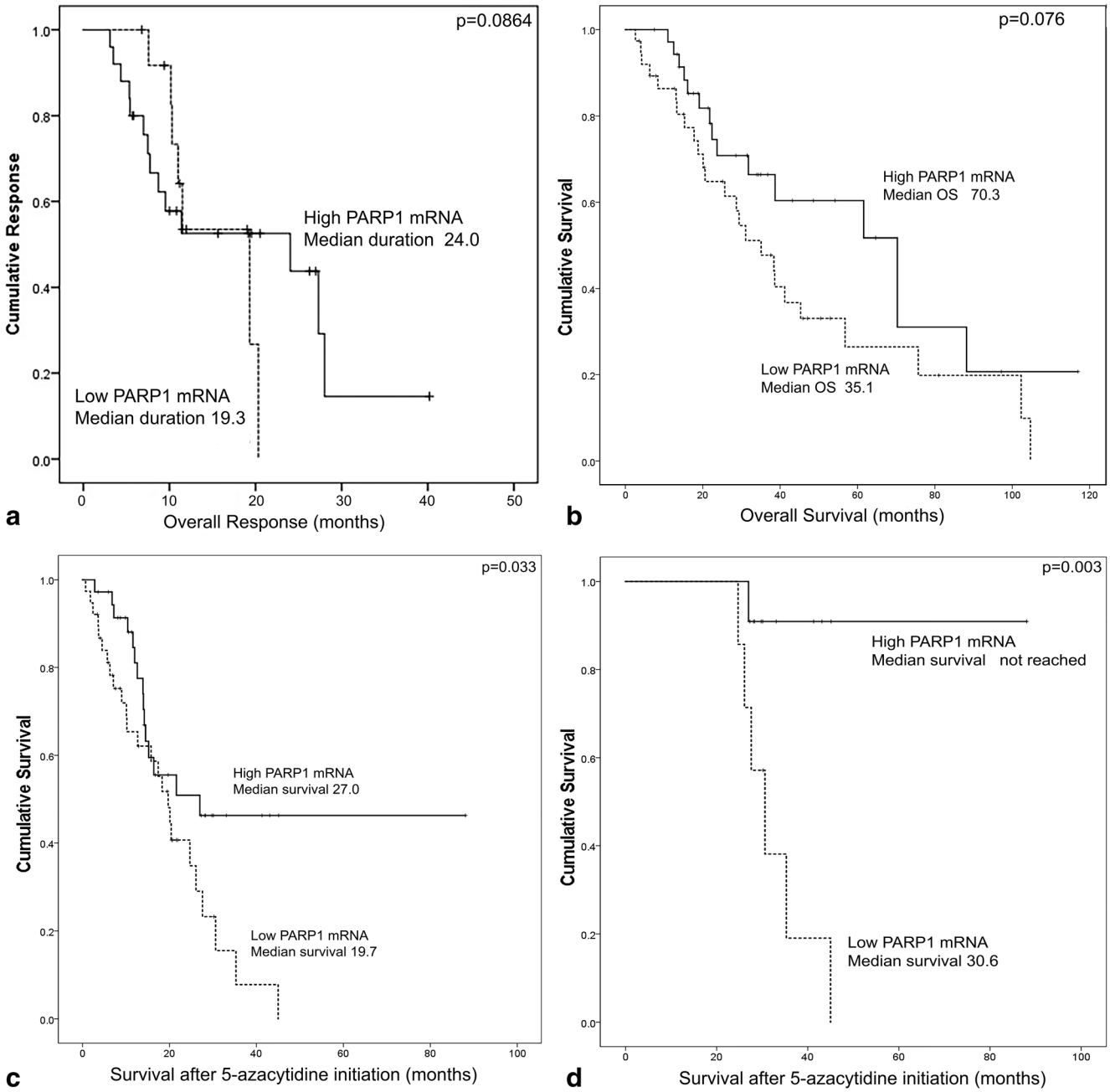

Fig. 1 Kaplan-Meier Curves for **a** duration of response to 5-azacytidine, **b** overall survival (OS), **c** survival after 5-azacytidine initiation, and **d** survival after 5-azacytidine initiation of long-term survivors

times higher than those of non-responders in this subgroup (independent samples Mann-Whitney *U* Test, two-sided $p = 0.045$). Interestingly, 6 (50%) of the long-term survivors with high PARP1 mRNA levels achieved a CR versus none (0%) of those with low PARP1 mRNA levels.

Cox regression analysis for OS_T revealed that PARP1 mRNA levels retained their significance as an independent prognostic factor for survival in two of the three tested models (Table 2, models D, E, and F). No other factor of those tested (IPSS, IPSS-R, components of IPSS, i.e., karyotype risk, bone marrow blast percentage, cytopenias) was found to have prognostic significance for OS_T.

NGS results

NGS was performed in 16 randomly selected patients and detected mutations of the following genes: *IDH2* in 3/16 (18.8%) patients, *SRSF2* in 3/16 (18.8%), *DNMT3A* in 2/16 (12.5%), *SF3B1* in 2/16 (12.5%), *ASXL1* in 1/16 (6.3%), *RUNX1* in 4/16 (25.0%), *TET2* in 2/16 (12.5%), *U2AF1* in

1/16 (6.3%), and *TP53* in 1/16 (6.3%) patients. As a whole, 11/16 (68.8%) patients carried one or more mutations.

No correlation was found between PARP1 mRNA levels and any of the detected mutations; nevertheless, the sample was small to extract conclusions for individual mutations. PARP1 mRNA levels did not differ between patients with and without splicing mutations (6/16 (37.5%) carried mutations of *SRSF2*, *SF3B1*, or *U2AF1*). Moreover, among patients with epigenetic mutations (8/16 (50.0%) carried mutations of *TET2*, DNMT3A, *ASXL1*, or *IDH2*), 6/8 (75.0%) patients had low PARP1 mRNA levels, but this result was not statistically significant (Fisher's exact test, two-sided $p = 0.608$). Response to 5-azacytidine was not correlated to the presence of mutations. Finally, there was no survival disadvantage attributable to the presence of splicing or epigenetic mutations, but again it should be pointed out that the sample was too small to extract solid conclusions.

PARP1 promoter methylation results

The methylation status (F_M and F_{UM}) of PARP1 promoter was quantified in 18 (23.4%) randomly selected patients (16 of these samples were also tested with NGS). The F_M was found to be > 60% in 4/18 (22.2%) patients, while the F_M of the remaining 14/18 (77.8%) was < 20%.

A Spearman's rank-order correlation was run to determine the relationship between PARP1 mRNA levels and the percentage of the unmethylated fraction of PARP1 promoter (F_{UM}). There was a weak non-statistically significant positive correlation between the levels of the tested parameters ($r_s = 0.239$, $p = 0.101$). The methylation status was not correlated to the response to 5-azacytidine, to its duration, and to OS or OS_T. The methylation status was not correlated to the mutation status of the patients (Suppl. Table 1).

Discussion

HMAs have been the mainstay of treatment for patients with higher risk MDS for the last few years. Response to 5-azacytidine and decitabine is achieved in up to 40% of patients, but most of them ultimately experience loss of response. Predicting response to HMAs in patients with MDS has been proven a difficult task, hence the lack of a widely accepted biomarker or other clinical/hematologic marker of response that would offer a valid pretreatment estimation of the anticipated benefit from the use of HMAs.

In the last few years, several studies have tried to address the problem of response prediction, and several factors have been proposed to serve as predicting factors of response to HMAs. Thus, lower uridine-cytidine kinase-1 (UCK1) expression has been correlated to lack of response to 5-azacytidine [24], ten-eleven translocation 2 (*TET2*) mutations have been correlated to better treatment response in two studies [25, 26] while in another study, both TET2 and DNMT3A were found to be independent predictors of response [27]. It has been recently shown that the pretreatment signal transducer and activator of transcription 3 and 5 (STAT3/5) signaling profiles in CD34+ cells correlated with response to 5-azacytidine and independently predicted event-free survival [28]. In the same study, *TET2* mutations were not found to be predictive of response. Moreover, previous low-dose cytosine arabinoside treatment, bone marrow blasts > 15%, and an abnormal karyotype had been also found predictive of lower response rates in patients treated with 5-azacytidine [29]. Another study showed that 5-azacytidine improves the outcome of higher risk patients with MDS and chromosome 7 abnormalities, although no clear differences were found in the response rate of patients with and without chromosome 7 abnormalities [30]. The presence of myeloid progenitors with an aberrant immunophenotype at baseline has been associated with lack of response to 5-azacytidine [31]. Finally, TP53 mutations have been correlated to better response to decitiabine in a population of patients with MDS and AML [32]. These studies are listed in Suppl. Table 2, along with comments about the populations studied (in most cases the authors used mixed populations with MDS, AML, and MDS/MPN), the HMA used (5-azacytidine, decitabine, or both), and the pretreatment status of the patients (pretreated with chemotherapy or not). In the same table, we list the corresponding features of the present study, highlighting its main advantages, being the homogeneous population consisting only of patients with MDS not previously treated with cytotoxic agents and homogeneously treated with 5-azacytidine monotherapy. Nevertheless, the main limitations of the present study are its retrospective nature and the fact that other possible prognostic factors such as *TET2* and *TP53* mutations were analyzed only in a small fraction of the studied population. In fact, no solid conclusions could be drawn from the statistical analysis of the NGS results due to the small sample size.

The accumulation of data about the identification of a possible biomarker of response to HMAs has not yet proven fruitful enough, and no widely accepted biomarker exists to date. Our study proposes a new biomarker with a high level of statistical significance and a plausible rationale. Indeed, our results imply that patients with MDS with high baseline levels of PARP1 mRNA respond better to treatment with 5-azacytidine and this response is translated into a survival benefit.

It has been previously shown by our team that PARP1 is an adverse prognostic factor for OS in patients with lower risk MDS [15], a group of patients usually not treated with 5-

azacytidine. In the present study, high PARP1 mRNA levels were correlated to a better response to treatment and this correlation was translated into a survival benefit. More specifically, PARP1 mRNA levels were clearly correlated to OS_T, with higher levels being correlated to higher survival rates. Moreover, long-term survivors were more likely to have high PARP1 mRNA levels, and 50% of long-term survivors with high PARP1 mRNA levels achieved a CR versus none (0%) of those with low PARP1 mRNA levels. The rationale behind these impressive correlations is probably that upregulation of PARP1 has been found to be correlated with DNA hypermethylation [16]; thus, HMAs are possibly more effective in environments with higher PARP1 and, therefore, higher methylation levels. Nevertheless, it seems that there is no standard pathway involving PARP1 and DNMTs since there are reports implying that PARP1 interacts with DNMT1, preventing its enzymatic activity [33, 34]; however, more data is needed. Another possible explanation could be that high PARP1 level is an indicator of increased endogenous DNA damage inducing PARP1 expression and probably cells with higher PARP1 levels are more susceptible to the azacytidine effect. The results of the assay on the methylation status of *PARP1* promoter in the tested subgroup of the cohort imply that lower methylation levels of the promoter are (not statistically significant) correlated with higher PARP1 mRNA levels, but the sample size was not large enough to further support this first result.

Multivariate analysis confirmed that PARP1 mRNA levels are the sole factor predicting response to 5-azacytidine. These results clearly indicate that PARP1 is a key factor in determining response and may serve as a biomarker that can be easily measured by a qRT-PCR in bone marrow samples of patients at diagnosis or before treatment initiation.

The fact that PARP1 is at the same time an adverse prognostic factor for OS in lower risk MDS, as well as a factor that determines response to HMAs is highly interesting. This fact makes PARP1 a potentially valuable treatment target since patients with higher PARP1 levels seem to have worse prognosis when not treated with 5-azacytidine, making those patients a possible target group for treatment with PARP1 inhibitors. Thus, our results about the prognostic value of PARP1 in MDS can set the basis for clinical trials evaluating the use of PARP1 inhibitors in patients with MDS, possibly in combination with HMAs. After all, synergistic lethality of 5-azacytidine with olaparib has been already proposed by in vitro studies in AML cell lines [13], but again, this result is not supported by other studies, where the simultaneous addition of a HMA to cell lines treated with PARP inhibitors did not add to the result [14].

The role of PARP1 in several solid tumors has been well studied for over 25 years, and PARP1 inhibitors have been approved as antitumor agents in malignancies such as metastatic breast cancer [35, 36] and advanced ovarian cancer [37–40], while more phase 2 and 3 studies on breast, ovarian, colorectal, non-small-cell lung cancer, and melanoma are on the way. Mutations of several tumor suppressor genes, mainly *BRCA1* and *BRCA2*, lead to deficits in DNA repair that can eventually lead to malignant transformation. PARP1 inhibitors in *BRCA1*- and *BRCA2*-mutated tumors prevent the efficient repair of DNA breaks eventually leading to death of the tumor cells. Thus, they can be used as monotherapy in *BRCA1/2*-positive tumors, or in combination with agents that act by damaging DNA, such as cytotoxic chemotherapy and radiotherapy, irrespective of the *BRCA* status of the tumor [41]. Cells with lower replication potential, such as normal cells, evade the effect of PARP1 inhibition and selectively survive [42]. PARP1 inhibitors are also tested in patients with relapsed or refractory non-Hodgkin lymphomas in clinical trials, [43] while there are no studies on MDS or AML so far.

Our study is the first to show a correlation between PARP1 and response to HMAs in clinical samples of patients with MDS, a fact that renders PARP1 a useful predictor of response to HMAs and survival of the patients. PARP1 mRNA may be easily quantified by a qRT-PCR, thus offering a simple and cost-effective tool for predicting response to HMAs. Nevertheless, further validation of our results is warranted, so that PARP1 may take its place as a guide to therapeutic decisions. Bearing that in mind, we recommend that PARP1 mRNA levels should be measured in the bone marrow of all patients that are due to be treated with HMAs. The results could guide the clinician's decision concerning the aggressiveness of the approach. Since there are no approved combinations of HMAs with other agents, patients with lower PARP1 mRNA levels could be good candidates for clinical trials evaluating the performance of such combinations.

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Compliance with ethical standards

Informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Ethics Committee.

Conflict of interest Argiris Symeonidis, Alexandra Kourakli, and Nora-Athina Viniou report investigational grants and personal fees for presentations and advisory roles from Celgene/Genesis Pharma. Ioannis Kotsianidis, Vassiliki Pappa, Athanasios Galanopoulos, Theodoros Vassilakopoulos, Maria Angelopoulou, Sotirios Papageorgiou, and Panayiotis Panayiotidis report personal fees for honoraria and advisory roles from Celgene/Genesis Pharma. Maria Dimou reports personal fees for advisory roles from Celgene/Genesis Pharma. The remaining authors declare that they have no conflict of interest.

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