

Can peripheral blood provide good DNA methylation biomarkers in myelodysplastic syndrome?

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Myelodysplastic syndromes (MDS) are a group of hematological stem cell malignancies strongly associated with aberrant epigenetic anomalies, namely DNA methylation. Blood-based specimens may be a potential source of noninvasive DNA methylation cancer biomarkers. Systemic methylation profile has been explored in solid tumors but is still largely unknown in hematological cancers. We compared DNA methylation status in bone marrow (BM) aspirates and peripheral blood (PB) in MDS patients at diagnosis. Using MSPCR, we compared DNA methylation status of nine tumor suppressor genes (TSG) *P15*, *P16*, *TP53*, *DAPK*, *MGMT*, and TRAIL receptors (*TRAIL-DcR1*, *-DcR2*, *-DR4*, and *-DR5*) genes. Statistical analysis was performed using the chi-square test, and Kappa statistics analyzed the concordance between BM and PB methylation. Overall survival was assessed by the Kaplan-Meier method. In MDS patients, we observed a high methylation status of the analyzed genes, mainly *P15* (64.7%) and *DAPK* (60.3%). Moreover, 75% of MDS patients presented more than two hypermethylated genes, and these patients had a significantly lower overall survival. We observed a good correlation between gene methylation patterns in PB and BM samples, mainly for *P15* (70.6% concordance; kappa = 0.344) and *TRAIL-DcR1* (75% concordance; kappa = 0.243). No patient presented *TP53* and *MGMT* methylated genes. Our results suggest that DNA methylation patterns measured in PB may have great potential as informative biomarkers of MDS-related tumor suppressor genes methylation.

Keywords

Myelodysplastic syndrome; DNA methylation; Peripheral blood; Bone marrow; Peripheral biomarkers

1. Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal hematopoietic stem-cell disorders mainly characterized by significant morphologic dysplasia, ineffective hematopoiesis, and peripheral blood cytopenias. MDS patients also have an increased probability of leukemic transformation [1–4]. Several mechanisms have been recognized to be involved in MDS development, including recurrent cytogenetic abnormalities, genetic mutations, and aberrant epigenetic modifications, which are also variable throughout the different MDS subtypes. Epigenetic modifications are very common in cancer and play a central role in MDS pathogenesis [5–7]. This process is a significant gene transcription regulator that occurs mainly in the promoter region of genes, particularly in tumor suppressor genes (TSG), leading to the silencing and inactivation of numerous TSG genes [8].

DNA methylation appears to have great potential as a nucleic acid-based biomarker for assessing cancer risk and early detection, prognosis, targeted therapy, and therapeutic responses [9–11]. There are several advantages to using hypermethylated genes as biomarkers: hypermethylation is a mechanism characteristic of neoplastic cells, different tumors show different methylation profiles with different clinical impact, and it can be measured by quick and sensitive techniques. Another advantage is the high stability of the DNA molecule that can be obtained from a wide variety of sources [12], such as peripheral blood where its recovery is highly efficient.

The peripheral blood has a rich content of several cellular and molecular elements that provide different information relative to health and disease status, making it an ideal sample source to develop noninvasive biomarkers for cancer detection and monitoring [13]. DNA methylation biomarkers from whole blood and blood leucocytes have been identified in patients with different solid tumors, such as breast, gastric, and colorectal cancer [9, 14–16]. However, despite the important role of DNA methylation in MDS pathogenesis and other hematopoietic cancers, methylation profiles of peripheral blood cells still largely unknown.

In the present study, we analyzed the DNA methylation status in peripheral blood (PB) and in the corresponding bone marrow (BM) samples from MDS patients in order to explore the existence of a correlation between these two samples. This analysis will provide the relevance of PB samples for DNA methylation detection in MDS patients.

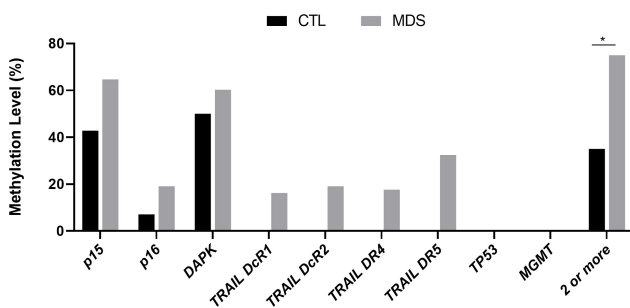


Fig. 1. Gene methylation status in bone marrow aspirates from controls (CTL) and myelodysplastic syndrome (MDS) patients presented as the percentage of patients and CTLs that show methylation in the nine analyzed genes. Differences between groups are evaluated by Pearson's chi-square analysis. * $P < 0.05$.

2. Material and methods

2.1 Ethical statement

The procedures performed in the present study were approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Portugal. Samples were collected after informed consent was obtained from the involved patients in accordance with the Helsinki Declaration.

2.2 Patients and samples

Peripheral blood samples and bone marrow aspirates from 68 MDS patients were collected at diagnosis between 2010 and 2015, in the Clinical Hematology Department of the Centro Hospitalar e Universitário de Coimbra (CHUC) and in the Medicine Service of the Hospital Distrital da Figueira da Foz (HDFF), and bone marrow aspirates were collected from 14 non-neoplastic controls referred to BM aspiration due to non-neoplastic diseases (e.g., immune thrombocytopenia). Patient diagnosis was performed through peripheral blood and bone marrow findings, and conventional cytogenetic analysis, according to the 2008 classification system of the World Health Organization.

Table 1. Demographic and clinical patients characteristics.

Patient Characteristics	MDS (n = 68)
Age (median, range)	74 (22-89)
Gender (Male/Female)	29/39
Blood routine (median, range)	
Hemoglobin (g/L)	10.6 (6.4-16.0)
White blood cell count ($10^9/L$)	3.6 (1.3-13.0)
Platelet count ($10^9/L$)	91.5 (10.0-317.0)
Absolute neutrophil count ($10^9/L$)	1.7 (0.1-9.4)
Blast (%)	1.0 (0.0-11.0)
WHO classification (n, %)	
RA	2 (2.9)
RN	3 (4.4)
RT	1 (1.5)
RARS	5 (7.4)
RCMD	40 (58.8)
RAEB-1	4 (5.9)
RAEB-2	2 (2.9)
5q-	2 (2.9)
CMML	9 (13.2)
IPSS (n, %)	
Low	30 (44.1)
Intermediate-1	28 (41.2)
Intermediate-2	5 (7.4)
High	1 (1.5)
N/A	4 (5.9)
Cytogenetic risk group (n, %)	
Normal	53 (77.9)
Intermediate	7 (10.3)
Poor	4 (5.9)
N/A	4 (5.9)

5q-, Myelodysplastic Syndrome associated with isolated del(5q); CMML, Chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; n.a., not available; RA, Refractory Anemia; RAEB-1, Refractory Anemia with Excess Blasts-1; RAEB-2, Refractory Anemia with Excess Blasts-2; RARS, Refractory Anemia with Ringed Sideroblasts; RCMD, Refractory Cytopenia with Multilineage Dysplasia; RN, Refractory Neutropenia; RT, Refractory Thrombocytopenia; WHO, World Health Organization 2008.

2.3 DNA methylation analysis

DNA methylation status of the tumor suppressor genes *P15*, *P16*, *TP53*, *DAPK* (Death-Associated Protein Kinase), and *MGMT* (*O*⁶-methylguanine-DNA methyltransferase), as well as of *TRAIL* (TNF-Related Apoptotic Inducing Ligand) receptor genes *TRAIL-DcR1* (-Decoy Receptor 1), *-DcR2* (-Decoy Receptor 2), *-DR4* (-Death Receptor 4), and *-DR5* (-Death Receptor 5) was evaluated in the peripheral blood and bone marrow aspirates. Genomic DNA was isolated from the samples according to Bartlett & White protocol [17]. Methylation analysis was performed using Methylation-Specific Polymerase Chain Reaction (MS-PCR) method [18]. Genomic DNA was treated with sodium bisulfite, using *EZ DNA Methylation-GoldTM* kit (Zymo Research, CA, USA) and amplified by PCR using *Supreme NZYtaq DNA polymerase* (NZYtech, Lisbon, PT) according to manufacturer instruc-

Table 2. Level of agreement of DNA methylation status between peripheral blood and corresponding bone marrow samples in Myelodysplastic Syndrome patients using Methylation Specific-Polymerase Chain Reaction.
Kappa statistic was used to evaluate the level of agreement.

	Concordance (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa Value	Agreement
<i>P15</i>	70.6	79.5	54.2	76.1	59.1	0.344	Fair
<i>P16</i>	69.1	38.5	76.4	27.8	84.0	0.129	Slight
<i>DAPK</i>	57.4	56.1	59.3	67.6	47.1	0.147	Slight
<i>TRAIL DcR1</i>	75.0	45.5	82.5	33.3	88.7	0.243	Fair
<i>TRAIL DcR2</i>	69.1	7.7	83.6	10.0	79.3	> 0.000	Poor
<i>TRAIL DR4</i>	73.5	8.3	87.5	12.5	81.7	> 0.000	Poor
<i>TRAIL DR5</i>	51.5	13.6	69.5	17.6	62.7	> 0.000	Poor

NPV, Negative Predictive Value; PPV, Positive Predictive Value.

tions, with previously reported primers [18–22]. PCR products were then analyzed on a 4% agarose electrophoresis gel.

2.4 Statistical analysis

Statistical analyses were performed using the software SPSS Windows (*Statistical Package for Social Sciences*) version 22.0 (SPSS Inc., Chicago, IL, USA). Numerical and categorical data were analyzed for statistical significance using *Kruskal-Wallis* and Pearson's chi-square analysis, respectively. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also determined. Kappa statistic was used to evaluate the degree of agreement between peripheral blood and bone marrow methylation results. Agreement is classified as *almost perfect* ($0.99 > \kappa > 0.81$), *substantial* ($0.8 > \kappa > 0.61$), *moderate* ($0.6 > \kappa > 0.41$), *fair* ($0.4 > \kappa > 0.21$), *slight* ($0.2 > \kappa > 0.01$) and *poor* ($\kappa < 0.01$). Kaplan-Meier analysis was used to assess the correlation between methylation and patient survival. A *P*-value under 0.05 was considered to indicate a statistically significant result.

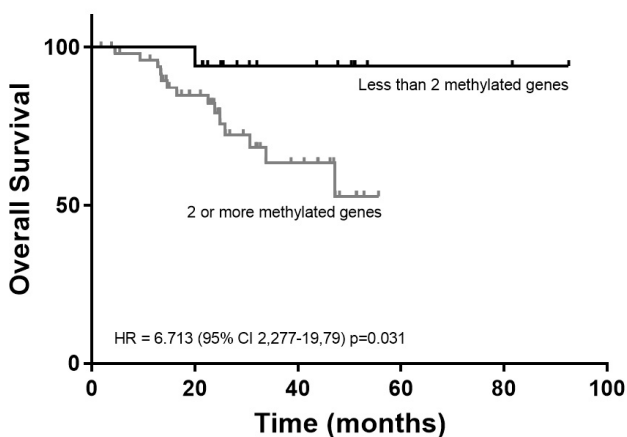


Fig. 2. Survival analysis according to DNA methylation in myelodysplastic syndrome (MDS) patients. Kaplan-Meier method was performed to evaluate overall survival between patients with less than two methylated genes and patients with two or more methylated genes.

3. Results

3.1 Patients characterization

A total of 68 MDS patients, 29 (43.0%) males and 39 (57.0%) females, were analyzed. All demographic and clinical characteristics of MDS patients included are shown in Table 1. The median age was 74 years, ranging between 22 to 89 years, wherein about 90% ($n = 61$) of the patients were more than 60 years old. Patients were classified according to World Health Organization (WHO) classification system: 58.8% ($n = 40$) were diagnosed with refractory cytopenia with multilineage dysplasia subtype (RCMD); 2.9% ($n = 2$) with refractory anemia (RA); 4.4% ($n = 3$) with refractory neutropenia (RN); 1.5% ($n = 1$) with refractory thrombocytopenia (RT); 7.4% ($n = 5$) with refractory anemia with ringed sideroblasts (RARS); 5.9% ($n = 4$) with refractory anemia with excess blasts-1 (RAEB-1); 2.9% ($n = 2$) with refractory anemia with excess blasts-2 (RAEB-2); 2.9% ($n = 2$) with MDS associated with isolated del(5q), and 13.2% ($n = 9$) with chronic myelomonocytic leukemia (CMML) subtype. Patients were also distributed in accordance to the International Prognostic Scoring System (IPSS): 44.1% ($n = 30$) were included in the low risk group, 41.2% ($n = 28$) in the intermediate-1 group, 7.4% ($n = 5$) in the intermediate-2, and 1.5% ($n = 1$) in the high risk group. In 5.9% ($n = 4$) of cases, the clinical information needed to assess risk was not available. The control group included 14 non-neoplastic individuals, from which 11 (78.6%) males and 3 (21.4%) females with a median age of 71 years, ranging from 34 to 86 years.

3.2 Methylation profile in myelodysplastic syndrome

Gene-specific DNA methylation was assessed in bone marrow aspirates from 68 MDS patients and 14 non-neoplastic controls (Fig. 1). Methylation of tumor suppressor genes *P15*, *P16*, and *DAPK* was detected in both control group (CTL) and MDS patients. However, the methylation frequency was higher in the patient group: *P15* methylation was found in 64.7% (44/68) of MDS patients and 42.8% (6/14) of CTL, *P16* in 19.1% of MDS patients, and 7.1% (1/14) of CTL and *DAPK* methylation was detected in 60.3% (41/68) of MDS patients and (50%) of CTL (3/6). In the group of MDS patients, the methylation frequency of TRAIL receptor genes was 16.2% (11/68) for *TRAIL DcR1*, 19.1% (13/68) for *TRAIL DcR2*, 17.6% (12/68) for *TRAIL DR4*, and 32.4% (22/68) for

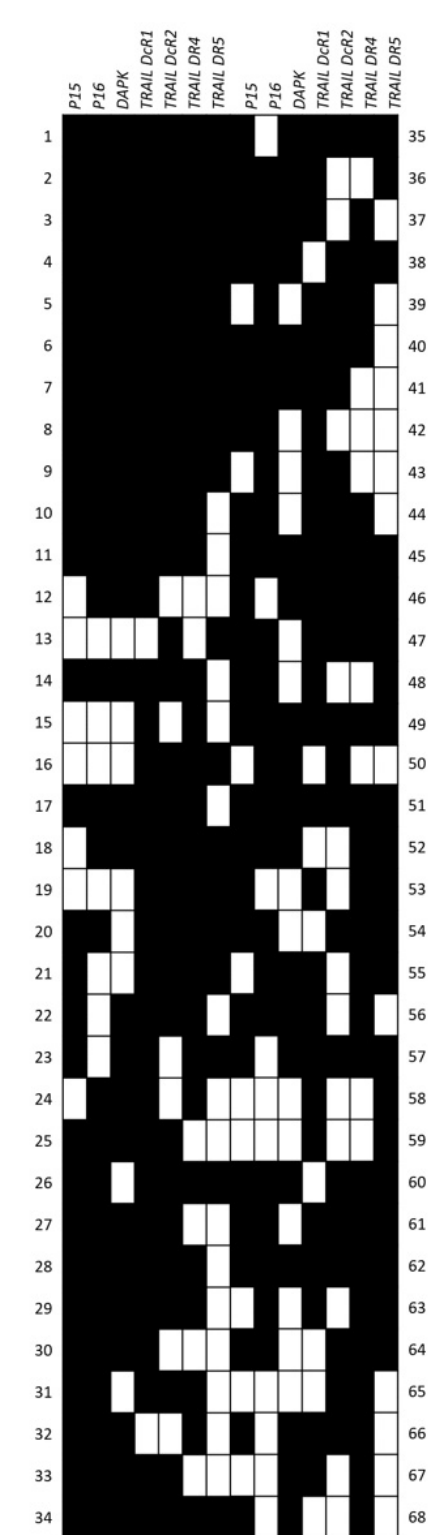


Fig. 3. Global concordance analysis between peripheral blood (PB) and bone marrow (BM) in the Myelodysplastic Syndrome (MDS) patients for each analyzed gene. Filled square-concordant samples; Empty square-discrepant samples.

TRAIL DR5. In the control group, no methylation was observed in genes encoding for TRAIL receptors. None of the samples analyzed presented methylation in *TP53* and *MGMT*

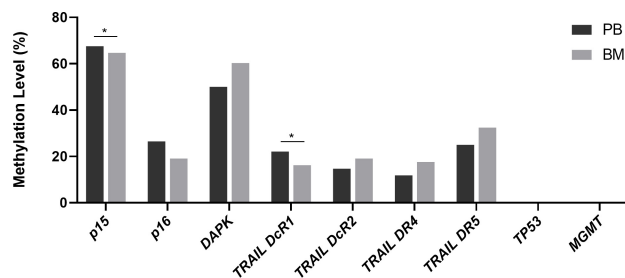


Fig. 4. Methylation status of the analyzed genes in peripheral blood (PB) and in the corresponding bone marrow (BM) samples from myelodysplastic syndrome (MDS) patients. Differences between the two groups were analyzed by Pearson's chi-square analysis. * $P < 0.05$.

genes. About 75% of MDS patients and 35% of CTL presented promotor methylation of at least two of the nine analyzed genes ($P = 0.004$). According to our results, promotor methylation of the analyzed genes did not influence MDS patients' overall survival (Fig. 2). However, MDS patients with two or more methylated genes presented a significantly lower overall survival than patients with less than two methylated genes ($P = 0.031$).

3.3 Methylation profile in peripheral blood and bone marrow samples

DNA methylation status of all genes was also assessed in peripheral blood of MDS patients. For tumor suppressor genes, the methylation of *P15* gene was found in 67.6% (46/68) of PB samples. *P16* gene methylation was observed in 26.5% (18/68), and *DAPK* gene methylation was observed in 50.0% (34/68) of peripheral blood samples. For the genes encoding TRAIL receptors, the methylation status in peripheral blood samples was 22.0% (15/68), 14.7% (10/68), 11.8% (8/68), and 26.5% (18/68) for *TRAIL DcR1*, *TRAIL DcR2*, *TRAIL DR4*, and *TRAIL DR5*, respectively. None of the patients presented *TP53* or *MGMT* promotor methylation in peripheral blood samples. A global view of BM and PB methylation for each patient is showed in Fig. 3.

3.4 Comparison of methylation status between peripheral blood and bone marrow samples

The comparison of the methylation status between bone marrow and peripheral blood samples was first performed by evaluating the methylation levels (Fig. 4). *P15* ($P = 0.005$) and *TRAIL DcR1* ($P = 0.041$) presented a significant similarity between both samples.

Concordance of the methylation status for each gene was then evaluated using the bone marrow methylation results as a reference standard against the peripheral blood results. As observed in Table 2, a global analysis revealed a concordance higher than 50% in all studied genes; specifically, *P15* gene presented a fair agreement between the two analyzed samples (70.6%, kappa = 0.344) with a PPV and NPV of 76.1% and 59.1%, respectively. Moreover, both *P16* and *DAPK* genes presented a slight agreement between BM and PB methylation results, with 69.1% and 57.4% of concordant samples, respectively (*P16*: kappa = 0.129; *DAPK*: kappa = 0.147). *P16*

gene methylation showed a sensitivity of 38.5%, a specificity of 76.4%, a PPV of 23.5%, and an NPV of 84%. *DAPK* methylation revealed a sensitivity of 56.1%, specificity of 59.3%, a PPV of 67.6%, and NPV of 47.1%.

Finally, *TRAIL DcR1* presented the most significant result with a fair agreement in methylation results (75%, kappa = 0.243), a sensitivity of 45.5%, a specificity of 82.5%, PPV of 33.3%, and NPV of 88.7%. The other three TRAIL receptors studied presented a poor agreement between samples with concordant results of 69.1% for *TRAIL DcR2* (kappa > 0), 73.5% for *TRAIL DR4* (kappa > 0) and 51.5% for *TRAIL DR5* (kappa > 0). These TRAIL receptor genes presented low sensitivity and PPV, as well as high specificity and NPV values.

4. Discussion

Noninvasive molecular tests for biomarker detection have become one of the most important issues in everyday clinical practice, not just for detecting and monitoring cancer but also for the diagnosis and follow-up of several other diseases. Peripheral blood samples and other body fluids seem to have great potential, as they can be obtained by noninvasive procedures with almost no harm to the patients. Utilizing these tests will allow the clinicians to perform a tighter follow-up with the patients since they can apply these methods more frequently along the disease progression. Several studies have already addressed the potential use of peripheral blood markers in myelodysplastic syndrome patients using different approaches. For instance, *WT1* mRNA expression levels in PB leucocytes have been shown to be potential markers for MDS diagnosis and risk evaluation [23, 24] and for the prediction of early relapse [25]. Fakhr *et al.* demonstrated that FISH analysis in PB for common MDS cytogenetic abnormalities could be useful for clinical monitoring of these patients [26]. Other groups have demonstrated that flow cytometry in PB testing might also be useful for diagnosing low-risk MDS patients based on neutrophil and monocyte immunophenotyping [27] and could also replace BM aspiration in some suspected cases of MDS [28].

Aberrant DNA methylation has been reported in many types of cancers and has been implicated in MDS pathogenesis, where disruption of several genes is known to affect different cellular processes [6, 29–31]. DNA methylation analysis is performed mainly in bone marrow samples, but in pathologies like MDS, methylation analysis in peripheral blood might give clinicians a large amount different information while avoiding invasive medullary aspirates.

The present results revealed that DNA methylation is a common process in MDS patients, with more than 75% of MDS patients presenting more than two hypermethylated genes. *P15* and *DAPK* presented a higher methylation status among our MDS group. *P15* gene is one of the most studied MDS genes, as its hypermethylation is present in 50 to 80% of MDS cases [32–35]. The inactivation of this cell cycle regulator plays a crucial role in MDS pathogenesis, lead-

ing to inappropriate progression of the cell cycle which induces the growth advantage of neoplastic cells that can result in leukemic transformation [35, 36]. *DAPK* promoter inactivation leads to apoptosis inactivation and a higher metastatic capacity in solid tumors, and is associated with a poor prognosis, for instance, in lung cancer [37, 38]. In hematological cancers such as monoclonal gammopathies, it was observed that *DAPK* methylation might be a marker of disease progression with a negative impact on disease prognosis [39]. A more recent study in lymphoma patients also demonstrated that *DAPK* promoter methylation might serve as a negative prognostic biomarker even though it is not associated with lymphoma progression [40]. Despite the impact of DNA methylation in several neoplasias, it is important to point out that there is also a link between DNA methylation and aging, showing the establishment of global hypomethylation and regions of CpG-island methylation with age [41]. The presence of comorbidities in some older patients and controls, such as type 2 diabetes, can also influence DNA methylation profile [42]. In our study, both analyzed groups are age-matched; however, information about the presence of comorbidities in all individuals in both patient and control groups were not available and might explain some differences between our results and other studies.

We also evaluated the correlation of DNA methylation in BM and PB samples of MDS patients. We observed the higher correlation rates in *P15* and *TRAIL DcR1* gene methylation. *TRAIL DcR1* gene is an anti-apoptotic TRAIL receptor similar to *TRAIL DcR2*; neither have the death domain, which makes them unable to transduce the death signal. For this reason, they are considered oncogenes [43, 44]. The methylation levels of *TRAIL DcR1* gene are not yet described in MDS, as well as the comparison between BM and PB samples. However, Shivapurkar (2004) demonstrated the hypermethylation of this TRAIL receptor in several solid neoplasias, as well as in lymphomas and multiple myelomas [44].

For *P15* and *TRAIL DcR1*, the level of agreement between samples was fair according to the kappa statistics (*P15*: k = 0.344; *TRAIL DcR1*: k = 0.243), and the concordance between the peripheral blood and bone marrow were also considerably high for these two genes (*P15*: 70.6%; *TRAIL DcR1*: 75.0%). Although this type of correlation had never been done in MDS patients, there are a few studies involving a simple comparison of *P15* gene promoter methylation between these different types of samples. One of these studies demonstrated that all patients with *P15* methylation in BM (n = 4) also presented the same result in PB samples [45]. Moreover, an analysis performed in our investigation group, using samples from monoclonal gammopathy patients, reported a strong correlation between BM and PB samples when evaluating the methylation status of *P15* and *DAPK* genes [46]. Even though we observed a good level of agreement between the two analyzed samples, a higher agreement would be expected. The agreement level obtained might result from the small sampling studied or even due to false-positive and/or

false-negative results that are a direct consequence of using a very sensitive detection method such as the MS-PCR [47]. *P15* also presented the highest sensitivity (79.5%), and *TRAIL DcR1* showed high specificity (82.5%). Although both of these parameters should present higher rates, these results might be explained by the number of methylated samples for each gene promoter, with *P15* having a high percentage of methylated samples (positive results) and *TRAIL DcR1* a high percentage of unmethylated samples (negative results). This fact is also supported by PPV and NPV values.

The other genes did not present any significant results when we compared their methylation status, though the level of methylated samples was relatively high in some of those genes, like *DAPK*. This outcome may result from the number of patients enrolled in this study. It is also important to note that none of the patients presented promoter methylation for *TP53* and *MGMT* genes. The lack of *TP53* promoter methylation was expected as its inactivation is usually due to genetic mutations [48] and it is more frequently associated with del(5q) cases [49, 50].

In conclusion, our study showed that promoter methylation is a common event in MDS patients, in both peripheral blood and bone marrow samples. *P15* and *TRAIL DcR1* methylation status might be used as potential cancer biomarkers in peripheral blood samples from MDS patients, mainly as part of a biomarkers panel. DNA methylation patterns measured in peripheral blood might have great potential as informative biomarkers of cancer risk and prognosis; however, large systematic and prospective studies must be performed, and other genes should be evaluated in single or combined approaches.

Author contributions

JJ and ABSR conceived of the study. EC, CG, AP, and LR recruited and provided the clinical information of the participants. JJ, ACP, and RA conducted the experiments. JJ and ACG performed the statistical analysis. RA, ACG, and JJ wrote the paper. JNNC and ABSR revised the manuscript. All authors read the final version and approved its submission.

Ethics approval and consent to participate

The procedures performed in the present study were approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra, Portugal. Samples were collected after informed consent was obtained from the involved patients in accordance with the Helsinki Declaration.

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Conflict of interest

The authors declare no competing interests.

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