Annals of Hematology (2019) 98:2719–2727
https://doi.org/10.1007/s00277-019-03775-y

ORIGINAL ARTICLE

Residual methylation of tumor suppressor gene promoters, RASSF6 and RASSF10, as novel biomarkers for minimal residual disease detection in adult acute lymphoblastic leukemia

Samareh Younesian[1,2] · Sepideh Shahkarami[1,3] · Parisa Ghaffari[1] · Shaban Alizadeh[4] · Roya Mehrasa[1] · Seyed H. Ghaffari[1]

Received: 1 February 2019 / Accepted: 4 August 2019 / Published online: 5 September 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Aberrant promoter methylation of RASSF6 and RASSF10 occurs at a high frequency in acute lymphoblastic leukemia (ALL). Because of the complexity of the current minimal residual disease (MRD) detecting-methods, the DNA methylation status of the RASSF6 and RASSF10 genes could potentially become biomarkers for the assessment of MRD levels in ALL patients. The promoter methylation status of RASSF6 and RASSF10 was assessed by using methylation-specific PCR (MSP) in the DNA isolated from 280 peripheral blood (PB) samples taken at the time of diagnosis, day 14, 28, and from the subsequent 30-month follow-ups of 45 adult ALL patients. The relative methylation level obtained during the follow-ups by MSP was compared to the MRD results obtained by the amplification of IG/TCR clonal rearrangements using the allele-specific quantitative-PCR (ASO-PCR) assay. Frequently, RASSF6 was methylated in B-ALL, and RASSF10 was methylated in T-ALL. The applicability and accuracy of the assays were increased when these markers were combined (91.1% and 93.8%, respectively). When a cutoff was defined for the PCR-MRD level, results of the 30 months of MRD detection showed a significant correlation between the PCR and MSP techniques ($r = 0.848$; $p < 0.001$). Due to the high applicability, the non-invasiveness, and promising prospect of longitudinal assessment, the DNA methylation status of the RASSF6 and RASSF10 genes could be potential biomarkers for the assessment of residual disease in PB of patients with ALL.

Keywords Minimal residual disease · RASSF6 · RASSF10 · Acute lymphoblastic leukemia · DNA hypermethylation · Tumor suppressor gene

Introduction

Acute lymphoblastic leukemia (ALL), sometimes called acute lymphocytic leukemia, is the most common form of leukemia found in children, accounting for about 30% of all pediatric cancer, which originates from the accumulation of clonal lymphoblast's B or T cells in the bone marrow [1]. Despite all the impressive progressions in the treatment of patients with ALL, the risk of relapse and cancer-related death is still reported to be high in pediatrics [2, 3]. In adult ALL, the risk stratification, which is based on the prognostic factors at the beginning of therapy, is critical for achieving improved clinical outcomes [4, 5]. The minimal residual disease (MRD) diagnosis as a prognostic factor throughout therapy is useful for the prediction of relapse and guidance of treatment decisions [6–10]. Thus far, the flow cytometry and the amplification of IG/TCR clonal rearrangements using allele-specific quantitative PCR (ASO-PCR)

✉ Seyed H. Ghaffari
shghaffari200@yahoo.com; shghaffari@tums.ac.ir

[1] Hematology, Oncology and Stem Cell Transplantation Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Hematology, School of Allied Medical Sciences, International Campus, Tehran University of Medical Sciences, Tehran, Iran

[3] Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran

[4] Department of Hematology, School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran

assays are considered as the gold standard methods for the monitoring of MRD in clinical laboratories [10].

The multiparameter flowcytometry (MFC) method for identifying leukemia-associated immunophenotypes (LAIPs) is regularly used in our hematology-oncology department. The MRD evaluation by Ig/TCR markers using ASO-PCR at the sequential follow-ups was also recently included, though, is it still under evaluation [11]. Despite the high sensitivity of ASO-PCR, the setup of the approach was laborious and time-consuming (4–5 weeks for the determination of the V/J usage, sequencing, and primer design) for the routine experiment. The high cost of testing for the patients was also deliberated as a problem with this system of testing. Therefore, we are in search for a reliable molecular marker for the determination of MRD that would be consistently sensitive, specific, and accurate as well as performing congruently fast and inexpensive. The recent studies suggested that the MRD in patients with leukemia, lymphoma, and neuroblastoma could be measured by using the promoter methylation status of only a small number of genes [12–16].

The DNA hypermethylation in the promoter of the tumor suppressor genes (TSGs) is a trait of the ALL blast cells [17, 18], which is generally associated with chemotherapy resistance in ALL patients [19]. Studies in both childhood and adult ALL cases have reported that the methylation levels of a subset of genes can be considered as a prognostic factor for the identification of patients at risk for disease relapse or complete remission [20–22]. Other studies, including our own, show that the DNA hypermethylation in the promoters of the RASSF6 and RASSF10 genes are found to be unique features of the ALL blasts, as well as specifying their prognostic value in the ALL patients [23, 24]. In a recent study, we have shown that RASSF6 and RASSF10 were methylated at a high frequency in adult ALL patients at the time of diagnosis [24]. The objective of the present study was to test whether the aberrant DNA methylation of the RASSF6 and RASSF10 genes could be useful as molecular markers for the monitoring of disease in adult patients with ALL. Here, we analyzed the aberrant RASSF6 and RASSF10 genes' methylation by the methylation-specific PCR (MSP) to detect the presence of minute amounts of residual cells. The results of the study obtained by using MSP were compared to the MRD results obtained from the Ig/TCR rearrangements ASO-PCR methods from our previous study [11].

Material and methods

Patients

Forty-five newly diagnosed adult patients with ALL (26 males and 19 females; age ranging 14–80 years) undergoing treatment with the Hyper-CVAD chemotherapy program in our Hematology, Oncology and Stem Cell Transplantation Research Center were included in this study. The diagnoses were established by oncologists based on the patients' standard morphological examinations and by using flowcytometric immunophenotyping. Samplings were performed according to the ethics approval of Tehran University of Medical Sciences. An informed consent was obtained from all the patients in accordance with the Declaration of Helsinki (IR.TUMS.REC.1394.2208).

Treatment regimens and sample collection

All the patients were given induction and consolidation therapy, according to the standard protocols; and the patients who had a proper donor were referred for allogeneic bone marrow transplantation (BMT). The prophylactic treatment of the CNS (central nervous system) was accomplished following chemotherapy or BMT. A total of 280 peripheral blood (PB) samples were collected for the monitoring of MRD at the time of diagnosis, day 14, day 28 (middle and end of the induction therapy), and subsequently at 1-month interval for the first year, followed by 3-month intervals thereafter until the time of death or at the end of the study. The PB samples of 20 healthy individuals were used as negative controls.

DNA extraction

Mononuclear cells (MNC) were separated from the PB samples by Ficoll-Hypaque (Lymphodex, Germany) density gradient centrifugation. DNA was isolated from the MNC by the DNA extraction mini kit (Yekta Tajhiz Azma, Iran). DNA was quantified by using NanoDrop 1000TMS spectrophotometer (OD 260 nm/OD 280 nm).

Methylation assay

The bisulfite treatment was performed according to the protocol of the EpiTect Fast DNA Bisulfite Kit (Qiagen, Hilden, Germany). A DNA protect reagent and bisulfite solution was added to 1 μg of genomic DNA and incubated at 95 °C for 5 min followed by 60 °C for 20 min, 95 °C for 5 min, and finally 60 °C for 20 min. Finally, each sample was desulfonated and eluted in a volume of 15 μl. The bisulfite-modified DNA was quantified using the NanoDrop 1000TMS spectrophotometer (OD 260 nm/OD 280 nm) and its quality was assessed using agarose gel electrophoresis. The DNA samples were frozen and stored at − 20 °C until the time of analysis.

The DNA methylation patterns of RASSF6 and RASSF10 promoters were determined by using two sets of primers for each gene; one set was used for amplifying methylated DNA and the other set was used for amplifying unmethylated DNA. The sequences of the primers used for MSP were previously reported [24]. The final volume for each MSP reaction was 25 μl, containing 2 μl of bisulfite-modified DNA template (40 ng/μl), 1 μl

of 10 μM of forward and reverse primers, 8 μl of Taq DNA polymerase master mix (Ampliqon), and 14 μl of ddH2O. The thermal cycling step was as follows; a 5-min hot start at 95 °C, followed by 35 cycles at 95 °C for 25 s, 60 °C for 40 s, 72 °C for 25 s, and a final extension at 72 °C for 10 min. All the MSP amplified products were loaded on a 2% agarose gel and a 6% polyacrylamide gel; in addition, they were visualized using the SYBR-safe and silver-nitrate staining (Sigma), respectively. Lastly, a semi-quantitative PCR analysis was performed on scanned gel images using the Multi-Analyst software version1.1 (Bio-Rad laboratories Inc., Life Science Group 2000).

Standard curve

To determine the sensitivity of the MSP assay, we used the Nalm-6 cell line (B-ALL cell) which has complete methylation of the RASSF6 promoter. DNA isolated from this cell line was serially diluted in DNA obtained from normal PB samples, bisulfite treated and amplified with RASSF6 methylation primers. Intensity of the bands was plotted against the log concentration of standards to generate a standard curve using the Multi-Analyst software. The PB samples of healthy individuals were used as negative control. In addition, DNA from Nalm-6 and AGS (human gastric cancer cell) were used as positive controls for RASSF6 and RASSF10, respectively.

Results

Patient characteristics

A total of 45 patients including 19 females and 26 males with the median age of 28 years (range 14 to 80 years) were enrolled in the present study for the molecular monitoring of MRD detection. Standard flowcytometry and cytomorphological criteria and the later data obtained from the PCR products confirmed 30 (66.66%) and 15 (33.33%) patients were B-ALL and T-ALL, respectively; 36 (80%) of the patients achieved CR, and 9 (20%) patients died during the induction therapy. The patients who achieved CR were monitored for MRD for approximately 30 months. During the follow-ups, 280 PB samples were collected, with a median number of 5 PB samples per patient (range 2–20).

Methylation status of RASSF6 and RASSF10 in the diagnostic samples

This is the first report using the promoter methylation status of RASSF6 and RASSF10 as molecular markers for the detection of MRD in ALL patients. For this purpose, their methylation status was assessed in the PB samples taken at the time of diagnosis from 45 adult ALL patients. The vast majority of the patients (41/45; 91.1%) demonstrated to have aberrant promoter methylation in either the RASSF6 (37, 82.2%) or RASSF10 (16, 35.6%) genes; 12 of the patients (26.7%) had both RASSF6 and RASSF10 promoter methylation at the same time; only four patients (8.88%) did not show any methylation. The clinical-laboratory features of these two methylated groups are summarized in Table 1.

RASSF6 was frequently hypermethylated in patients diagnosed with B-ALL (27/30, 90%) as compared to T-ALL (10/15, 66.7%); whereas, RASSF10 methylation was more confined to T-ALL (12/15, 80%) as compared to B-ALL (4/30, 13.3%). The overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the markers in the PB samples taken at the time of diagnosis were analyzed when tested independently or in combinations (Table 2). The overall sensitivity (the true positive rate) of RASSF6 and RASSF10 (82.2% and 35.6%) was increased to 91.1% when RASSF6 was combined with the RASSF10 marker ($p < 0.05$). The PPV, NPV, and overall accuracy were 100, 83.3%, and 93.8, respectively. None of the healthy controls ($n = 20$) revealed any RASSF6 or RASSF10 promoter methylation (Fig. 1a). There was no significant difference between the sensitivity of RASSF6 and RASSF10 with respect to the patient's gender or clinical-laboratory features. Finally, the MSP had a sensitivity of 10^{-3} for the detection of the methylated allele (Fig. 1c), and the specificity of MSP was demonstrated by the inclusion of appropriate positive and negative controls (Fig. 1a, b).

Methylation status of RASSF6 and RASSF10 during the serial follow-ups for MRD detection

The patients who were methylated in the RASSF6 and/or RASSF10 genes at the time of presentation and achieved CR were monitored for the residual methylation-based MRD detection by using MSP assay for up to 30 months. The relative intensity of the bands detected at each follow-up point was compared to the intensity of the bands detected at the time of diagnosis.

As seen in Fig. 2, the residual methylation-based MRD detection estimates the levels of residual leukemic cells during the follow-ups. In 9 of the 15 (60%) cases of T-lineage ALL, both the RASSF6 and RASSF10 genes were methylated; whereas in 3 of the 30 (10%) cases of B-lineage ALL, both of the genes were methylated. The promoter methylation patterns in both genes from the follow-up samples were quite similar in the patients having two methylated genes (Fig. 3b).

Comparison of the results obtained by MSP and ASO-PCR

To determine the validity of the MRD results obtained using the promoter methylation status as a marker, the MSP results were compared with those of the PCR amplification of Ig/TCR rearrangements, which were recently published and available for all the patients [11]. Figure 3 shows the sequential MRD detection

Table 1 A summary of the studied clinical-laboratory features in adult patients with ALL monitored for the evaluation of MRD (41 patients)

Clinical factor	Aberrantly methylated genes in ALL patients	
	RASSF6 methylation	RASSF10 methylation
Number	37	16
Age (years)	32.64	25.37
Gender		
Male (22 patients)	21 (95.45%)	9 (40.9%)
Female (19 patients)	16 (84.21%)	7 (36.84%)
Blast (%)	74.13	77.44
WBC ($\times 10^9$/l)	67.54	88.54
Hb (g/dl)	9.04	8.88
Plt ($\times 10^9$/l)	58.37	91.94
Overall survival (months)	8.95 (1–30)	9.06 (1–18)
Relapse (17 patients)	13 (76.47%)	8 (47.05%)
Deceased (21 patients)	18 (85.71%)	7 (33.33%)
Types		
B-lineage ALL (28 patients)	27 (96.42%)	4 (14.28%)
T-lineage ALL (13 patients)	10 (76.92%)	12 (92.3%)
Achieved CR:		
Yes (32 patients)	28 (87.5%)	14 (43.75%)
No (9 patients)	9 (100%)	2 (22.22%)
Relapse (after CR, 32 patients):		
Yes (17 patients)	13 (76.47%)	8 (47.05%)
No (15 patients)	15 (100%)	6 (40%)
Mortality (after CR, 32 patients):		
Yes (12 patients)	9 (75%)	5 (41.66%)
No (20 patients)	19 (95%)	9 (45%)
Mortality (41 patients):		
During the induction therapy (9)	9 (100%)	2 (22.2%)
During the follow-up (12)	9 (75%)	5 (41.66%)
None (20)	19 (95%)	9 (45%)

WBC, white blood cell; *Plt*, platelet; *Hb*, hemoglobin; *B-lineage ALL*, B-lineage acute lymphoblastic leukemia; *T-lineage ALL*, T-lineage acute lymphoblastic leukemia; *CR*, complete remission

in the PB samples of four representative patients performed by the promoter methylation status of RASSF6 and RASSF10 and are compared with those of the ASO-PCR amplification of Ig/TCR rearrangements. The comparative results showed that the results obtained from the MSP technique were to some extent comparable to those obtained from the ASO-PCR method.

As shown in Fig. 3a and Fig. b, sequential MRD levels in two patients: patient 7 with a good response to the initial induction chemotherapy, with a significant decrease in MRD level; patient 3 with no response to chemotherapy, having persistently high levels of MRD. Figure 3c represents the patient 17 with an initial decrease and a subsequent re-increase during chemotherapy. This patient was considered to be in a clinical remission; however, the MRD levels remained detectable by both techniques. Patients 1 achieved complete remission after initial induction chemotherapy, but the level of MRD started to increase again on day 148 (M5). Molecular relapse could be predicted by both methods about 2 months before the clinical relapse on day 208 (M7).

Concordance between MSP and ASO-PCR detection of MRD

The two methods were first compared to determine their ability to detect the presence of any residual leukemic DNA. Overall,

Table 2 The overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of RASSF6 and RASSF10 when tested independently or in combinations in the blood samples of ALL patients

	Markers	Sensitivity (%)*	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
B-ALL ($n = 30$)	RASSF6	90.0 (27/30)	100.0	100.0	87.0	94.0
	RASSF10	13.3 (4/30)	100.0	100.0	43.5	48.0
	RASSF6 + RASSF10	93.3 (28/30)	100.0	100.0	90.9	96.0
T-ALL ($n = 15$)	RASSF6	66.7 (10/15)	100.0	100.0	80.0	85.7
	RASSF10	80.0 (12/15)	100.0	100.0	87.0	91.4
	RASSF6 + RASSF10	86.7 (13/15)	100.0	100.0	90.9	94.3
B- & T-ALL ($n = 45$)	RASSF6	82.2 (37/45)	100.0	100.0	71.4	87.7
	RASSF10	35.6 (16/45)	100.0	100.0	40.8	55.4
	RASSF6 + RASSF10	91.1 (41/45)	100.0	100.0	83.3	93.8

*Sensitivity (the true positive rate)

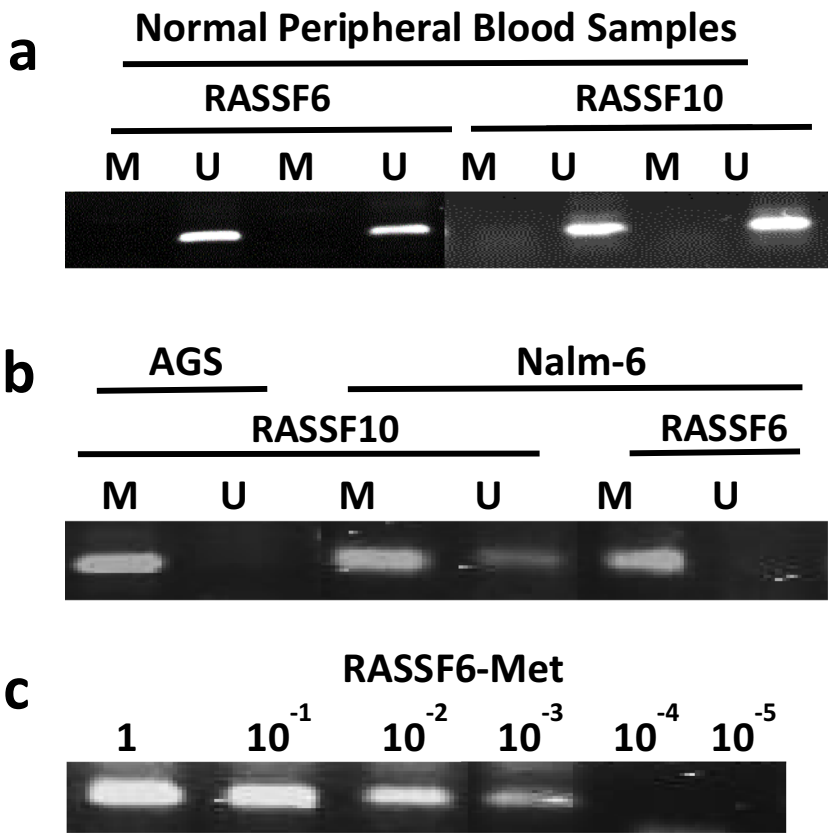

Fig. 1 The sensitivity and specificity of MSP. The representative MSP results of RASSF6 and RASSF10 in **a** normal human PB samples, **b** Nalm-6 cell line (B-ALL cell line), and AGS (gastric cancer cell line). **c** The sensitivity of MSP, showing the detection of the methylated RASSF6 at 10^{-3}. M, methylated; U, unmethylated

the concordance rate was 134 out of 280 (47.9%); the discordance was more prevalent in the MSP-negative and low-level ASO-PCR-positive (146/280, 52.1%) samples, whereas there were no MSP-positive and ASO-PCR-negative samples.

In the previous study, we established a precise cutoff of 100 for the ASO-PCR method. Regarding the MRD threshold, patients could be divided into two distinct groups at each of the time intervals: low-risk and high-risk groups. The MRD-levels

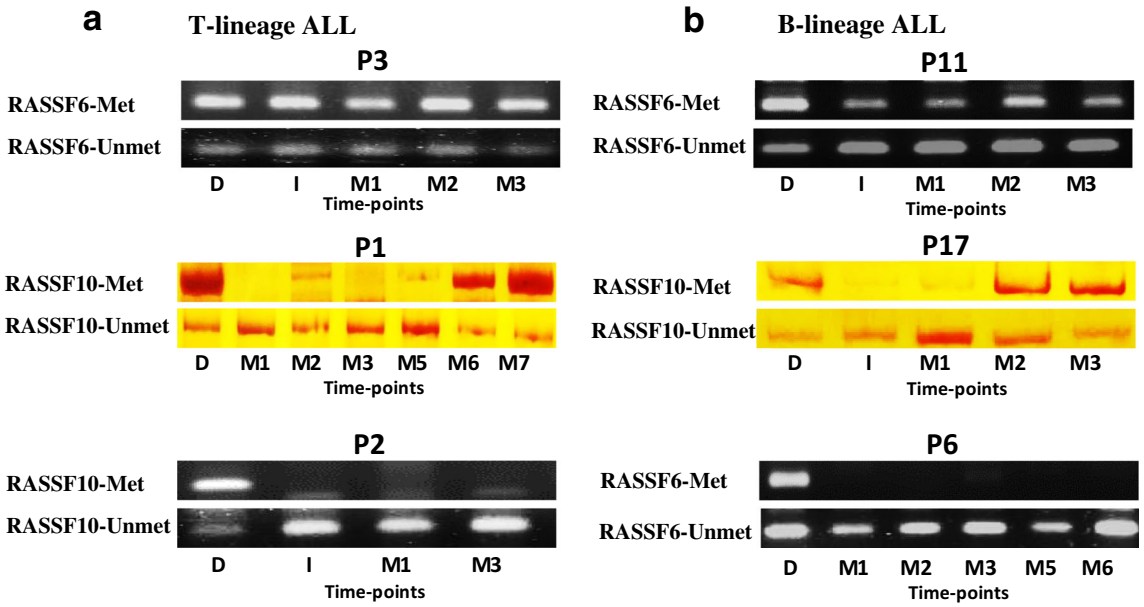

Fig. 2 The sequential monitoring of MRD using the promoter methylation status of RASSF6 and RASSF10 in adult ALL. The representative MRD results with the use of the promoter methylation status in the PB samples collected during the followups from **a** T-lineage ALL, **b** B-lineage ALL patients. Met, methylated; Unmet, unmethylated; P, patient; D, at diagnosis; i, day 14 of induction therapy; M1, day 28; M2, day 58; M3, day 88; M5, day148; M6, day 178; M7, day 208

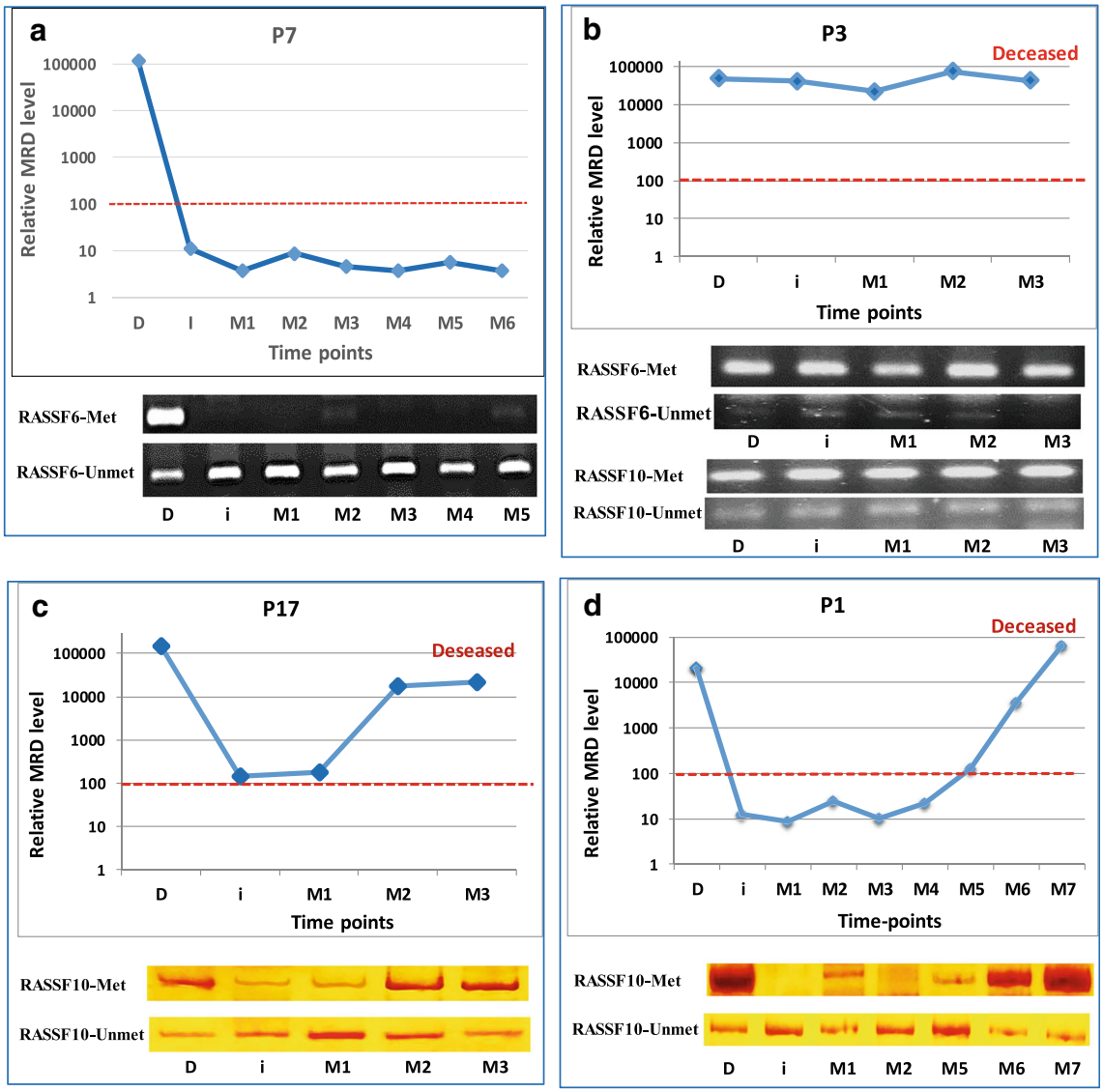

Fig. 3 The comparison of sequential MRD detection by MSP and ASO-PCR in PB samples of patients with ALL. To determine the validity of the MRD results obtained by the promoter methylation status of RASSF6 and RASSF10 as markers, the representative MSP results were compared with those of the ASO-PCR amplification of Ig/TCR rearrangements in the PB samples collected at the time of diagnosis and during the follow-ups of patients with adult ALL. The threshold level (dotted line) may identify low- and high-risk patients, persistent increase above this level, patients may be destined for clinical relapse. **a** The response curves and MSP-gel image of a patient with a good response to the initial induction chemotherapy, with a significant decrease in the MRD level, and with no relapse during the follow-up time. **b** The sequential MRD levels in a patient with no response to chemotherapy, having persistently high levels of MRD. **c** The response curves of patients with an initial decrease and a subsequent re-increase during chemotherapy. This patient was considered to be in a clinical remission; however, the MRD levels remained detectable by both techniques. **d** The patient achieved complete remission after initial induction chemotherapy, but the level of MRD started to increase again in M5. Both of the methods could predict molecular relapse about 2 months before clinical relapse. Met, methylated; Unmet, unmethylated; P, patient; D, at diagnosis; i, day 14 of induction therapy; M1, day 28; M2, day 58; M3, day 88; M4, day 118; M5, day148; M6, day 178; M7, day 208

lower than the cutoff level at any time-points were associated with a significantly increased overall survival (OS). In contrast, the patients with higher MRD on these points and a subsequent increase in the next follow-ups usually led to a progressively worse outcome. We applied the cutoff of 100 for the ASO-PCR assay and compared it with the MSP results in all the patients' follow-ups. These two methods gave significantly higher concordant results above the cutoff (concordance rate 134/147, 91.1%). Of 147 samples above the cutoff, only 13 samples were negative by the MSP, while, they were positive by the PCR (dis-concordance rate 13/147, 8.84%).

The direct comparison of MRD estimated by the PCR and MSP techniques at each time point is shown in Fig. 4. The analysis shows a significant correlation between the ASO-PCR and MSP (Spearman correlation coefficient: $r = 0.848$; $p < 0.001$). It is interesting that, this level of assessment by the

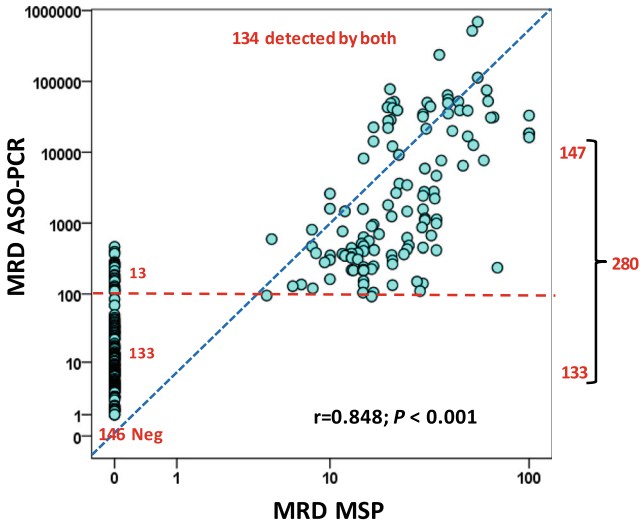

Fig. 4 The correlation between MSP and ASO-PCR detection of MRD. The scatter plot shows the direct comparison of 280 paired MRD results estimated by the PCR and MSP, during the 30 months of the follow-up period; 134/280 (47.8%) samples were positive in both assays. When the previously defined cutoff level ($\geq$ 100) was applied to the PCR assays, from 147 samples detected by PCR, 134 were also positive by MSP (concordance rate of 91.1%). The correlation coefficient was calculated with the Spearman's rank correlation test, which showed a significant correlation between the ASO-PCR and MSP methods ($r = 0.848$; $P < 0.001$). The threshold level (red dotted line $\geq$ 100) may identify the low- and high-risk patients; persistent increase above this level, patients may be destined for clinical relapse

MSP was equivalent to the PCR cutoff level. Below these levels, no concordance between the MSP and ASO-PCR assays was detected.

Discussion

The quantification of IG/TCR clonal rearrangements using allele-specific quantitative PCR (ASO-PCR) is currently recognized as the gold standard for the detection of MRD in ALL. Despite the high sensitivity of the ASO-PCR, setup of the approach is laborious, time-consuming, and expensive for regular routine practice. Nonetheless, efforts persist to identify an appropriate marker for the diagnosis of MRD in ALL. The objective of the present study was to evaluate the detection of the residual methylated DNA of RASSF6 and RASSF10 gene promoters by using MSP as an alternative method for the evaluation of the MRD levels in the follow-up PB samples of patients with ALL. Our results showed that the MSP analysis allows the accurate determination of the residual leukemic DNA in the PB follow-up samples. This technique was applicable for 91.1% (41/45) of our patients. In addition, when comparing these two techniques for MRD detection, the applicability of MSP was to some extent comparable to those obtained by the ASO-PCR methods.

Previous studies have shown that there is a good correlation between the residual methylation of DNA and the occult disease of the bone marrow (BM) samples of neuroblastoma, natural killer cell lymphoma, and diffuse large B cell lymphoma (DLBCL) [14–16]. The DNA hypermethylation in the promoter of the tumor suppressor genes (TSGs) has recently been applied as a prognostic factor in various forms of human cancers; however, its use in the evaluation of MRD is still under investigation. Its predictive power for monitoring MRD before the presentation of clinical relapse was not reported in the serial follow-up time points. So far, no study has attempted to evaluate the RASSF6 and RASSF10 promoter methylation patterns for the detection of MRD in either PB or BM samples of ALL patients by the utilization of the MSP technique.

RASSF6 and RASSF10 are tumor suppressors, which are highly methylated in ALL patients [23, 24]. In this study, we have shown that RASSF6 was frequently methylated in patients diagnosed with B-ALL (90%) as compared to T-ALL (66.7%); whereas, RASSF10 methylation was more confined to T-ALL (80%) as compared to B-ALL (13.3%). The applicability and accuracy were increased when RASSF6 was combined with the RASSF10 marker (91.1 and 93.8%, respectively; $p < 0.05$). Since, they are not methylated in normal human PB samples, the use of the methylation pattern of these novel markers could potentially become markers for the detection of MRD in ALL patients [24].

During the follow-ups, there was a significant correlation between the residual DNA methylation in the clinical remission and the subsequent risk of relapse. The MSP of RASSF6 and RASSF10 genes methylation was able to estimate the levels of residual leukemia cells, and the residual methylation levels were gradually increased several months before the occurrence of the clinical and morphological relapse. According to the previous studies, DNA hypermethylation of the RASSF6 and RASSF10 genes has never been reported to be found in normal samples [23, 24]. Therefore, the presence of residual methylation during the follow-ups is a sign of the existence of leukemic cells; thus, the likelihood of a false positive result is very low.

We compared the sensitivity of detecting MRD by the MSP study with the Ig/TCR rearrangement RT-PCR based study in the PB samples obtained from the same ALL patients. The utilization of MSP had a sensitivity of 10^{-3} for the detection of methylated alleles, which was approximately 2-log less sensitive than the usage of PCR. In the previous study, we established a defined cutoff for the MRD level in the ASO-PCR method. Based on this threshold, our patients were divided into two groups (low- and high-risk groups). During the follow-ups, a molecular remission or relapse was indicated by decreasing or re-increasing the MRD below or above the cutoff level, respectively [11]. Interestingly, in the present study, the MSP detection limit was equivalent to the cutoff level which was defined by the ASO-PCR; thus, during the follow up, when a sample became MSP-positive, the patient was regarded as a high-risk patient.

The sequential MRD detections by MSP were compared with those of the ASO-PCR amplification in the PB samples collected at the time of diagnosis and during the follow-ups. There was a significant correlation between the results obtained from the Ig/TCR rearrangement technique and the MSP technique (Spearman correlation coefficient: $r = 0.848$; $p < 0.001$). Of the 280 PB samples studied by the ASO-PCR assay at the time of diagnosis and during the follow-ups, 147 samples were above the cutoff points (as determined in the previous study [11]), also, 134 of these samples were also positive by the MSP assay (concordance rate 134/147, 91.1%). The concordance rate observed between these two assays were all above the cutoff level as defined by the ASO-PCR; below that, no concordance was detected between the MSP and ASO-PCR.

The evaluation of the treatment response through MRD detection is usually determined by investigating the leukemic cells through bone marrow (BM) biopsy. Nevertheless, several studies have shown that peripheral blood (PB) can replace the BM for the detection of MRD as well as provide valuable prognostic information in ALL and acute myeloid leukemia (AML) [25–29]. Even though the BM is the most appropriate and sensitive sample for MRD measurement [10], it is still a relatively invasive and dreadful process, thus, implying the need for more expertise in PB sample collections. Since short interval time-points and regular sampling for monitoring the MRD-level is recommended for the prediction of early clinical relapse which allows better clinical management of the disease, the application of PB sampling could be a useful alternative for detecting MRD. Furthermore, in our previous study, the MRD data obtained from the PB highlights the practicality of using the PB as a sensitive source for MRD detection. This suggests that the PB could be an advantageous alternative source for the monthly monitoring of MRD.

The advantages of the MSP technique are that it is simple, fast, and affordable, and it requires only a pair of primers for each gene. Our setup techniques exhibited the sufficient sensitivity and specificity potentially applicable for the routine evaluation of MRD in ALL patients with aberrantly methylated RASSF6 and RASSF10 genes at the time of their diagnosis. The setup of the Ig/TCR rearrangement-based technique for each individual patient is more challenging than the MSP-based technique. Accordingly, this finding suggests that by considering the results obtained from the MSP technique, it is possible to monitor the ALL patients for longer periods simply by performing repeated PB samplings and sequential MSP analyses to monitor the MRD levels during the follow-ups. In addition, since we were able to detect these molecular markers in the early stages of the disease when compared to the long process of setup for the Ig/TCR rearrangement technique, the use of the MSP technique could be a valuable tool for the screening of the early stages of the disease. Likewise, this technique can be used to monitor the effect of treatment in the PB samples of the ALL patients. As well, MSP can be used as a complementary method to monitor the MRD along with the multi-parameter flow cytometry assays. Given the rapidity of which the results can be obtained in the routine practice, MSP could be especially valuable for the evaluation of the treatment response during induction therapy, and also during the follow-up, it identifies the patients who are at a higher risk in whom may require intensification of treatments before the manifestation of clinical relapse.

In summary, for the first time, we introduced the aberrant promoter methylation status of the two tumor suppressors, RASSF6 and RASSF10, as potentially valuable molecular markers for the monitoring of disease in the PB of patients with ALL. Since, RASSF6 was frequently methylated in B-ALL and RASSF10 in T-ALL, the applicability and accuracy of the assays were increased when these markers were combined. The results of the 2.5-year study of MRD detection in the PB samples of patients showed a significant correlation between the results obtained by the Ig/TCR rearrangements ASO-PCR and the MSP techniques. Even though, these two techniques cannot simply substitute each other, the DNA methylation status of the RASSF6 and RASSF10 genes could prove to be potential biomarkers for the assessment of the MRD in ALL patients especially in many labs where the ASO-PCR technique is highly difficult and expensive to setup and use in the routine practice. Hence, due to the high applicability, the non-invasiveness, and promising prospect of longitudinal assessment, the detection of MRD in PB by RASSF genes warrants further contemplation as a tool for monitoring MRD in patients with ALL.

Funding information This study was supported by the Hematology, Oncology and Stem Cell Transplantation Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran. (Grant number: 95-01-36-31971).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Hoffbrand AV, Higgs DR, Keeling DM, Mehta AB (2016) Postgraduate haematology. Wiley
2. Locatelli F, Schrappe M, Bernardo ME, Rutella S (2012) How I treat relapsed childhood acute lymphoblastic leukemia. Blood 120(14):2807–2816. https://doi.org/10.1182/blood-2012-02-265884
3. Mullighan CG, Willman CL (2011) Advances in the biology of acute lymphoblastic leukemia—from genomics to the clinic. Journal of adolescent and young adult oncology 1(2):77–86. https://doi.org/10.1089/jayao.2011.0012

4. Gökbuget N, Hoelzer D (2009) Treatment of adult acute lymphoblastic leukemia. In: Seminars in hematology, vol 1. Elsevier, pp 64–75. https://doi.org/10.1053/j.seminhematol.2008.09.003
5. Bassan R, Hoelzer D (2011) Modern therapy of acute lymphoblastic leukemia. J Clin Oncol 29(5):532–543. https://doi.org/10.1200/JCO.2010.30.1382
6. Brüggemann M, Raff T, Flohr T, Gökbuget N, Nakao M, Droese J, Lüschen S, Pott C, Ritgen M, Scheuring U (2006) Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. Blood 107(3):1116–1123. https://doi.org/10.1182/blood-2005-07-2708
7. Bassan R, Spinelli O, Oldani E, Intermesoli T, Tosi M, Peruta B, Rossi G, Borlenghi E, Pogliani EM, Terruzzi E (2009) Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). Blood 113(18):4153–4162. https://doi.org/10.1182/blood-2008-11-185132
8. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, Linda S, Martin PL, Pullen DJ, Viswanatha D (2008) Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's oncology group study. Blood 111(12):5477–5485. https://doi.org/10.1182/blood-2008-01-132837
9. Vora A, Goulden N, Wade R, Mitchell C, Hancock J, Hough R, Rowntree C, Richards S (2013) Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. Lancet Oncol 14(3):199–209. https://doi.org/10.1016/S1470-2045(12)70600-9
10. van Dongen JJ, van der Velden VH, Brüggemann M, Orfao A (2015) Minimal residual disease diagnostics in acute lymphoblastic leukemia: need for sensitive, fast, and standardized technologies. Blood 125(26):3996–4009. https://doi.org/10.1182/blood-2015-03-580027
11. Shahkarami S, Mehrasa R, Younesian S, Yaghmaie M, Chahardouli B, Vaezi M, Rezaei N, Nikbakht M, Alimoghaddam K, Ghavamzadeh A (2018) Minimal residual disease (MRD) detection using rearrangement of immunoglobulin/T cell receptor genes in adult patients with acute lymphoblastic leukemia (ALL). Ann Hematol:1–11. https://doi.org/10.1007/s00277-018-3230-z
12. Agrawal S, Unterberg M, Koschmieder S, zur Stadt U, Brunnberg U, Verbeek W, Büchner T, Berdel WE, Serve H, Müller-Tidow C (2007) DNA methylation of tumor suppressor genes in clinical remission predicts the relapse risk in acute myeloid leukemia. Cancer Res 67(3):1370–1377. https://doi.org/10.1158/0008-5472.CAN-06-1681
13. Wang MX, Wang H-Y, Zhao X, Srilatha N, Zheng D, Shi H, Ning J, Duff DJ, Taylor KH, Gruner BA (2010) Molecular detection of B-cell neoplasms by specific DNA methylation biomarkers. Int J Clin Exp Pathol 3(3):265
14. Hagiwara K, Li Y, Kinoshita T, Kunishma S, Ohashi H, Hotta T, Nagai H (2010) Aberrant DNA methylation of the p57KIP2 gene is a sensitive biomarker for detecting minimal residual disease in diffuse large B cell lymphoma. Leuk Res 34(1):50–54. https://doi.org/10.1016/j.leukres.2009.06.028
15. Siu LL, Chan JK, Wong KF, Choy C, Kwong YL (2003) Aberrant promoter CpG methylation as a molecular marker for disease monitoring in natural killer cell lymphomas. Br J Haematol 122(1):70–77
16. Stutterheim J, Ichou FA, den Ouden E, Versteeg R, Caron HN, Tytgat GA, van der Schoot CE (2012) Methylated RASSF1a is the first specific DNA marker for minimal residual disease testing in neuroblastoma. Clin Cancer Res 18(3):808–814
17. Dunwell TL, Hesson LB, Pavlova TV, Zabarovska V, Kashuba VI, Catchpoole D, Chiaramonte R, Brini AT, Griffiths M, Maher ER (2009) Epigenetic analysis of childhood acute lymphoblastic leukemia. Epigenetics-Us 4(3):185–193. https://doi.org/10.4161/epi.4.3.8752
18. Garcia-Manero G, Yang H, Kuang S-Q, O'Brien S, Thomas D, Kantarjian H (2009) Epigenetics of acute lymphocytic leukemia. In: Seminars in hematology, vol 1. Elsevier, pp 24–32. https://doi.org/10.1053/j.seminhematol.2008.09.008
19. Bhatla T, Wang J, Morrison DJ, Raetz EA, Burke MJ, Brown P, Carroll WL (2012) Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. Blood 119(22):5201–5210
20. Garcia-Manero G, Bueso-Ramos C, Daniel J, Williamson J, Kantarjian HM, Issa J-PJ (2002) DNA methylation patterns at relapse in adult acute lymphocytic leukemia. Clin Cancer Res 8(6):1897–1903
21. Nordlund J, Milani L, Lundmark A, Lönnerholm G, Syvänen A-C (2012) DNA methylation analysis of bone marrow cells at diagnosis of acute lymphoblastic leukemia and at remission. PLoS One 7(4):e34513. https://doi.org/10.1371/journal.pone.0034513
22. Garcia-Manero G, Bueso-Ramos C, Xiao L, Hoshino K, Rosner G, Pierce S, Yang H (2006) Detection of residual p73 DNA methylation predicts for shorter disease free and overall survival in patients (pts) with Philadelphia (Ph) chromosome negative acute lymphocytic leukemia (ALL) in remission. Am Soc Hematology
23. Hesson LB, Dunwell TL, Cooper WN, Catchpoole D, Brini AT, Chiaramonte R, Griffiths M, Chalmers AD, Maher ER, Latif F (2009) The novel RASSF6 and RASSF10 candidate tumour suppressor genes are frequently epigenetically inactivated in childhood leukaemias. Mol Cancer 8(1):42. https://doi.org/10.1186/1476-4598-8-42
24. Younesian S, Shahkarami S, Ghaffari P, Alizadeh S, Mehrasa R, Ghavamzadeh A, Ghaffari SH (2017) DNA hypermethylation of tumor suppressor genes RASSF6 and RASSF10 as independent prognostic factors in adult acute lymphoblastic leukemia. Leuk Res 61:33–38. https://doi.org/10.1016/j.leukres.2017.08.016
25. Brisco MJ, Sykes PJ, Hughes E, Dolman G, Neoh SH, Peng LM, Toogood I, Morley AA (1997) Monitoring minimal residual disease in peripheral blood in B-lineage acute lymphoblastic leukaemia. Br J Haematol 99(2):314–319
26. van Rhee F, Marks DI, Lin F, Szydlo RM, Hochhaus A, Treleaven J, Delord C, Cross NC, Goldman JM (1995) Quantification of residual disease in Philadelphia-positive acute lymphoblastic leukemia: comparison of blood and bone marrow. Leukemia 9(2):329–335
27. van der Velden VH, Jacobs DC, Wijkhuijs AJ, Comans-Bitter WM, Willemse MJ, Hahlen K, Kamps WA, van Wering ER, van Dongen JJ (2002) Minimal residual disease levels in bone marrow and peripheral blood are comparable in children with T cell acute lymphoblastic leukemia (ALL), but not in precursor-B-ALL. Leukemia 16(8):1432–1436. https://doi.org/10.1038/sj.leu.2402636
28. Coustan-Smith E, Sancho J, Hancock ML, Razzouk BI, Ribeiro RC, Rivera GK, Rubnitz JE, Sandlund JT, Pui CH, Campana D (2002) Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia. Blood 100(7):2399–2402. https://doi.org/10.1182/blood-2002-04-1130
29. Zeijlemaker W, Kelder A, Oussoren-Brockhoff YJ, Scholten WJ, Snel AN, Veldhuizen D, Cloos J, Ossenkoppele GJ, Schuurhuis GJ (2016) Peripheral blood minimal residual disease may replace bone marrow minimal residual disease as an immunophenotypic biomarker for impending relapse in acute myeloid leukemia. Leukemia 30(3):708–715. https://doi.org/10.1038/leu.2015.255

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.