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Minimal Residual Disease Detection Using Gene Scanning Analysis, Fluorescent Fragment Analysis, and Capillary Electrophoresis for *IgH* Rearrangement in Adult B-Lineage Acute Lymphoblastic Leukemia: A Cross-Sectional Study

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Abstract

Objective: Minimal residual disease (MRD) is considered the greatest prognostic factor in acute lymphoblastic leukemia (ALL). MRD is a valuable tool for anticipating impending relapse and treatment response assessment. The objective of the present study was to investigate whether the detection of *IgH* gene rearrangement using polymerase chain reaction (PCR)-based GeneScan analysis could be a complementary method to monitor MRD along with the quantitative real-time PCR (qPCR).

Materials and Methods: In this cross-sectional study, we valued the MRD levels, based on the GeneScanning analysis (GSA), and then compared the data with quantitative real-time polymerase chain reaction at different time points in peripheral blood (PB) samples of adult B-lineage ALL patients (n=35). The specific polymerase chain reaction (PCR) primers for *IGH* gene FR-1 and fluorescence-labeled J-primer were used and analyzed by capillary gel electrophoresis on a sequencer. The results of this study were compared with the previously reported MRD results obtained by the *IGH* rearrangements allele-specific oligonucleotide (ASO) -qPCR methods.

Results: The total concordance rate was 86.7%, with a P<0.001. MRD results obtained by GSA and ASO-qPCR methods were concordant in all diagnostic samples and samples on the 14th and 28th days of induction therapy. The results of these 2.5 years' follow-ups demonstrated a significant correlation between the two techniques (r=0.892, P<0.001).

Conclusion: It seems that the PCR-based GeneScan analysis of *IGH* gene rearrangement detection may be a valuable molecular technique to distinguish monoclonality from polyclonality. And also, it may be a precise tool to detect the residual leukemic DNA in the PB follow-up samples of patients.

Keywords: Acute Lymphoblastic Leukemia, Capillary Gel Electrophoresis, Immunoglobulin Heavy Chain, GeneScanning, Minimal Residual Disease

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Introduction

Acute lymphoblastic leukemia (ALL), a malignant disorder, is caused by the proliferation and accumulation of clonal lymphoblasts in the bone marrow that can be of derived cell lines, including B-cell (80-85%) or T-cell (20-25%) (1). Despite high rates of complete remission (CR) achievement with current treatment regimens, approximately 30 to 50% of adult ALL patients and 10 to 20% of pediatric ALL patients with CR show various degrees of residual leukemia (2-6). The residual leukemic cells resistant to chemotherapy, termed "minimal residual disease (MRD)", are the major cause of relapse and treatment failure in patients with ALL. Sequential monitoring of MRD can reliably predict the response to treatment and the risk of relapse (7-9).

A meta-analysis of 39 studies, including 13637 patients with ALL, concluded that 10-year event-free survival for MRD negativity vs. MRD positivity was 64 vs. 21% for adults and 77 vs. 32% for pediatrics, respectively. It seems achieving a negative MRD result is vital for these patients, adult and pediatric (10). In addition, MRD-based

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risk stratification is one of the critical components of pediatric ALL treatment protocols (11, 12), and may play a similar role in adult ALL patients (13-15). Therefore, it is necessary to use techniques with sufficient specificity and sensitivity to detect leukemic cells that allow the definition of different risk group patients who may benefit from reduction or intensification of therapy based on different MRD values (13, 14, 16, 17).

In patients with ALL, flow cytometric analysis of leukemia-associated immunophenotypes and polymerase chain reaction (PCR) amplification of different fusion genes or antigen-receptor genes are broadly used to diagnose MRD (18). In a recent study, we demonstrated that monitoring MRD in the adult ALL patients using allele-specific oligonucleotide (ASO)-PCR technique is a beneficial tool for identifying patients at risk of relapse (19). However, the most limitation of the ASO-qPCR technique, along with the drawbacks such as the need for broad skill, being time-consuming, a determination 30-35 day of the V/J usage, sequencing, and primer design , cost and also, false negative result due to the clonal evolution during disease (20). Therefore, there is still a need for a technique that can overcome the ASO-qPCR technique limitations.

PCR-based GeneScan analysis (PCR-GSA) by capillary gel electrophoresis on a sequencer can detect the size of *IG/TCR* gene rearrangements fragments within a single base pair, which facilitates the identification of clonal rearrangements within a polyclonal background (21, 22).

In addition, a follow-up PCR-GSA can identify the emergence of novel clones and clonal changes/evolution changes, which causes the relapse occurrence (23-25). A study to date has evaluated the specificity of PCR-GSA for monitoring MRD at diagnosis and subsequent relapse in childhood precursor-B-ALL patients (23). Its predictive power for MRD detection before the manifestation of clinical relapse at serial follow-up time points has not yet been reported in B-ALL patients. Due to the rapidity with which the results can be achieved in routine practice by PCR-GSA, its use may be able to overcome the ASO-qPCR technique limitations. The objective of the present study was to investigate whether the detection of an *IGH* gene rearrangement using PCR-GSA could be a complementary method to monitor MRD along with the ASO-qPCR.

Material and Methods

This cross-sectional study was performed according to the Declaration of Helsinki and was approved by the Regional Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.REC.1394.2175). Written informed consent was obtained from the patients and/or their guardians.

Patients and clinical samples

Forty-four newly diagnosed adult B-lineage ALL patients undergoing treatment with the Hyper-CVAD (hyperfractionated cyclophosphamide, vincristine, doxorubicin [Adriamycin], and dexamethasone) chemotherapy regimes in the Bone Marrow Transplantation Research Center of Shariati Hospital were included in this prospective study for MRD detection. The diagnosis was confirmed by standard morphological examinations and immunophenotyping.

The patients who were alive at the end of induction therapy and achieved clinical remission were included in this study and monitored for approximately 30 months. A total of 256 peripheral blood (PB) samples was obtained from 35 patients at the time of diagnosis, 14th day, 28th day (middle and end of the induction therapy), and subsequently at 30th day intervals for the first year, followed by 90th day intervals afterward until the time of death or at the end of the study. The PB samples of 20 healthy individuals were used as controls in this study.

Fluorescent fragment analysis to assess MRD

The unique sequence of rearranged junction regions in *IGH* genes was amplified by a multiplex PCR using a set of primers for the V segments and fluorescently labeled (HEX-labeled) consensus primers for the J segments. Primer sequences were selected according to the European BIOMED-2 PCR collaborative study instructions (26). The size of the PCR products was determined by capillary gel electrophoresis on a sequencer (ABI Prism 3130xL Genetic analyzer, Applied Biosystems, USA).

All samples were amplified in a final volume of 25 µl including 2 µl of DNA sample (40 ng/µl), 1 µl of 10 µM of forward and reverse primers, 10 µl of Taq DNA Polymerase Master Mix 2x (Cat. No. A190303, Ampliqon, Denmark), and 12 µl of ddH_2O. The cycling condition was as follows: initial denaturing, 5 minutes at 95˚C, followed by 35 cycles of 30 seconds at 95˚C, 30 seconds at 60˚C, and 30 seconds at 72˚C. After the last cycle, an additional extension step of 7 minutes at 72˚C was performed. Afterward, 2 µl of PCR product was mixed with a 7.5 µl HiDi™formamide (Cat. No. 4311320, Applied Biosystems, USA), and 0.5 µl of the GeneScan™ 500 LIZ™ Size Standard (Cat. No. 4322682, Applied Biosystems, USA) was included for determination of the amplicon size. After 10 minutes of denaturation at 94˚C, the DNA fragments were separated on POP-7 polymer (Cat. No. 4393708, Applied Biosystems, USA) and analyzed by 310 GeneScan 3.1 software (Applied Biosystems Foster City, CA, USA). Typical running times were 4 hours with a 7-cm long electrophoresis gel.

PCR reactions included a sample containing monoclonal DNA as a positive control and a sample containing polyclonal DNA obtained from five healthy controls to assess specificity. And also, a non-template control sample, as a negative control, was considered in each PCR reaction to exclude possible contaminations.

Monoclonal cell samples give rise to a distinct fluorescent peak in intensity higher than the background, within the appropriate size ranges; whereas, in polyclonal samples, many different *IGH* PCR products are formed, which display a characteristic Gaussian size distribution. The relative size of the peak identified at each follow-up time point was compared to the size of the peak identified at the time of diagnosis.

Statistical analysis

The MRD results of this study were compared with the previously reported MRD results obtained by the *IGH* rearrangements ASO-qPCR techniques (19). The correlation analysis between the results of PCR-GSA and ASO-qPCR technique was accomplished using the Spearman's rank correlation coefficient. The SPSS software package version 21.0 (Chicago, IL, USA) was used for statistical analysis. A $P<0.05$ was considered statistically significant.

Results

Patient characteristics

Initially, 44 B-lineage ALL patients were enrolled in this study. The 9 patients deceased during induction therapy. The 35 patients, including 15 females and 20 males, who were alive at the end of induction therapy and achieved clinical remission were included in this study and monitored for approximately 30 months. The median age of patients was 28 years (range: 14 to 80 years). According to the ALL immunophenotyping, these B-lineage ALL patients were divided into two groups, pre-B- (26/35, 74%) and B-ALL (9/35, 26%). A total of 256 PB samples was obtained from the patients for MRD diagnosis by the PCR-GSA, with an average number of five samples per patient (range 2-20).

Fluorescent fragment analysis of scanning profiles

MRD quantification through fluorescent fragment analysis in the follow-up samples was carried out considering the size of a clone-specific marker primarily identified in diagnostic samples. When the patient's pattern changed to a pattern of normally distributed peaks (Gaussian curve), it was considered MRD negative. In all our patient's diagnostic samples, the PCR products showed only a dominant fluorescent peak in the fade background signals indicating a specific clonal *IGH* gene rearrangement (mono-clonal), and it was interpreted as a cancer clone. Depending on the rearranged alleles, the nucleotide size of clones usually ranged between 100-400 nucleotides. All diagnostic DNA samples in this study revealed products of *IGH,* which were distinguished by one dominant peak in the GeneScan electropherogram profile, showing the presence of monoclonal proliferation.

During the follow-up, in each patient, the height of the PCR peak in the follow-up sample was compared to the peak pattern and the height of the peak that was present in the respective diagnostic sample. A follow-up sample was considered MRD positive, when a monoclonal peak of a size identical to that demonstrated in the diagnosis sample was detected in its follow-up sample; whereas specific clonality was gradually cleared in the patients who entered continuous complete remission. During the entire follow-up period, relapses occurred in some patients. These patients had an initial decrease in specific clonality, but in later follow-ups, they showed a rising MRD level before clinical relapse (usually 1-2 months before clinical relapse). During the follow-up period, none of the patients showed oligoclonality or acquired a new clone other than a clone already detected at presentation.

Comparison of MRD results determined by PCR-GSA and ASO-qPCR

To confirm the validity of the MRD results achieved from the PCR-GSA technique, the results of GSA were compared with the MRD results achieved from the *IGH* ASO-qPCR rearrangement method from our previous study (19). Figure 1 displays the sequential MRD pattern in the diagnosis and follow-up samples of three representative subjects carried out by the PCR-GSA method, compared with those of the ASO-qPCR amplification of *IGH* rearrangements. This picture illustrated the MRD level in a case (P6) who achieved continued remission after induction therapy; a patient (P2) who showed a significant reduction in the level of MRD and achieved clinical remission but subsequently experienced a relapse on the 58^{th} day (M3); and a refractory subject (P18) with no response to chemotherapy, respectively. Also, the data in relapsed patients showed that both methods were able to predict molecular relapse at least 1-2 months before the clinical relapse.

Concordance rate between PCR-GSA and ASO-qPCR detection of MRD

The ability of both techniques to detect the presence of any residual leukemic DNA was compared; the overall concordance rate between PCR-GSA and ASO-qPCR MRD results in 256 PB samples at diagnosis and during 2.5-year follow-up was 86.7% (222 out of 256 samples). Figure 2 shows a direct comparison of MRD levels estimated by both methods at each follow-up time point. The correlation analysis represents that there is a significant correlation between the PCR-GSA and ASO-qPCR with a Spearman correlation coefficient of $r=0.876$ ($P<0.001$). MRD results obtained by GSA and ASO-qPCR methods were concordant in all diagnostic samples and samples on the 14^{th} and 28^{th} days of induction therapy. Of 256 total samples, 34 (13.3%) were positive by ASO-qPCR but negative by PCR-GSA, and all negative cases by the GSA were also negative by the ASO-qPCR. Of 34 discordant results, 4 were observed during consolidation therapy on day 58, 5 on day 88, and 25 from M4 onwards. So, this amount of discordance rate (13.3%) between the two tests is not unexpected, because the GSA may fail to detect the very small amount of residual disease due to its lower sensitivity (10^{-3} to 10^{-4}) versus the highly sensitive ASO-qPCR assay with 10^{-4} to 10^{-5} sensitivity. The concordance rate between the results of the two methods at different time points is shown in Table 1.

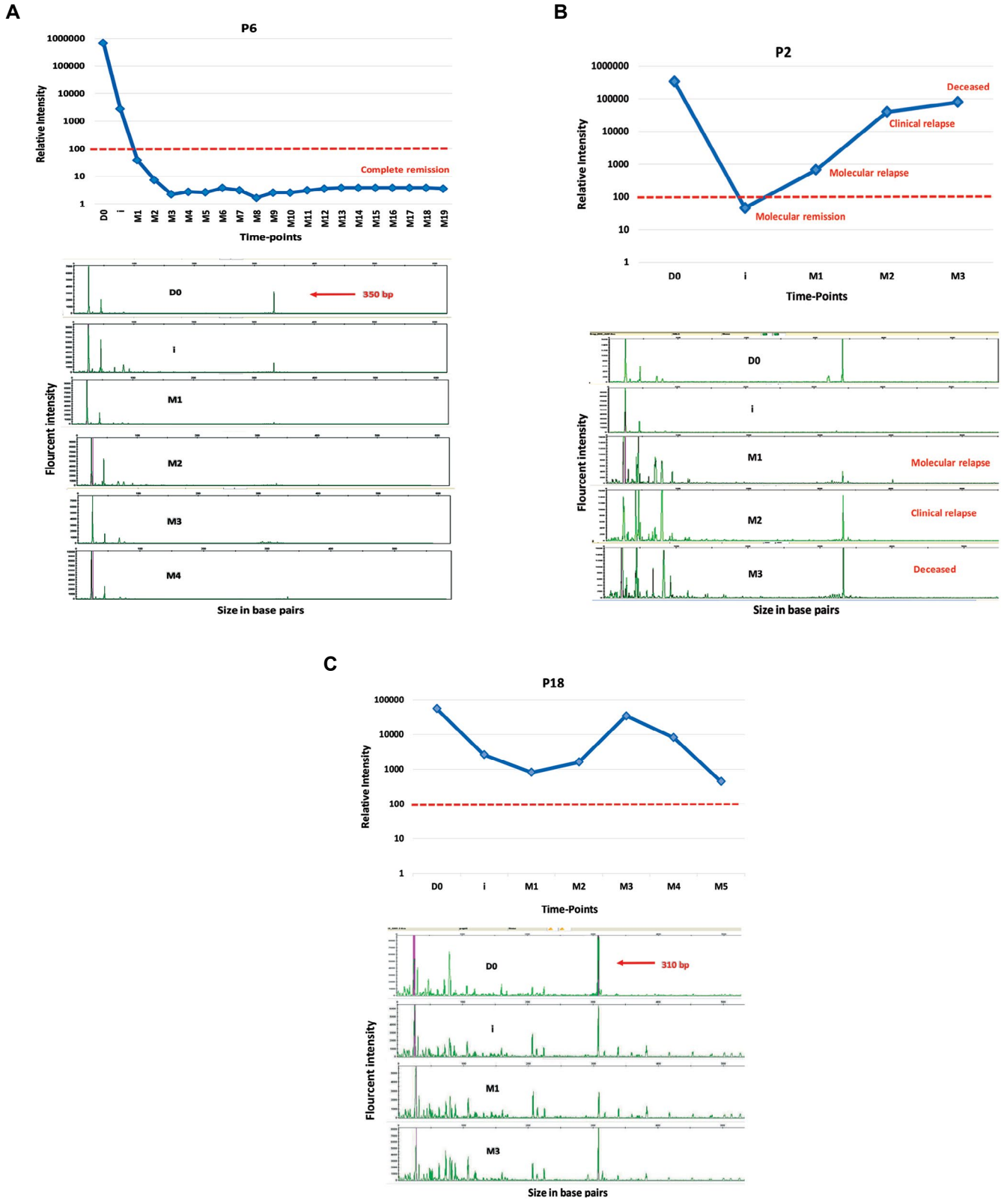

Fig.1: The results of sequential MRD detection by GSA and ASO-qPCR. MRD results were obtained by both PCR-GSA and ASO-qPCR for 256 follow-up PB samples. In electropherograms, the size of each product (in base pairs) is exhibited on the x-axis with its relative fluorescence intensity on the y-axis. The threshold level in the ASO-qPCR method (red dotted line) identifies low and high-risk patients. **A.** The response curves and electropherograms of a pre-B ALL patient that responds to the initial induction chemotherapy, with a significant reduction in the MRD level and without relapse during the follow-up period. **B.** The B-ALL patient attained complete remission after initial induction chemotherapy, but the MRD levels began to rise again in M1. Diagnostic and relapse samples showed identical (clonal VH3–JH) PCR products. **C.** The sequential MRD levels in a pre-B ALL patient without response to chemotherapy, having persistently high levels of MRD. This patient was considered to be in clinical remission; however, the level of MRD remained detectable by both techniques at all time points. P; Patient, D0; 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, MRD; Minimal residual disease, GSA; GeneScanning analysis, ASO-qPCR; Allele-specific oligonucleotide-quantitative PCR, PB; Peripheral blood, and ALL; Acute lymphoblastic leukemia.

Table 1: Concordance between GeneScan PCR and ASO-PCR analyses for MRD detection

Samples	ASO-qPCR and GSA (n)	GSA+/ASO+	GSA-/ ASO-	GSA-/ ASO+	Concordance (%)
Total samples	256	135	87	34	86.7
Diagnosis	35	35	0	0	100.0
During induction, day 14	35	30	5	0	100.0
End of induction, day 28	35	27	8	0	100.0
Consolidation treatment, day 58	33	18	11	4	87.9
Day 88	30	12	13	5	83.3
Other time-points	88	13	50	25	71.6

ASO-PCR; Allele-specific oligonucleotide-quantitative polymerase chain reaction, MRD; Minimal residual disease, and GSA; GeneScanning analysis.

In the previous study, we defined a cut-off level for PCR-based MRD, and our patients were divided into two groups based on this MRD threshold; low- and high-risk groups. During the follow-up period, patients with MRD levels lower or higher than the cut-off level were significantly associated with increased overall survival (OS) or with a progressively worse outcome, respectively (19). The concordance between these two methods was significantly higher above the cut-off; 32 out of 34 dis-concordance were below this cut-off line (Fig.2).

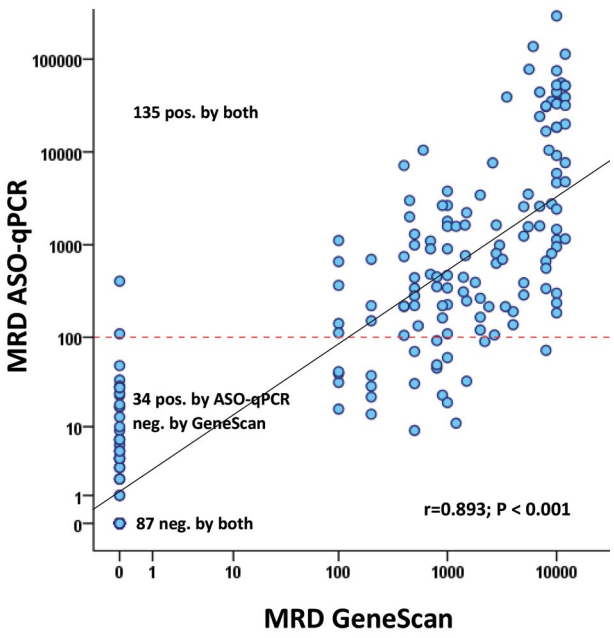

Fig.2: Comparison of MRD results, as obtained by GSA and ASO-PCR. The scatter plot indicates that the overall concordance rate of PCR-GSA and ASO-qPCR MRD results in 256 PB samples at diagnosis and during 2.5-year follow-up was 86.7% (222 out of 256 samples). Most of the discordant results were samples with low level of disease. When the previously defined cut-off level (red dotted line≥ 100) was applied to the ASO-qPCR assay to define high and low-risk patients, only 2 (0.8%) samples were identified above the cut-off level as discordant among the high-risk group. The correlation coefficient was calculated with Spearman's rank correlation test, which indicated a significant correlation between the ASO-PCR and GSA techniques (r=0.893, P<0.001). MRD; Minimal residual disease, GSA; GeneScanning analysis, ASO-qPCR; Allele-specific oligonucleotide-quantitative polymerase chain reaction, and PB; Peripheral blood.

Discussion

Risk stratification is still a puzzling matter of ALL patient management. Patient classification according to regular detection of MRD levels at different time points gives supervision to the clinicians for personalized medicine and adjusting the therapy base on the molecular status of each individual. PCR-based approaches based on molecular rearrangements (*IG/TCR* gene rearrangements) are extensively used as targets for tracing residual leukemic cells in patients with ALL (18), and it has been widely standardized within the European Study Group on MRD diagnosis in ALL (ESG-MRD-ALL) that established guidelines for the analysis and interpretation of RQ-PCR data (27). In our previous study, we designed an ASO for each patient based on the sequence data of complementarity-determining regions (CDR) and used them to quantify MRD levels. The set-up technique was a sensitive and reliable method for the diagnosis of MRD and the result showed a significant relationship between MRD values and clinical outcomes (19). However, this approach was high-cost and labor time-consuming to use in routine practice. In this study, we set up the GeneScanning method which is another PCR-based approach with lower cost and greater ease for the first time for the Iranian ALL patients. The results of MRD detection obtained by GS assay were compared with the ASO-qPCR, to evaluate and validate the capability of the GS technique to routinely employ for MRD detection.

Our results showed that PCR-GSA is an accurate procedure in discriminating monoclonal leukemia-specific *IGH* rearrangements from the normal polyclonal background and it has wide applicability for long-term MRD monitoring in PB samples of ALL patients. In contrast with the ASO-qPCR method which requires nucleotide sequencing analysis and designing patient-specific primers, the PCR-GSA does not need prior knowledge of the type of V and J usage in the VDJ rearrangement, a multiplex PCR with a set of v-region primers with a universal fluorescence-labeled J primer can be used to distinguish monoclonality from polyclonality.

This feature makes it feasible to be performed in large numbers of samples within one working day and enables us to monitor MRD levels in patients without the need for individualized procedures.

The ability of both techniques for the precise detection of the residual leukemic DNA was compared; GSA had a sensitivity of 10^{-3}-10^{-4} for the diagnosis of leukemic cells, which was about 2-log less sensitive than the ASO-qPCR. The overall concordance rate of PCR-GSA and ASO-qPCR MRD results in 256 PB samples at diagnosis and during 2.5-year follow-up was 86.7% (222 out of 256 samples). The correlation analysis demonstrates that there is a significant correlation between the PCR-GSA and ASO-qPCR with a Spearman correlation coefficient of r=0.893.

Under several leading investigations, the presence of MRD following therapy is an important prognostic marker in ALL patients (11). MRD levels at different time points after the therapy can identify patients with different prognostic values. MRD assessment to monitor therapeutic efficacy following the month of the induction therapy has become increasingly important. It has been reported that patients with rapid tumor clearance (on day 28) are associated with an incredibly lower risk of recurrence, while patients with any persevering MRD at the end of consolidation therapy are associated with a remarkably poor prognosis (28-30). In a previous study, we have shown that a low MRD level on day 28 has a statistically significant association with higher OS. In the present study, we achieved 100% agreement for MRD detection between GSA and ASO-qPCR methods in diagnostic samples and samples at the end of induction therapy (day 28). It is interesting to point out that OS on day 28 regarding the patient stratification based on GSA was in line with ASO-qPCR results since most of the patients who were categorized into the high/low-risk groups based on the ASO-qPCR assay were placed in the same group by GSA method.

In all our relapsed cases, the *IGH* gene rearrangement fragments as detected by PCR-GSA at the time of diagnosis and relapse were completely similar nucleotide size. The appearance of one conserved rearranged clonal target in our relapsed patients confirms that the relapse was probably related to the diagnostic clone and no secondary subclone was detected in any of our patients. The false negative result due to clonal evolution phenomena and ongoing secondary rearrangement processes during the course of the disease could confine the effectiveness of VDJ junction sequences as targets for MRD detection (31). An alternative strategy to reduce false-negative results is using two *IGH* rearrangements as ASO-qPCR targets for MRD detection in the clinical setting. However, using two ASO-qPCR targets might be unrealistic, particularly for considerable routine diagnostic laboratories, especially those with restricted technical and financial resources. An alternative more affordable strategy to reduce false-negative results is using GSA along with ASO-qPCR. GSA has the advantage of allowing us to identify the emergence of novel clones (detecting clonal change/evolution) during follow-up that might be neglected during disease by using ASO-qPCR (23-25), causing the relapse occurrence.

The molecular response is greater predictive before the relapse occurrence in patients with ALL, according to several research projects that classified the cases based on the level of MRD (20, 29, 30). In agreement with the extensive report by Germano et al. (23), our data demonstrated that GSA employed in this investigation has great adequacy as a predictive marker. They have shown that GSA sensitivity had been vastly reasonable at diagnosis as well as at relapse. Our study confirms the efficiency of GSA not only in the identification of *IGH* clonal targets for MRD detection at various time points but also in the prediction of relapse. When we compared the obtained data of GSA with the data of our previous project regarding the power of ASO-qPCR in clinical relapse prediction (19), the linearity of results supported the capacity of PCR-GSA to predict the risk of relapse and treatment failure. In both methods, rising MRD levels above the threshold (molecular relapse) on serial monitoring almost always correlated with clinical relapse. The period between molecular and clinical relapse due to the emerging leukemia-specific clonality in PB samples was approximately one month using GSA, which shows this method is less powerful than RT-PCR but still valuable.

Conclusion

The PCR-GSA technique is a rapid technique that could be a valuable tool for monitoring the effectiveness of treatment during induction therapy. Likewise, this method can be used to detect MRD during follow-up and identify high-risk patients who may need to intensify treatments before the occurrence of clinical relapse. Hence, GSA can be served alone or as a complementary method for monitoring MRD along with the ASO-qPCR or multi-parameter flow cytometry assays.

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Authors' Contributions

S.Sh., S.H.Gh.; Conceptualization and Formal analysis. S.Sh., S.Y., Sh.R., F.K., D.B., M.V.; Investigation. S.Sh., Sh.R.; Validation. S.Sh., S.H.Gh., S.Y.; Writing-Original Draft. S.Sh., S.H.Gh., S.Y., D.B., M.V.; Writing-Review and Editing. D.B., M.V., S.H.Gh.; Supervision. S.H.Gh.; Project administration and Funding acquisition. All authors read and approved the final manuscript.

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