Monitoring immunoglobulin heavy chain and T-cell receptor gene rearrangement in cfDNA as minimal residual disease detection for patients with acute myeloid leukemia

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Abstract. The present study aimed to examine whether monoclonal immunoglobulin heavy chain (IGH) or T-cell receptor (TCR) gene rearrangement in cell-free DNA (cfDNA) may be used for minimal residual disease (MRD) monitoring in patients with acute myeloid leukemia (AML). Monoclonal IGH and TCR rearrangement in cfDNA were monitored in patients with AML. A total of 94 (40%) patients had monoclonal IGH or TCR rearrangements in cfDNA at diagnosis; 84% of these patients (79 cases) achieved complete remission following 1-3 courses of induction chemotherapy. Among these cases, 89.9% were negative for monoclonal IGH or TCR rearrangement in cfDNA following consolidation chemotherapies. A total of 8 patients with consistently positive monoclonal IGH or TCR rearrangement in cfDNA relapsed within 6-10 months. During follow up, 39 patients demonstrated positive monoclonal IGH or TCR rearrangement in cfDNA and relapsed. Recurrence of monoclonal IGH or TCR rearrangement in cfDNA was observed 1-3 months earlier than bone marrow relapse and 11 patients with solitary extramedullary relapse demonstrated positive monoclonal IGH or TCR rearrangement recurrence in cfDNA. In conclusion, the detection of monoclonal IGH and TCR rearrangement in cfDNA may represent a useful tool for MRD monitoring in patients with AML.

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Abbreviations: MRD, minimal residual disease; IDAC, intermediate-dose cytarabine; HDAC, high dose cytarabine; AML, acute myeloid leukemia; IHG, immunoglobulin heavy chain; TCR, T-cell receptor; PB, peripheral blood; BM, bone marrow; CD, cluster of differentiation; cfDNA, circulating tumor DNA; DSS, disease-specific survival

Key words: circulating tumor DNA, immunoglobulin heavy chain, T-cell receptor gene, minimal residual disease, rearrangement

Introduction

Circulating tumor DNA, which is tumor-derived soluble cell-free DNA (cfDNA) found in plasma, contains the same mutations as the cellular DNA of the tumor. In 1977, Leon *et al* (1) found that the concentration of cfDNA in patients with cancer was much higher than in normal controls. Several studies have shown that tumor-derived DNA is released into blood and enriched in plasma. As tumor-derived cfDNA contains the same mutations as tumor cellular DNA (2,3), the former may be used as evidence of the presence of a tumor. Using cfDNA as a diagnostic sample represents a novel, convenient, and noninvasive method for tumor detection, as cfDNA derived from tumors possesses mutations specific to these tumors (4). Furthermore, such analyses are expected to provide information regarding minimal residual disease (MRD).

Acute myeloid leukemia (AML) is a highly aggressive hematologic malignancy; MRD monitoring is crucial for the successful management of this disease (5). Currently, two strategies are employed for MRD monitoring of AML-detection of specific gene abnormalities in leukemia cells by quantitative polymerase chain reaction (qPCR) and detection of phenotypic abnormal tumor cells by flow cytometry (6). Several patients with AML harbor recurrent genetic abnormalities of prognostic significance, such as PML-RARα, AML1-ETO, and CBFβ-MYH11. The quantitative measurement of PML-RARα by real-time polymerase chain reaction (PCR) has been extremely useful for MRD monitoring of AML. However, not all AML patients may be monitored using qPCR, and flow cytometry is less satisfactory than qPCR for MRD monitoring (6).

Leukemia cells represent the clonal outgrowth of hematopoietic stem cells arrested at early stages of myeloid differentiation. Numerous patients with AML display surface antigens associated with lymphoid development. In the last few decades, several studies have reported the prognostic utility of lymphoid antigen expression in AML (7). Furthermore, monoclonal rearrangements of immunoglobulin heavy chain (IGH) and/or T-cell receptor (TCR) have been detected in AML (8,9). For most patients with AML without recurrent genetic abnormalities, monoclonal rearrangements of IGH and/or TCR represent useful tools for MRD monitoring. In

the present study, we aimed to use clonal rearrangement of IGH and the TCR gene in cfDNA as MRD markers in AML. Additionally, we determined the incidence of monoclonal IGH and TCR gene rearrangement in patients with AML, using cfDNA as well as DNA from bone marrow (BM) and peripheral blood (PB) samples. Finally, we examined the prognostic utility of these variables and their relationship with early relapse for MRD monitoring.

Patients and methods

Patients. We recruited 235 adult patients diagnosed with AML at our hospital between September 2009 and September 2014 according to the World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissue (4th edition) criteria. Patients with acute promyelocytic leukemia were excluded. PB, BM, and plasma samples were collected and archived before induction chemotherapy, after two and four courses of consolidation chemotherapies, and every 3 months thereafter. Control samples were obtained from 40 patients without malignant hematologic disease. The present study was approved by the ethics committee of Sichuan Academy of Medical Science and Sichuan Provincial Peoples' Hospital (Sichuan, China) according to the Declaration of Helsinki. All patients provided written informed consent.

DNA extraction. PB samples were drawn and then immediately centrifuged at 3,000 rpm for 5 min. Plasma and cells were collected and stored in liquid nitrogen. The mononuclear cells of BM samples were isolated as previously described (8) and stored in liquid nitrogen.

DNA from the PB was extracted using an SBS DNA extraction kit (SBS, Beijing, China) according to the manufacturer's instructions. cfDNA and DNA from BM samples were extracted using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. To enrich the cfDNA, we modified the extraction protocol by adding 10% PEG 8000 solution. Briefly, 20 μl of proteinase K was added to 200 μl of thawed plasma, and the mixed samples were incubated at 56˚C for 30 min. Then, after being cooled to room temperature, an equal volume of 10% PEG 8000 solution was added and the samples were placed at 4˚C for 30 min. Then, 200 μl of 100% ethanol was added to the samples, which were subsequently eluted using the QIAamp spin column, in two equivalent increments. The column was then washed with three different buffers (AW1, AW2, AE; Qiagen GmbH). DNA was eluted to a volume of 50 μl with the final buffer; 10 μg of cfDNA was obtained from 3 ml PB.

PCR. The DNA products from plasma, PB, and BM were analyzed for clonality using the strict quality control protocols established by Kwok and Higuchi (10). In addition, for each experiment, cellular DNA from the normal and empty controls was used as a double-negative control. Raji and Jurkat cells were used as positive controls for monoclonal IGH and TCR rearrangement, respectively. PCR was performed in a total volume of 25 μl containing 12.5 μl of 2x reaction mix (Takara Biotechnology Co., Ltd., Dalian, China), 0.1 μl of golden DNA polymerase, 25 μM primer mix (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; Table I), DNA template (<0.5 μg), and dH_2O. A Bio-Rad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for PCR; the final PCR products were separated on 8% polyacrylamide gels and confirmed by DNA sequencing. A sample was defined as monoclonal if the assay showed a distinct, single band in the appropriate region. If there was no band or smear, the sample was considered polyclonal. Globin was set as an internal reference. Touchdown PCR consisted of an initial denaturation step of 3 min at 95˚C; followed by five cycles of denaturation at 94˚C for 30 sec, annealing for 30 sec from 60˚C to 55˚C decreasing by 1˚C every cycle, and extension at 72˚C for 40 sec; and then 30 cycles of 30 sec at 94˚C, 30 sec at 55˚C, and 40 sec at 72˚C, with a final extension step of 10 min at 72˚C. The *IGH* gene was amplified by PCR using a mixture of oligonucleotides specific for each of the VH leader sequences of the VH1-6 (11) gene families, together with a mixture of oligonucleotides complementary to all possible JH gene segments (JH1-6). Amplification consisted of an initial denaturation step of 5 min at 94˚C for 1 min, annealing at 61˚C for 1 min, and extension at 72˚C for 1 min, with a final extension step of 10 min at 72˚C. In the second round (nested PCR), 1 μl of amplified DNA (first-round product) was re-amplified using oligonucleotides representative of framework regions (FWR)-2 and -3 (FWR2/FWR3) together with the mixture of JH1-6 oligonucleotides as primers. PCR was carried out as described above. Amplification consisted of an initial denaturation step of 5 min at 94˚C, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, and extension at 72˚C for 1 min, with a final extension step of 10 min at 72˚C. Touchdown PCR for the TCR gene consisted of an initial denaturation step of 3 min at 95˚C; followed by five cycles of denaturation at 94˚C for 30 sec, annealing for 30 sec from 60˚C to 55˚C decreasing by 1˚C every cycle, extension at 72˚C for 40 sec; and then 30 cycles of 30 sec at 94˚C, 30 sec at 55˚C, 40 sec at 72˚C, with a final extension step of 10 min at 72˚C.

PCR product analysis. PCR products were analyzed by 5% agarose electrophoresis. A distinct band was interpreted as a clonal rearrangement. If the results were unclear, polyacrylamide gels were used to confirm monoclonal rearrangement. DNA sequencing was performed to validate that the PCR products corresponded to clonal IGH or TCR rearrangements, and that the initial monoclonal rearrangement reappeared in follow-up samples. Furthermore, in follow-up samples, clone conformity of AML samples at diagnosis and before relapse was tested through capillary electrophoresis.

Multiparameter flow cytometry. Assessment of MRD by multi-parameter flow cytometry (MFC) was conducted as previously described (12). A minimum of 1000,000 events were acquired to achieve a potential sensitivity level of 10-4. In each tube, after excluding debris and doublets, an initial wide gate (CD45dim gate) is drawn around the CD45dim blast and monocyte regions on a traditional CD45/SSC display, followed by back-gating to identify the CD34+ population, as well as CD117 cells on the CD45/SSC plot. CD19 is used as an exclusion gate on most plots, to separate out normal immature precursor B cells and plasma cells from the CD45dim gate.

Table I. Clinical characteristics at presentation for patients in the *IGH*neg and *TCR*neg, *IGH*posor *TCR*pos, *IGH*pos and *TCR*pos genotype groups.

Characteristics	IGHneg and TCRneg	IGHposor TCRpos	IGHpos and TCRpos	P-value
Hemoglobin (g/dl)				
Median	86.0	77.5	74.0	0.094
Range	59-192	64-131	5-106	
Platelets (10^9/l)				
Median	42	37	24	0.16
Range	6-208	4-135	2-87	
WBC (10^9/l)				
Median	14.29	40.99	213.23	0.010
Range	1.13-39.37	19.86-114.65	63.18-277.80	
PB Blast (%)				
Median	52	71	81	0.05
Range	0-96	11-90	69-91	
BW blasts (%)				
Median	61	64	79	0.29
Range	23-87	37-85	50-82	
Serum LDH				
Median	369	560	1725	0.010
Range	92-949	225-1203	581-5450	
Immune marker				
CD34	80	90	100	0.40
HLA-DR	80	90	100	0.40
CD13	76	90	100	0.39
CD33	75	88	100	0.36
CD117	68	87	100	0.35
CD7	36	40	100	0.024
CD19	15	17	75	0.016
CD56	0	6	50	0.010
CD4	0	0	25	0.011
Hepatomegaly	4	6	11	0.32
Splenomegaly	4	13	12	0.17
Lymphadenopathy	13	6	11	0.86
Gum hypertrophy	23	12	12	0.61
Skin infiltrates	9	12	12	0.87

WBC, white blood cell; PB, peripheral blood; BM, bone marrow; LDH, lactate dehydrogenase; CD, cluster of differentitation; IGH, immunoglobulin heavy chain; TCR, T-cell receptor.

qPCR. qPCR was used for analysis of the status of rearranged IGH and TCR over time in remittent patients, as well as to monitor MRD. The primers used in the present study are shown in Table II. The results of DNA sequencing were inputted into VBASE database or IMGT database to acquire missed or inserted bases. Primer3.0 and BLAST were used to design primers and probes. The procedure for amplification included establishment of standard curves by both serial dilution of DNA templates from patients undertaking initial therapy (10:1 to 10:6) targeting specific IGH/TCR rearrangements and serial dilution of DNA templates from normal individuals (10:1 to 10:4) targeting the internal control gene encoding albumin. qPCR was performed using the following protocol: 95˚C for 60 sec; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The standard curves of IGH/TCR for each patient and the control gene encoding albumin were obtained after qPCR.

qPCR was performed using TaqMan probe detection of both genes at different time points during therapy, and further quantification was carried out based on the expression and proportion of both genes, reflecting the MRD level. The positive standard for MRD was relative IGH/TCR expression of at least 10:4 according to the European Study Group criteria (13).

Therapeutic regimen. All patients received standard induction chemotherapy, i.e., doxorubicin 60 mg/m^2 or idarubicin 12 mg/m^2 administered intravenously (i.v.) for 3 days and

Table II. Oligonucleotide primers used for PCR.

Gene name	Primer	Sequence (5'-3')
β-Globin	Globin-F	GATCTGTCCACTCCTGATGCTG
	Globin-R	ATCAAGCGTCCCATAGACTCAC
IGH	VH1	CAGGRGCAGCTGGTGCAGTCTGG
	VH2	CAGGTCAACTTAAGGGAGTCTGG
	VH3	GAGGTGCAGCTGGTGGAGTCTGG
	VH4	CAGGTGCAGCTGCAGGAGTCGGG
	VH5	GAGGTGCAGCTGTTGCAGTCTGC
	VH6	CAGGTACAGCTGCAGCAGTCAGG
	JH1-2	TGAGGAGACGGTGACCAGGGTGCC
	JH3	TGAAGAGACGGTGACCATTGTCCC
	JH4-5	TGAGGAGACGGTGACCAGGGTTCC
	JH6	TGAGGAGACGGTGACCGTGGTCCC
	FWR2	TGGATCCGACAGGCCCCAGGG
	FWR3	ACACGGCCGTGTATTACTGT
TCRB	Vβ2:	AACTATGTTTTGGTATCGTCA
	Vβ4:	CACGATGTTCTGGTACCGTCAGCA
	Vβ5/1:	CAGTGTGTCCTGGTACCAACAG
	Vβ6a/11:	AACCCCTTTATTGGTACCGACA
	Vβ6b/25:	ATCCCTTTTTTGGTACCAACAG
	Vβ6c:	AACCCTTTATTGGTATCAACAG
	Vβ7:	CGCTATGTATTGGTACAAGCA
	Vβ8a:	CTCCCGTTTTCTGGTACAGACAGAC
	Vβ9:	CGCTATGTATTGGTATAAACAG
	Vβ10:	TTATGTTTACTGGTATCGTAAGAAGCC
	Vβ11:	CAAAATGTACTGGTATCAACAA
	Vβ12a/3/13a/15:	ATACATGTACTGGTATCGACAAGAC
	Vβ13b:	GGCCATGTACTGGTATAGACAAG
	Vβ13c/12b/14:	GTATATGTCCTGGTATCGACAAGA
	Vβ16:	TAACCTTTATTGGTATCGACGTGT
	Vβ17:	GGCCATGTACTGGTACCGACA
	Vβ18:	TCATGTTTACTGGTATCGGCAG
	Vβ19:	TTATGTTTATTGGTATCAACAGAATCA
	Vβ20:	CAACCTATACTGGTACCGACA
	Vβ21:	TACCCTTTACTGGTACCGGCAG
	Vβ22:	ATACTTCTATTGGTACAGACAAATCT
	Vβ23/8b:	CACGGTCTACTGGTACCAGCA
	Vβ24:	CGTCATGTACTGGTACCAGCA
	Jβ1.1:	CTTACCTACAACTGTGAATCTGGTG
	Jβ1.2:	CTTACCTACAACGGTTAACCTGGTC
	Jβ1.3:	CTTACCTACAACAGTGAGCCAACTT
	Jβ1.4:	CATACCCAAGACAGAGCTGGGTTC
	Jβ1.5:	CTTACCTAGGATGGAGAGTCGAGTC
	Jβ1.6:	CATACCTGTCACGATGAGCCTG
	Jβ2.2:	CTTACCCAGTACGGTCAGCCT
	Jβ2.6:	CTCGCCCAGCACGGTCAGCCT
	Jβ2.7:	CTTACCTGTAACCGTGAGCCTG
	Jβ2.1:	CCTTCTTACCTAGCACGGTGA
	Jβ2.3:	CCCGCTTACCGAGCACTGTCA
	Jβ2.4:	CCAGCTTACCCAGCACTGAGA
	Jβ2.5:	CGCGCACACCGAGCAC
TCRγ	Vγ1f:	GGAAGGCCCCACAGCGTCTT
	Vγ10:	AGCATGGGTAAGACAAGCAA

Table II. Continued.

Gene name	Primer	Sequence (5'-3')
	Vγ9:	CGGCACTGTCAGAAAGGAATC
	Vγ11:	CTTCCACTTCCACTTTGAAA
	J γ1.1/2.1:	TTACCAGGCGAAGTTACTATGAGC
	J γ1.3/2.3:	GTGTTGTTCCACTGCCAAAGAG
albumin	abm-F	GCT GTC ATC TCT TGT GGG CTG T
	abm-R	AAA CTC ATG GGA GCT GGT T
	abm-P	CCT GTC ATG CCC ACA CAA ATC TCT CC

PCR, polymerase chain reaction; F, forward; R, reverse; abm, albumin; V, variable zone; H, heavy chain; J, J chain; IGH, immunoglobulin heavy chain; TCRB, T cell receptor beta chain; TCRG, T cell receptor gamma chain; FR, frame region.

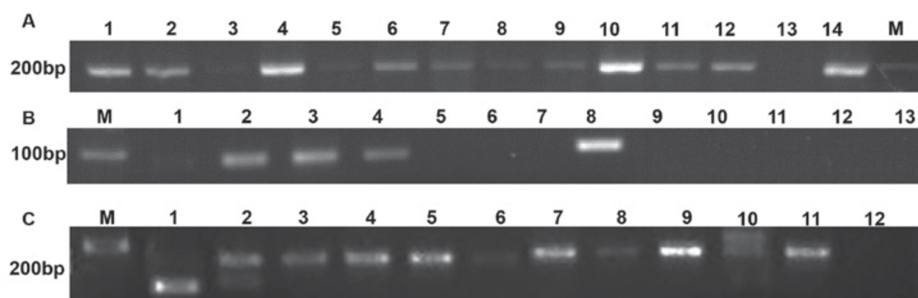

Figure 1. Example of monoclonal IGH and TCR rearrangement in cfDNA. (A) Detection of Globin DNA in the plasma of patients with AML. Lane M, DNA marker. Lanes 1, 2, 4, 6-12, 14 PCR product of cfDNA from AML patients. Lanes 3, 5 and 13 PCR product of cfDNA from normal control. (B) Detection of monoclonal TCR rearrangement in cfDNA of patients with AML. Lane M, DNA marker. Lane 1, negative result from AML patient. Lane 8, positive control (Raji cells). Lanes 2-4, positive results from AML patients. Lanes 5-7 and 9-13, negative results from AML patients. (C) Detection of monoclonal IGH rearrangement in the cfDNA of patients with AML. Lane M, DNA marker. Lanes 6 and 8, negative result from AML patient. Lanes 1-5 and 9-11, positive result from AML patients. Lane 12, negative control. Lane 7, positive control (Jurkat cells). IGH, immunoglobulin heavy chain; TCR, T-cell receptor; AML, acute myeloid leukemia; cfDNA, cell free DNA; PCR, polymerase chain reaction.

cytarabine 200 mg/m^2 in 24-h continuous i.v. infusion for 7 days. Dual induction was administered if BM blasts decreased more than 50% but with residue blasts exceeding 10% at 7 days after completion of first induction. High-dose cytarabine was administered for eligible patients if BM blasts were reduced to less than 50% at 7 days after completion of initial induction. After complete remission (CR), 4-6 cycles of intermediate-dose cytarabine or high-dose cytarabine were given for consolidation. Patients with suitable donors received allogeneic hematopoietic stem cell transplantation.

Statistical analysis. Categorical variables, such as cluster of differentiation (CD) and leukemic involvement of skin, gum, liver, and spleen, were compared using the chi squared test and Fisher's exact tests. Continuous variables, such as platelets, hemoglobin, and white blood cell (WBC) count, were compared using the Kruskal-Wallis test followed by a Nemenyi multiple comparisons post hoc test. Pairwise comparisons of cfDNA vs. BM DNA and cfDNA vs. PB DNA were conducted using McNemar's tests. Overall survival (OS) was determined from the date of diagnosis to death or last follow-up. The Kaplan-Meier estimator was used to estimate OS rates, and log-rank tests were used for comparison. All statistical analysis was performed using IBM SPSS v22.0 software (IBM Corp., Armonk, NY, USA).

Results

Detection of monoclonal IGH and TCR rearrangements at diagnosis. cfDNA was successfully isolated from all 235 patients, and no cfDNA was extracted from the normal control group (Fig. 1A). In cfDNA samples, 94 cases showed monoclonal IGH and/or TCR rearrangement (40%, 94/235); among these cases, 73 showed monoclonal IGH rearrangement (31.1%, 73/235), 21 showed monoclonal TCR rearrangement (8.9%, 21/235), and nine showed both monoclonal IGH and TCR rearrangement (Fig. 1B and C). Monoclonal rearrangement was present at a significantly higher frequency in patients with AML-M4 and -M5 than other type AML (P=0.01).

There were no differences between cfDNA and BM DNA in terms of IGH (χ^2=1.32, P=0.25) or TCR (χ^2=0.45, P=0.5) rearrangement, and there were no significant differences between cfDNA and PB DNA in terms of IGH (χ^2=1.32, P=0.25) or TCR (χ^2=2.71, P=0.1) rearrangement.

Clinical characteristics of patients with AML having different genotypes. Next, we compared the clinical characteristics of patients with IGH-negative (IGH^{neg})/TCR-negative (TCR^{neg}), IGH-positive (IGH^{pos}) or TCR-positive (TCR^{pos}), and IGH^{pos}/TCR^{pos} genotypes. There were no significant differences in hemoglobin, platelets, and other clinical characteristics

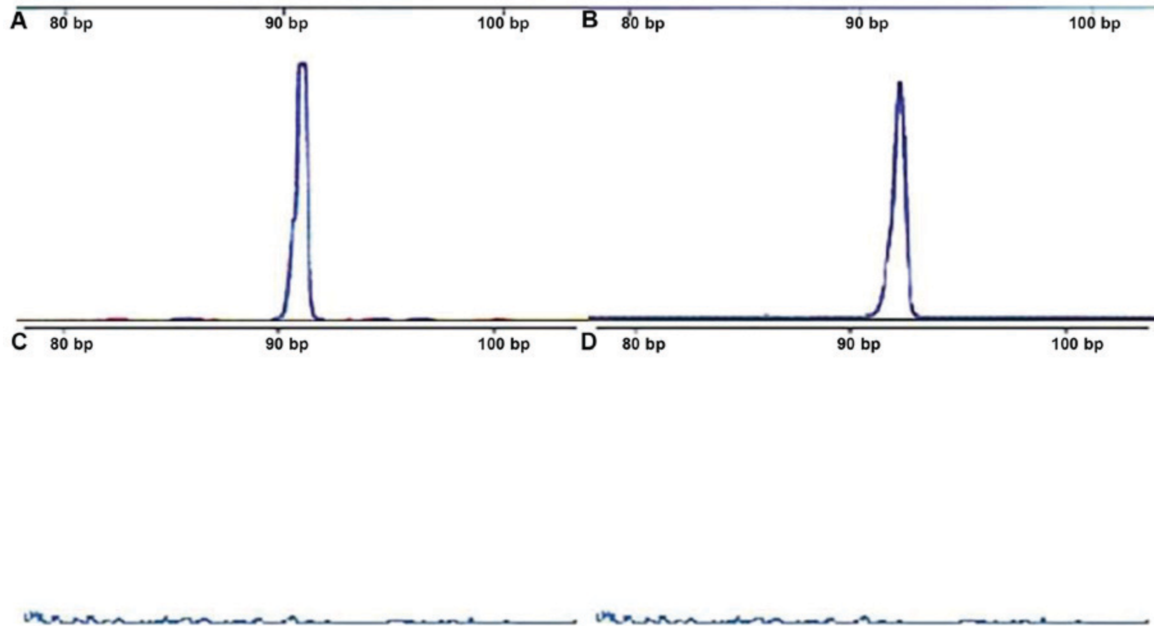

Figure 2. Demonstration of a case with dynamic monitoring minimal residual disease by cfDNA. (A) Monoclonal IGH rearrangement in a BM sample at diagnosis. (B) Identical monoclonal IGH rearrangement in cfDNA prior to relapse. (C) Negative result of BM sample at the same time. (D) Negative result of PB sample at the same time. cfDNA, cell free DNA; BM, bone marrow; IGH, immunoglobulin heavy chain; PB, peripheral blood.

such as gum hypertrophy, lymphadenopathy, splenomegaly, and hepatomegaly, across all three groups (Table I). The median WBC counts were significantly different between the three groups, ranging from $14.29x10^9$/l to a maximum of $213.23x10^9$/l. However, the increases in the percentages of PB leukemic blasts and BM blasts were not statistically significant. Serum lactate dehydrogenase (LDH) levels increased from 369 IU/l in patients in the IGH^{neg}/TCR^{neg} group to 560 IU/l in patients in the IGH^{pos} or TCR^{pos} group and reached a maximum in patients in the IGH^{pos}/TCR^{pos} group (1725 IU/l, P=0.01). Only CD7, CD19, CD56, and CD4 showed differential expression in the three groups, with increased frequency observed in the IGH^{pos}/TCR^{pos} group. No association between CD7, CD19, CD56 and CD4 expression on blast cells and clinical outcomes was observed (data not shown).

Detection of monoclonal IGH and TCR rearrangements in remission. Among 94 patients with monoclonal IGH and/or TCR rearrangements, 44 exhibited abnormal cytogenetics; 5 patients had t(8;21), 2 had inv(16), 7 had complex abnormality, 7 had del5(q31), 3 had -5, 6 had del7(q22), 4 had -7, 2 had -y, 1 had der(1;14) and +1, 2 had t(8;16), 3 had t(6;11), and 3 had t(9;11). There were 6 patients for whom cytogenetic results could not be obtained as unqualified samples, and the remaining 44 patients had normal cytogenetic results. The molecular genetic results for these 94 patients were as follows: 12 patients carried the AML1-ETO fusion gene, 3 carried the CBFβ-MHY11 fusion gene, 3 carried the MLL-AF6 fusion gene, 1 carried the CEBPA double mutation, 2 carried the CEBPA single mutation, 13 carried the NPM1 mutation, 4 carried the FLT3-ITD mutation, 4 carried the IDH2R140 mutation, 8 carried the IDH2R170 mutation, 9 carried the DNMT3A mutation, 14 carried the TET2 mutation, 3 carried the TP53 mutation, and 1 carried the NRAS/RUNX1 mutation. Thirty-one patients showed normal results for molecular genetic tests; for 9 patients, results could not be obtained as the samples were unqualified. There were 21 low-risk, 40 intermediate-risk, and 33 high-risk patients according to ELN category (13) based on the cytogenetic and molecular genetic abnormalities detected.

After one to two courses of induction chemotherapy 79 patients achieved CR. Among these, 24 patients received allo-HSCT and 55 patients received chemotherapy only. The donors for patients who received allo-HSCT were as follows: One unrelated matched donor, six related matched donors, and 17 related HLA-haplotype-mismatched donors. The conditioning regimens and GVHD prophylaxes were as previously reported, with minor modification (we used short-term cyclophosphamide 600 mg/m^2 on day +1, 400 mg/m^2 on days +3, +5, +11 instead of methotrexate) (14). The median follow-up was 27 months and the range was 7.5 to 60 months. There were 37 recorded relapses and 34 deaths. During follow up, 71 patients were negative for monoclonal IGH and TCR rearrangements in PB DNA, BM DNA, and cfDNA after 2-4 courses of consolidation chemotherapy, and eight patients with continuous positive monoclonal rearrangements relapsed within 6-10 months. Thirty-two patients were continuously negative for monoclonal IGH and TCR rearrangements during follow-up both from cfDNA and BM cells, and were in continuous CR with negative MRD confirmed by BM aspiration and flow cytometry. For the remaining 39 patients, monoclonal IGH or TCR rearrangements were positive both in cfDNA and in BM DNA prior to clinical relapse (median 28 days prior to overt clinical relapse, range: 15-59 days). There were 11 patients with extramedullary relapse who showed positive IGH/TCR rearrangements in cfDNA but not in BM DNA or PB DNA (Fig. 2). The change occurred earlier in cfDNA than in BM cells (median, 31 days; Fig. 3).

Prognostic impact of monoclonal IGH and TCR rearrangement status in cfDNA on OS and LFS. For patients who achieved CR,

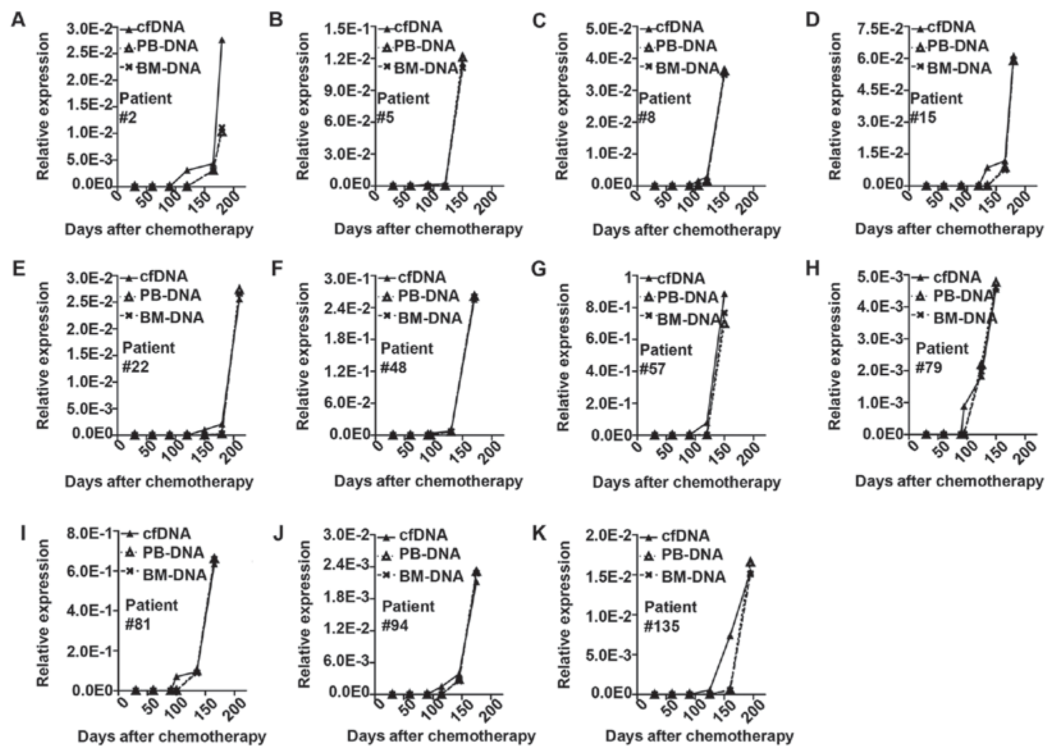

Figure 3. Representative cases of minimal residual disease monitored by monoclonal IGH or TCR rearrangement in cfDNA, BM and PB. The solid line represents change in cfDNA. The long dotted line represents change in BM. The short dotted line represents change in PB. (A) Patient no. 2, monoclonal IGH rearrangement in cfDNA increased at the 120 day after chemotherapy, while in BM and PB it increased at day 165. The clinical relapse occurred at day 180. (B) Patient no. 5 IGH in cfDNA at day 90, in BM and PB day 120, clinical relapse day 150. (C) Patient no. 8 TCR in cfDNA day 105, in BM and PB day 120, clinical relapse day 150. (D) Patient no. 15 IGH in cfDNA day 135, in BM and PB day 165, clinical relapse day 180. (E) Patient no. 22IGH in cfDNA day 150, in BM and PB day 180, clinical relapse day 210. (F) Patient no. 48 IGH in cfDNA day 95, in BM and PB day 130, clinical relapse day 170. (G) Patient no. 57 IGH in cfDNA day 90, in BM and PB day 120, clinical relapse day 150. (H) Patient no. 79 IGH in cfDNA day 95, in BM and PB day 125, clinical relapse day 150. (I) Patient no. 81 IGH in cfDNA day 100, in BM and PB day 135, clinical relapse day 165. (J) Patient no. 94 IGH in cfDNA day 115, in BM and PB day 145, clinical relapse day 175. (K) Patient no. 135 IGH in cfDNA day 125, in BM and PB day 160, clinical relapse day 195. IGH, immunoglobulin heavy chain; TCR, T-cell receptor; cfDNA, cell free DNA; BM, bone marrow; PB, peripheral blood.

we performed survival analysis based on the monoclonal IGH and TCR rearrangement status in cfDNA. We found that the monoclonal IGH and/or TCR rearrangement status in cfDNA was in agreement with MRD status (Table III). And there were more negative status in cfDNA been found in low risk patients, followed by intermediate risk patients, and least in high risk patients (Table III). We found that patients with monoclonal IGH or TCR rearrangement in cfDNA at any time point had worse outcomes, with a 21 months median LFS and 27 months median OS, whereas patients with persistent negative monoclonal IGH or TCR rearrangement showed significantly improved outcomes (median LFS and OS not reached; $P<0.01$; Fig. 4A and B). The difference in survival based on monoclonal IGH or TCR rearrangement was similar to that of MRD, as indicated by flow cytometry analysis (Fig. 4C and D).

Discussion

In the present study, we found that tumor-derived DNA could be collected from the plasma of patients with AML whose tumor cells were informative on PCR analysis. cfDNA cannot be detected in healthy individuals (limit of detection <100 μg/ml) (15). Some reports have described the successful amplification of genomic or tumor-associated DNA from fresh or archived plasma samples, which supports our observations (16). Moreover, cfDNA may be associated with highly proliferative tumor cells. Treatment of patients with cytotoxic drugs is followed by rapid clearance of DNA from the PB. These findings suggest that soluble tumor-derived DNA can only be tested during specific disease stages and may be highly predictive of resistance to treatment and impending relapse (17). Degradation of clonal DNA by nucleases *in vitro* was shown to be one cause of false-negative PCR results. This technical drawback may be overcome by adding a nuclease inhibitor such as ethylenediaminetetraacetic acid (EDTA). Thus, the use of EDTA-anticoagulant tubes is recommended.

PCR amplification of IGH and TCR rearrangement may be helpful for diagnosing and predicting the prognosis of hematologic malignancies. If DNA shows a distinct band with the same electrophoretic mobility, the sample is defined as monoclonal. Normal DNA and reactive hyperplastic DNA are polyclonal with smeared bands (18). Recent studies have reported that the positive ratios may be as high as 99% for IGH and 94% for TCR using multiplex primers (19). However, the specific frequency of antigen receptor gene rearrangement in AML is difficult to calculate from previously reported data (20) because of the selection of specific AML subtypes and the use of different experimental protocols with markedly different sensitivities. Based on the experiences of our group and others, we adopted a sensitive two-round amplification method; touchdown PCR may also be performed for analysis of large samples.

Table III. Association between cfDNA status and MRD as determined by flow cytometry and ELN risk group based on molecular genetic and cytogenetic alterations.

	MRD status n (%)		ELN risk group n (%)		
cfDNA status	MRD-	MRD+	Low risk	Intermediate risk	High risk
cfDNA-	23 (71.8)	9 (28.2)	14 (73.7)	8 (38.1)	10 (25.6)
cfDNA+	12 (25.5)	35 (74.5)	5 (26.3)	13 (61.9)	29 (74.4)

cfDNA, cell free DNA; MRD, minimal residue disease; ELN, Europe leukemia net.

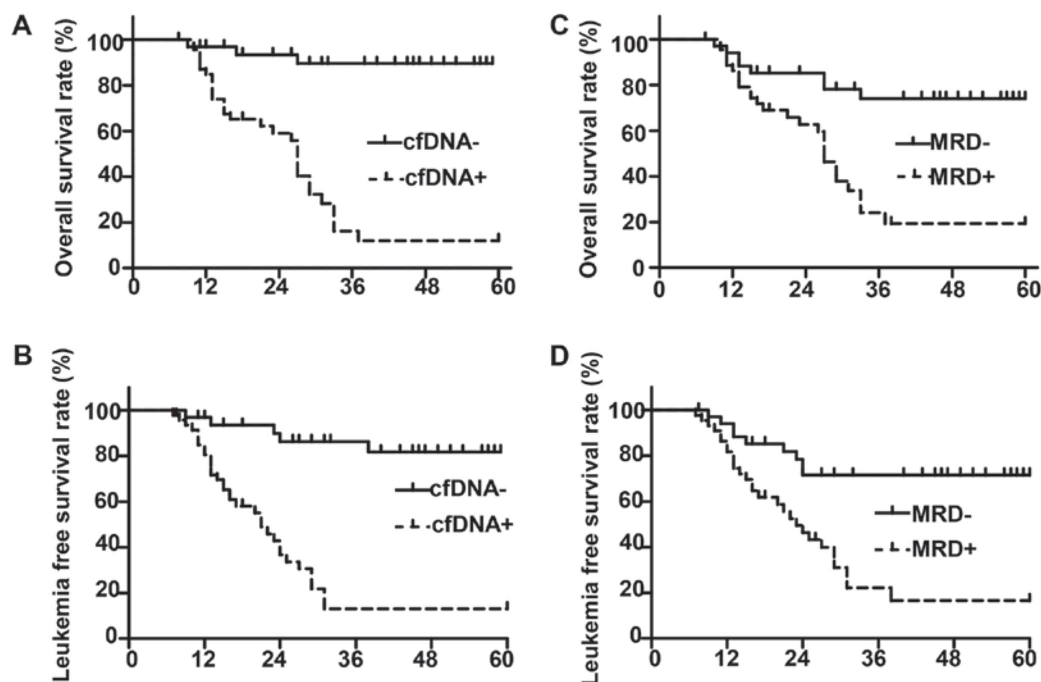

Figure 4. Survival according to IGH and TCR results in cfDNA. (A) Overall survival according to cfDNA status. (B) Overall survival according to MRD status. (C) Leukemia free survival according to cfDNA status. (D) Leukemia free survival according to MRD status. IGH, immunoglobulin heavy chain; TCR, T-cell receptor; MRD, minimal residual disease; AML, acute myeloid leukemia; MRD+, AML patients with positive MRD by flow cytometry at any time point; MRD-, AML patients with persistent negative MRD by flow cytometry; cfDNA-, AML patients with persistent negative monoclonal IGH or/and TCR rearrangement; cfDNA+, AML patients with positive monoclonal IGH or/and TCR rearrangement at any time point.

Our results confirmed that some patients with AML harbor monoclonal IGH and TCR rearrangements; this phenomenon is referred to as distortion of series or lineage promiscuity. There are three explanations for this phenomenon: first, most scholars believe that distortion may be related to pluripotent stem cells. Cells with crossed rearrangement originate from pluripotent stem cells; further, tumors may promote changes in stem cells, allowing them to develop into myeloid cells. Therefore, based on morphology, tumor cells still exhibit myeloid characteristics. However, transient mixed gene expression is still observed (21). Second, tumor cells usually exhibit disruption of normal proliferation and differentiation. Hematopoietic stem cells in patients with hematological malignancies may only exhibit gene clonal rearrangement during the process of maturation to a specific lineage; however, these cells do not successfully differentiate into the specific cell lineage, resulting in functional disorders (22). Consequently, IGH and TCR monoclonal rearrangement may represent markers for the monitoring of some patients with AML, specifically those without recurrent genetic abnormalities.

Cen *et al* (23), proposed that IGH and TCR rearrangements rarely occur in M4 and M5 subtypes. In contrast, Boeckx *et al* (24) suggested that IGH and TCR rearrangements are irrelevant to FAB classification. Our results showed that these rearrangements were more frequent in M4 and M5 subtypes. However, additional large-scale studies are needed. These above-described results suggest that malignant clones tend to behave abnormally, providing evidence for biological differences between cases lacking clonally rearranged IGH and TCR genes. Importantly, we did not find any healthy individuals positive for monoclonal rearrangements. Patients with the IGHpos/TCRpos genotype showed far higher WBC counts and serum LDH levels and exhibited more frequent occurrence of lymphoid-associated markers such as CD7, CD19, and CD56. Their disease-specific survival rates were also significantly

lower. These findings supported the poor prognoses of these patients. Similarly, Qiu *et al* (25) reported that IGH and TCR gene rearrangements were adverse factors in patients with AML. Therefore, detection and monitoring of IGH and TCR rearrangements are critical in patients with AML; cfDNA may have applications in such monitoring procedures. The use of cfDNA as a sample to detect IGH and TCR rearrangements is also a more convenient and noninvasive method than the use of other types of samples, and is expected to yield the same clinical information as biopsy samples.

Furthermore, we found that gene rearrangements identified using cfDNA were correlated with pathological results, suggesting that the use of cfDNA as a sample enables high accuracy. Importantly, IGH and TCR rearrangements were detected earlier in cfDNA than in BM cells. In a total of 11 patients, significantly increased IGH and TCR rearrangements were detected only in cfDNA, but not in BM cell DNA, prior to clinical relapse. Moreover, the relapsed patients who had increased IGH and TCR rearrangements in BM cells prior to clinical relapse succumbed to their disease. However, the elevated levels of monoclonal IGH or TCR rearrangement could be detected earlier and more reliably using cfDNA than using BM cells for the monitoring of MRD. These data suggest that plasma is more enriched with leukemic cell nucleotides than with normal nucleotides. BM cell aspirates almost always contain residual normal cells in addition to leukemic cells, with variations dependent on the stage of the disease. Unfortunately, it is impossible to determine the minimum number of leukemic cells required in the BM to yield positive plasma PCR results. We assumed that even extramedullary leukemic cells provide nucleotides that may be detected during cfDNA analysis. Data from studies of solid tumors support the concept that plasma/serum samples are enriched with tumor-specific cfDNA (26,27). This enrichment is perhaps attributable to the high turnover of tumor cells compared with that in normal tissues. Plasma samples from patients with leukemia may be more enriched with leukemia-specific cfDNA than those from patients with solid tumors, as the cells in the former have greater contact with circulating blood. Furthermore, monitoring cfDNA may be more important for predicting leukemic extramedullary relapse than BM relapse. The extramedullar tissues or organs may show evidence of relapse, while the BM still shows CR. Extramedullary relapse is common in the central nervous system, reproductive system, and skin infiltrations such as green tumors, and can exist alone, but often predicts overall leukemia recurrence. Treatment of the impending relapse should be initiated in advance, if possible. Overall there was no difference between cfDNA and BM in terms of predicting an upcoming relapse when analyzing remission samples. At present, the methods usually used to isolate bone marrow mononuclear cells (BMNCs) are density gradient centrifugation, flow cytometry and immunomagnetic beads. The purity of BMNCs was higher using immunomagnetic beads or flow cytometry than density gradient centrifugation but with much higher cost. We used Ficoll density gradient centrifugation to concentrate BMNC with lower cost, and then used qPCR for amplification in order to assure the sensitivity and specificity. And the other advantage of using cfDNA for MRD detection compared with MFC is that the sampling procedure for cfDNA is simpler than flow cytometry since we need only blood to extract cfDNA while we need BM for flow cytometry. As shown in our results for patients with solitary extramedullary relapse the cfDNA is superior compared to flow cytometry.

There are some limitations of our study. First with much larger sample size the association of monoclonal IGH and/or TCR rearrangement and CD markers of blast and cytogenetics and molecular abnormalities would be clearer; second using Ficoll density gradient centrifugation may lead to contamination by lymphocytes which may cause false positive result due to oligo clones of reactive lymphocytes, hopefully a repeated test would lower the risk; last bias would be limited to the minimum with a clinical trial cohort.

In conclusion, the use of circulating tumor DNA may provide a useful, noninvasive approach for the detection of tumor cells that secrete DNA. In hematologic malignancies, particularly leukemia, tumor cells frequently circulate in the blood; this enables the direct examination of blood cells for the presence of molecular markers at the DNA/RNA level or aberrant protein expression. Our findings suggest that analysis of circulating plasma DNA may be useful in cases in which PB cellular analysis is negative and the BM is positive for the disease.

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Availability of data and material

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and TJ conceived the study and wrote the manuscript. LZ and YXL performed the majority of the experiments. TJ, JC and XBH collected and analyzed the clinical data. YXL, JC and XBH critically revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sichuan Academy of Medical Science and Sichuan Provincial Peoples' Hospital (Sichuan, China). All patients provided written informed consent prior to their inclusion within the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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