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Comparison of spatial chromosomal organization between bone marrow and peripheral blood in acute myeloid leukemia

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Abstract. Acute myeloid leukemia associated with t(8;21)(q22;q22)/runt related transcription factor (RUNX)1-RUNX1 translocation partner 1 has been reported to exhibit a favorable outcome. The quantitative polymerase chain reaction is a reliable method for assessing minimal residual disease persistence, and peripheral blood (PB) samples are as informative as bone marrow (BM) samples during follow-up monitoring. However, few studies have compared the spatial organization of leukemia-specific chromosomes between BM and PB. In the present study, paired BM and PB samples were extracted from 6 patients with acute myeloid leukaemia-M2 and compared using three-dimensional fluorescence *in situ* hybridization. Cells were classified into three types: Normal, proximal and malignant. Comparisons of proportions (% of all cells) of different cell types revealed no significant difference between BM and PB samples. The relative radial positions (RRPs; *d*/*R*) of chromosomes 8 and 21 were consistent for 2/3 of BM and PB samples. The RRPs of chromosomes in proximal pairs were more interior in nuclei compared with chromosomes in normal pairs for BM and PB samples. The consistency of the spatial organization of chromosomes between BM and PB suggests that PB may be an alternative to BM for research and clinical diagnosis.

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Abbreviations: NP, normal pair; PP, proximal pair; NC, normal cell; PC, proximal cell; MC, malignant cell; RRP, relative radial position

Key words: acute myeloid leukemia, spatial organization, bone marrow, peripheral blood, three-dimensional fluorescence *in situ* hybridization

Introduction

Core-binding factor (CBF) acute myeloid leukemia (AML) associated with t(8;21; q22;q22)/runt related transcription factor (RUNX)1-RUNX1 translocation partner 1 (T1) or inv(16; p13q22)/t(16;16; p13;q22)/CBFβ subunit-myosin heavy chain 11 has been reported to exhibit a favorable outcome (1-3). Jourdan *et al* (4) reported that, compared with gene mutations, minimal residual disease (MRD) levels play a more important role in predicting early relapse in patients with CBF-AML. Two popular approaches to detect MRD persistence are quantitative polymerase chain reaction (qPCR) and multiparametric flow cytometry, which possess increased submicroscopic sensitivity compared with that possessed by conventional morphology (5-7). Although multiple studies are attempting to stratify patients into low- or high-risk relapse groups, the threshold to do so remains controversial (8-11). Since it is affected by many technical and clinical factors, a universal cut-off remains to be agreed upon at present. Furthermore, for children with AML, PCR and multiparametric flow cytometry have been associated with inconsistent results (5,7).

Spatially proximal heterogeneous chromosomes are more likely to cause chromosomal translocations compared with spatially distant heterogeneous chromosomes, and these translocation-prone chromosomes are frequently located towards the center of the nucleus (12-15). To further understand the clinical features and treatment response, our previous study followed up with a patient with AML with maturation to delineate the spatial organization of leukemia-specific chromosomes using three-dimensional fluorescence *in situ* hybridization (3D-FISH) as a means to detect MRD persistence (16). Disease deterioration was detected using 3D-FISH [with 37% malignant cells (MCs); $P<0.05$] 4 months prior to final relapse, whereas the PCR and 2D-FISH results were negative with no gene fusion transcripts or chromosomal translocations. 3D-FISH analysis in the study provided information that may assist in predicting early relapse (16).

Studies using PCR to monitor MRD persistence in AML have demonstrated that peripheral blood (PB) samples were as informative as bone marrow (BM) samples in identifying

patients at high risk of relapse and may represent an alternative source of cells for diagnosis (17-20). To further assess the similarities and differences between BM and PB, the present study compared the spatial organization of leukemia-specific chromosomes between BM and PB samples from 6 patients with AML-M2 using 3D-FISH with a confocal laser scanning microscope system.

Materials and methods

Materials. Paired BM and PB samples were collected from 6 patients (aged 24-47 years, including 5 males and 1 female) with AML-M2, including 5 RUNX1-RUNX1T1^{+} patients (numbered as 1-5 in Table I) at three different stages (initial diagnosis, post-chemotherapy and remittent) and 1 RUNX1-RUNX1T1^{-} patient at initial diagnosis (numbered as 6 in Table I), who were treated at Peking University Third Hospital (Beijing, China) between December 2012 and September 2015. All patients provided written informed consent to participate in the present study. The present study was approved by the Ethics Committee of Peking University Third Hospital.

Reverse transcription-quantitative poylermase chain reaction (RT-qPCR). Mononuclear cells (MNCs) from the BM were isolated using Ficoll-Hypaque lymphocyte separation medium (Lonza Group, Ltd., Basel, Switzerland). Furthermore, RNA was extracted from MNCs using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's guidelines. cDNA was generated using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cycling conditions were 25˚C for 10 min, followed by 50˚C for 15 min and the reaction was terminated at 85˚C for 5 min. RT-qPCR was performed to monitor the RUNX1-RUNX1T1 transcripts according to the manufacturer's standard protocol (RUNX1-RUNX1T1 Fusion gene Quant® kit; YuanQi Biomedical, Ltd., Shanghai, China) using the ABI PRISM 7500 DNA Sequence Detection system (Applied Biosystems; http://home.appliedbiosystems.com). The reference gene was Abelson (ABL). The fluorophore of TaqMan and primers (RUNX1-RUNX1T1 fusion gene: Forward, 5'-AATCACAGTGGATGGGCCC-3' and reverse, 5'-GAGTCTGGCATTGTGGAGTGC-3'. ABL gene: Forward, 5'-TGGAGATAACACTCTAAGC-3' and reverse, 5'-GATGTAGTTGCTTGGGACCCA-3') were all included in the kit. Cycling conditions were 42˚C for 30 min, initial denaturation at 94˚C for 5 min, followed by 40 cycles of denaturation (at 94˚C for 15 sec) and annealing (at 60˚C for 15 sec). Clinical RT-qPCR results showed that the relative RUNX1-RUNX1T1 transcripts, which were calculated using the $2^{-\Delta\Delta Cq}$ method (7,21), for BM samples from the 6 patients were 52.00, 0.09, 0.05, 0.00, 1.80 and 0.00%, which approximately described the disease conditions of the patients that the initial diagnosed patient showed high RUNX1-RUNX1T1 transcripts (52.00%) and remitted or RUNX1-RUNX1T1^{-} patients showed low RUNX1-RUNX1T1 transcripts (0.09, 0.05, 0.00, 1.80 and 0.00%).

3D-FISH and imaging. MNCs were obtainedd using Ficol-Hypaque density gradient centrifugation as previously described (22), and ≥60 interphase cells from each sample were analyzed in 3D-FISH experiments, which were performed as previously described (22). Suspended MNCs were adhered onto the rough surface of a microscope slide following incubation for 150 min at 37˚C with 5% CO_2. Subsequently, MNCs were fixed in 4% paraformaldehyde for 10 min at room temperature and then underwent certain pre-treatments (22) to increase the likelihood of successful hybridization. Whole chromosome 8 probes (green; Kreatech Diagnostics; Leica Biosystems, Wetzlar, Germany) labeled with fluorescein isothiocyanate (FITC) and whole chromosome 21 probes (red; Kreatech Diagnostics; Leica Biosystems) labeled with tetramethylrhodamine isothiocyanate were used to label chromosome 8 and 21, respectively, in a hybridization oven (ThermoBrite S500; StatSpin, Inc., Westwood, MA, USA; www.statspin.com) with denaturation for 5 min at 75˚C and then hybridization for 48 h at 37˚C. Following washing with Igepal CA-630 (21), 10 μl diamidinophenylindole (blue; 0.1 μg/ml) was used to counterstain the nuclei at -20˚C for more than 15 min.

Image stacks were acquired using a Nikon A1Rsi confocal microscope system with a Plan Apo 100x/1.4 NA oil immersion objective lens. Excitation spectrums for diamidinophenylindole, FITC, and tetramethylrhodamine isothiocyanate were 405, 488, and 561 nm, respectively. The size of the x-y optical sections was 512x512 pixels with a pixel size of 80x80 nm, and the axial step was 500 nm.

Statistical analysis. The 3D views of the nuclei were reconstructed using Amira 5.4.3 software (FEI; Thermo Fisher Scientific, Inc.; Fig. 1). Green signals indicated chromosome 8 and red signals referred to chromosome 21. The blue spheres represented the nuclei. Only cells that exhibited ≥2 green signals and 2 red signals were considered in the analysis. According to the number and spatial relative positions of the green/red signals, the present study classified the cells into three types: Normal cells (NCs; Fig. 1A), proximal cells (PCs; Fig. 1B) and malignant cells (MCs; Fig. 1C and D). Cells that exhibited 3 green or red signals (3g or 3r) were identified as MCs with chromosomal translocations and breakages. Cells that possessed 2 red and 2 green signals (2r2g) were further categorized according to the characterization of the physical contact between the heterogeneous chromosomes. In cells that exhibited 2r2g signals, heterogeneous chromosomes touching or intermingling with each other were considered proximal and categorized as proximal pairs (PPs); chromosomes located distantly from each other were categorized as normal pairs (NPs). Cells that exhibited 2r2g signals were classified as NCs when all heterogeneous chromosomes were NPs, and were classified as PCs when there was at least one PP in the nucleus.

The present study calculated the relative radial positions [RRPs; the distance from the center of mass of chromosome 8 or 21 to the center of mass of the nucleus (d)/the radius of the nucleus (R)] of chromosomes 8 and 21 in all cell nuclei. No less than 60 cells were calculated for each sample during the analysis. RRPs of chromosomes are presented as mean ± standard error of the mean. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The frequencies of different types of cell were compared between BM and PB samples using Pearson's χ^2 test. Chromosomal RRPs were compared between

Table I. P-values for comparisons of occurrences of three types of cells and RRP of chromosomes 8 and 21 between BM and PB samples from 6 patients with acute myeloid leukemia-M2.

Variables	Initial diagnosis	Post-chemotherapy		Remittent stage		Negative
Patient	1	2	3	4	5	6
Occurrences	0.416	0.420	0.779	0.115	0.744	0.431
Chromosome 8-RRP	0.054	0.894	/[a]	/[a]	0.069	/[a]
Chromosome 21-RRP	0.435	0.511	0.459	0.897	0.899	0.024[b]

[a]Heterogeneity of variance; [b]P<0.05 (comparison of RRP of chromosomes 21 between BM and PB). RRP, relative radial position; BM, bone marrow; PB, peripheral blood.

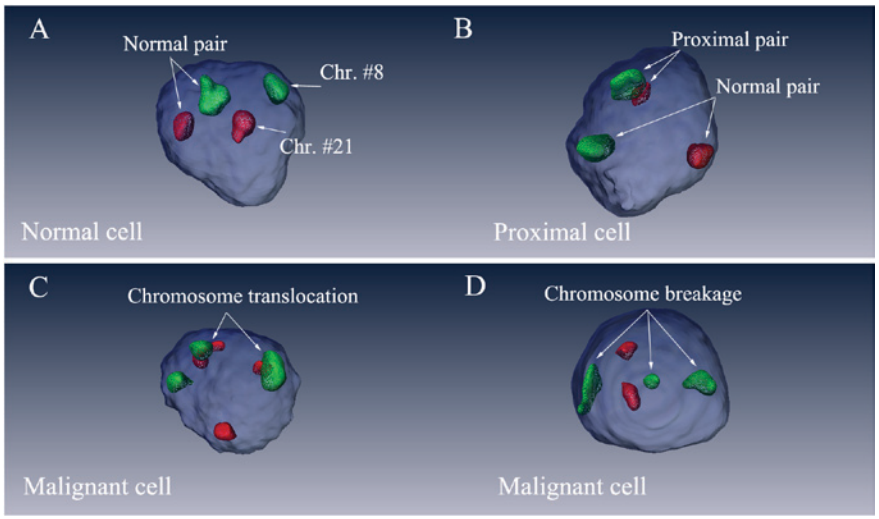

Figure 1. Cell classification. (A) Normal cell (2r2g; no proximal pairs). (B) Proximal cell (2r2g; ≥1 proximal pair). (C) Malignant cell (3r3g) with chromosome translocation. (D) Malignant cell (3r2g or 2r3g) with chromosome breakage. Chr, chromosome; r, red; g, green.

BM and PB samples using one-way analysis of variance with the least significant difference post hoc test. RRPs were compared between NPs and PPs using the two-sample, two-sided Kolmogorov-Smirnov (K-S) test. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparing the occurrence (% of all cells) of different types of cell in BM and PB samples. Cells were classified into three types (Fig. 1): NCs, PCs and MCs. The occurrence (% of all cells) of different types of cell in the BM and PB samples from the 6 patients was provided (Fig. 2). Histograms of sparse, medium and dense left-inclined diagonals referred to the occurrence of NCs, PCs and MCs in the BM samples, respectively (Fig. 2A). Histograms of sparse, medium and dense right-inclined diagonals referred to the occurrence of NCs, PCs and MCs in the PB samples, respectively (Fig. 2B). P-values for comparisons of the proportions between BM and PB samples, as determined using Pearson's χ^2 tests, were provided (Table I). The comparisons revealed no significant differences (P>0.05 for all patients), which suggested that the occurrence (% of all cells) of the different types of cell in the BM samples were generally consistent with those in the PB samples.

Comparing chromosomal RRPs between BM and PB samples. RRPs (*d*/*R*) of chromosomes 8 and 21 were calculated for all cell nuclei. Chromosomal RRPs were compared between BM and PB samples from the 6 patients (Fig. 3; Table I). Histograms of sparse, medium and dense left-inclined diagonals (Fig. 3A and B) referred to the RRPs of chromosomes in NCs, PCs and MCs from the BM samples, respectively. Histograms of sparse, medium and dense right-inclined diagonals (Fig. 3C and D) referred to the RRPs of chromosomes in NCs, PCs and MCs from the PB samples, respectively. Chromosomal RRPs were compared between the BM and PB samples using one-way analysis of variance (Table I). The comparison of RRPs of chromosome 21 between the BM and PB samples revealed a significant difference only for the RUNX1-RUNX1T1$^-$ patient (P=0.024; number 6 in Fig. 3).

However, the RRPs of chromosomes 8 and 21 in the PCs were generally more proximate to the centre of the nucleus compared with those in the NCs. P-values for the comparison of RRPs of chromosome 8 in NC and PC nuclei ranged from 0.089-0.508 (median, 0.378) for the 6 BM samples and from 0.010-0.964 (median, 0.583) for the 6 PB samples. P-values for the comparison of RRPs of chromosome 21 in NC and PC nuclei ranged from 0.024-0.980 (median, 0.334) for the 6 BM samples. For the 6 PB samples, 50% of the chromosome 21

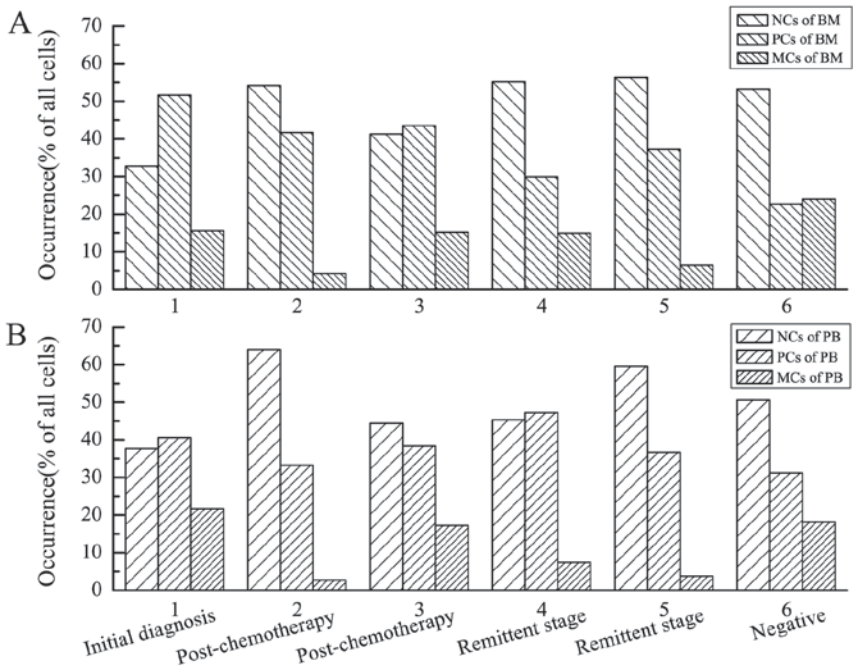

Figure 2. Occurrence (% of all cells) of the different types of cell in BM and PB samples from 6 patients with acute myeloid leukemia-M2. (A) Occurrence of NCs, PCs and MCs in BM samples. (B) Occurrence of NCs, PCs and MCs in PB samples. NC, normal cell; PC, proximal cell; MC, malignant cell; BM, bone marrow; PB, peripheral blood.

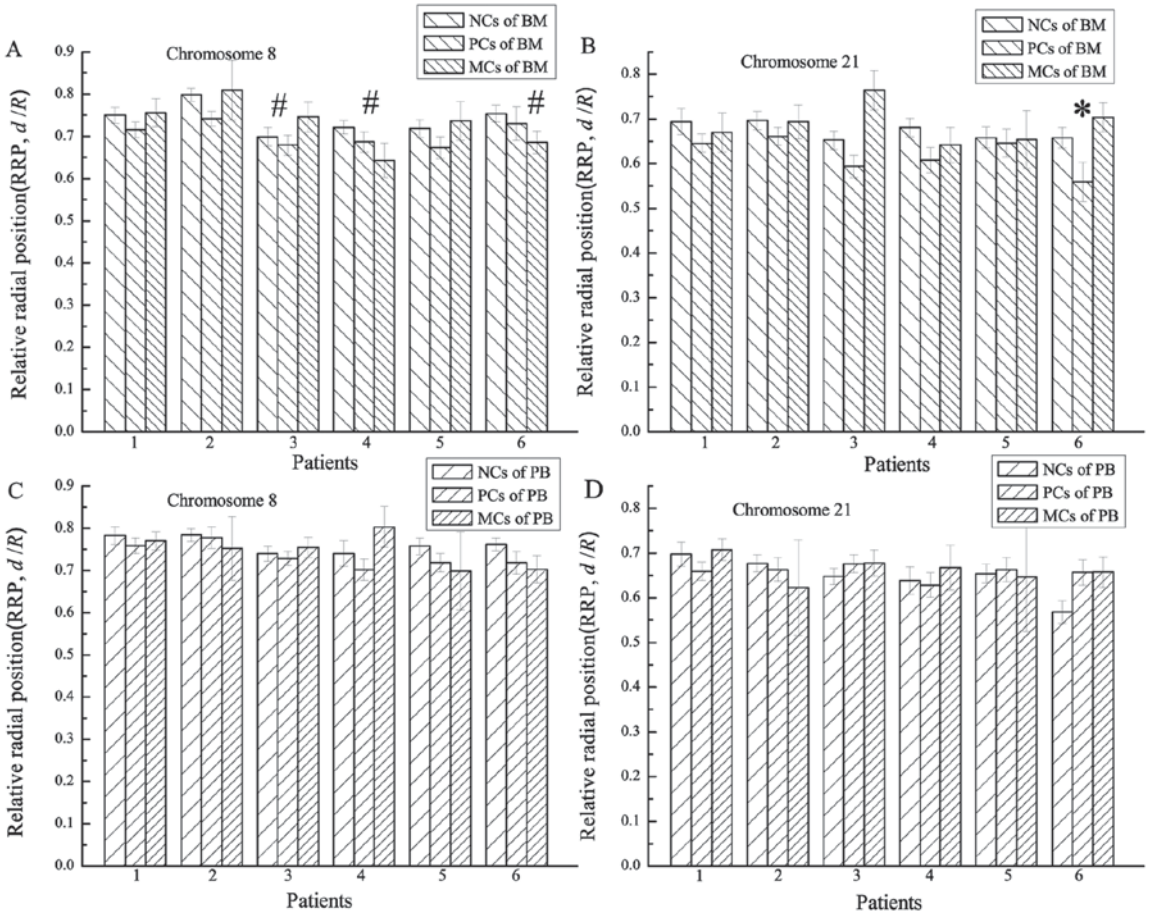

Figure 3. Chromosomal RRPs for the different types of cell in BM and PB samples from 6 patients with acute myeloid leukemia-M2. (A) RRPs of chromosome 8 for the different types of cell in BM samples. (B) RRPs of chromosome 21 for the different types of cell in BM samples. (C) RRPs of chromosome 8 for the different types of cell in PB samples. (D) RRPs of chromosome 21 for the different types of cell in PB samples. Data represents the mean ± standard error of the mean. *Significant difference between BM and PB samples. #P<0.05, heterogeneity of variance in the comparisons. RRP, relative radial position; d, the distance from the center of mass of chromosome 8 or 21 to the center of mass of the nucleus; R, the radius of the nucleus; BM, bone marrow; PB, peripheral blood.

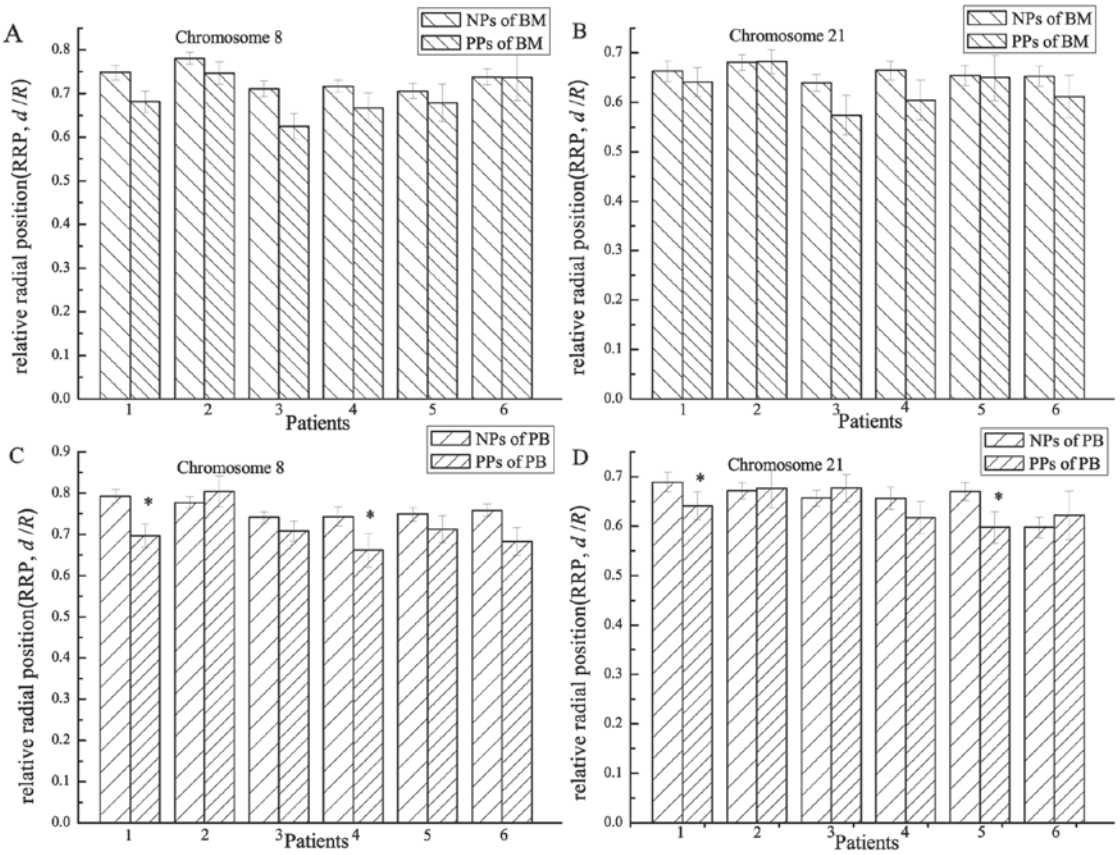

Figure 4. Chromosomal RRPs for different chromosomal pairs in BM and PB samples from 6 patients with acute myeloid leukemia-M2. (A) RRPs of chromosome 8 for the BM samples. (B) RRPs of chromosome 21 for the BM samples. (C) RRPs of chromosome 8 for the PB samples. (D) RRPs of chromosome 21 for the PB samples. Data represents the mean ± standard error of the mean. *P<0.05, significant difference between NPs and PPs. RRP, relative radial position; d, the distance from the center of mass of chromosome 8 or 21 to the center of mass of the nucleus; R, the radius of the nucleus; BM, bone marrow; PB, peripheral blood; NP, normal pair; PP, proximal pair.

RRPs in PC nuclei were more interior in the nuclei compared with those in NC nuclei (P=0.234, 0.289 and 0.607), and 50% were not (P=0.104, 0.608 and 0.014). Comparisons were made using the two-sample, two-sided K-S test.

Comparing chromosomal RRPs between NPs and PPs. Heterogeneous chromosomes were classified into NPs and PPs according to their relative physical positions and whether they were touching or intermingling with each other or not. RRPs of chromosomes 8 and 21 in NPs and PPs for all BM and PB samples (12 in total) were provided (Fig. 4). Chromosomal RRPs were compared between NPs and PPs using the two-sample, two-sided K-S test. For 11/12 samples (6/6 for BM and 5/6 for PB), chromosome 8 was more interior (P=0.004-0.664; median, 0.232) in nuclei in PPs compared with that in NPs. For 8/12 samples (5/6 for BM and 3/6 for PB), chromosome 21 was more interior (P=0.029-0.923; median, 0.308) in nuclei in PPs compared with that in NPs.

Discussion

The spatial distribution of chromosomes in interphase is non-random, and gene-dense chromosomes are more likely to be located proximate to the center of the nucleus (15,23). Furthermore, chromosomes spatially proximate to each other are more prone to translocation compared with those that are spatially distant (12-15,24). Therefore, studying the 3D distribution of chromosomes, even if the patient exhibits a normal karyotype, is essential. The 3D-FISH technique is useful since it may preserve the chromatin architecture at the level of the 1 Mb chromatin domains (25,26). In addition, a 3D view of interphase chromosomes may assist in detecting complex or alternate translocations or masked runt related transcription factor 1 (AML1)/RUNX1T1 fusions presenting as chromosomal breakages in MCs (27-29).

A previous study revealed that MCs accounted for <20% of all cells from healthy donors or patients in the remittent stage (16). However, in the present study, MCs in BM samples from the patient with AML1/RUNX1T1$^-$ AML-M2 represented ~24% of all cells derived from this patient. All these MCs exhibited chromosomal breakage (3r or 3g) rather than chromosomal translocation (3r3g). Therefore, chromosomal breakages may serve a critical function in disease occurrence. However, 20% of MCs in BM samples does not represent a universal cut-off for sorting patients into low- or high-risk relapse groups. For the patient initially diagnosed in the present study with AML1/RUNX1T1$^+$ AML-M2, the proportion of MCs in BM samples was only 16%. We previously also monitored an AML-M2 AML1/RUNX1T1$^+$ patient following hematopoietic stem cell transplantation and demonstrated a continuous increase in MC proportion in the patient prior to final relapse (30). Therefore, the proportion of MCs may be

associated with patient condition, and should be assessed for variation during follow-up rather than tested only once.

Fritz *et al* (31) suggested that pairwise border distance rather than pairwise center distance may prove preferential for studying chromosomal interaction. Improving upon our previous study (16), PPs were defined as heterogeneous chromosomes touching or intermingling with each other in the present study, thereby avoiding the complexity of considering chromosomal size and structural orientation when calculating the relative distance between the centers of heterogeneous chromosomes. Previous studies have demonstrated that PPs were positioned more interiorly in the nuclei than NPs (12,14,15,24). The present study compared the RRPs (*d*/*R*) of chromosomes observed in PPs with those only observed in NPs using the two-sample, two-sided K-S test. In general, chromosomes 8 and 21 in PPs were more interior in the nuclei compared with those in NPs, which is consistent with our previous study (16) and indicated that the spatial organization of PPs in the nucleus was non-random.

Cells were classified into three types: NCs, PCs and MCs. Occurrences (% of all cells) of the different types of cell in BM samples were generally consistent with those in PB samples ($P>0.05$), which indicated that the PB samples were as informative as BM samples in disease detection using 3D-FISH and may represent an alternative source of cells for studies using 3D-FISH. The results of the present study may be implicated in cases where only PB is available for study and MRD detection due to decreased BM availability (32).

To conclude, the 3D-FISH technique may be used to detect the spatial organization of chromosomes *in situ* and distinguish the chromosomal morphology of NPs, PPs and chromosomal translocations or breakages. Quantitative analysis of spatial chromosomal organization may provide significant information for clinical diagnosis of acute myeloid leukemia. The spatial organization of leukemia-specific chromosomes in nuclei were generally consistent between BM and PB samples in the present study. Therefore, PB may represent an alternative to BM in disease follow-up or studies using 3D-FISH.

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Competing interests

The authors declare that they have no competing interests.

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