with chromosome 5 abnormalities. Therefore, we screened for the *NPM1* gene exon 12 mutations in MDS and AML patients with chromosome 5 abnormalities.

Materials and methods

Patients and samples

Clinical samples from 28 patients with MDS or AML at the Tokyo Women's Medical University Hospital were analyzed. Diagnosis was made according to the WHO classification [24]. Cytogenetic analysis was performed by G-banding method. After informed consent, mononuclear cells were isolated from bone marrow (25 patients) or peripheral blood samples (three patients) at diagnosis by a Ficoll-Conray gradient centrifugation and stored in liquid nitrogen until the analysis. Genomic DNA was extracted from the mononuclear cells by using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacture's instructions. In some cases, total RNA was extracted using ISOGEN reagent (Nippongene, Tokyo, Japan) followed by a guanidinium thiocyanate-phenol chloroform extraction method, and then cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

PCR for exon 12 of the NPM1 gene, subcloning, and sequencing

Sequences of the primers to amplify the coding region in exon 12 of the *NPM* gene are as follows: 5′-TTAACTCTCTGGTGCTAGAATGAA (forward) and 5′-CAAGACTATTTGCCATTCCTAAC (reverse) for genomic DNA, 5′-GGTTGTTCTCTG GAGCAGCGTTC (forward) and 5′-CCTGGAC AACATTTATCAAACACGGTA (reverse) for cDNA. A PCR was carried out using 100 ng of genomic DNA as a template in 50 μL of a reaction mixture containing 5 μL of 10× buffer, 1.5 mM of MgCl2, 250 nM of each deoxynucleotide, 0.2 unit of TaKaRa Taq DNA polymerase (Takara, Ohtsu, Shiga, Japan), and 300 nM of each primer. Thirty-five cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 60 s were performed. For the analysis of cDNA, a PCR was carried out under the same conditions as for genomic DNA, except for the amount of template (50 μg) and the addition of an initial incubation at 94°C for 5 min prior to the first cycle. After confirming the amplification by agarose gel electrophoresis, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and subjected to direct sequence analysis using a DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham, Buckinghamshire, UK). PCR products were also cloned into pGEM-T vector (Promega, Madison, WI, USA). After bacterial transformation, colonies formed on LB agar containing ampicillin (100 μg/mL) were picked up, suspended into LB culture media, and then subjected to a PCR to amplify the cloned exon 12 of the *NPM1* gene in the pGEM-T vector, using primer set designed corresponding to the vector sequence. The PCR product was purified with a QIAquick PCR Purification Kit (Qiagen) and then sequenced. Both strands of each PCR product were sequenced using the MegaBASE DNA Analysis System (Amersham) according to the manufacturer's instruction.

Results

Twenty-eight patients, 16 with MDS and 12 with AML patients, were included in this study. Table I shows the characteristics of these patients. All AML cases in this study were classified into AML with multilineage dysplasia in the WHO classification because of dysplastic features of bone marrow cells. Among 12 patients with AML, 10 patients had antecedent disease (two with refractory anemia, two with refractory cytopenia with multilineage dysplasia, four with refractory anemia with excess blasts-1, two

Table I. Characteristics of the 28 patients in this study.

Median age, years (range)	70 (29–85)
Sex (*n*) male/female	17/11
Diagnosis, *n* (%)	
MDS	
RA	3 (10.7)
RCMD	1 (3.6)
RARS	1 (3.6)
RAEB-1	3 (10.7)
RAEB-2	5 (17.9)
5q-syndrome	3 (10.7)
AML with multilineage dysplasia	
With antecedent MDS	10 (35.7)
Without antecedent MDS	2 (7.1)
Karyotype, *n* (%)	
Chromosome 5 abnormalities	
Deletion of 5q	20 (71.4)
Monosomy 5	4 (14.3)
Others	4 (14.3)
Other chromosomes abnormalities	
Monosomy 7	5 (17.9)
Trisomy 8	4 (14.3)
Deletion of 20q	3 (10.7)

Abbreviations: MDS, myelodysplastic syndrome; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ringed sideloblasts; RAEB-1, refractory anemia with excess blasts–1; RAEB-2, refractory anemia with excess blasts–2; AML, acute myeloid leukemia.

with refractory anemia with excess blasts-2). At the time of sample collection, none of the patients had been treated with cytotoxic drugs. Cytogenetic analyses indicated that all 28 cases had any chromosome 5 abnormalities with or without other chromosomes abnormalities. Deletion of chromosome 5q was seen in 20 (71.4%) patients including six (21.4%) patients with an isolated chromosome 5q deletion. Monosomy of chromosome 5 was found in four patients (14.3%). In addition to chromosome 5 abnormalities, monosomy 7, trisomy 8, and deletion 20q were seen in five (18.8%), four (14.3%), and three (10.7%) patients, respectively.

To investigate whether *NPM1* exon 12 mutations are present, we performed PCR amplification and direct sequencing of the genomic DNA or cDNA. The results were confirmed by sequencing of at least three cloned PCR products. Sequence results revealed that *NPM1* mutations were not detected in the 28 patients examined.

Discussion

This study demonstrates that *NPM1* exon 12 mutations are absent in MDS and AML patients with chromosome 5 abnormalities. Frequent deletion of chromosome 5q in MDS cases indicates that the tumor suppressor gene(s), which are involved in pathogenesis of MDS, are located on this chromosome region. Allelotype analyses also demonstrate that allelic loss on chromosome 5q is found in 40% of MDS cases [14–17]. Although several putative suppressor genes have been identified on chromosome 5q, their implication in MDS is still unclear and controversial [18]. We hypothesized that the *NPM1* gene is a candidate tumor suppressor gene, which is inactivated by the loss of one allele and mutation of another allele in MDS patients with chromosome 5 abnormalities. The present results, however, indicate that exon 12 mutations do not play a role for inactivation of the *NPM1* gene.

In the mouse model, NPM haploinsufficiency causes an MDS-like disorder, suggesting that quantitative abnormality of NPM play s a major role in the pathogenesis of MDS [19]. Although it is unclear whether an MDS-like disorder observed in $NPM1^{+/-}$ mice could be an actual model of human MDS, NPM haploinssuficiency rather than exon 12 mutations may imply in the development of human MDS. It should be necessary to examine the expression level of *NPM1* in cells from MDS patients.

$NPM1^{+/-}$ mouse model also demonstrates that mouse embryonic fibroblasts with decreased dosage of NPM exhibit genomic instability in a dose-dependent manner [19]. Genomic instability caused by decreased amount of NPM results in chromosome abnormalities, and then may lead to development of an MDS-like disorder in mice. In contrast, most of AML patients carrying *NPM1* exon 12 mutations show a normal karyotype [7–13]. Unlike the quantitative abnormality of NPM, *NPM1* exon 12 mutations itself may not cause genomic instability. The pathological role of *NPM1* exon 12 mutations may be different from that of decreased dosage of NPM.

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Multidrug resistance protein expression of adult T-cell leukemia/lymphoma

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Abstract

In adult T-cell leukemia/lymphoma (ATL), it is difficult to achieve remission and the reason for the resistance to chemotherapeutic agents may be linked to the presence of multidrug resistance (MDR) proteins. Lung resistance-related protein (LRP), multidrug resistance-associated protein and *P*-glycoprotein are three MDR proteins which we examined in ATL cells using multiparametric flow cytometry and real-time RT-PCR. LRP was highly expressed and suppressing LRP function increased doxorubicin accumulation in nuclei. This indicates LRP may be contributing to drug resistance in ATL patients, and the suppression of LRP function could be a new strategy for ATL treatment.

Keywords: Adult T-cell leukemia/lymphoma; Multidrug resistance protein (MDR); Lung resistance-related protein (LRP); Multidrug resistance-associated protein (MRP); *P*-glycoprotein (*P*-gp)

1. Introduction

Adult T-cell leukemia/lymphoma (ATL) is a malignant tumor of mature helper T-cells infected with human T-cell leukemia virus type I (HTLV-I) [1–3]. In ATL, it is difficult to induce remission, and the prognosis for ATL has been known to be poor [4]. One of the reasons for resistance to chemotherapeutic agents may be the presence of multidrug resistance (MDR) proteins. Lung resistance-related protein (LRP) is an MDR protein which was separated from *P*-glycoprotein (*P*-gp) negative small cell lung carcinoma cell lines by Scheper et al. [5]. LRP was shown to be the major vault protein. Vaults are localized in nuclear pore complexes and involved in the nucleocytoplasmic transport of drugs [6]. Multidrug resistance-associated protein (MRP), which was isolated from a *P*-gp negative lung cancer cell line [7], has been proven to confer drug resistance using human carcinoma cells [8]. The clinical significance of MDR proteins such as LRP or MRP in ATL has not been examined extensively. Recently, overexpression of *LRP* or *MRP1* in ATL cells was shown using RT-PCR and Northern/Slot blot analysis [9–11]. The poor response to chemotherapy of ATL cases was partially attributed to the expression of *P*-gp or *MDR1* RNA [12,13] in early reports, though, there was a report that *P*-gp was not overexpressd in most ATL cells [9] when examined by RT-PCR, thus no definite conclusion has yet been drawn.

Multiparametric flow cytometry has proven to be a reliable and sensitive approach to analyse MDR proteins [14]. Real-time RT-PCR is a recognized method to examine gene expression quantitatively [15]. There are no reports in which measuring the presence of MDR proteins or gene expression in ATL cells has been done using these techniques. In the present study, we used three color immunofluorescence analysis and real-time RT-PCR to clarify the expression of LRP, MRP and *P*-gp in ATL. In addition to this, we performed an LRP function assay to clarify whether LRP affects doxorubicin concentration in nuclei.

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2. Materials and methods

2.1. Cell line

KK-1, an interleukin-2 (IL-2) dependent cell line established from an ATL patient by Yamada et al. [16], was maintained with 0.25 U/ml of recombinant IL-2 (kindly provided by Takeda Pharmaceutical, Tokyo, Japan). The cell surface phenotype of KK-1 was CD3+ CD4+ CD8+ CD25+ as previously reported [16]. Adriamycin resistant sublines K562/ADM and HL60/AD of K562 and HL60, respectively, were maintained in RPMI1640 medium (Life Science, Grand Island, NY) containing 10% fetal calf serum (FCS; Intergen, Purchase, NY). HL60/AD was kindly provided by Dr. Takemura [17].

2.2. Cell separation from ATL patients

Peripheral blood or bone marrow samples were taken from patients with ATL under written informed consent. The diagnosis of ATL was made based on a positive test for the antibody for HTLV-I and on the pathology of bone marrow or peripheral blood, and all patients were diagnosed as acute type ATL [18]. Mononuclear cells of each sample were separated through Ficoll–Conray density gradient centrifugation (density 1.077). Cells were suspended in alpha-modified Eagle's minimal essential medium (α-MEM; Flow Laboratories, McLean, VA), and then were either used immediately or cryopreserved in liquid nitrogen with 10% dimethylsulphoxide (DMSO) and 50% fetal calf serum (FCS) until use, or 1×10^7 cells were frozen after washing with phosphate-buffered saline (PBS) until RNA extraction.

2.3. Detection of LRP, MRP and P-gp by flow cytometry

Cells were stained with a three color immunofluorescence method as follows. For detection of the intracellular epitopes of LRP and MRP, 1×10^6 cells were incubated with 100 μl fixation medium (Caltag Laboratories, Burlingame, CA) for 15 min at room temperature, then washed with PBS, and incubated with 10% rabbit serum (R&D Systems, McKinley Place, MN) and permeabilization medium (Caltag Laboratories) for 5 min. Cells were then incubated for 1 h at 4 °C with anti-LRP monoclonal antibody (MoAb) LRP56, with anti-MRP MoAb MRPm6 (both from Kamiya Biotech, Thousand Oaks, CA) or with mouse isotype-matched control MoAb. For detection of *P*-gp, 1×10^6 cells were incubated with human γ-globulin for blocking, and then with MRK16, anti-*P*-gp MoAb (Kyowa Medix, Tokyo, Japan) or subclass-matched IgG2a MoAb (Beckman Coulter, Miami, FL) for 30 min at 4 °C as in our previous reports [19,20]. Cells were then washed and incubated with a secondary antibody, goat anti-mouse IgG conjugated with phycoerythrin (PE), for 30 min at 4 °C. After washing and blocking with mouse IgG (Beckman Coulter), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 MoAb (Immunotech, Marseille, France) and phycoerythrin–cyanine 5 (PC5)-conjugated anti-CD25 MoAb (Immunotech) for another 30 min, then washed and analysed.

The LRP, MRP or *P*-gp expression on ATL cells was analysed by gating on CD4+ and CD25+ populations using an EPICS-XL system II flow cytometer (Beckman Coulter). MDR positivity was determined with a CD4 and CD25 double positive population, thus we could identify MDR positivity of leukemic ATL cells without contamination of normal T-cells. No difference was observed in LRP, MRP or *P*-gp positivity between fresh and cryopreserved samples.

2.4. Detection of LRP, MRP1 and MDR1 mRNA by real-time RT-PCR

2.4.1. RNA isolation and cDNA synthesis

Total RNA was isolated by a guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [21], using ISOGEN reagent (Nippongene, Tokyo, Japan). After being checked by gel electrophoresis, the RNA of each sample was stored at −80 °C until use. One microgram of total RNA was converted to single-stranded cDNA using a random primer and reverse transcriptase under conditions as described by the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The resulting cDNA was diluted in diethylprocarbonate water and stored at −80 °C.

2.4.2. Quantitative real-time RT-PCR

Measuring mRNA levels of *LRP*, *MRP1* and *MDR1* was based on the TaqMan probe method [22] using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). The mRNA levels of an internal control gene, human β-actin (TaqMan endogeneous control kit, Applied Biosystems) was also measured and used to normalize the mRNA levels of the drug resistance genes. PCR products were detected using a dual-fluorescent non-extendable probe labeled with the reporter dye FAM and the quencher dye TAMRA for all reactions, except for the human β-actin reaction in which FAM and TAMRA were replaced by VIC and MGB, respectively. All primer pairs and probes were made according to the reports as follows [23,24].

LRP
Forward primer 5′-CAG CTG GCC ATC GAG ATC A-3′
Reverse primer 5′-TCC AGT CTC TGA GCC TCA TGC-3′.
Probe 5′-CAA CTC CCA GGA AGC GGC GGC-3′

MRP1
Forward primer 5′-CAA TGC TGT GAT GGC GAT G-3′
Reverse primer 5′-GAT CCG ATT GTC TTT GCT CTT CA-3′
Probe 5′-AGA CCA AGA CGT ATC AGG TGG CCC AC-3′

MDR1
Forward primer 5′-GCT ATA ATG CGA CAG GAG ATA GGC T-3′
Reverse primer 5′-CAT TCC AAT TTT GTC ACC AAT AAC TT-3′
Probe 5′-CAC GAT GTT GGG GAG CTT AAC ACC CG-3′.

β-Actin (Applied Biosystems)

Table 1
Clinical data of ATL patients and expression of MDR proteins

Patient	Age/sex	WBC ($\times 10^9$/l)	ATL cells (%)	CD2 (%)	CD3 (%)	CD4 (%)	CD8 (%)	CD25 (%)	CD4+ 25+ LRP+ (%)	CD4+ 25+ MRP+ (%)	CD4+ 25+ *P*-gp+ (%)
1	27/F	207.0	77	99.4	96.4	97.2	3.8	96.9	81.9	1.4	2.3
2	58/M	29.3	49	76.5	43.4	58.9	10.3	86.7	57.2	0.5	1.6
3	54/F	30.6	45	93.3	84.8	88.4	4.8	47.5	13.6	2.0	ND
4	67/F	43.6	46	21.0	82.0	81.0	10.0	74.1	73.1	0.5	0.3
5	64/M	48.8	42	96.5	87.2	94.3	3.3	93.7	41.9	3.5	3.4
6	58/F	3.6	47	95.0	80.0	71.0	19.0	60.0	45.1	5.5	2.1
7	61/F	18.3	61	97.6	90.2	96.9	1.3	94.1	80.1	0.4	1.1
8	59/M	130.3	80	98.0	97.1	93.6	9.6	90.2	89.8	2.1	0.6
9	63/F	30.4	11	91.4	61.3	53.1	15.0	74.8	45.0	0.7	1.5
10	62/M	19.2	72	67.4	52.9	47.9	16.7	22.6	11.6	0.3	0.3
11	65/F	103.0	78	99.1	79.6	98.4	1.3	62.4	5.6	0.3	0.4

Peripheral blood was used except for case number 8 (pleural effusion) and case number 10 (bone marrow aspirate). ND, not done because of insufficient cell number.

The real-time amplifcation reaction was performed in the presence of 300 nM of each forward and reverse primer, 200 nM of each TaqMan probe, and 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. In the reaction, 2.5 μl of diluted cDNA was used as a template in triplicate for all samples. Samples were heated for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C. Under this assay, serially diluted cDNA prepared from KK-1, K562/ADM or HL60/AD was used as a positive control for each reaction. To compare the expression levels among different patient samples, the relative expression of the MDR genes and β-actin levels were calculated using the comparative standard curve method [25]. From each standard curve, which showed a linear detection range of 6–7 logs, the threshold cycle values were set for each target gene and β-actin. We then obtained the mean relative gene expression value for each sample. The difference between

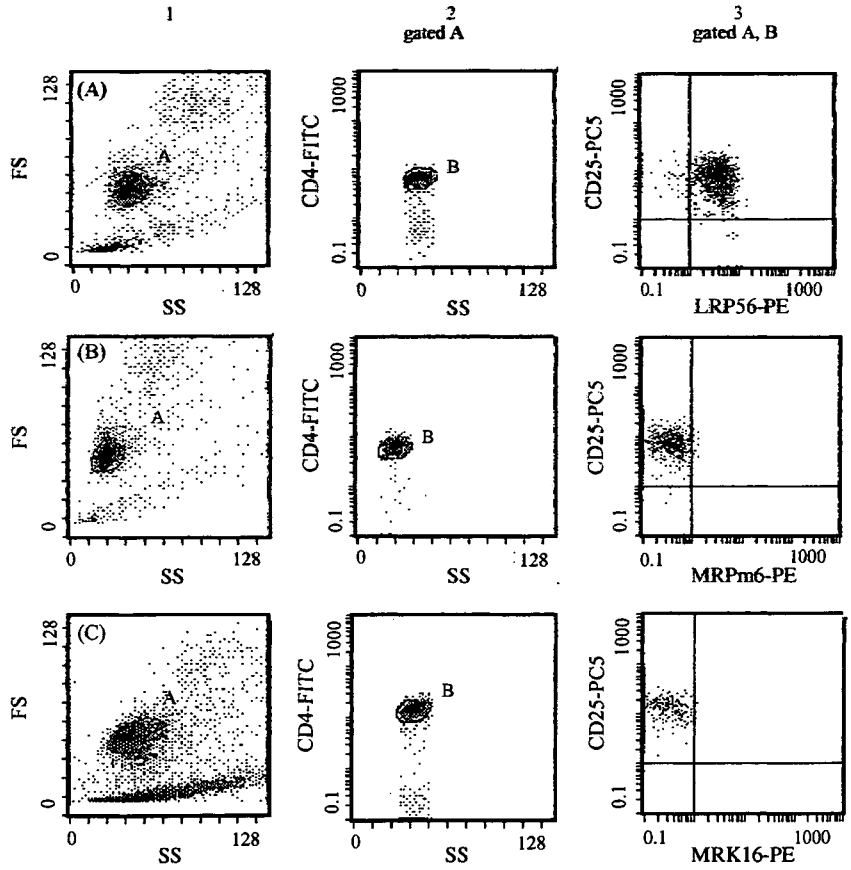

Fig. 1. Expression of LRP, MRP and *P*-gp by using three color flowcytometric analysis. Flowcytometric analysis of case number 8. A1–A3, B1–B3 and C1–C3 represent CD4+ CD25+ cell populations expressing LRP (89.8%), MRP (2.1%) and *P*-gp (0.6%), respectively.

the slopes of each target gene and β-actin was <0.1 in every experiment.

2.5. Measurement of [^{14}C] doxorubicin in isolated nuclei

To investigate whether LRP affects doxorubicin accumulation in nuclei, the nuclei of ATL cells were isolated according to the report by Newmeyer and Wilson [26]. Nuclei were suspended in solution A [250 mM sucrose; 1 mM dithiothreitol; 80 mM KCl; 15 mM EDTA; 15 mM piperazine diethanesulfonic acid, NaOH (pH 7.4); 0.5 mM spermidine; 0.2 mM spermine; 1mM phenylmethylsulfonyl fluoride] (Wako, Osaka, Japan). To inhibit the function of LRP, anti-LRP MoAb LRP56 or LRP mediated reversing agent PAK-104P (2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide) (provided by Dr. Akiyama [27]), was added just before 1 μM of [^{14}C] doxorubicin (Amersham Biosciences, Buckinghamshire, UK) to the nuclei suspension, which was then incubated for 10 min at 37 °C. The nuclei were washed twice with ice cold solution A, suspended in Aqueous Counting Scintilant (Amersham Biosciences) and radioacitivity was determined with the liquid scintillation counter system (LSC-700 Aloka., Tokyo, Japan) after 10 min. In our preliminary experiment, the MoAb LRP56 caused a 1.4-fold increase in [^{14}C] doxorubicin accumulation in KK-1 cell nuclei.

3. Results

3.1. Characterization of ATL patients

The hematological findings of 11 patients and cell surface phenotype of ATL cells are summarized in Table 1. As CD4 and CD25 were positive in all 11 ATL patients' leukemic cells, we examined MDR expression of ATL cells in the CD4 and CD25 double positive cell population. In normal mononuclear cells from healthy individuals the CD4 and CD25 double positive cell population was less than 1%.

3.2. Expression of MDR proteins in ATL cells

As shown in Table 1, LRP was positive in 8 out of 11 samples and the positivity varied from 41.9 to 89.8%. For two of the remaining three, case numbers 3 and 10, positivity was 13.6 and 11.6% and CD25 expression was relatively low. The LRP positivity was 5.6% in case number 11 in spite of the 62% positivity of CD25 expression. On the other hand, there was no case with significant MRP or *P*-gp expression. Fig. 1 shows a representative result of three color immunofluorescence analysis of CD4 and CD25 with LRP, MRP or *P*-gp. KK-1 cells were positive for LRP in 97% of the cells, for MRP in 8.2%, and for *P*-gp in 12%.

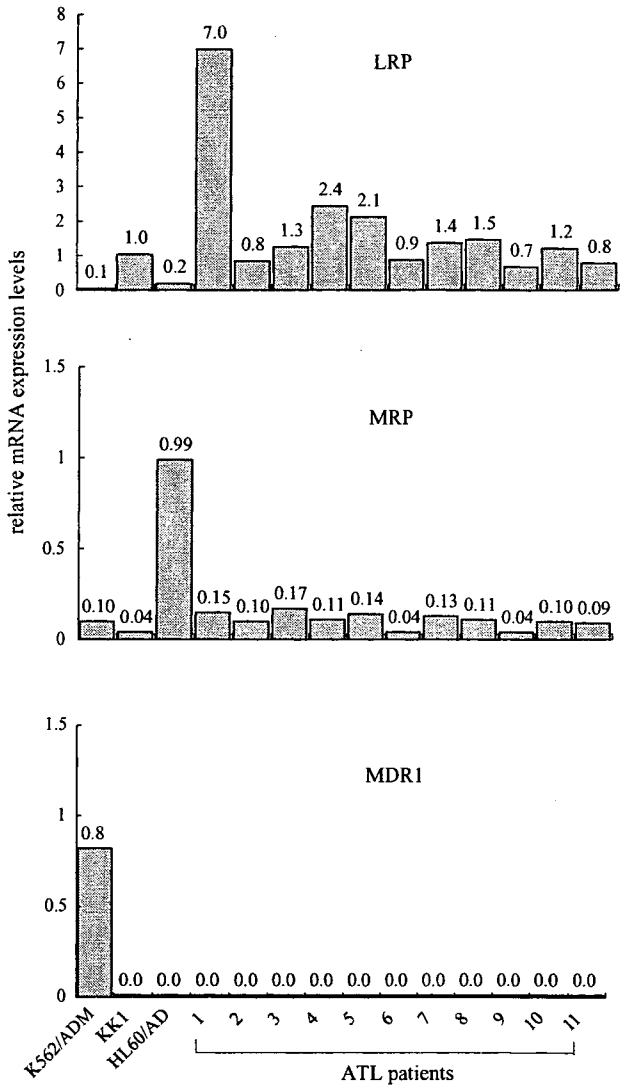

Fig. 2. Relative mRNA expression levels of MDR genes. Relative mRNA expression levels were calculated as follows: MDR protein expression/β-actin expression. KK-1, HL60/AD and K562/ADM were used as positive controls of *LRP*, *MRP1* and *MDR1*, respectively.

3.3. MDR gene expression in ATL cells

The relative mRNA expression levels of MDR by real-time RT-PCR is shown in Fig. 2. *LRP* was expressed on all of the cases. The relative mRNA expression levels of *LRP* varied from 0.7 to 7.0 compared to the KK-1 cells. *LRP* expression was higher than the KK-1 cells in 7 of 11 patients (64%). *MRP1* was weakly expressed in all cases with a lower magnitude of expression than that of HL60/AD. Expression of *MDR1* was not observed in any of the ATL cells.

3.4. Accumulation of [^{14}C] doxorubicin in isolated nuclei

Fig. 3 shows accumulation of [^{14}C] doxorubicin in isolated nuclei from five patients in which enough cells were avail-

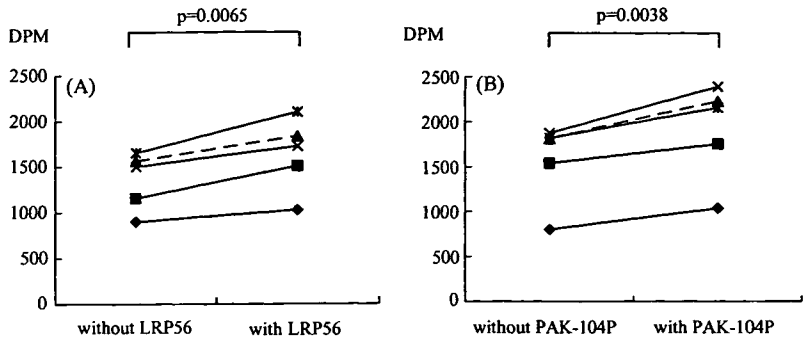

Fig. 3. Accumulation of [^{14}C] doxorubicin in isolated nuclei. Anti-LRP antibody (A) LRP reversing agent PAK-104P (B) significantly increased accumulation of [^{14}C] doxorubicin. Each value represents the mean of triplicate measurements (DPM).

able. LRP56 or PAK-104P resulted in a significant increase of [^{14}C] doxorubicin accumulation compared with an absence of LRP inhibitor (paired *t*-test).

4. Discussion

Our present results indicate that LRP is highly expressed in leukemic cells in most ATL patients. We used three color flow cytomeric analysis, which can eliminate the effect of residual normal T-cells, and it revealed high expression of LRP in ATL cells. The results of real-time RT-PCR also demonstrated overexpression of *LRP*. For each case, however, these two test results do not always correlate. The reason for the discrepancy may be due to translation regulation, glycosylation [28], degradation, contamination by normal cells, etc. LRP is thought to be a drug transporter between the nucleus and cytoplasm. There are two reports [9,10] that show leukemic cells from chronic ATL patients overexpressed mRNA of the *LRP* gene. Sakaki et al. [29] reported that transfection of the Tax gene activated *LRP* expression in leukemic clones established from ATL patients. They demonstrated Tax transfected cells acquired drug resistance to doxorubicin and vincristine, suggesting that *LRP* expression caused the drug resistance in ATL cells. As well, we also showed that an anti-LRP MoAb or PAK-104P, an LRP inhibitor, increased doxorubicin accumulation in nuclei, suggesting that LRP confers drug resistance. Reversing LRP function may augment efficacy of anti-leukemic agents.

Our results show that MRP expression was low in the CD4+ CD25+ population of ATL cells and also *MRP1* gene expression was low using real-time RT-PCR. The MRP efflux function tests with calcein acetoxymethyl ester, which becomes fluorescent calcein by cleavage of the ester bond by intracellular esterases, was not affected in ATL cell lines by probenecid, a specific modulator of MRP [14,30] (data not shown). MRP appears not to have a role with drug resistance in ATL cells. Ohno et al. [11] reported that expression of *MRP1* mRNA was higher with patients who have large numbers of peripheral blood abnormal lymphocytes, and with patients in chronic phase. Our results did not show any correlation between the *MRP1* mRNA and leukemic cell counts. Ikeda et al. [9] showed a difference in MRP expression between the chronic type and control sample, and Ohno et al. [11] showed a difference between the chronic and lymphoma types, but neither found a significant difference between the acute type and others. Some of the acute type ATL cases may have high MRP expression, as in Ohno's report (3 of 32 cases) [11]. Ikeda et al. [9] reported the correlation of *MRP1* and *LRP* mRNA levels in chronic ATL cells. Although the *LRP* gene is localized close to the *MRP1* gene, on the short arm of chromosome 16, there is a report that *MRP1* and *LRP* genes are rarely coamplified and are not normally located within the same amplicon [31]. Our cases are all acute type, and there was no correlation between *LRP* and *MRP1* mRNA levels.

For *P*-gp and *MDR1* expression in ATL cells, there has been no definite conclusion from other studies [9,12,13]. Our results demonstrated no expression of *P*-gp or *MDR1* in ATL cells.

In conclusion, our present results suggest that LRP overexpression may contribute to MDR in ATL patients. LRP mediated reversing agents such as PAK-104P or MoAb against LRP may be useful to improve the effect of chemotherapy for patients with LRP positive ATL cells. The suppression of LRP function would be a new strategy in ATL treatment.

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ORIGINAL ARTICLE

Unbalanced translocation der(1;7)(q10;p10) defines a unique clinicopathological subgroup of myeloid neoplasms

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The unbalanced translocation, der(1;7)(q10;p10), is one of the characteristic cytogenetic abnormalities found in myelodysplastic syndromes (MDS) and other myeloid neoplasms. Although described frequently with very poor clinical outcome and possible relationship with monosomy 7 or 7q- (−7/7q-), this recurrent cytogenetic abnormality has not been explored fully. Here we analyzed retrospectively 77 cases with der(1;7)(q10;p10) in terms of their clinical and cytogenetic features, comparing with other 46 adult −7/7q- cases without der(1;7)(q10;p10). In contrast with other −7/7q- cases, where the abnormality tends to be found in one or more partial karyotypes, der(1;7)(q10;p10) represents the abnormality common to all the abnormal clones and usually appears as a sole chromosomal abnormality during the entire clinical courses, or if not, is accompanied only by a limited number and variety of additional abnormalities, mostly trisomy 8 and/or loss of 20q. der(1;7)(q10;p10)-positive MDS cases showed lower blast counts ($P<0.0001$) and higher hemoglobin concentrations ($P<0.0075$) at diagnosis and slower progression to acute myeloid leukemia ($P=0.0043$) than other −7/7q- cases. der(1;7)(q10;p10) cases showed significantly better clinical outcome than other −7/7q cases ($P<0.0001$). In conclusion, der(1;7)(q10;p10) defines a discrete entity among myeloid neoplasms, showing unique clinical and cytogenetic characteristics.

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Introduction

The unbalanced translocation, der(1;7)(q10;p10), is a nonrandom chromosomal abnormality found in myelodysplastic syndromes (MDS) as well as acute myeloid leukemia (AML), and less frequently in myeloproliferative disorders (MPD).[1] It is presented typically with an International System for Chromosome Nomenclature (2005) description,[2] 46,XY(or XX), +1,der(1;7)(q10;p10) and characterized by the presence of one of the two possible derivative chromosomes with two copies of apparently normal chromosome 1 and a single copy of the intact chromosome 7, leading to allelic imbalance of trisomy 1q and monosomy 7q. It was found consistently in 1.5–6% in MDS, 0.2–2.1% in AML and rarely in MPD depending on the literature.[3–9] Detailed molecular studies using quantitative fluorescent *in situ* hybridization analysis disclosed that the translocation is likely to occur through a mitotic recombination within the large clusters of homologous centromere alphoid sequences, D1Z7 on chromosome 1 and D7Z1 on chromosome 7. The breakpoints are widely distributed within these alphoids clusters but typically spare the short arm third of both alphoids, suggesting that it may not be a dicentric chromosome but that der(1;7)(q10,q10) is a more appropriate karyotypic description for this translocation.[10]

On the other hand, however, the clinical aspect of this translocation is less clearly defined compared with its genomic structure. While the common clinical features thus far reported include a previous history of chemo- and/or radiotherapies in more than half cases,[11–13] presence of eosinophilia,[13] trilineage dysplasia, high rates of progression to AML in MDS cases and poor prognosis with less than 1 year of median survival,[3,14,15] these features have not been fully investigated because of the rarity of this translocation compared with other well-characterized cytogenetic groups found in AML/MDS. Also it is unclear that its pathogenetic link to monosomy 7 or partial deletion of 7q (−7/7q-) other than der(1;7)(q10;p10), although some authors speculated that der(1;7)(q10;p10) represents a mere variant of the former.

In this paper, to clarify these points, we analyzed retrospectively 77 cases with der(1;7)(q10;p10) for their clinical and genetic/cytogenetic characteristics in comparison with other 46 cases with −7/7q-. A literary review of 125 der(1;7)(q10;p10) cases was also provided.

Materials and methods

Patients

The patients included in this study are those who were diagnosed and treated as having MDS, AML and MPD at one of the collaborating hospitals during 1 January 1990 through 30 September 2005, for whom at least one cytogenetic report showed either der(1;7)(q10;p10), monosomy 7, or other unbalanced abnormalities showing loss of genetic materials of the long arm of chromosome 7. −7/7q- was required to be

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confirmed in at least three independent karyotypes, whereas other abnormalities were found in at least two karyotypes.[2] Complex karyotypes were defined by the presence of more than two independent karyotypes with at least three unrelated aberrations according to the previous studies.[16,17] Clinical records were reviewed retrospectively at each hospital according to the above criteria and after fully anonymized, the cases who met the criteria were reported according to an indicated form with regard to patients' age, sex, diagnosis (French–American–British (FAB)), date of diagnosis, cytogenetics (G-banding), histories of chemo-radiotherapies, cellularity and blast counts of bone marrow, complete peripheral blood counts at diagnosis, presence or absence of leukemic transformation, and date and cause of death. The list of the collaborating hospitals is the Hematology unit of Akita University Hospital and Dokkyo University Hospital, Honma Hospital, Jichi Medical School Hospital, National Kyushu Cancer Center, Nishio Municipal Hospital, Saitama Medical School Hospital, Showa University Fujigaoka Hospital, Tokyo Medical School Hospital, the Hospital of Tokyo Women's Medical University and the University of Tokyo Hospital. The diagnosis and sub-classification of MDS and AML at each collaborating hospital were made according to the FAB criteria.[18] Mutation study was performed for 20 cases each with der(1;7)(q10;p10) and −7/7q-, for which genomic DNA from bone marrow was available. Another 20 MDS cases showing normal karyotypes were also subjected to the mutation analysis, according to the approval from the ethical committee, University of Tokyo (Approval No. 948-1).

Statistical analysis

The statistical difference in each clinical feature between der(1;7)(q10;p10) and other −7/7q cases was tested by 2×2 contingency tables using the Fischer's exact test or the Student's *t*-test. Overall survival was estimated using the Kaplan–Meier method and the statistic significance was calculated using log-rank tests. After possible association with overall survival was individually tested for a number of variables, including age, bone marrow blasts, peripheral blood counts, chromosomal abnormalities and history of anticancer therapies, proportional hazard modeling was used for identifying the independent risk factors that influence overall survival, where those factors that showed potential significance in the univariate tests with $P<0.10$ were subjected to the multivariate analysis using backward stepwise selection of covariates. All *P*-values were two-sided and *P*-values of 0.05 or <0.05 were considered statistically significant.

Mutation analysis

Mutation status of the *runx1/AML1 and N-ras* genes was tested on aforementioned 60 cases. Exons 3, 4, 5, 6, 7b and 8 of the *runx1* gene and exons 1 and 2 of the *N-ras* gene were amplified from genomic DNA by polymerase chain reaction (PCR) as described previously.[19,20] Sequencing was performed using an ABI Prism 3100 Genetic Analyzer with the same primers as used in PCR amplification.

Literary review of der(1;7)(q10;p10) cases

Reported cases of der(1;7)(q10;p10) were retrieved from the Mitelman Database of Chromosome Aberrations in Cancer 2006[21] according to the following karyotypic descriptions: der(1;7)(q10;p10); der(1)t(1;7)(p11;p11); +t(1;7)(p11;p11),−7; der(1;7)(p10;q10); and dic(1;7)(p11;q11).

Results

Patients' characteristics

In total, 123 cases, including 22 AML, 98 MDS and 3 MPD, were analyzed. The demographic features of these 123 cases are summarized in Table 1. Seventy-seven cases were der(1;7)(q10;p10), which contain 20 cases reported previously.[22,23] The other 46 cases had −7/7q- other than der(1;7)(q10;p10). Strong male predominance was evident in both groups, especially in der(1;7)(q10;p10). The cases having der(1;7)(q10;p10) accounted for 2.3% (10 cases) of 427 cases who were diagnosed as having AML or MDS at the University of Tokyo Hospital. −7/7q- was found in 38 cases among the 427 cases, of which 22 and 16 cases were diagnosed as having MDS and AML, respectively. Both showed a high median age of the disease onset but it was higher in der(1;7)(q10;p10) than for −7/7q- ($P=0.0027$). In our series, 32.9% of der(1;7)(q10;p10) and 25.0% of −7/7q- cases had one or more prior histories of chemotherapies and/or radiotherapies for some malignancies. In both groups, more cases are diagnosed as MDS than as AML. Increased eosinophil counts in der(1;7)(q10;p10) have been underscored in some literatures,[13,24,25] but in our series it was not so conspicuous and only six der(1;7)(q10;p10) and one −7/7q- cases had prominent eosinophilia ($>450/\mu l$).

Cytogenetic features

Karyotypic analysis revealed a number of cytogenetic features which contrast der(1;7)(q10;p10) to other 7q- abnormalities (Supplementary Table 1 and 2). der(1;7)(q10;p10) was always present at the time of clinical diagnosis and even when multiple subclones exist, it involves all the abnormal karyotypes. In 45 (58.4%) cases, it appeared as a sole chromosomal abnormality during the observation periods. The remaining 32 cases had additional chromosomal abnormalities, but they were limited in number and mostly consisted of trisomy 8 (18 cases) and/or loss of 20q (10 cases). In contrast, −7/7q- appeared as the solitary abnormality was less common (28.3%) and it was more typically accompanied by other frequently complex abnormalities, where 24 of the 46 −7/7q- cases showed more than four additional unrelated chromosomal abnormalities. Indeed, the mean number of additional abnormalities was significantly higher in the −7/7q cases than in der(1;7)(q10;p10) cases ($P<0.0001$) (Table 1). Moreover, in some cases, it appeared only in partial karyotypes (−7/7q- cases 5, 6, 22, 23, 28, 31 and 35) and evolved during the course of the diseases. 5q- was the most common abnormality that was found in association with −7/7q- (23/46), whereas trisomy 8 and 20q- were very rare in this group.

der(1;7)(q10;p10) in MDS

Since the majority of cases in this study were diagnosed as MDS (FAB classification[18]), it is of interest to focus the analysis on those cases having MDS (Table 2). In fact, clinical features were considerably different between both cytogenetic groups in MDS. According to the FAB classification, der(1;7)(q10;p10) cases were more likely to be classified into RA ($P=0.0002$), whereas RAEB/RAEB-t were the leading diagnosis in the −7/7q- group ($P=0.0003$). Similarly, der(1;7)(q10;p10) cases were more frequently classified into favorable risk groups than −7/7q- cases ($P<0.0001$) in the International Prognostic Scoring System (IPSS),[16] where more Int-1-risk cases ($P<0.0001$) and less high-risk cases ($P<0.0001$) were diagnosed in der(1;7)(q10;p10) than in −7/7q- cases. In accordance with this was that bone marrow

Table 1 Clinical characteristics of patients with der(1;7) and −7/7q-

	der(1;7)	*−7/7q-*	P-*value***	*der(1;7) Mittelman**	P-*value****
Number of patients	77	46		125	
Male/female	68/9	36/10	0.1963	74/51	<0.0001
Median age	67 (17–88)	58 (21–78)	0.0027	58 (7–86)	<0.0001
Positive prior history of chemoradiotherapy	25/76 (32.9%)	10/40 (25.0%)	0.4045		
Time to diagnosis (month)	105 (8–224)	76 (15–135)	0.1057		
Diagnosis					
MDS	64	34	0.2512	77	0.0015
AML	10	12	0.0888	33	0.0329
M0	2	1		2	
M1	1	2		3	
M2	3	4		4	
M4	2	1		8	
M5	0	0		0	
M6	0	3		0	
M7				3	
AML, NS	1	0		13	
MLL(Ph+)	1	1		0	
MPD	3	0		13	
Additional chromosomal abnormalities					
Total	32 (41.6%)	33 (71.7%)	0.0015	45 (36.0%)	0.4581
Trisomy 8	18	1	0.0014	25	0.5983
Trisomy 21	2	1		8	0.3235
Trisomy 9	1	0		4	0.6513
del(20q)	10	4	0.5666	2	0.0014
−5/5q-	1	23	<0.0001	3	
Number of additional chromosomal abnormalities					
1	23	7	0.0837	29	0.3223
2	7	2	0.4935	7	0.3975
3	1	4	0.0645	4	0.6513
⩾4	2	24	<0.0001	5	0.7108
PB					
WBC ($\times 10^3/\mu l$)	3.0 (0.8–39)	3.35 (0.3–56.9)	0.1072		
Hb (g/dl)	9.4 (2.8–13.8)	7.9 (3.8–15.0)	0.0150		
PLT ($\times 10^4/\mu l$)	8.1 (1.3–87)	5.75 (0.6–70.8)	0.2542		
Eosino ($/\mu l$)	45 (0–16932)	0 (0–740)	0.2720		
$>450/\mu l$	6	1			
BM					
Hypercellular	16/69	17/42	0.0585		
Normocellular	31/69	16/42	0.5544		
Hypocellular	22/69	9/42	0.2795		

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; Eosino, Median eosinophil count; Hb, median hemoglobin level; MDS, myelodysplastic syndrome; MLL, mixed lineage leukemia; MPD, myeloproliferative disorders; NS, not specified; PB, peripheral blood; PLT, median platelet count; WBC, median white blood cell count.
*Non-Asian cases from the Mitelman database of chromosome aberrations in cancer.
**P*-values are calculated between der(1;7)(q10;p10) and −7/7q- cases in the current series or ***P*-values between ours and Mittelman's der(1;7)(q10;p10) cases.

blast counts were significantly lower in der(1;7)(q10;p10) cases than in −7/7q- cases ($P<0.0001$). Also anemia tended to be less severe at the time of diagnosis in der(1;7)(q10;p10) than in −7/7q- cases ($P=0.0075$). More than half cases in both groups were transformed into AML but der(1;7)(q10;p10) cases showed significantly slower progression to AML than −7/7q- cases ($P=0.0043$). Accordingly more patients tend to have been treated by chemotherapy in −7/7q- group than in der(1;7)(q10;p10) group ($P=0.049$). In total, 57 deaths had occurred during the observation period. Infection was the most common cause of deaths in the der(1;7)(q10;p10) group without leukemic transformation (11 of 16 informative cases), whereas MDS patients were more frequently transformed to AML before death in the −7/7q- group (14/22 in −7/7q- vs 14/35 in der(1;7)(q10;p10)).

Allogeneic stem cell transplantation was performed in two der(1;7)(q10;p10) and three −7/7q- cases. The two der(1;7)(q10;p10) cases survived 1184 and 1508 days after transplantation, whereas two of the three −7/7q- cases succumbed to death within a year due to relapse or complication of the transplantation. Among non-transplanted cases, the der(1;7)(q10;p10) group showed significantly better clinical outcome than other −7/7q- cases, although there was significant heterogeneity with regard to therapies. The median overall survivals were 710 and 272 days in der(1;7)(q10;p10) and other −7/7q- cases, respectively ($P<0.0001$) (Figure 1). In univariate analyses of all MDS cases, >10% blast counts ($P=0.0006$) and cytopenia in two or more lineages ($P=0.0189$) were also extracted as significant risk factors. However, after the backward step-wise liner regressions, only −7/7q- karyotypes ($P<0.0001$)

and 60 years or more ages ($P=0.0148$) were extracted as independently significant risk factors, indicating der(1;7)(q10;p10) and −7/7q- karyotypes define separate risk groups (Table 3).

Table 2 Clinical characteristics of MDS patients with der(1;7) and −7/7q-

	der(1;7)	*−7/7q-*	*P-value*
MDS	64	34	
Male/female	57/7	27/7	0.2310
Median age	67 (17–88)	60 (22–78)	0.0974
De novo MDS	44	20	0.9999
Positive prior history of chemoradiotherapy	20	8	
WBC ($\times 10^3/\mu l$)	3.0 (0.8–34.0)	2.4 (0.3–24.0)	0.7043
Hb (g/dl)	9.0 (2.8–13.7)	7.8 (3.8–12.1)	0.0075
PLT ($\times 10^4/\mu l$)	8.0 (1.3–87)	8.1 (0.8–71)	0.9293
BM blast (%)	3.0 (0–14.0)	7.2 (0–29)	<0.0001
Trisomy 8	17	1	0.0048
del(20q)	10	4	0.7651
−5/5q-	0	20	<0.0001
Diagnosis			
RA	39	7	0.0002
RAEB	9	12	0.0203
RAEBt	6	9	0.0379
CMMoL	4	3	0.6908
MDS, NS	6	3	0.9999
IPSS			
High	5	14	0.0002
Int-2	29	17	0.6766
Int-1	31	2	<0.0001
Therapy			
Chemotherapy	11/59	10/24	0.0491
Allo HSCT	2	3	
Transformation to AML (%)	33 (51.6%)	22 (64.7%)	0.2853
Median time to transform (month)	12.0	4.23	0.0043
Median overall survival (month)	23.7	9.07	<0.0001
Death (infection)	39 (18)	22 (8)	

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CMMoL, chronic myelomonocytic leukemia; Hb, median hemoglobin level; HSCT, hematopoietic stem cell transplantation; IPSS, international prognostic scoring system; MDS, myelodysplastic syndrome; NS, not specified; PLT, median platelet count; RA, refractory anemia; RAEB, refractory anemia with excess of blasts; RAEBt, RAEB in transformation; WBC, median white blood cell count.

Mutation of runx1 *and* N-ras *genes*

Since high rates of *runx1* mutations have recently been reported in association with −7/7q- cases,[19,26,27] we examined mutation status of *runx1* in 20 der(1;7)(q10;p10) as well as 20 −7/7q- cases together with additional 20 MDS cases without cytogenetic abnormalities. More *runx1* mutations were found in der(1;7)(q10;p10), but it was not significant (7 der(1;7)(q10;p10), 2 −7/7q- and two other cases with normal karyotypes). There were no correlations between mutations and specific FAB subtypes, blast counts or over all survivals, although the sample numbers being too small. *N-ras* mutaions were found in one der(1;7)(q10;p10) cases (at codon 12), two −7/7q- cases (at codon 12) and two normal karyotypes (at codons 12 and 13), which are comparable to the rates reported previously in MDS.[20,28,29]

Literary review of 125 der(1;7)(q10;p10) cases reported previously in the literatures

In total, 164 entries were retrieved from the Mitelman database as having der(1;7)(q10;p10), of which 39 Asian cases were excluded from the further analyses to prevent duplicated retrievals and to explore the ethnic difference in the clinical and cytogenetic pictures of this translocation (Table 1). They had almost similar demographic and cytogenetic features to the current series, but still showed marked difference in several respects. Compared with Asian cases, the male preponderance

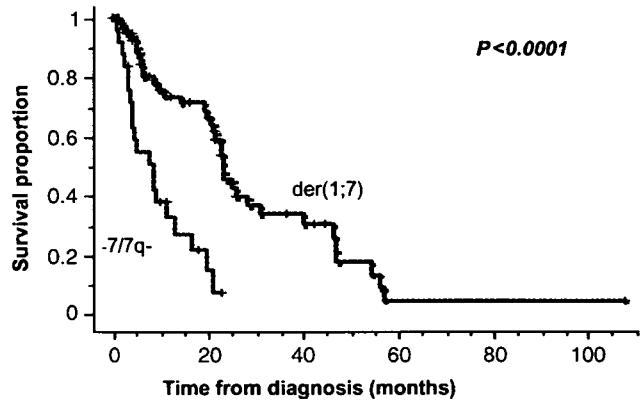

Figure 1 Comparison of survival between der(1;7)(q10;p10) and other −7/7q- cases in MDS. Kaplan–Meier curves of overall survival in MDS cases are compared between der(1;7)(q10;p10) and other −7/7q-. *P*-values in log rank tests are shown.

Table 3 Factors on overall survival

	Univariate		*Multivariate*	
	P-value	*HR (95% CI)*	*P-value*	*HR (95% CI)*
MDS cases				
der(1;7) vs −7/7q-	<0.0001	4.240 (2.202–8.164)[a]	<0.0001	4.787 (2.455–9.333)[a]
Age >60	0.0655	1.724 (0.959–3.096)	0.0148	2.088 (1.155–3.774)
Cycytopenia[b] 2/3	0.0189	1.984 (1.107–3.546)		
BM blast >10%	0.0006	2.810 (1.518–5.205)		
Additional cytogenetic changes	0.1598	1.470 (0.856–2.526)		
Secondary MDS	0.1872	1.473 (0.836–2.470)		

Abbreviations: BM, bone marrow; CI, cumulative interval; HR, hazard ratio; MDS, myelodysplastic syndrome.
[a]Hazard ratio of −7/7q- to der(1;7) group.
[b]Found in more than two lineages as defined by neutrophil count <1800/μl, platelets <10,000/μl, Hb <10 g/dl.

is not prominent and diagnosis of MDS and del(20q) abnormalities seem to be less common in the Western cases.

Discussion

We retrospectively analyzed 77 der(1;7)(q10;p10) cases in order to disclose clinical and cytogenetic features of this translocation, focusing on comparison to other −7/7q- cases, since loss of 7q material is a cardinal consequence of this unbalanced translocation as well as gain of 1q material. In spite of the common 7q loss, we found significant differences between both cytogenetic groups in terms of hematological pictures, clinical outcome and cytogenetic characteristics.

der(1;7)(q10;p10) cases tend to be presented with milder anemia, lower blast counts, thus more RA diagnosis, and to show slower progression to AML and have significantly better clinical outcome than other −7/7q- cases. The cytogenetic profiles exhibit a more striking contrast between both groups. der(1;7)(q10;p10) appears as the sole chromosomal abnormality in more than half cases and if not, the additional abnormalities are limited in number and variation, consisting mostly of trisomy 8 and loss of 20q, although the latter has not been described in the Western literatures.[14] In contrast, −7/7q- as the sole abnormality is less common in adult cases but more likely to coexist with other frequently complex abnormalities of partial karyotypes.[4] Common additional abnormalities in −7/7q- cases include −5/5q-, which predicts grave clinical outcomes among −7/7q- group[30] but is rarely found in der(1;7)(q10;p10) cases.

Comparison with der(1;7)(q10;p10) cases reported from the Western countries mostly confirmed the results from our series, but also disclosed a marked contrast between both series. Extreme male predisposition and the common 20q abnormality in our series may indicate that genetic or ethnic background play an important role in the pathogenesis of der(1;7)(q10;p10) positive neoplasms. In our series only two of nine female patients with der(1;7)(q10;p10) are *de novo* cases.

In the past studies, involving relatively small numbers of patients, reported very poor prognosis for this translocation, typically showing less than 1 year of median survival.[3,14,15] However, in the current study including 64 der(1;7)(q10;p10) cases, it appears to have better clinical outcome than reported previously, where the survival curve is roughly overlapped with that of the Int-2 IPSS category with a median survival of 23 months for der(1;7)(q10;p10) cases, which was significantly longer than 9 months of −7/7q-positive MDS cases. This may be explained by the fact that −7/7q- cases showed higher bone marrow blast counts and lower hemoglobin concentration than der(1;7)(q10;p10) cases, which were extracted as significant risk factors in univariate analyses (Table 3). Unexpectedly, however, in multivariate analysis using backward stepwise selection of covariates, only the −7/7q- karyotypes and 60 years or more ages were selected as independent risk factors. Indeed, when the effects of blast counts and cytopenia were adjusted by a proportional hazard model, difference in karyotypes still remains to be significant ($P=0.0034$). These observations suggest that der(1;7)(q10;p10) defines a separate prognostic group that shows significantly better clinical outcome than −7/7q- cases and also indicate a possibility that the der(1;7)(q10;p10) may be more appropriately assigned to an intermediate rather than high-risk karyotype in the IPSS[16] or GCEGCH[17] scoring system for better prediction of prognosis. Unfortunately, however, we were not able to test the latter possibility using our series due to a small number of the cases.

With regard to the pathogenetic role of der(1;7)(q10;p10), no specific molecular targets have been identified for this translocation. Since their breakpoints are distributed widely within the large (~0.5–3 Mb) alphoid cluster regions on the centromeres of chromosome 1 and chromosome 7,[10] no specific gene target at the breakpoints is likely to be involved in the recombination event, but loss of 7q and/or gain of 1q should play a role in the pathogenesis of this translocation. In this point of views, it may be worth mentioning that gains of 1q material are also among recurrent chromosomal abnormalities in MDS.[6,31] Of particular note is that similar 'dicentric' translocations are found in MDS and most frequently involve chromosome 1, resulting in trisomy 1q.[15]

On the other hand, it is still open to questions whether additional genetic hits are required to develop der(1;7)(q10;p10)-positive MDS. According to the cytogenetic profiles of der(1;7)(q10;p10) and −7/7q- cases, different pathogenetic models might be postulated in both cytogenetic groups. der(1;7)(q10;p10) represents a relatively early genetic event and a few additional genetic insults, including +8 and/or 20q-, could be involved in the neoplastic evolution. On the other hand, −7/7q- is likely to be shared by more heterogeneous subgroups and could be a later genetic event during neoplastic process which shows discrete cytogenetic profiles from those involved in der(1;7)(q10;p10). *Runx1* and *N-ras* genes seem to be among common targets of both cytogenetic groups. Especially, a relatively high incidence of *runx1* mutations (7/20) in our der(1;7)(q10;p10) series should be further confirmed, considering a possible link between runx1 mutations and 7q loss.[19,26]

Conclusion

der(1;7)(q10;p10) defines a unique clinicopathological entity of myeloid neoplasm having a distinct cytogenetic profile and clinical picture. In our study on heterogeneous MDS patients, their clinical outcome is not as bad as reported previously, but still poor with 23 months of median survival, which argues for importance of stem cell transplantation as a potentially curative treatment, and also for development of novel therapeutic approaches.

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ARTICLE

Highly Sensitive Method for Genomewide Detection of Allelic Composition in Nonpaired, Primary Tumor Specimens by Use of Affymetrix Single-Nucleotide–Polymorphism Genotyping Microarrays

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Loss of heterozygosity (LOH), either with or without accompanying copy-number loss, is a cardinal feature of cancer genomes that is tightly linked to cancer development. However, detection of LOH is frequently hampered by the presence of normal cell components within tumor specimens and the limitation in availability of constitutive DNA. Here, we describe a simple but highly sensitive method for genomewide detection of allelic composition, based on the Affymetrix single-nucleotide–polymorphism genotyping microarray platform, without dependence on the availability of constitutive DNA. By sensing subtle distortions in allele-specific signals caused by allelic imbalance with the use of anonymous controls, sensitive detection of LOH is enabled with accurate determination of allele-specific copy numbers, even in the presence of up to 70%–80% normal cell contamination. The performance of the new algorithm, called "AsCNAR" (allele-specific copy-number analysis using anonymous references), was demonstrated by detecting the copy-number neutral LOH, or uniparental disomy (UPD), in a large number of acute leukemia samples. We next applied this technique to detection of UPD involving the 9p arm in myeloproliferative disorders (MPDs), which is tightly associated with a homozygous *JAK2* mutation. It revealed an unexpectedly high frequency of 9p UPD that otherwise would have been undetected and also disclosed the existence of multiple subpopulations having distinct 9p UPD within the same MPD specimen. In conclusion, AsCNAR should substantially improve our ability to dissect the complexity of cancer genomes and should contribute to our understanding of the genetic basis of human cancers.

Genomewide detection of loss of heterozygosity (LOH), as well as copy-number (CN) alterations in cancer genomes, has drawn recent attention in the field of cancer genetics,[1–3] because LOH has been closely related to the pathogenesis of cancers, in that it is a common mechanism for inactivation of tumor suppressor genes in Knudson's paradigm.[4] Moreover, the recent discovery of the activating Janus kinase 2 gene (*JAK2* [MIM *147796]) mutation that is tightly associated with the common 9p LOH with neutral CNs, or uniparental disomy (UPD), in myeloproliferative disorders (MPDs)[5–8] uncovered a new paradigm—that a dominant oncogenic mutation may be further potentiated by duplication of the mutant allele and/or exclusion of the wild-type allele—underscoring the importance of simultaneous CN detection with LOH analysis. On this point, Affymetrix GeneChip SNP-detection arrays, originally developed for large-scale SNP typing,[9] provide a powerful platform for both genomewide LOH analysis and CN detection.[10–12] On this platform, the use of large numbers of SNP-specific probes showing linear hybridization kinetics allows not only for high-resolution LOH analysis at ~2,500–150,000 heterozygous SNP loci but also for accurate determination of the CN state at each LOH region.[12–14] Unfortunately, however, the sensitivity of the currently available algorithm for LOH detection by use of SNP arrays may be greatly reduced when they are applied to primary tumor specimens that are frequently heterogeneous and contain significant normal cell components.

In this article, we describe a simple but highly sensitive method to detect allelic dosage (CNs) in primary tumor specimens on a GeneChip platform, with its validations, and some interesting applications to the analyses of primary hematological tumor samples. It does not require paired constitutive DNA of tumor specimens or a large set of normal reference samples but uses only a small number of anonymous controls for accurate determination of allele-specific CN (AsCN) even in the presence of significant

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proportions of normal cell components, thus enabling reliable genomewide detection of LOH in a wide variety of primary cancer specimens.

Material and Methods

Samples and Microarray Analysis

Genomic DNA extracted from a lung cancer cell line (NCI-H2171) was intentionally mixed with DNA from its paired lymphoblastoid cell line (LCL) (NCI-BL2171) to generate a dilution series, in which tumor contents started at 10% and increased by 10% up to 90%. The ratios of admixture were validated using measurements of a microsatellite (*D3S1279*) within a UPD region on chromosome 3 (data not shown). The nine mixed samples, together with non-mixed original DNAs (0% and 100% tumor contents), were analyzed with GeneChip 50K Xba SNP arrays (Affymetrix). Microarray data corresponding to 5%, 15%, 25%,..., and 95% tumor content were interpolated by linearly superposing two adjacent microarray data sets after adjusting the mean array signals of the two sets. Both cell lines were obtained from the American Type Culture Collection (ATCC). Genomic DNA was also extracted from 85 primary leukemia samples, including 39 acute myeloid leukemia (AML [MIM #601626]) samples and 46 acute lymphoblastic leukemia (ALL) samples, and was subjected to analysis with 50K Xba SNP arrays. Of the 85 samples, 34 were analyzed with their matched complete-remission bone marrow samples. DNA from 53 MPD samples—13 polycythemia vera (PV [MIM #263300]), 21 essential thrombocythemia (ET [MIM #187950]), and 19 idiopathic myelofibrosis (IMF [MIM #254450])—43 of which had been studied for *JAK2* mutations,[8] were also analyzed with 50K Xba SNP arrays. Microarray analyses were performed according to the manufacturer's protocol,[15] except with the use of LA *Taq* (Takara) for adaptor-mediated PCR. Also, DNA from 96 normal volunteers was used for the analysis. All clinical specimens were made anonymous and were incorporated into this study in accordance with the approval of the institutional review boards of the University of Tokyo and Harvard Medical School.

AsCN Analyses Using Anonymous Control Samples (AsCNAR)

SNP typing on the GeneChip platform uses two discrete sets of SNP-specific probes, which are arbitrarily but consistently named "type *A*" and "type *B*" SNPs, at every SNP locus, each consisting of an equal number of perfectly matched probes (PM_As or PM_Bs) and mismatched probes (MM_As or MM_Bs). For AsCN analysis, the sums of perfectly matched probes (PM_As or PM_Bs) for the *i*th SNP locus in the tumor (tum) sample and reference samples (ref1, ref2,..., ref*N*),

$$S^{\mathrm{tum}}_{A,i} = \sum \mathrm{PM}^{\mathrm{tum}}_{A,i}\ ,\quad S^{\mathrm{tum}}_{B,i} = \sum \mathrm{PM}^{\mathrm{tum}}_{B,i}$$

and

$$S^{\mathrm{ref}l}_{A,i} = \sum \mathrm{PM}^{\mathrm{ref}l}_{A,i}\ ,\quad S^{\mathrm{ref}l}_{B,i} = \sum \mathrm{PM}^{\mathrm{ref}l}_{B,i}\ ,\quad (l = 1,2,3,\ldots,N)\ ,$$

are compared separately at each SNP locus, according to the concordance of the SNP calls in the tumor sample (O^{tum}_i) and the SNP calls in a given reference sample ($O^{\mathrm{ref}l}_i$),

$$R^{\mathrm{ref}l}_{A,i} = \frac{S^{\mathrm{tum}}_{A,i}}{S^{\mathrm{ref}l}_{A,i}} \qquad (\text{for } O^{\mathrm{tum}}_i = O^{\mathrm{ref}l}_i),$$

$$R^{\mathrm{ref}l}_{B,i} = \frac{S^{\mathrm{tum}}_{B,i}}{S^{\mathrm{ref}l}_{B,i}}$$

and the total CN ratio is calculated as follows:

$$R^{\mathrm{ref}l}_{AB,i} = \begin{cases} R^{\mathrm{ref}l}_{A,i} & \text{for } O^{\mathrm{tum}}_i = O^{\mathrm{ref}l}_i = AA \\ R^{\mathrm{ref}l}_{B,i} & \text{for } O^{\mathrm{tum}}_i = O^{\mathrm{ref}l}_i = BB \\ \frac{1}{2}(R^{\mathrm{ref}l}_{A,i} + R^{\mathrm{ref}l}_{B,i}) & \text{for } O^{\mathrm{tum}}_i = O^{\mathrm{ref}l}_i = AB \end{cases} (l = 1,2,3,\ldots,N)\ .$$

For CN estimations, however, $R^{\mathrm{ref}l}_{AB,i}$, $R^{\mathrm{ref}l}_{A,i}$, and $R^{\mathrm{ref}l}_{B,i}$ are biased by differences in mean array signals and different PCR conditions between the tumor sample and each reference sample and need to be compensated for these effects to obtain their adjusted values $\hat{R}^{\mathrm{ref}l}_{AB,i}$, $\hat{R}^{\mathrm{ref}l}_{A,i}$, and $\hat{R}^{\mathrm{ref}l}_{B,i}$, respectively (appendix A).[16]

These values are next averaged over the references that have a concordant genotype for each SNP in a given set of references (K), and we obtain $\bar{R}^K_{AB,i}$, $\bar{R}^K_{A,i}$, and $\bar{R}^K_{B,i}$. Note that $\bar{R}^K_{A,i}$ and $\bar{R}^K_{B,i}$ are calculated only for heterozygous SNPs in the tumor sample (see appendix A for more details).

A provisional total CN profile Λ_K is provided by

$$\Lambda_K = \{\bar{R}^K_{AB,i}\}\ ,$$

and provisional AsCN profiles are obtained by

$$\Lambda^{\mathrm{large}}_K = \{\max(\bar{R}^K_{A,i}, \bar{R}^K_{B,i})\}$$

$$\Lambda^{\mathrm{small}}_K = \{\min(\bar{R}^K_{A,i}, \bar{R}^K_{B,i})\}\ .$$

These provisional analyses, however, assume that the tumor genome is diploid and has no gross CN alterations, when the coefficients are calculated in regressions. In the next step, the regressions are iteratively performed using a diploid region that is truly or is expected to be diploid, to determine the coefficients on the basis of the provisional total CN, and then the CNs are recalculated.

Finally, the optimized set of references is selected that minimizes the SD of total CN at the diploid region by stepwise reference selection, as described in appendix A.

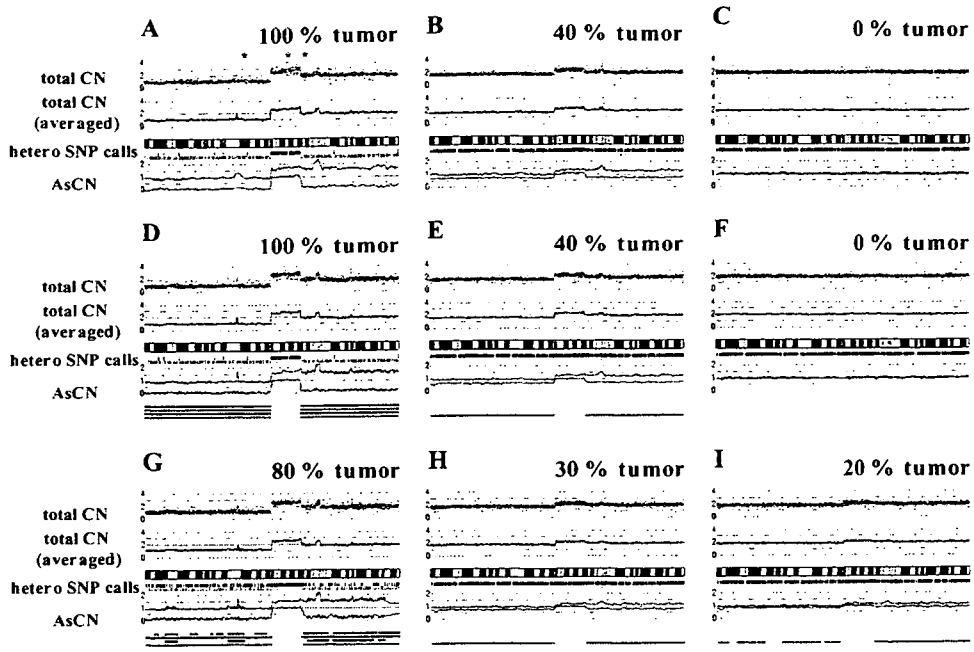

Figure 1. AsCN analysis with or without paired DNA. DNA from a lung cancer cell line (NCI-H2171) was mixed with DNA from an LCL (NCI-BL2171) established from the same patient at the indicated percentages and was analyzed with GeneChip 50K Xba SNP arrays. AsCNs, as well as total CNs, were analyzed using either the paired reference sample (NCI-BL2171) (*upper panels, A–C*) or samples from unrelated individuals simultaneously processed with the tumor samples (*middle and lower panels, D–I*). On each panel, the upper two graphs represent total CNs and their moving averages for the adjacent 10 SNPs, whereas moving averages of AsCNs for the adjacent 10 SNPs are shown below (*red and green lines*). Green and pink bars in the middle are heterozygous (hetero) calls and discordant SNP calls between the tumor and its paired reference, respectively. At the bottom of each panel, LOH regions inferred from AsCNAR (*orange*), SNP call–based LOH inference of CNAG (*blue*), dChip (*purple*), and PLASQ (*light green*) are depicted. Asterisks (*) indicate the loci at which total CNs were confirmed by FISH analysis (data not shown). The calibrations of CN graphs are linearly adjusted so that the mean CNs of null and single alleles should be 0 and 1, respectively.

Allele-specific analysis using a constitutive reference, refSelf, is provided by

$$\Lambda^{\text{large}} = \left|\max\left(\hat{R}_{A,i}^{\text{refSelf}}, \hat{R}_{B,i}^{\text{refSelf}}\right)\right|$$

and

$$\Lambda^{\text{small}} = \left|\min\left(\hat{R}_{A,i}^{\text{refSelf}}, \hat{R}_{B,i}^{\text{refSelf}}\right)\right| .$$

Computational details of AsCNAR are provided in appendix A.

Comparison with Other Algorithms

dChip[17] and PLASQ[18] were downloaded from their sites, and the identical microarray data were analyzed using these programs. Since PLASQ requires both Xba and Hind array data, microarray data of mixed tumor contents for Hind arrays were simulated by linearly superimposing the tumor cell line (NCI-H2171) and LCL (NCI-BL2171) data at indicated proportions.

Statistical Analysis

Significance of the presence of allelic imbalance (AI) in a given region, Γ, called as having AI by the hidden Markov model (HMM), was statistically tested by calculating *t* statistics for the difference in AsCNs, $|\log_2 \tilde{R}_{A,i}^{K} - \log_2 \tilde{R}_{B,i}^{K}|$, between Γ and a normal diploid region, where the tests were unilateral. Significance between the numbers of UPDs detected by the SNP call–based method and by AsCNAR was tested by one-tailed binominal tests. *P* values for AI detection by allele-specific PCR were calculated by one-tailed *t* tests, comparing triplicates of the target sample and triplicates of five normal samples that have heterozygous alleles in the SNP.

Detection of the JAK2 *Mutation and Measurements of Relative Allele Doses*

The *JAK2* V617F mutation was examined by a restriction enzyme–based analysis, in which PCR-amplified *JAK2* exon 12 fragments were digested with *Bsa*XI, and the presence of the undigested fragment was examined by gel electrophoresis.[5] Relative allele dose between wild-type and mutated *JAK2* was determined by measuring allele-specific PCR products for wild-type and mutated *JAK2* alleles by

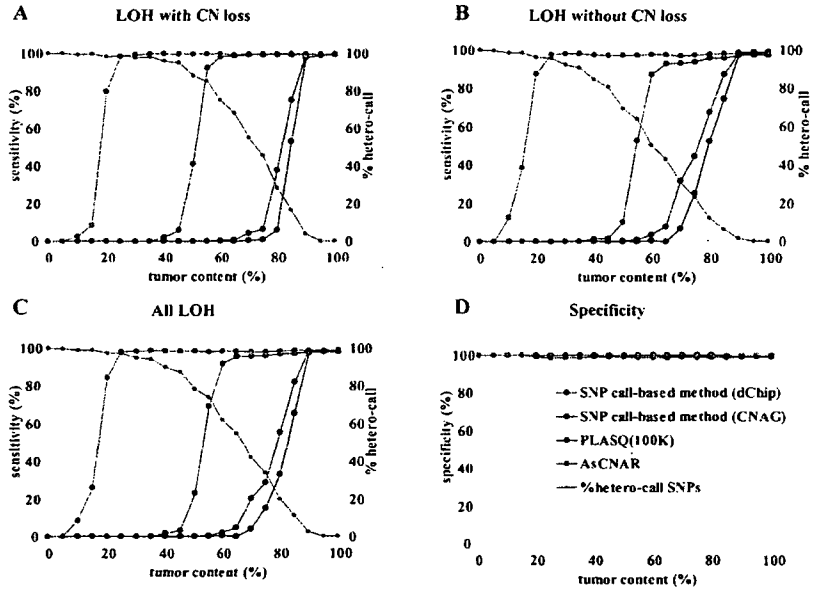

Figure 2. Sensitivity and specificity of LOH detection for intentionally mixed tumor samples. Sensitivity of detection of LOH with or without CN loss (*A* and *B*) in different algorithms were compared using a mixture of the tumor sample (NCI-H2171) and the paired LCL sample (NCI-BL2171). The results for all LOH regions are shown in panel C, and the specificities of LOH detection are depicted in panel D. For precise estimation of sensitivity and specificity, we defined the SNPs truly positive and negative for LOH as follows. The tumor sample and the paired LCL sample were genotyped on the array three times independently, and we considered only SNPs that showed the identical genotype in the three experiments. SNPs that were heterozygous in the paired LCL sample and were homozygous in the tumor sample were considered to be truly positive for LOH, and SNPs that were heterozygous both in the paired LCL sample and in the tumor sample were considered to be truly negative. Proportions of heterozygous SNP calls (%hetero-call) that remained in LOH regions of each sample are also shown in panels A–C.

capillary electrophoresis by use of the 3100 Genetic Analyzer (Applied Biosystems), as described in the literature.[19] Likewise, the fraction of tumor components having 9p and other UPDs was measured by either allele-specific PCR or STR PCR,[7,19] by use of the primers provided in appendix B [online only]. The percentage of UPD-positive cells (%UPD(+)) was also estimated as the mean difference of AsCNs for heterozygous SNPs within the UPD region divided by that for homozygous SNPs within an arbitrary selected normal region:

$$\%\mathrm{UPD}(+) = \frac{E(|\tilde{R}^K_{A,i} - \tilde{R}^K_{B,i}|_{i\in\text{hetero SNPs in UPD region}})}{E(|\tilde{R}^K_{A,j} - \tilde{R}^K_{B,j}|_{j\in\text{homo SNPs with normal CN}})},$$

where AsCNs for the denominator were calculated as if the homozygous SNPs were heterozygous. However, in those samples with a high percentage of UPD-positive components, the heterozygous SNP rate in the UPD region decreased. For such regions, we calculated the percentage of UPD-positive cells by randomly selecting 30% (the mean heterozygous SNP call rate for this array) of all the SNPs therein and by assuming that they were heterozygous SNPs. Cellular composition of *JAK2* wild-type (wt) and mutant (mt) homozygotes (wt/wt and mt/mt) and heterozygotes (wt/mt) in each MPD specimen was estimated assuming that all UPD components are homozygous for the *JAK2* mutation. The fractions of the wt/mt heterozygotes in cases with a 9p gain were estimated assuming that the duplicated 9p alleles had the *JAK2* mutation. Throughout the calculations, small negative values for wt/mt were disregarded.

FISH

FISH analysis was performed according to the previously published method, to confirm the absolute total CNs in NCI-H2171.[20] The genomic probes were generated by whole-genome amplification of FISH-confirmed RP11 BAC clones 169N13 (3q13; CN = 2), 227F7 (8q24; CN = 2), 196H14 (12q14; CN = 2), 25E13 (13q33; CN = 2), 84E24 (17q24; CN = 2), 12C9 (19q13; CN = 2), 153K19 (3q13; CN = 3), 94D19 (3p14; CN = 1), 80P10 (8q22; CN = 1), and 64C21 (13q12-13; CN = 1), which were obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute in Oakland, California.

Results

SNP Call–Based Genomewide LOH Detection by Use of SNP Arrays

When a pure tumor sample is analyzed with a paired constitutive reference on a GeneChip Xba 50K array, LOH is easily detected as homozygous SNP loci in the tumor spec-

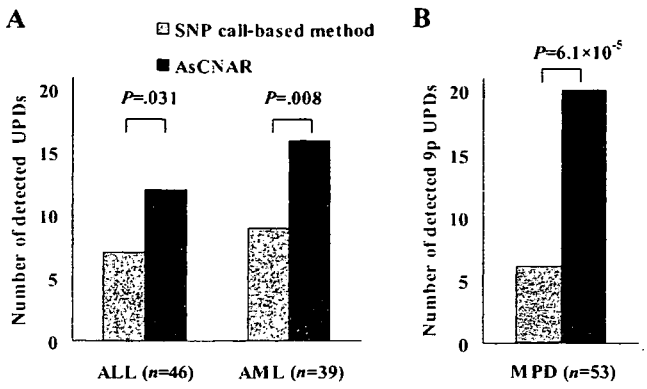

Figure 3. The number of UPD regions for acute leukemia and MPD samples detected by either the SNP call–based method or AsCNAR. The number of UPD regions for ALL and AML samples detected by the SNP call–based method or by AsCNAR is shown in panel A, and the number of 9p UPDs for MPD samples detected by the two methods is shown in panel B. Some samples have more than one UPD region. Details of UPD regions are given in table 1. Significance between the numbers of UPDs detected by the SNP call–based method and by the AsCNAR method was tested by one-tailed binomial tests.

imen that are heterozygous in the constitutive DNA (fig. 1*A*, pink bars). In addition, given a large number of SNPs to be genotyped, the presence of LOH is also inferred from the grossly decreased heterozygous SNP calls, even in the absence of a paired reference (fig. 1*D*). The accuracy of the LOH inference would depend partly on the algorithm used but more strongly on the tumor content of the specimens. Thus, our SNP call–based LOH inference algorithm in CNAG (appendix C), as well as that of dChip,[17] show almost 100% sensitivity and specificity for pure tumor specimens. But, as the tumor content decreases, the LOH detection rate steeply declines (fig. 1*G*), and, with <50% tumor cells, no LOH can be detected, even when complete genotype information for both tumor and paired constitutive DNA is obtained (fig. 1*B*, 1*E*, 1*H*, and 1*I*).

LOH Detection Based on AsCN Analysis

On the other hand, the capability of allele-specific measurements of CN alterations in cancer genomes is an excellent feature of the SNP array–based CN-detection system that uses a large number of SNP-specific probe sets.[16,18,21] When constitutive DNA is used as a reference, AsCN analysis is accomplished by separately comparing the SNP-specific array signals from the two parental alleles at the heterozygous SNP loci in the constitutive genomic DNA.[16] It determines not only the total CN changes but also the alterations of allelic compositions in cancer genomes, which are captured as the split lines in the two AsCN graphs (fig. 1*A* and 1*B*). In this mode of analysis, the presence of LOH can be detected as loss of one parental allele, even in specimens showing almost no discordant calls (fig. 1*B*).

AsCNAR

The previous method for AsCN analysis, however, essentially depends on the availability of constitutive DNA, since AsCNs are calculated only at the heterozygous SNP loci in constitutive DNA.[16] Alternatively, allele-specific signals can be compared with those in anonymous references on the basis of the heterozygous SNP calls in the tumor specimen. In the latter case, the concordance of heterozygous SNP calls between the tumor and the unrelated sample is expected to be only 37% with a single reference. However, the use of multiple references overcomes the low concordance rate with a single reference, and the expected overall concordance rate for heterozygous SNPs and for all SNPs increases to 86% and 92%, respectively, with five unrelated references (appendix D [online only]). Thus, for AsCNAR, allele-specific signal ratios are calculated at all the concordant heterozygous SNP loci for individual references, and then the signal ratios for the identical SNPs are averaged across different references over the entire genome. For the analysis of total CNs, all the concordant SNPs, both homozygous and heterozygous, are included in the calculations, and the two allele-specific signal ratios for heterozygous SNP loci are summed together. Since AsCNAR computes AsCNs only for heterozygous SNP loci in tumors, difficulty may arise on analysis of an LOH region in highly pure tumor samples, in which little or no heterozygous SNP calls are expected. However, as shown above, such LOH regions can be easily detected by the SNP call–based algorithm, where AsCNAR is formally calculated assuming all the SNPs therein are heterozygous. Thus, the AsCNAR provides an essentially equivalent result to that from AsCN analysis using constitutional DNA, with similar sensitivity in detecting AI and LOH (compare fig. 1*A* with 1*D* and 1*B* with 1*E*).

As expected from its principle, AsCNAR is more robust in the presence of normal cell contaminations than are SNP call–based algorithms. To evaluate this quantitatively, we analyzed tumor DNA that was intentionally mixed with its paired normal DNA at varying ratios in 50K Xba SNP arrays, and the array data were analyzed with AsCNAR. To preclude subjectivity, LOH regions were detected by an HMM-based algorithm, which evaluates difference in AsCNs in both parental alleles (appendix E).[22] As the tumor content decreases, the SNP call–based LOH inference fails to detect LOH because of the appearance of heterozygous SNP calls from the contaminated normal cell component (fig. 1*E* and 1*G*–1*I*), but these heterozygous SNP calls, in turn, make AsCNAR operate effectively.

Table 1. CN-Neutral LOH in Primary Acute Leukemia

The table is available in its entirety in the online edition of *The American Journal of Human Genetics.*