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Automated JAK2^{V617F} quantification using a magnetic filtration system and sequence-specific primer-single molecule fluorescence detection

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Abstract We established an automated mutational analysis detection system using magnetic filtration and the sequence-specific primer-single molecule fluorescence detection (SSP-SMFD) assay to identify the janus activating kinase-2 (JAK2)^{V617F}. DNA was extracted from 100 μL of whole blood automatically by a magnetic filtration system. The JAK2 1849G→T mutation occurs in chronic myeloproliferative disorder (CMPD), and the detection of this change has diagnostic potential. To detect and semiquantitate this mutation, we used two artificial oligonucleotides (wild-type specific and mutated-type specific) and performed the SSP-SMFD assay using an automated fluorescence cell sorter measuring device. The SSP-SMFD assay can detect the presence of a minimum of 5% of the mutated artificial oligonucleotide, thus indicating that this technique is available in detecting contamination of at least 5% cells with the homozygous JAK2^{V617F} mutation. Based on this technique, we analyzed 94 patients with CMPD and compared with the results obtained by the polymerase chain reaction (PCR)-direct sequence. Two homozygous JAK2^{V617F} patients were identified as heterozygous JAK2^{V617F} by the PCR-direct sequence, and four patients judged as wild-type JAK2 by the PCR-direct sequence were identified as heterozygous JAK2^{V617F} by the SSP-SMFD method. Our automated system is simple and suitable for high-throughput analysis in detecting JAK2^{V617F} with a threshold detection limit of 5%.

1. Introduction

The janus kinase-2 (JAK2) 1849G→T mutation results in valine to phenylalanine substitution at amino acid 617 (JAK2^{V617F}), and this molecular change induces constitute signaling of JAK2 in chronic myeloproliferative disorder (CMPD) [1–5]. The JAK2 mutation occurs in most patients with polycythemia vera (PV) and approximately 50% of patients with essential thrombocythemia (ET) or chronic idiopathic myelofibrosis [6,7]. The detection of JAK2 mutational status is important because the presence of this mutation and mutational status (i.e., heterozygous or homozygous JAK^{V617F}) is linked to hematologic and clinical complication in CMPD patients [8–13]. However, the reported incidence of JAK2^{V617F} ranged from 63–91%, and the frequency of heterozygous JAK2^{V617F} was approximately double that of homozygous JAK2^{V617F} in PV patients, ranging from 1.4 to 3.5 [6,7]. The differences among reports may result from technical differences or diagnostic criteria of CMPD, or both.

Methods for detecting the JAK2^{V617F} mutation included direct sequencing with a detection limit around 25% [1,3,4], as well as allele-specific polymerase chain reaction (PCR) [2] or amplification-refractory mutational sequencing (ARMS) PCR with a detection limit of 3% [13]. Another report dealing with AMRS-PCR and capillary electrophoresis showed a detection limit of 1% [14]. Since the detection of JAK2^{V617F} is essential for the diagnosis of PV, the simple and reliable assay system for clinical practice with acceptable detectability would be useful. We set out to develop and evaluate an automated assay system for mass-screening of the JAK2 mutational status.

2. Materials and methods

2.1. Extraction of DNA

DNA was extracted from 100 μL of whole blood by a Magtraction system (SX-6GCN; Precision System

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Science, Chiba, Japan) [15]. Magtraction is a magnetic filtration system for automated magnetic particle-based reaction systems. The automation of nucleic acid extraction mediated by magnetic particles has been done in a robot as follows. A sample is first drawn up into a pipette tip, which is loaded into a nozzle. The sample is then dispensed into a well containing reagent and mixed by repeated aspiration and dispensing. Keeping the apical end of the tip soaked in liquid prevents air bubbles or splashes from coming up. The magnetic particles can be effectively separated and captured on the inner wall of the pipette tip by a magnet that comes into contact with the tip at the part with optimal diameter during aspiration or dispensing. Moving the separated particles to the next well and repeating the separating /dispensing operation allows effective resuspending or washing of the magnetic particles (Fig. 1).

2.2. Detection of $JAK2^{V617F}$ mutation by sequence-specific primer-single molecule fluorescence detection (SSP-SMFD)

For the SSP-SMFD study, the 10 ng of extracted DNA was amplified by first-round PCR, and then extended by the fluorescence-labeled single-nucleotide polymorphism (SNP)-specific primer. In this SSP-SMFD method [16,17], DNA containing mutation was amplified by the first-round PCR, and then mutation allele–specific and wild-type allele–specific oligonucleotide labeled by fluorescence was reacted as primers, as described elsewhere [16]. If the sample DNA contains the $JAK2^{V617F}$ mutation (T), the mutation allele–specific oligonucleotide can bind to it and start to extend (Fig. 2, A and B). Fluorescence of PCR products was measured by an automated fluorescence cell sorter measuring device (MF 20; Olympus, Tokyo) (Fig. 1).

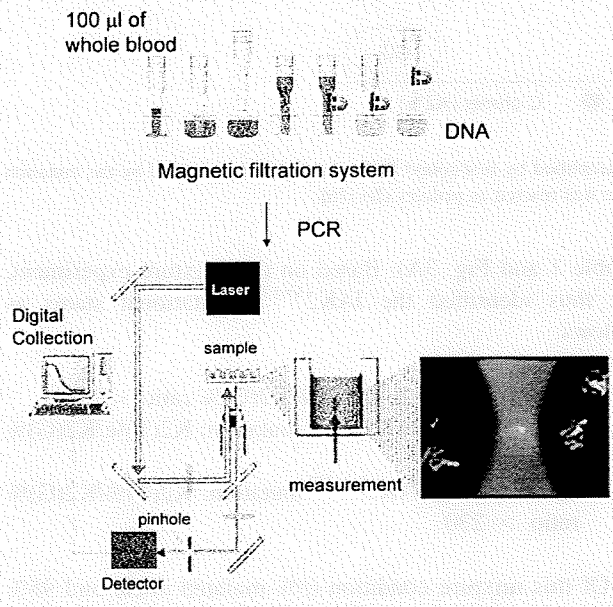

Fig. 1. Workstation of the automated $JAK2^{V617F}$ quantification using a magnetic filtration system and the SSP-SMFD technique.

The first-round forward primer was 5′-TCC TTC TTT GAA GCA GCA AGT ATG ATG AGC AAG CTT TCT CACA.

The first reverse primer was 3′-TAG AAG AGT CCT ACA GTG TTT TCA GTT TCA AAA ATA CTT AAC.

The allele-specific forward primer was 5′-AGC ATT TGG TTT TAA AAT TAT GGA GTATATT.

The allele-specific reverse primer was 3′-TGA AAC TGA AAA CTG TAGGACTATTCAG.

To quantitate the T allele–specific extension, we conducted a plasmid containing mutation allele–specific oligonucleotide (puc18_T: inserted 366-bp mutation-allele-specific-oligonucleotide into puc18) or wild-type allele–specific oligonucleotide (puc18_G), and then used that as a reference. A serial dilution of reference (puc18_T and puc18_G) was used as a standard curve. To confirm the results by SSP-SMFD, the PCR sequence technique was performed with BigDye Termination ver3.1 (Perkin-Elmer Cetus).

2.3. Clinical samples

We studied $JAK2^{V617F}$ in 94 patients with CMPD, including 34 patients with PV, 51 with ET, and 9 patients with idiopathic myelofibrosis, as well as 15 samples obtained from healthy volunteers. We collected frozen marrow cells or peripheral blood (100 µL) from patients after obtaining their written informed consent [17–19], using a SSP-SMFD assay. For detection of $JAK2^{V617F}$ mutation, we used the JAK2 muation kit (NovusGene, Tokyo, Japan).

2.4. Statistical analysis

Continuous data were compared by the two-sample *t*-test and one-way ANOVA for approximately normally distributed data, and the nonparametric Wilcoxon rank-sum test for other distributions. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Validation of SSP-SMFD assay using puc18-T and puc18-G

We first validated the SSP-SMFD assay using two plasmids, puc18_T (Cy-5 labeled) and puc18_G (TAMRA labeled), which contained sequences with mutated or wild-type JAK2. To obtain reproducible results and to clarify the quantitative relationship between the mutated allele and the wild-type allele in the sample, we calculated the ratio of K2(T)%/K2(G)% × 100 [K2(T)%/K2(G)%]. We mixed two references with various ratios (percentages of puc18_T: 0–100%), and measured K2(T)% or K2(G)% (Table 1 and Fig. 3A). In the presence of 5% Cy-5–labeled puc 18_T mutated allele, the K2(T)% increased to 4.6%, while the K2(G)% was 55.2%, then the K2(T)%/K2(G)% ratio [K2(T)%/K2(G)% × 100] was 27.717. Without the puc

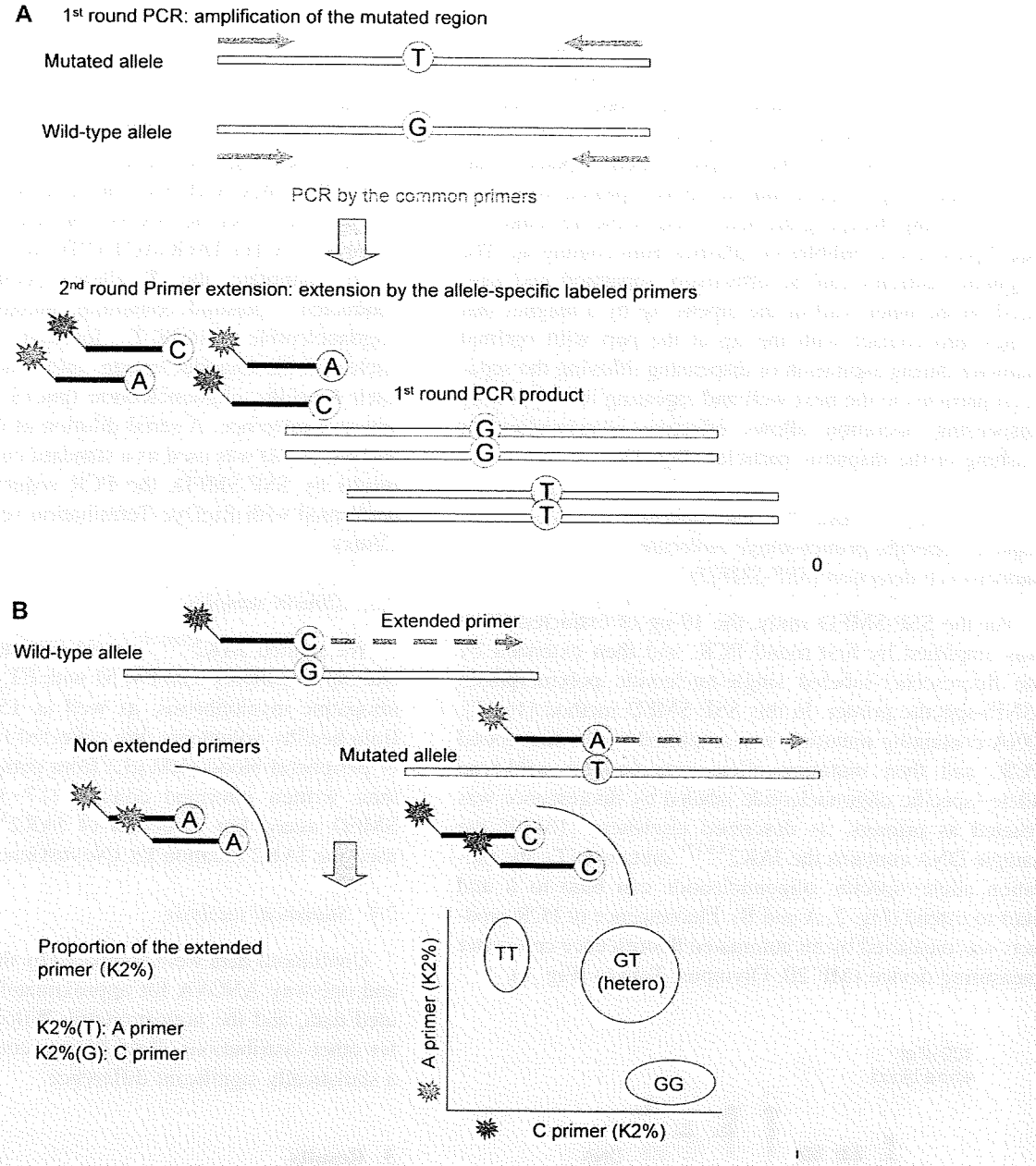

Fig. 2. Schematic presentation of the SSP-SMFD assay. The extracted DNA was amplified by first-round PCR (A), and then extended by the mutation allele–specific and wild-type allele–specific oligonucleotide labeled by fluorescence was reacted as primers (B) [16].

18_T mutated allele, the K2(T)%/K2(G)% ratio was 8.288, therefore we determined that the presence of the 5% mutated allele in the sample could be separated by the K2(T)%/K2(G)% ratio of 30. A K2(T)%/K2(G)% ratio less than 30 was thus considered to be wild-type JAK2. In the presence of 80% Cy-5-labeled puc 18_T mutated allele, the K2(T)%/K2(G)% ratio was 169.431, while the K2(T)%/K2(G)% ratio of 90% of mutated allele was 230.519. Therefore, we tentatively determined that the homozygous $JAK2^{V617F}$ mutation was a K2(T)%/K2(G)% ratio of 230 or more because it was difficult to separate the mutated allele between 40% [K2(T)%/K2(G)% ratio was 128.235] and 80% [K2(T)%/K2(G)% ratio was 169.431] mixture (Table 1 and Fig. 3A). Based on this mixture experiment, we thus identified the $JAK2^{V617F}$ mutational status as follows:

- Wild-type JAK2: K2(T)%/K2(G)% ratio < 30
- Heterozygous $JAK2^{V617F}$ mutation: K2(T)%/K2(G)% ratio 30–230
- Homozygous $JAK2^{V617F}$ mutation: K2(T)%/K2(G)% ratio > 230

Of this mixture condition (5% mutated allele and 95% wild-type allele), the K2(T)%/K2(G)% ratio was 27.717, thus we considered that this method could detect at least

Table 1
Mixture experiment using Cy-5-labeled puc 18_T mutated allele and TAMRA-labeled puc 18_G wild-type allele

Percentage of mutated	Measure d T%	Log K2(T)%	Measure d G%	Log K2(G)%	K2(T)%/K2(G)% × 100
0%	4.60%	0.663	55.50%	1.744	8.288
5%	15.30%	1.185	55.20%	1.742	27.717
10%	33.90%	1.53	57.50%	1.76	58.957
20%	49.30%	1.693	57.80%	1.762	85.294
40%	65.40%	1.816	51.00%	1.708	128.235
80%	71.50%	1.854	42.20%	1.625	169.431
90%	71%	1.851	31%	1.489	230.519
100%	70.90%	1.851	3.70%	0.568	1961.216

5% mixture of the T-specific population [i.e., at least 10% of the population with heterozygous JAK2^{V617F} (Fig. 3B).

3.2. *JAK2^{V617F} mutation in clinical specimens*

By the SSP-SMFD assay, 9/34 PV patients diagnosed by the Polycythemia Vera Study Group (PVSG) had wild-type JAK2, 18 had heterozygous JAK2^{V617F}, and 7 homozygous JAK2^{V617F} (Fig. 4, A and B). There was no particular difference in the K2(T)%/K2(G)% ratio among patients with secondary erythrocytosis (n = 8: 10.9 ± 5.8), those with PV without the World Health Organization (WHO) criteria (n = 7: 8.3 ± 4.9), and those with WHO-PV with wild-type JAK2 (n = 2: 10.4 ± 8). Of the seven patients with homozygous JAK2^{V617F} (TT) by the SSP-SMFD assay, two were identified as heterozygous JAK2^{V617F} (GT) by the PCR-direct sequence. Thirty-two ET patients (62.7%) had JAK2^{V617F} with 31 heterozygous and 1 homozygous JAK2^{V617F} by the SSP-SMFD, and 4 ET patients judged as wild-type JAK2 by the PCR-direct sequence were identified as heterozygous JAK2^{V617F} by the SSP-SMFD method. All the specimens obtained from healthy volunteers showed wild-type JAK2 [n = 15; K2(T)%/K2(G)% ratio = 10 ± 3.6].

4. Discussion

The detection of JAK2^{V617F} is important in diagnosing PV patients because the current PV criteria included the presence of this mutation [20]. To detect this mutation, the PCR-direct sequence method was first applied, but the limitation of detectability was about 25% of mutated alleles. Vannucchi et al. [14] reported a quantitative assay for JAK2 using ARMS-PCR with capillary electrophoresis with a detection limit of around 1%. Capillary electrophoresis is powerful tool to resolve different amplicons and therefore to detect a minor population of JAK2 mutated allele (around 1%), but it might be a complex problem in clinical practice. Poodt et al. [20] developed a semi-quantitative real-time PCR test to detect JAK2^{V617F}. With this assay, quantities down to 0.8% JAK2 ^{V617F} among wild-type DNA could be detected reliably, and they concluded that the JAK2^{V617F} qPCR assay is quick, effective, simple,

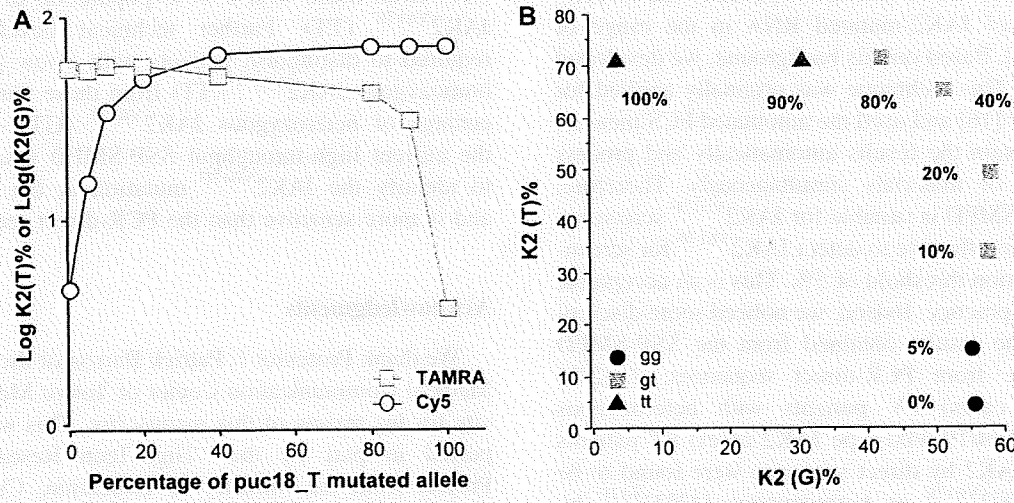

Fig. 3. (A) Quantitative relationship between percentage of mutated alleles and genotypes. The mixture experiment used puc18_T and puc18_G: puc18_T was labeled with Cy5 and puc18_G was labeled with TAMRA. Each fluorescence signal was expressed as K2%. The mixed percentage of puc18_T is indicated in the horizontal axis and log K2(T)% or log K2(G)% are shown in the vertical axis. (B) Scattergram of K2(T)% (vertical axis) and K2(G)% (horizontal axis) of the SSP-SMFD assay in a mixture experiment reference for mutation allele–specific (Cy5) and wild-type allele–specific (Cy3). Percentages in the figure indicate the mixed percentage of mutation-allele-specific reference. Separation is based on the K2(T)%/K2(G)% ratio [=K2(T)%/K2(G)% × 100]. K2(T)%/K2(G)% ratio below 30 are designated as wild-type JAK2 (solid dots), K2(T)%/K2(G)% ratio between 30 and 230 are as heterozygous JAK2^{V617F} (solid squares), and those with 230 or more are considered as homozygous JAK2^{V617F} (solid triangles).

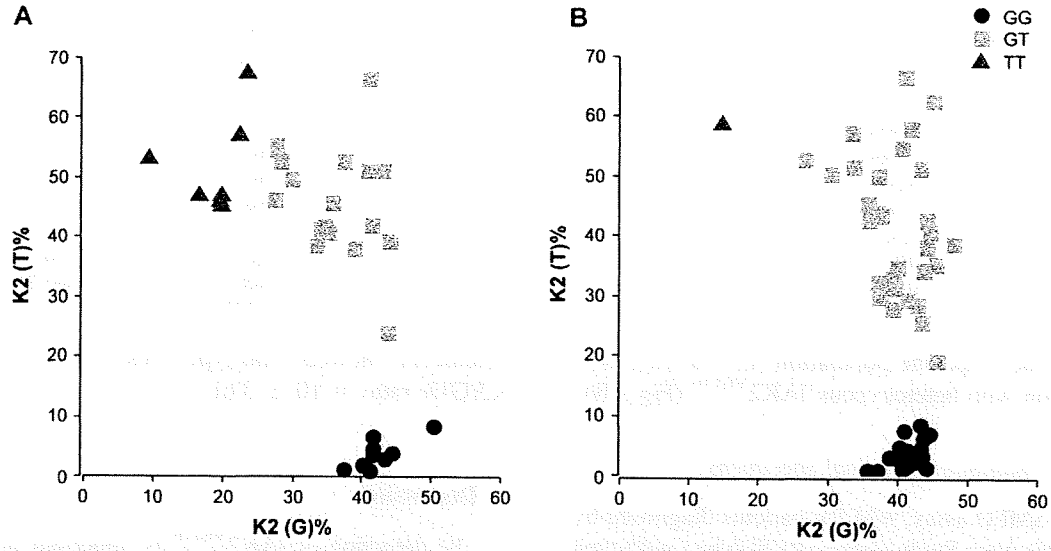

Fig. 4. Scattergram of K2(T)% (vertical axis) and K2(G)% (horizontal axis) of the SSP-SMFD assay in patients with PV diagnosed by PVSG criteria (A) or those with ET (B). Separation of the JAK2 mutational status was based on the K2(T)%/K2(G)% ratio [=K2(T)%/K2(G)% × 100]. Patients with a K2(T)%/K2(G)% ratio less than 30 are designated as wild-type JAK2 (solid dots), those with a K2(T)%/K2(G)% ratio between 30 and 230 are designated as heterozygous $JAK2^{V617F}$ (solid squares), and those with 230 or more as homozygous $JAK2^{V617F}$ (solid triangles).

and more sensitive than direct sequencing, restriction fragment length polymorphism (RFLP), ARMS assay, and other methods published so far to detect $JAK2^{V617F}$ [20]. Lay et al. also reported a simple and sensitive method to detect this mutation via PCR and probe dissociation analysis using the LightCycler platform, and they concluded that the LightCycler method offered advantages of speed, reliability, and more straightforward interpretation over the RFLP and sequencing approaches [21]. The detection of a minor population of around 1% of mutated JAK2 allele might be necessary in only a limited number of patients who actually had low levels of JAK2-mutated RNA in the range of 1–4% [14,20,21]. Based on this background, we developed the SSP-SMFD. This technique was originally applied for detection of SNP [16] and used the automated FCS measuring device to obtain the results automatically and process large quantities of materials simultaneously. Therefore, the current SSP-SMFD is suitable for $JAK2^{V617F}$ screening. Our SSP-SMFD is effective to detect $JAK2^{V617F}$ for screening, with a detection threshold of 5%. This is an acceptable level for clinical practice. Indeed, we noticed some discrepancy between the results obtained from the SSP-SMFD assay and those from PCR-direct sequences; 2 (2/25 JAK2 mutated cases) PV patients with heterozygous $JAK2^{V617F}$ and 4 (4/19 wild-type JAK2 cases) ET patients with wild-type JAK2 by direct sequence were found to be homozygous $JAK2^{V617F}$ or heterozygous $JAK2^{V617F}$ by SSP-SMFD, respectively [18]. We therefore concluded that the SSP-SMFD assay may be more sensitive for detection of mutated alleles than the PCR-direct sequence assay. The frequency of $JAK2^{V617F}$ in PV patients due to technical differences is also currently closed up, but we must be cautious in understanding the biologic significance of this mutation with a very low percentage of cells with $JAK2^{V617F}$ in CMPD [22].

In the current study, we tentatively assigned patients with homozygous $JAK2^{V617F}$ mutation as those with a K2(T)%/K2(G)% ratio of more than 230. Based on the mixture experiments using artificial oligonulcotides, this level represents more than 90% of cells with homozygous $JAK2^{V617F}$ (TT). The SSP-SMFD assay detects only a rough percentage of $JAK2^{V617F}$ mutated cells, therefore it is difficult to separate cells with 100% heterozygous $JAK2^{V617F}$ (GT) from those with a 50% population of homozygous $JAK2^{V617F}$ (TT). Further technical developments are required to distinguish patients with a low percentage of homozygous $JAK2^{V617F}$ (TT) from those with a high percentage of heterozygous $JAK2^{V617F}$ (GT). Nevertheless, the current high-throughput SSP-SMFD assay is suitable to identify the $JAK2^{V617F}$ mutation at the clinical level and is more sensitive than the PCR-direct sequence.

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achieved morphologic and cytogenetic remission after 2 months of treatment with lenalidomide therapy and a second patient who achieved a platelet response after only 1 month of therapy before receiving an unrelated allogeneic stem cell transplant. Both patients remain without any evidence of relapse with a maximum follow-up of 8 months.

Both radiation therapy and traditional DNA-interactive antineoplastics, such as alkylating agents and topoisomerase II inhibitors, are known genotoxins with the potential to induce MDS or acute myeloid leukemia (AML) that commonly harbors a chromosome 5q deletion with high frequency of evolution to AML and short overall survival.[4–6] Our findings indicate that lenalidomide has therapeutic potential in patients with secondary MDS with complex karyotype accompanied by chromosome 5q deletion.

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Recurrent chromosomal aberration at 12q15 in chronic idiopathic myelofibrosis with or without JAK2^{V617F} mutation

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Mutation of JAK2^{V617F} is currently known to play a potential role in the development of chronic myeloproliferative disorders (CMPD);[1] 78% (393/506) of polycythemia vera (PV) cases have JAK2^{V617F}, while only 43% (55/127) of reported chronic idiopathic myelofibrosis (CIMF) cases have JAK2^{V617F}.[2] Thus the question naturally arises whether the CIMF patients without JAK2^{V617F} might have another pathway towards myelofibrosis or a common pathogenic factor may exist with or without the

Table 1 JAK2-V617F status and cytogenetic results at the time of myelofibrosis

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Idiopathic myelofibrosis			
JAK2_0048	41/male	46,XY,**t(1;12)(p34;q15)**[10]	G/G
JAK2_0039	56/male	46,XY,del(11)(q13)[18]/46,XY[3]	G/G
JAK2_0057	78/female	46,XX[21]	G/G
JAK2_0098	67/male	46,XY,del(20)(q11)[5]/46,XY[3]	G/G
JAK2 0112	33/male	46,XY[23]	G/G
JAK2_0163	63/female	46,XX,**t(12;20)(q15;q11)**[7]/47,XX,+9[10]	G/T
JAK2_0036	54/male	46,XY,del(20)(q11)[2]/ 46,XY,idem,t(2;17)(q24;q22)[13]/ 46,XY,idem,i(17q)[5]	G/T
JAK2_0105	70/male	46,XY,add(9)(p21)[16]	G/T
JAK2_0148	56/male	46,XY[21]	T/T
Myelofibrosis with prior history of myelodysplastic syndrome			
JAK2_0021	70/male	46,XY,**t(4;12)(q27;q15)**[22][a]	G/T
Polycythemia vera developing myelofibrosis			
JAK2_0042	55/female	46,XX,del(7)(q22)[9]/45,X,add(X)(p22),−18[6]/ 46,XX[5]	G/T
JAK2_0061	60/female	46,XX,tan(1q12–1qter)[8]/46,XX[1]	G/T
JAK2_0065	43/female	43,XX,−1,−3,−7,−9,−10,−12,−13, −16,+5m[13]/46,XX[8]	T/T
JAK2_0118	46/female	46,XX[20]	T/T
JAK2_0141	62/male	46,XY[20]	T/T

Table 1 *Continued*

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Essential thrombocythemia developing myelofibrosis			
JAK2_0013	58/female	46,XX[13]	G/G
JAK2_0035[b]	49/male	46,XY,t(2;5)(p16;q14),add(11)(q23)[23]	G/G
JAK2_0005[b]	57/male	46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[20]/ 46,XY,idem,add(18)(p11)[2]/46,XY[4]	G/T
JAK2_0034	67/male	46,XY,i(7q)[6]/48,XY,+8,+21[9]/46,XY[6]	G/T
JAK2_0054[b]	59/male	46,XY,+1,der(1;7)(q10;p10)[9]/46,XY[3]	G/T
JAK2_0055	76/male	46,XY,add(18)(p11)[20]	G/T
JAK2_0158	46/female	46,XX[17]	G/T

[a]This case was reported as myelodysplastic syndrome developing myelofibrosis with a 6-year interval;[5] however, the continuity of the disease is uncertain.[3]
[b]JAK2_0035 was UPN-12, JAK2_0005 was UPN-6 and JAK2_0054 was UPN-8 in Hsiao *et al.*[6]
Bold signifies chromosomal translocations involving 12 of 15 region.

presence of $JAK2^{V617F}$. During the series of mutational assay of $JAK2^{V617F}$ in CMPD using the sequence-specific primer-single molecule fluorescence detection assay,[3] we studied cytogenetic changes in nine patients with CIMF, 51 with essential thrombocythemia (ET) and 34 with PV. Myelofibrosis developed in seven of 51 patients (13.7%) with ET and five of 34 patients (14.7%) with PV, and we compared clinical and cytogenetic changes between patients with CIMF and those with myelofibrosis developing in PV/ET.

The group of patients with myelofibrosis associated with PV/ET had high incidences of history of thrombosis (4/12 versus 0/9; $P=0.0542$), requirement of cytoreductive chemotherapy (12/12 versus 5/9; $P=0.0103$) and acute leukemia development (7/12 versus 1/9; $P=0.0274$) compared with the CIMF group. Depending on the status of $JAK2^{V617F}$, the group of patients with myelofibrosis associated with PV/ET with GT/TT mutation of JAK2 had a high incidence of chemotherapy requirement (10/10 versus 2/4; $P=0.0157$) and tended to have a frequent thrombosis history (4/10 versus 0/4; $P=0.1345$) (Supplementary Table 1). However, there was no particular difference in the percentage of abnormal karyotypes at the time of myelofibrosis according to CIMF diagnosis or the mutational status of $JAK2^{V617F}$. We also noted a high frequency of myelofibrosis development in patients with $JAK2^{V617F}$ in PV (wild-type JAK2/heterozygous $JAK2^{V617F}$/homozygous $JAK2^{V617F}$: 0/9 versus 2/18 versus 3/7: $P=0.0460$), but not in ET (2/18 versus 5/30 versus 0/1: $P=0.7970$), in agreement with the report by Kralovics *et al.*[4]

In myelofibrosis patients, we noticed that two of the nine patients with CIMF had chromosome abnormalities at the 12q15 region; one had t(1;12)(p34;q15), while the other had t(12;20)(q15;q11?) (Table 1 and Figure 1). Another patient (JAK2_0021) reported as showing t(4;12)(q31;q21) had a prior history of myelodysplastic syndrome (MDS)-refractory anemia with a normal karyotype 6 years before myelofibrosis.[5] Re-assessment by the spectral karyotypic analysis revealed that this anomaly was t(4;12)(q27;q15) (Supplementary Figure 1). In contrast to the results of CIMF, no patients with myelofibrosis developing from PV/ET had 12q15 anomaly. Of the 12 patients with myelofibrosis associated with PV/ET, four had −7/7q−, including two with der(1;7)(q10;p10); both patients with der(1;7)(q10;p10) had a prior ET diagnosis[6] with heterozygous $JAK2^{V617F}$ (Table 1). Of the nine patients with CIMF and one myelofibrosis associated with MDS, four had heterozygous and

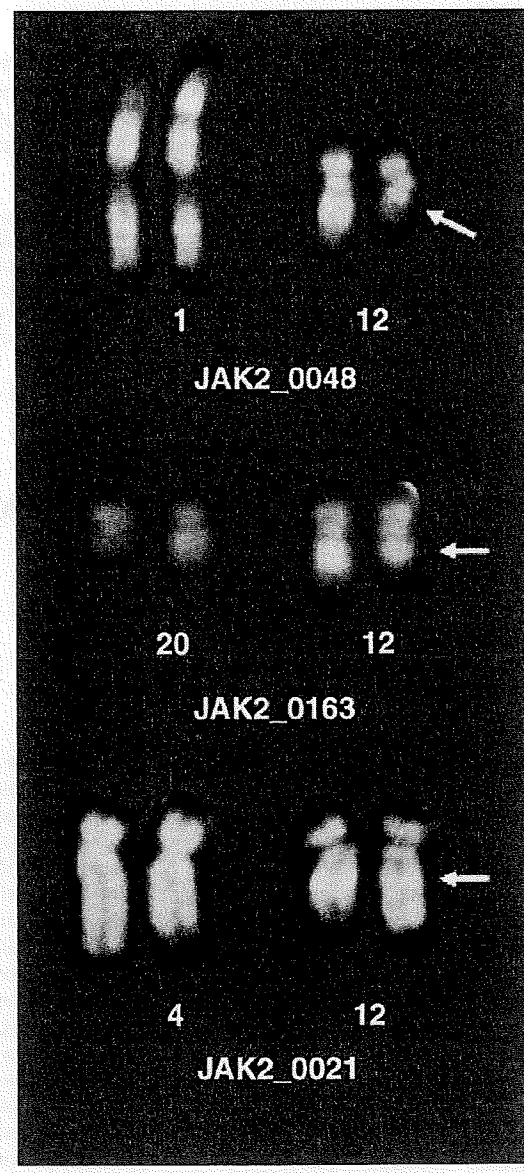

Figure 1 Partial quinacrine-banded karyotypes obtained from two patients with chronic idiopathic myelofibrosis (JAK2_0048 and JAK2_0163) and one patient with myelofibrosis with prior history of myelodysplastic syndrome (JAK2_0021) showing 12q15 anomalies, that is, t(1;12)(p34;q15), t(12;20)(q15;q11?) and t(4;12)(q27;q15). Arrows indicate possible breakpoint of 12q15.

one had homozygous $JAK2^{V617F}$; the 12q15 anomaly was detected in one patient with wild-type JAK2, while two had heterozygous $JAK2^{V617F}$.

Cytogenetic changes in CIMF are well documented: +8, del(20q), −7/7q−, del(11q) and del(13q) are known to be recurring nonspecific cytogenetic abnormalities, and some of them are also detectable in PV or ET patients. In the literature, Andrieux *et al.*[7] reported a possible role in the association between *HMGA2* and translocation involving 12q15 in CIMF. In the current study, we found that 12q15 anomaly does not depend on the JAK2 mutational status; thus genetic anomaly, independent to $JAK2^{V617F}$, may exist in CIMF, and molecular study on the 12q15 region, including *HMGA2*,[8] may disclose another pathogenetic pathway in CIMF. The 12q15 chromosomal abnormality was recurrently detected in patients with CIMF, while der(1;7)(q10;p10) was only noted in ET patients who had myelofibrosis with $JAK2^{V617F}$.[6] These findings clearly indicate that myelofibrosis among CMPD might be cytogenetically heterogeneous.

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Neighboring adipocytes participate in the bone marrow microenvironment of multiple myeloma cells

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Multiple myeloma (MM) is a hematological malignancy, characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). MM disease progression has been recently recognized as the result of an evolving crosstalk between different cell types within the BM. Although genetically abnormal plasma cells define the tumor compartment itself, the surrounding and interwoven stroma provides the supporting framework of the tumor. This framework includes extracellular matrix proteins, secreted growth factors and cellular interactions with fibroblasts, macrophages, endothelial cells, osteoblasts and osteoclasts.[1] Little attention has been given to another cell type present in the BM cavity: namely the adipocyte. These are absent in the BM of a new-born individual; however, their number increases with advancing age, resulting in adipocytic deposits occupying up to 70% of the BM cavity in elderly persons. MM is typically a disease of the elderly with a median age of diagnosis of 65 years and the incidence increases with age. Knowing that with advancing age, the BM cavity is filled with adipocytes and that MM cells closely interact with their neighboring cells, we assumed functional interactions between BM adipocytes and MM cells. We studied these interactions using the 5T33MM model and the human MM5.1 cell line. In this study, we further tried to characterize the secreted cytokines and explored the potential role of leptin in mediating the effects of adipocytes. We finally evaluated the expression of leptin receptor on both murine and human MM cells and tried to correlate this with different clinical parameters.

From the observation that MM cells, at interstitial disease stages, can be found in close contact with adipocytes, functional interactions between these cells are reasonable and prompted us to start *in vitro* tests. The murine BM adipocytic cell line 14F1.1 (obtained from Professor Zipori D, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) and primary isolated human adipocytes were used. The 14F1.1 cells initially have a

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Lack of nucleophosmin mutation in patients with myelodysplastic syndrome and acute myeloid leukemia with chromosome 5 abnormalities

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Abstract
Nucleophosmin (*NPM1*) gene exon 12 mutations are frequently present in patients with acute myeloid leukemia (AML) with normal karyotype. The *NPM1* gene is located on chromosome 5q35, which is often affected in myeloid malignancies including myelodysplastic syndrome (MDS). This suggests that the *NPM1* gene is a one of the target genes affected by chromosome 5 abnormalities and play a role in the development of MDS. It has not been clarified whether *MPM1* mutations are present in patients with MDS and AML with chromosome 5 abnormalities. Therefore, we carried out a mutational analysis on the *NPM1* gene exon 12. *NPM1* mutations were not detected in the 28 patients with MDS and AML with chromosome 5 abnormalities.

Keywords: *Nucleophosmin, MDS, AML, chromosome 5*

Introduction

Nucleophosmin (NPM) is a nuclear phosphoprotein, which functions as a molecular chaperon, and shuttles between the nucleus and cytoplasm [1–3]. Although the biological function of NPM has not been fully elucidated, it is thought to be involved in cell growth and differentiation. Several studies indicate that NPM plays a role in a p53-mediated cellular process, and it may function as a tumor suppressor gene product [4–6]. Recently, it has been reported that mutations in exon 12 of the nucleophosmin (*NPM1*) gene are frequently present in the patients with acute myeloid leukemia (AML) with a normal karyotype [7]. Since the initial report, other groups have reported similar research results, confirming implication of *NPM1* exon 12 mutations in human leukemogenesis [8–13]. The *NPM1* gene is located on chromosome 5q35, which is often affected in myeloid disorders including myelodysplastic syndrome (MDS) and AML [14–17], and the *NPM1* gene is lost in some MDS and AML cases with chromosome 5 rearrangement [18]. In addition, *NPM1* heterozygous ($NPM1^{+/-}$) mice develop MDS-like disorder at 6–10 months of age [19]. These facts suggest implication of *NPM1* abnormality in the development of human MDS. Two recent studies demonstrated that the *NPM1* gene mutations are uncommon in MDS [20,21]. Interpretation of the rare cases of MDS with *NPM1* mutations [20,21] is still a subject of controversy, since multilineage involvement is a distinguishing feature of AML with *NPM1* mutations [22]. Thus, these cases could well represent examples of de novo *NPM1*-mutated AML exhibiting multilineage involvement and dysplastic features [23]. Moreover, it has not been clarified whether the *NPM1* gene mutations are present in MDS and other myeloid malignancies

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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

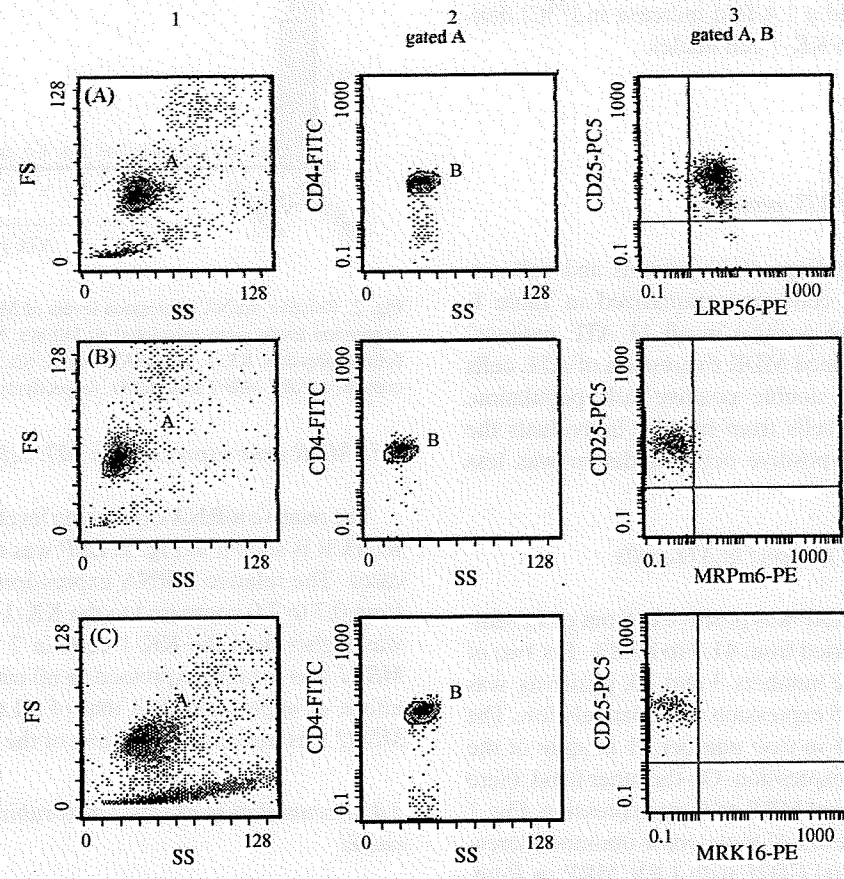

Fig. 1. Expression of LRP, MRP and *P*-gp by using three color flowcytometric analysis. Flowcytometric analysis of case number 8. A1–A3, B1–B3 and C1–C3 represent CD4+ CD25+ cell populations expressing LRP (89.8%), MRP (2.1%) and *P*-gp (0.6%), respectively.

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%.

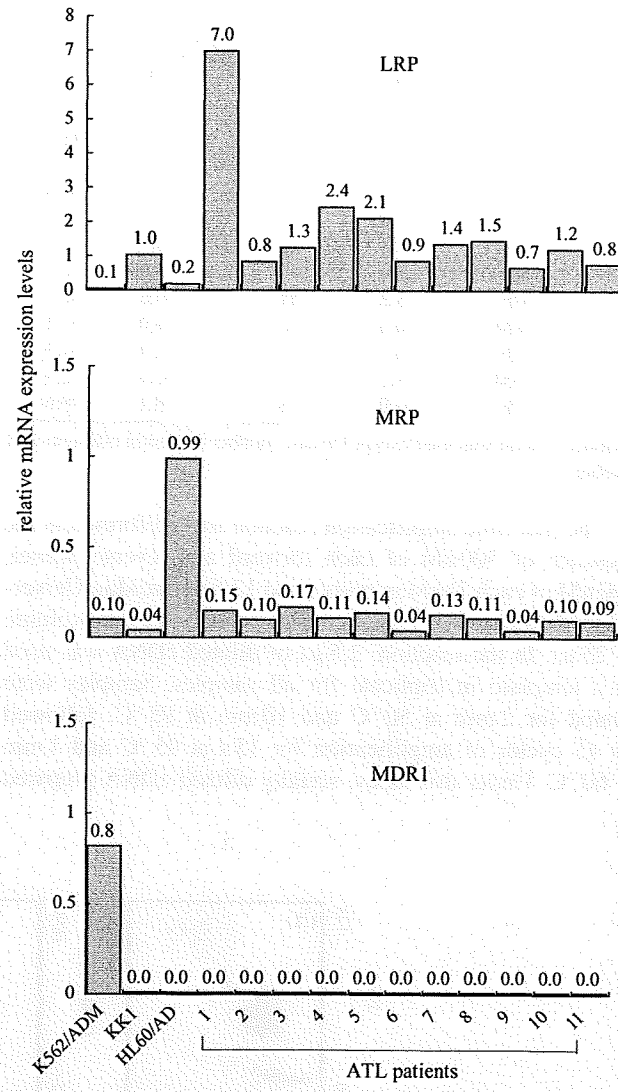

Fig. 2. Relative mRNA expression levels of MDR genes. Relative mRNA expression levels were calculated as follows: MDR protein expression/β-actin expression. KK-1, HL60/AD and K562/ADM were used as positive controls of *LRP*, *MRP1* and *MDR1*, respectively.

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-

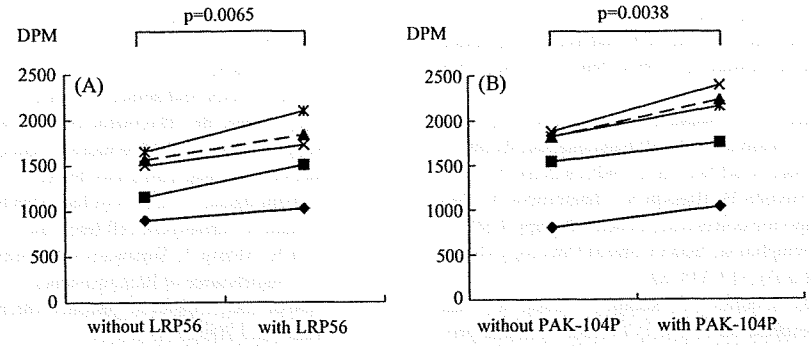

Fig. 3. Accumulation of [^{14}C] doxorubicin in isolated nuclei. Anti-LRP antibody (A) LRP reversing agent PAK-104P (B) significantly increased accumulation of [^{14}C] doxorubicin. Each value represents the mean of triplicate measurements (DPM).

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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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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