Table 4 Reasons for not visiting hospitals and the view of future attendance

Why don't you regularly consult a physician? ($n = 56$)	Number of respondents
The physician-in-charge said I did not need to visit hospital anymore	33
I don't need to consult a physician because I am in good health	26
I did not have time	7
A cessation of public financial support	2
Changes in the physician-in-charge	2
I had moved away	1
What do you think of regular consultations in the future? ($n = 56$)	
I have to consult a physician if I have any trouble with my condition	23
I should regularly visit a hospital	16
I want to consult a physician but there are no appropriate hospitals to visit	13
It is enough to undergo a school or office medical examination	13
A regular consultation is difficult because medical expenses are high	3

respondent did not answer this question. Fifteen (48.4%) of 31 respondents had consulted a physician because of late effects of cancer treatment, including the need for hormone replacement therapy for gonadal dysfunction. However, no respondents reported receiving survivor-focused care that included advice about risk reduction or a discussion of screening tests.

Fifty-six respondents not visiting hospitals regularly were asked about their reasons for not visiting and their view of future attendance. As shown in Table 4, 33 (58.9%) answered that the physician-in-charge said that they did not need to visit hospitals anymore because they had been cured, and 26 (46.4%) felt that they did not need to consult a physician because they were in good health.

Also, 23 (41.1%) of 56 respondents answered that they only had to consult a physician if they had any trouble with their condition.

When asked what kind of hospitals they would like to visit regularly in the future, 76 of 88 (86.4%) respondents answered. Thirty of them (39.5%) wanted to visit a long-term follow-up clinic, 25 (32.9%) wished to attend the hospital where they received their cancer treatment, and 24 (31.6%) chose the hospital where their physician-in-charge worked. One (1.3%) of the guardians wanted her child to visit a hospital near her house.

Discussion

Adult survivors of childhood cancer are at risk for adverse effects later in life. According to the Childhood Cancer Survivor Study (CCSS), it has been estimated that by 30 years after their cancer diagnosis, 73% of survivors will develop at least one chronic physical health condition, and 42% will develop a severe, life-threatening, or disabling condition that could result in death.[2] A risk-based approach to health care with a systemic plan for lifelong screening, surveillance, and prevention that incorporates risks based on previous cancer, cancer therapy, genetic predispositions, lifestyle factors, and comorbid health conditions is important, even if survivors have no late effects when their cancer therapy is completed. In another report from the CCSS, only 31.5% of survivors reported receiving care that focused on their prior cancer (survivor-focused care), and 11.2% received no medical care during the preceding 2 years.[4] In our institutions, we could not confirm hospital attendance of 396 (61.8%) among 641 survivors during the preceding 2 years. The ratio of non-attendees is extremely high, compared with CCSS participants. It is important to understand the background and reasons patients choose not to attend follow-up visits to develop a better long-term follow-up system in Japan.

In our study, two key reasons for non-attendance at hospitals were that the physician-in-charge said that the patient did not need to visit the hospital anymore because the cancer was cured or because the patient was in good physical condition. In addition, survivors thought that they did not need to consult a physician because they were in good health. Therefore, it is important to educate physicians and survivors about the late effects of cancer and its treatment and the importance of long-term follow up.

In an investigation of breast cancer surveillance practices among female childhood cancer survivors, screening rates were higher among women who reported a physician's recommendation to undergo screening than those who did not.[5] Having medical care providers communicate sufficient knowledge to patients about potential late effects of cancer and its treatment may play an important role in increasing attendance at follow-up visits.

Our results showed that only 75% of patients had been informed about their childhood cancer, and only 30% had been told about the late effects of cancer and its treatment. These results were lower than we expected.

Kadan-Lottick *et al.*[6] reported that 91% of the CCSS participants accurately reported their diagnosis and approximately 90% of them understood their treatment history. However, the proportion of patients who could recall details of their receiving therapy was low. The author concluded that survivors should be better educated about their medical history to be motivated to pursue appropriate follow up, and that treatment summaries must be given to patients in a form that is accessible, even many years later. That conclusion should also apply to the subjects of this study who are less knowledgeable than CCSS participants because their diagnosis and details about their cancer treatment were not always explained to them formerly in Japan. However, telling the children about their disease has become more common in recent years and the number of childhood cancer survivors who understand their disease will increase in the future.

Eiser *et al.*[7] surveyed survivors and their parents about the appropriateness of and need for long-term follow up. Survivors' main reasons for attending follow-up visits were to gain reassurance that they were well and to obtain information about the disease. However, survivors reported differences between the information they would like to be given at the clinic and the information they remembered being offered. In other words,

survivors wanted more information, and many clinic attendees were motivated by a wish to know more about the disease and understand the implications for their future. According to their study,[7] parents were generally more positive about follow-up visits compared with their children. Survivors reported that they would try to forget about the implications for their future and that they were likely to visit a clinic only when worried. Therefore, Eiser *et al.*[7] concluded that it was important to offer information about treatment history and probable late effects to survivors and to motivate them to attend hospitals.

In this study, a few survivors indicated that they should regularly visit a hospital, and wanted to consult a physician regularly, but that there was no appropriate hospital to visit. Therefore, we think that the survivors' views about regular consultations are not always negative. Our study revealed that the hospital where they received the cancer treatment or where their physician-in-charge worked was listed as the hospital where the respondent wanted to undergo follow-up consultations. This is likely due to the close relationship that develops between the physician and patient during cancer treatment. In some cases, it may be difficult for patients to establish a similarly close relationship with a different doctor. However, considering the need for life-long follow up and the fact that survivors themselves often choose not to attend follow-up visits, such a close relationship between patients and their physicians-in-charge may be a barrier to long-term follow up.

Oeffinger mentioned that there were three overlapping categories of barriers to health care for adult survivors of childhood cancer:[8] survivor-related barriers, physician-related barriers, and health-care-system-related barriers. Survivor-related barriers reflect the fact that most survivors are not aware of potential late effects and future health risks and that they often do not know the details of their cancer and their cancer therapy. Physician-related barriers reflect the fact that there is a lack of capacity for survivor care within cancer-treating institutions, and there are not enough physicians trained in survivorship care and appropriate long-term follow-up programs. In addition, communication between cancer centers and primary care physicians is often poor. Health-care-system-related barriers reflect issues with the health-care system itself. For example, in the USA, as survivors enter adulthood, they are no longer covered under their parent's health policy or the public assistance programs.

The situation is similar in Japan. In our study, some survivor-related barriers and physician-related barriers have emerged. It is necessary for parents to inform children about their disease and to explain the possible late effects of the disease and its treatment as well as the importance of long-term follow up. In addition, in this survey, physicians in charge told survivors that they no longer needed to visit hospitals. Therefore, it is important for medical staff to change their views regarding the need for long-term follow-up care among cancer survivors. Follow-up guidelines and educational programs for physicians and nurses involved with childhood cancer treatment are also needed. In addition, the smooth transition from pediatric to adult care and education for adult oncologists and primary care providers must be addressed in the future.

The findings of our study must be interpreted within the context of the study limitations. First, subjects of our study live in a limited area in Japan, and there was a bias of the primary disease. Specifically, approximately 70% of patients had hematological malignancies, and no patient had a brain tumor. Second, although there were no significant differences between the characteristics of 114 subjects who agreed to the third survey and those of 282 subjects who did not participate in the survey, except the time from diagnosis and cancer diagnosis of acute lymphoblastic leukemia and Wilms's tumor, these results may not accurately reflect non-attendees. Third, 30 of 88 respondents were guardians of survivors. According to Eiser *et al.*[7] there are some discrepancies between survivors and their parents regarding the reasons they felt it was important to attend follow-up visits and what they would be most likely to do if they were worried in the future. Thus, attitudes regarding present and future attendance at follow-up visits may not reflect only those of the survivors themselves. However, there has been no survey of non-attendees in Japan. Therefore, understanding the background of non-attendees in Japan, which may be different from those in the USA or Europe, is valuable. Considering the differences between the health-care system in Japan and those of other countries, it will be difficult to introduce long-term follow-up systems into our country. In the future, we need to develop original risk-based follow-up guidelines that consider the health-care system in Japan, resulting in providing appropriate two-way communication between follow-up centers and local treatment institutions or clinics to optimize survivorship care for childhood cancer survivors.

In conclusion, this study revealed that many Japanese cancer survivors stop attending follow-up visits after their treatment is complete. Both survivor-related and physician-related barriers contribute to the reasons that cancer survivors stop follow-up visits. To help survivors continue their physician visits, it is necessary to educate them, their families, and medical staff about the late effects of cancer and its treatment and the importance of long-term follow up to minimize morbidity and mortality.

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CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome

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Juvenile myelomonocytic leukemia (JMML) is a rare form of myeloproliferative disorder, which is characterized by excessive myelomonocytic proliferation.[1] Gene mutations on RAS signaling pathways are a hallmark of JMML and are thought to be central to the pathogenesis of JMML. Mutations of *NF1*, *NRAS*, *KRAS* and *PTPN11* genes are found in approximately 70% of JMML patients, and are implicated in the aberrant RAS signaling.[1] On the other hand, the remaining approximately 30% of JMML patients have no known mutations.

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because these *CBL* mutations were shown to result in aberrant tyrosine kinase signaling, which would lead also to activation of RAS signaling pathways.[2–4] We[2] and others[3,4] reported that *CBL* mutations occurred in a variety of myeloid neoplasms in adults, especially in chronic myelomonocytic leukemia,[2–4] which prompted us to search for possible *CBL* mutations in JMML cases as well as pediatric myelodysplastic syndrome (MDS) patients.

In total, 40 primary JMML and 24 pediatric MDS specimens were examined for CBL mutations. The median age at diagnosis of JMML was 1 year and 10 months (range, 2 months to 8 years and 4 months), and the study included 25 male and 15 female patients. MDS patients included 9 patients with refractory anemia, 14 with refractory anemia with excess of blasts and 1 with secondary MDS. As *CBL* mutations thus far reported almost exclusively involved exons 7–9 that encode linker/RING finger domains,[2–4] we confined our analysis to these exons using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). Of the 40 JMML samples, 24 were also analyzed using Affymetrix GeneChip 250K *Nsp*I (Santa Clara, CA, USA).[5] Genome-wide detection of copy number abnormalities or allelic imbalances was performed using CNAG/AsCNAR software (http://www.genome.umin.jp),[5] which enabled sensitive detection of copy number neutral loss of heterozygosity (or acquired uniparental disomy (aUPD)).[5] Mutations of *RAS* and *PTPN11* were also examined as previously reported.[6] The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

CBL mutations were identified in 3 out of 40 JMML samples (7.5%), which were located in the linker sequence (Y371H in UPN1) or in the RING finger domain (C416Y in UPN18 and C419R in UPN38). The origin of the mutations has not been determined owing to the lack of appropriate normal tissues. All 3 *CBL*-mutated cases accompanied 11q-aUPD, whereas heterozygosity of 11q was preserved in the remaining 21 patients with wild-type *CBL*, recapitulating a strong association of *CBL* mutations with 11q-aUPD as previously described.[2] In UPN18, the chromatogram exclusively showed a mutated sequence, indicating that the mutation (C416Y) was homozygous. In the remaining patients, chromatograms were apparently heterozygous (Figure 1). Nevertheless, this did not necessarily exclude the possibility of homozygous mutations in the aUPD-positive cells, which were found in a small fraction within these tumor samples, as estimated from allele-specific copy numbers.

Recently, Loh *et al.*[7] have reported on *CBL* mutations in 27 (17%) of 159 JMML patients, which were exclusive with regard to *RAS/PTPN11* mutations. They also showed that Y371H mutations represented nearly 50% of all *CBL* mutations in JMML,[7] which were also found in a patient in our series. Mutations of *NRAS, KRAS* and *PTPN11* genes were found in 18, 10 and 34% of patients in our series, respectively, which did not co-exist with three *CBL* mutations (Table 1). In our study, no *CBL* mutations were found in 24 pediatric MDS patients. Combining both studies, the prevalence of *CBL* mutations is expected to be significantly lower in pediatric MDS patients than in JMML patients (0/24 vs 30/199; $P=0.025$), although the number of patients is still small.

CBL is a negative modulator of tyrosine kinase signaling and, as such, was demonstrated to act functionally and genetically as a tumor suppressor. On the other hand, CBL mutants in myeloid neoplasms have clear oncogenic properties, because they strongly transform fibroblasts. These CBL mutants inhibit the E3 ubiquitin ligase activity of both wild-type CBL and CBL-B, leading to prolonged tyrosine kinase activity after cytokine stimulation and hypersensitive proliferative responses of hematopoietic progenitors to a wide variety of cytokines.[8]

We have recently shown that the biological consequence of *CBL* mutations is prolonged activation of tyrosine kinases after cytokine/growth factor stimulations, which is further augmented under the *CBL*-null background as caused by aUPD.[2] Thus, *CBL* mutations associated with 11q-aUPD in JMML could provide a feasible explanation to the hypersensitivity to GM-CSF, which is

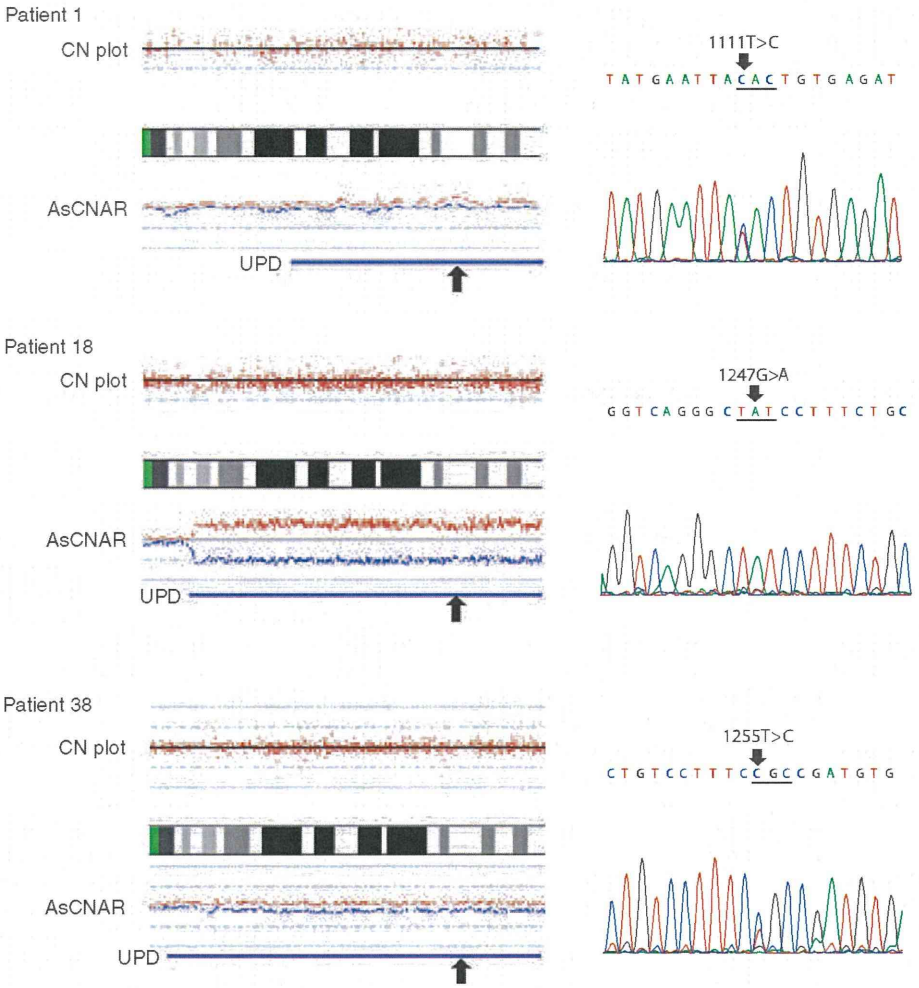

Figure 1 Identification of acquired isodisomy of 11q in JMML and *CBL* mutations. CNAG output for JMML UPN 1, UPN 18 and UPN 38. Left: Total copy numbers (tCNs) (red plot) are shown above the cytoband, and the result of allele-specific copy number analysis with anonymous reference (AsCNAR) plots are shown below the cytoband. The larger allele is presented in red line, and the smaller allele is presented in blue line. Allele-specific analysis showed 11q-aUPD (blue line), which contained the *CBL* region (arrow). Right: All samples of JMML with 11q-aUPD carried mutated *CBL*.

Table 1 Characteristics of three JMML patients with *CBL* mutations

Patient no.	*Age/sex*	*Karyotype*	*Location*	*Nucleotide change*[a]	*Amino-acid change*	*PTPN11*	*RAS*	*SCT*	*Survival*
UPN 1	3 m/F	46, XX, 5q-	Exon 8	1111T>C	371Tyr>His	—	—	—	+12 m
UPN 18	2 m/M	46, XY	Exon 9	1247G>A	416Cys>Tyr	—	—	—	+68 m
UPN 38	2 m/M	46, XY	Exon 9	1255T>C	419Cys>Arg	—	—	U-BMT	32 m[b]

Abbreviations: F, female; m, month; M, male; SCT, stem cell transplantation; U-BMT, unrelated-bone marrow transplantation; UPN, unique patient number; +, alive.
[a]GenBank accession number of CBL for nucleotide change: NM_005188.
[b]Died due to chronic graft versus host disease.

a cardinal feature of JMML. Although *NF1*, *RAS* or *PTPN11* mutations lead to deregulated RAS signaling, the outcome of *CBL* mutations could be more profound, also activating other downstream pathways of tyrosine kinases, including STAT5 and PI3K. Our data suggest that *CBL* is mutated in a subset of JMML patients without *RAS/PTPN11* mutations. Our findings suggest that 11q-aUPD-associated *CBL* mutations may define a unique subset of JMML.

Conflict of interest

The authors declare no conflict of interest.

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Trisomy 11: prevalence among 22 403 unique patient cytogenetic studies and clinical correlates

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Trisomy 11 is a rare cytogenetic abnormality and is yet reported to be one of the most frequent autosomal trisomies in acute myeloid leukemia (AML).[1] In a Cancer and Leukemia Group B study, isolated trisomy 11 was identified in 13 cases (0.9%) among 1496 consecutive adult patients with AML.[2] The majority of the patients with isolated trisomy 11 were older than 60 years and 46% achieved a complete remission after induction chemotherapy.[2] However, only one patient remained in first complete remission after undergoing allogeneic bone marrow transplantation. In a recent *Leukemia* paper, Wang *et al.*[3] identified 42 cases (0.008%) with trisomy 11 among ~5000 patients with myelodysplastic syndrome (MDS) or MDS with myeloproliferative features. Seventeen of the 42 patients (median age, 75 years) had trisomy 11 as a sole abnormality ($n=10$) or together with one or two additional abnormalities. Specific diagnoses in these 17 patients were refractory anemia with excess of blasts (RAEB)-2 in 8 patients, RAEB-1 in 5, refractory cytopenia with multilineage dysplasia in 1, therapy-related MDS in 1 and chronic myelomonocytic leukemia-2 in 1; bone marrow was not available for review in the remaining 1 patient. The authors compared their trisomy 11 MDS patients with historical controls and found their survival to be similar to that of high-risk MDS patients. Accordingly, they concluded that trisomy 11 should be considered a high-risk cytogenetic abnormality in MDS.

In the current study, we sought to clarify the prevalence of trisomy 11 in an unselected series of cytogenetic studies performed at the Mayo Clinic over the last 20 years and describe their clinical and pathological features. Between January 1988 and December 2008, unique patient cytogenetic studies were performed in 22 403 adults (age $\geqslant$18 years). Among them, we identified 19 patients (~0.08%) with abnormalities that included trisomy 11; WHO (World Health Organization)-defined[4] clinical diagnosis at the first sighting of trisomy 11 was AML in 14 patients and MDS in 5 (Table 1). Among the former, 10 cases constituted *de novo* AML and 4 constituted relapsed or secondary AML. Among the five MDS patients, three had RAEB-2 and two had RAEB-1. Trisomy 11 occurred as a sole abnormality in 10 patients with AML, but in only one patient with MDS (RAEB-2).

The median age at detection of trisomy 11 in AML was 71 years and in MDS was 67 years (range, 64–86). Median (range) values in AML included 8.7 g/100 ml (6.5–11.8) for hemoglobin, 6×10^9/l (1.2–123) for leukocytes and 96×10^9/l (12–444) for platelets. The corresponding values in MDS were 9.2 g/100 ml (8.1–11.3), 1.8×10^9/l (1.1–3.2) and 129×10^9/l (78–199), respectively. Approximately 50% of the AML patients were exposed to either cytotoxic or radiation therapy before the detection of trisomy 11 (Table 1). AML transformation was documented in one patient with RAEB-2 after 23 months of follow-up. In all the patients with relapsed AML and in one

Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome–negative myeloproliferative neoplasms

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Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera, essential thrombocythemia, and primary myelofibrosis show an inherent tendency for transformation into leukemia (MPN-blast phase), which is hypothesized to be accompanied by acquisition of additional genomic lesions. We, therefore, examined chromosomal abnormalities by high-resolution single nucleotide polymorphism (SNP) array in 88 MPN patients, as well as 71 cases with MPN-blast phase, and correlated these findings with their clinical parameters. Frequent genomic alterations were found in MPN after leukemic transformation with up to 3-fold more genomic changes per sample compared with samples in chronic phase ($P < .001$). We identified commonly altered regions involved in disease progression including not only established targets *(ETV6, TP53,* and *RUNX1)* but also new candidate genes on 7q, 16q, 19p, and 21q. Moreover, trisomy 8 or amplification of 8q24 *(MYC)* was almost exclusively detected in *JAK2V617F*$^-$ cases with MPN-blast phase. Remarkably, copy number–neutral loss of heterozygosity (CNN-LOH) on either 7q or 9p including homozygous *JAK2V617F* was related to decreased survival after leukemic transformation ($P = .01$ and $P = .016$, respectively). Our high-density SNP-array analysis of MPN genomes in the chronic compared with leukemic stage identified novel target genes and provided prognostic insights associated with the evolution to leukemia. (*Blood.* 2010; 115(14):2882-2890)

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) are defined as clonal hematopoietic stem cell disorders and characterized by increased proliferation of terminally differentiated myeloid cells. The tyrosine kinase *JAK2* is directly linked to the pathogenesis of MPN with the identification of *JAK2V617F* as a recurring gain-of-function mutation.[1,2] Almost all cases with PV, and roughly 50% of patients with ET and PMF, carry this specific mutation localized on chromosome 9p24.

The long-term outcome of patients with acute myeloid leukemia (AML) secondary to MPN, myelodysplastic syndrome (MDS), or treatment with cytotoxic agents is relatively poor compared with patients with de novo AML. Patients with de novo and secondary AML have a similar spectrum of cytogenetic abnormalities, but the occurrence of cytogenetic changes associated with unfavorable risk such as 5q−, −7/7q−, trisomy 8, or complex karyotype is higher in secondary AML.[3,4] However, so far only a small number of studies with limited number of cases have explored the chromosomal alterations and/or clinical markers associated with acceleration to blast phase of patients with MPN.

Previously, we developed the copy number analyzer for Affymetrix GeneChip (CNAG) program and the new algorithm allele-specific copy number analysis using anonymous references (AsCNAR).[5,6] These techniques in combination with high-density single nucleotide polymorphism (SNP) array provide a robust and

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An Inside *Blood* analysis of this article appears at the front of this issue.

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Table 1. Clinical features of MPN/MPN-blast phase cases (unmatched and matched)

	Unmatched MPN	Unmatched MPN-blast phase	Matched MPN	Matched MPN-blast phase
All cases, no. (%)	77 (56)	60 (44)	11 (50)	11 (50)
MPN diagnosis no. (%)				
PV samples	21 (27)	17 (28)	2 (18)	2 (18)
ET samples	31 (40)	18 (30)	1 (09)	1 (09)
PMF samples	25 (33)	25 (42)	8 (73)	8 (73)
Sex, M:F				
PV samples	1:2	1:1	1:1	1:1
ET samples	1:2	1:1	0:1	0:1
PMF samples	2:1	2:1	2:1	2:1
Mean age at diagnosis, y, ± SD*				
PV	57 ± 5	68 ± 5	—	—
ET	59 ± 6	69 ± 7	—	—
PMF	57 ± 6	65 ± 9	59 ± 9	65 ± 7
Mean blast count in bone marrow, ± SD, no. (%)*				
PV samples	< 5%	70 ± 20	—	—
ET samples	< 5%	66 ± 23	—	—
PMF samples	< 5%	70 ± 21	< 5%	66 ± 24
***JAK2V617F* (+) no. (%)**				
PV samples	21/21 (100)	14/17 (82)‡	2/2 (100)	1/2 (50)
ET samples	18/31 (58)	6/18 (33)	0/1 (0)	0/1 (0)
PMF samples	16/25 (64)	12/25 (48)	5/8 (62.5)	4/8 (50)
***c-MPL* mutation positive, no. (%)**				
PV samples	1/21 (5)†	0/17 (0)	0/2 (0)	0/2 (0)
ET samples	0/31 (0)	1/18 (6)	0/1 (0)	0/1 (0)
PMF samples	3/25 (12)	2/25 (8)	1/8 (12.5)	1/8 (12.5)

MPN indicates myeloproliferative neoplasm; PV, polycythemia vera; ET, essential thrombocytosis; M, male; F, female; and PMF, primary myelofibrosis.

*Data are available for 27 unmatched MPN (10 PV, 10 ET, and 7 PMF) and 54 unmatched MPN-blast phase (15 PV, 18 ET, and 21 PMF) cases, and 8 matched MPN (PMF) cases.

†This *c-MPL* mutation in a PV patient has already been validated and reported by Kawamata et al.[8]

‡Significantly fewer cases with *JAK2V617F* in blast phase vs chronic phase ($P = .045$).

detailed approach to detect large and small copy number changes, as well as copy number–neutral loss of heterozygosity (CNN-LOH). To obtain a comprehensive profile of genomic alterations associated with leukemic transformation in MPN, we applied this interrogational method and performed a systemic analysis of 159 samples obtained from patients either in chronic phase or blast phase of MPN.

Methods

Patients and clinical samples

In total, samples from 148 patients were analyzed by SNP-array. One hundred fifty-nine samples were obtained, of which 88 (55%) were diagnosed with MPN in chronic phase (23 PV, 32 ET, 33 PMF) and 71 (45%), with MPN in blast phase (19 PV, 19 ET, 33 PMF). Diagnosis was based on the World Health Organization criteria,[7] and an overview of patients, including clinical data, is given in Table 1. This study received institutional review board approval from the Cedars-Sinai Medical Center, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Given the relatively high incidence of homozygous *JAK2V17F* patients diagnosed with ET (3/18 in chronic phase, 2/6 in blast phase), which is usually lower for this disorder,[9] we suggest that at least some cases diagnosed with ET may have been incorrect.

Samples were provided by (1) Department of Hematology, Mayo Clinic (n = 35); (2) Brigham and Women's Hospital, Harvard University, School of Medicine (n = 46); (3) Department of Hematology, Archet Hospital (n = 44); (4) MLL Munich Leukemia Laboratory, (n = 14); (5) Division of Hematology-Oncology, Chang Gung Memorial Hospital (n = 14); and (6) Division of Hematology, Sheba Medical Center and Sackler School of Medicine, Tel-Aviv University (n = 6).

SNP-Chip analysis

A total of 159 tumor specimens (MPN and/or MPN-blast phase) were analyzed on GeneChip SNP genotyping microarrays (GeneChip Mapping 50K and/or 250K arrays; Affymetrix) as described previously.[5,6] After appropriate normalization of mean array intensities, signal ratios were calculated between tumors and anonymous normal references in an allele-specific manner. Genome-wide determination of allele-specific copy numbers (AsCNs) and detection of CNN-LOH at each SNP were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR algorithms (http://www.genome.umin.jp).[5,6] For clustering of patient samples with regard to the status of copy number changes, as well as CNN-LOH, CNAG-Graph software (Tokyo University) was used. Size, position, and location of genes were identified with the University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu)[10] and Ensemble Genome Browser (http://www.ensembl.org).[11] Germline copy number changes previously described as copy number variants at Database of Genomic Variants (http://projects.tcag.ca/variation)[12] and UCSC Genome Browser were excluded. SNP-array data used in this study are available in the Gene Expression Omnibus (GEO) database under accession number GSE19647.[13]

Comparison of 50K versus 250K SNP-Chip analysis in MPN chronic phase

SNP-array analysis of 46 of our MPN samples (10 PV, 20 ET, 16 PMF; kindly provided by D.G.G. at Brigham and Women's Hospital, Harvard University) has already been reported by our group.[8] At that time, only 50K arrays were available, whereas later in this study, the 250K arrays were accessible and used to analyze additionally 42 MPN and 71 MPN-blast phase samples. Because no significant differences in either number of deletions, duplications/amplifications, or CNN-LOH per case were found as analyzed by the 50K compared with 250K array (supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), we combined the analysis of both platforms in our

results. Supplemental Table 2 lists all individual samples and the array that was used.

Cytogenetics

Routine cytogenetic analysis with conventional banding techniques was performed in 35 of 88 MPN (10/23 PV, 10/32 ET, 15/33 PMF) and 63 of 71 MPN-blast phase (15/19 PV, 18/19 ET, 30/33 PMF) cases according to standard procedures as previously described.[14] No routine fluorescent in situ hybridization (FISH) panel was applied, but in some cases, however, FISH analysis was performed to supplement conventional cytogenetic analysis (supplemental Table 2).

Allele-specific PCR for *JAK2V617F* mutation

For the detection of *JAK2V617F*, allele-specific polymerase chain reaction (PCR) was performed according to the previously reported method.[15]

Direct mutation screening

Primers were designed to amplify and sequence coding exons and splice junctions of the following genes: *TET2*, *c-CBL*, *TP53*, and *RUNX1*. We screened only the 11 matched samples that showed genomic changes in the particular gene regions. Primer details are available from the corresponding author (N.H.T.).

We evaluated all MPN and MPN-blast phase patients with 1pCNN-LOH for the *MPLW515* mutation (exon 10) by direct sequencing. If no mutation was detected in this cohort, we also screened the other coding exons of the *c-MPL* gene previously shown to be mutated in MPN.[8]

Validation of acquired genomic copy number changes including CNN-LOH

To confirm the somatic origin of genomic copy number changes, quantitative genomic real-time (QG RT)–PCR was performed on the genomic DNA from the hybridized MPN and matched MPN-blast phase samples according to the calculation method described by Weksberg et al.[16] For example, we used primers for the *RUNX1* gene (21q22.12; supplemental Figure 1A) as well as *TET2* gene (4q24; data not shown) and a random region on chromosome 21q21.1 and 4p15.1, respectively, as a reference in patient 121.

Detection of acquired CNN-LOH was also validated by QG RT-PCR and subsequently by nucleotide sequencing. Three SNP sequences (rs919275, rs10854117, and rs10854117) on chromosome 19p in case 36 at diagnosis of PV, as well as at leukemic transformation, were determined (supplemental Figure 1B). The genomic region of each SNP site was amplified, and products were purified and sequenced (supplemental Figure 1C). In addition, we confirmed loss of CNN-LOH on 9p after leukemic transformation in matched case 120 using SNP sequences rs3858029, rs1360461, and rs10818814 on chromosome 9 (data not shown).

Homozygous deletions of *CUTL1* and *SH2B2* (case 138) as well as *PIG-A* (case 121) in both MPN and/or MPN-blast phase samples were also confirmed by QG RT-PCR (supplemental Figure 2). Primers for these experiments will be provided upon request.

Statistical analysis

Wilcoxon rank sum tests were used to assess differences in continuous variables, and categoric variables were assessed using chi-square tests, all with a significance level of $\alpha = .05$. The methods of estimations included the standard deviation ($\pm$ SD) of the sampling distribution. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($^*P < .05$; $^{**}P < .001$). Survival analysis was performed using the Kaplan-Meier method, and survival curves were compared using the log-rank test.

Results

Lower frequency of *JAK2V617F* and 9p alterations after leukemic transformation

In the present study, we examined 159 samples (88 MPN and 71 MPN-blast phase) from a total of 148 patients. An overview of the clinical features of matched and unmatched cases including sex, age, leukemic blast infiltration, and mutational status (*JAK2V617F, c-MPL*) is provided in Table 1. The sex ratio of male and female patients in chronic phase was 1:2 for PV and ET, whereas after transformation, the ratio was balanced with 1:1. For PMF patients, the male-to-female ratio was 2:1 in both MPN chronic and blast phase.

Overall, the incidence of *JAK2V617F* was almost 20% less in the blast phase compared with the chronic phase for both the matched and unmatched MPN cases (unmatched cases: $P < .05$; Figure 1A). Cases that were negative for *JAK2V617F* were also exclusively negative for 9p duplication, trisomy 9, or 9pCNN-LOH in the chronic as well as leukemic stage of MPN. 9pCNN-LOH was noted approximately 3 to 4 times more often than 9p duplication and/or trisomy 9 in *JAK2V617F*$^+$ MPN cases during either the chronic or blast phase (Figure 1A), but the frequency of 9pCNN-LOH was significantly less in the blast crisis compared with the chronic phase of unmatched PMF and PV patients (supplemental Table 3). In contrast, unmatched ET cases had about the same frequency of 9pCNN-LOH in the chronic phase versus the blast phase of the disease. Furthermore, in the analysis of the 11 matched MPN cases, 7 were positive for *JAK2V617F* (64%), 4 had 9p CNN-LOH (37%), and 1 had 9p duplication (9%) at first diagnosis (Figure 1A). In comparison, 2 of these patients were *JAK2V617F*$^+$ with either trisomy 9 or 9pCNN-LOH during their chronic phase (1 PV, 1 PMF), but no longer had detectable *JAK2V617F* with a normal chromosome 9 after leukemic evolution (Figure 1B).

JAK2V617F mutational status had no impact on time to transformation or survival

In the evaluation of clinical data for MPN-blast phase patients, no significant correlation was noted between the prevalence of *JAK2V617F* at transformation and either age, percentage of leukemic blast cells in the marrow, or pretreatment with alkylating agents and/or hydroxyurea (data not shown). Moreover, we found no statistical association between either time to leukemic transformation or overall survival and the *JAK2V617F* status at transformation in PV, ET, or PMF patients. The overall survival of MPN-blast phase patients with *JAK2V617F* versus blast phase patients without this mutation is provided in Figure 2A ($P = .6$). In addition, with respect to the comparably low frequency of *MPLW515*-positive MPN-blast phase patients (6%), we noted no impact of the *c-MPL* mutational status on either time to transformation (data not shown) or the overall survival in MPN patients who underwent leukemic transformation ($P = .5$; Figure 2B).

However, regardless of the mutational status of MPN-blast phase patients, we noted that the time from diagnosis of MPN to leukemic transformation was significantly shorter in those with pre-existing PMF (median, 58 months) compared with patients with either prior PV (median, 98 months) or ET (median, 110 months; $P = .01$). This earlier transformation resulted in a decreased overall survival from the time of diagnosis of the underlying MPN in leukemic patients with preceding PMF patients compared with preceding PV or ET ($P = .02$; Figure 2C), which is congruent with previously published results.[17]

Increased number of additional genomic changes after leukemic transformation

Altogether, a relatively low number of genomic alterations was found by SNP-array analysis in the chronic phase of the MPN samples (Figure 3A). In contrast, 2 to 3 times more abnormalities per sample were detected after leukemic evolution in both matched and unmatched cases with MPN (both $P < .001$; Figure 3A). We

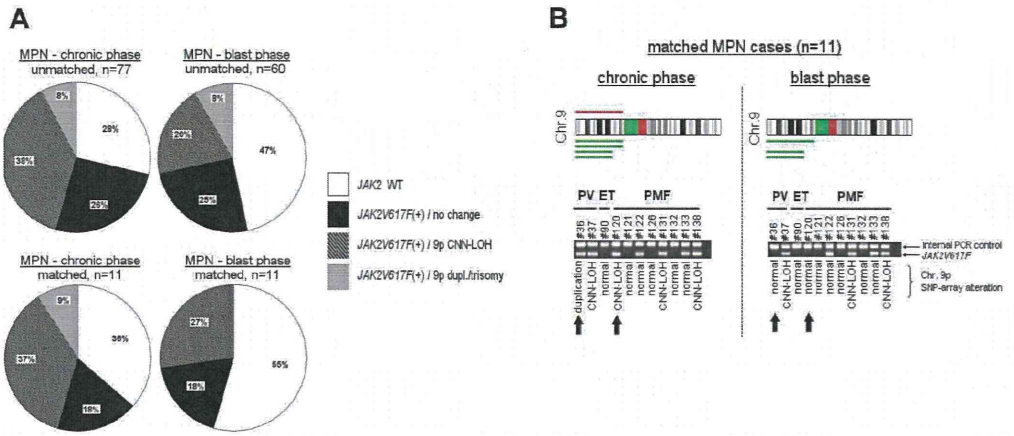


Figure 1. Frequency of *JAK2V617F* and associated alterations on chromosome 9. (A) Diagrams represent matched and unmatched MPN cases in chronic versus blast phase. Indicated are frequencies of *JAK2V617F* and association to 9p duplication (dupl)/trisomy 9 or 9pCNN-LOH. Data and statistical evaluation for underlying MPN subgroups are shown in supplemental Table 3. (B) CNAG software represents duplication (red) and CNN-LOH (green) on 9p detected in 11 patients with matched samples (chronic MPN vs MPN-blast phase). In addition, allele-specific PCR for the detection of *JAK2V617F* was performed in these samples. Arrows indicate 2 MPN patients who were initially positive for *JAK2V617F* in association with 9p imbalances; leukemic transformation was accompanied with loss of *JAK2V617F* and a normal chromosome 9.

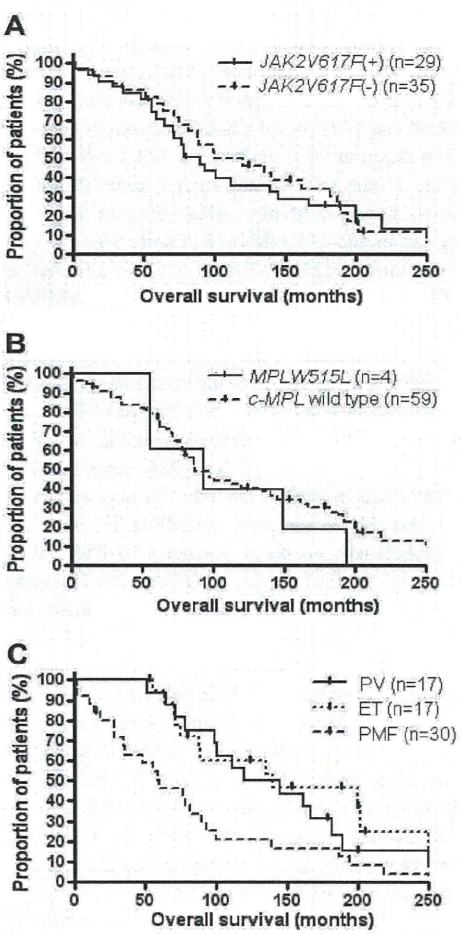


Figure 2. Overall survival of MPN patients with subsequent transformation to blast crisis. Kaplan-Meier plots of all MPN-blast phase patients from the diagnosis of pre-existing MPN were stratified for (A) the presence or absence of a *JAK2V617F* mutation at transformation, (B) the presence or absence of a *MPLW515L* mutation at transformation, and (C) the underlying type of MPN.

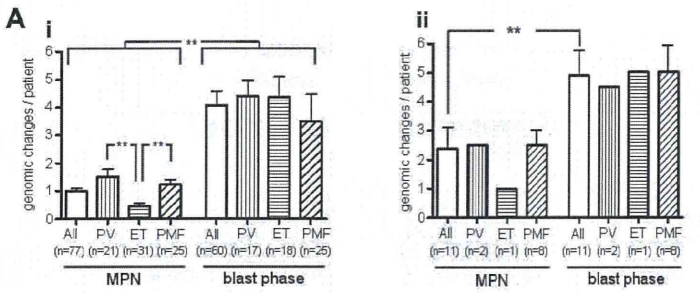
found no statistical relationship between the *JAK2V617F* status and the number of genomic changes in matched as well as unmatched samples (data not shown). However, samples from ET patients had fewer copy number changes than those from either PV or PMF patients in the chronic phase, which was highly significant in the unmatched cases ($P < .001$; Figure 3A, supplemental Figure 3A). After leukemic transformation, a similar number of SNP-array changes occurred in cases with prior ET compared with those with pre-existing PV and PMF (unmatched cases: $P = .59$). Statistical evaluation of the matched samples divided into each subentity was not possible because of the small number of cases (Figure 3Aii and supplemental Figure 3B). A subanalysis of the number of either deletions, duplications/amplifications, or CNN-LOH per case, matched and unmatched, is shown in supplemental Figure 3.

Compared with the cytogenetic data, SNP-array analysis detected more than 2-fold of additional chromosomal changes in the MPN samples of either chronic or blast phase, whereas SNP-array practically captured all cytogenetic abnormalities (Figure 3B).

Candidate genes involved in leukemic transformation of MPN patients

SNP-chip analysis detected several additionally altered regions in patients after leukemic evolution compared with the MPN chronic phase in both unmatched (Figure 4; supplemental Figure 4) and matched (Figure 5A) cases. The altered regions included chromosome 8q *(MYC)*, 12p *(ETV6)*, 17p *(TP53)*, and 21q *(RUNX1)*, which are already known to be involved in leukemogenesis.[18-22] Trisomy 8 was detected in 12% of unmatched and 9% of matched cases in MPN-blast phase; interestingly, almost all these samples were negative for *JAK2V617F*. PMF patient 148, who was also *JAK2V617F*$^-$, showed amplification of 8q24.21 in blast crisis involving the *MYC* gene. MPN-blast phase patients with trisomy 8 did not show an inferior outcome compared with cases without this abnormality ($P = .11$; data not shown).

In 20% of unmatched cases in MPN-blast phase, deletions (12%) or CNN-LOH (8%) occurred on chromosome 17 including *TP53* at p13.1. Deletions on the short arm of chromosome 17 were detected significantly often in MPN-blast phase patients who received prior treatment with hydroxyurea with or without the addition of alkylating agents ($P = .035$, Table 2). Supplemental



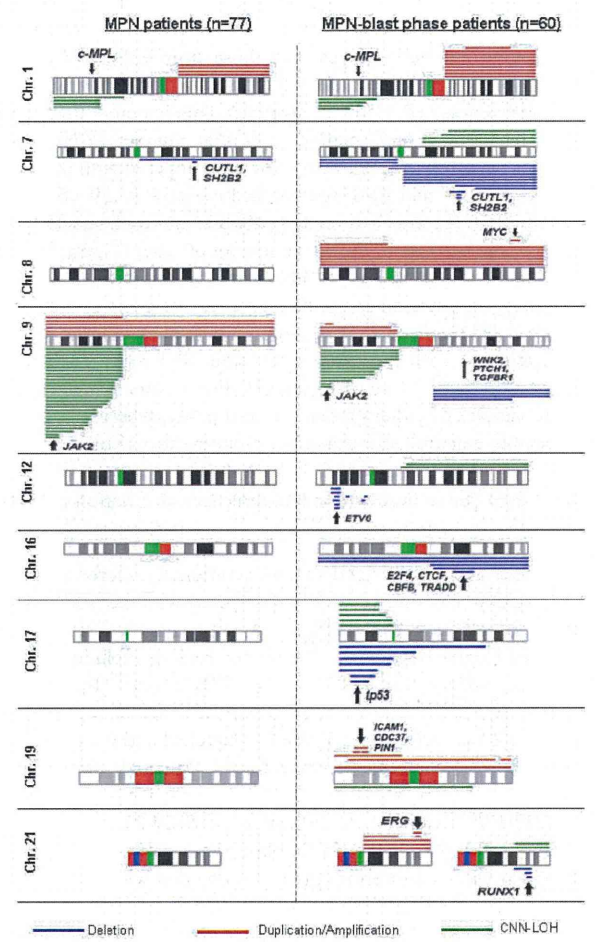


Figure 4. Overview of gains and losses detected by CNAG software. Indicated are the most common altered regions in unmatched MPN-blast phase patients (n = 60; right-sided cytobands) compared with unmatched MPN patients (n = 77; left-sided cytobands). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). Candidate genes of the minimal altered regions are highlighted by arrows.

Figure 3. Genomic alterations per MPN patient in chronic versus blast phase. (A) Mean of SNP-array alterations per patient in MPN versus MPN-blast phase with (i) unmatched samples and (ii) matched samples (± SD); $^{**}P < .001$. (B) Mean of SNP-array aberrations compared with cytogenetic alterations per patient in chronic versus blast phase with (i) unmatched samples and (ii) matched samples (± SD).

Table 4 indicates pretreatment (hydroxyurea and/or alkylating agents) of 47 MPN-blast phase patients and their individual SNP-array findings. Deletion or CNN-LOH on 17p in unmatched blast phase cases was associated with either complex karyotype or isochromosome 17 ($P = .01$), and significantly decreased survival (with 17p deletion: $P = .012$; with 17p CNN-LOH: $P = .018$). One of the 11 matched MPN samples (case 120) acquired a 17p deletion at diagnosis of blast phase (Figure 5A), resulting in a hemizygous mutant *TP53* (M133K; Figure 5Bi).

On chromosome 21, SNP-chip analysis revealed either deletions or CNN-LOH in 8% of unmatched cases in MPN-blast phase involving the transcription factor *RUNX1* at q22.12. Patient 121 acquired a small deletion of that locus in the leukemic sample (Figure 5A) associated with a mutation of the Runt domain of the *RUNX1* gene on the remaining allele (Q392Stop codon; Figure 5Bii).

Deletion or CNN-LOH on 4q24 spanning the *TET2* gene was detected in 6% of unmatched blast phase cases and 1% in chronic phase. One *TET2* mutation was found by nucleotide sequencing in the matched MPN samples. *JAK2V617F*$^-$ case 121 had no genomicimbalances on 4q at diagnosis of PMF, but acquired a microdeletion (1 Mbp) on 4q24 (*TET2*) after leukemic evolution 1 year later (Figure 5A). The remaining allele had a *TET2* frameshift mutation (N486T; Figure 5Biii), and the mutation was absent in the matched PMF sample.

CNN-LOH involving 11q23.3, which has been shown to be strongly associated with *c-CBL* mutations,[23] had an even lower frequency, with only 2% of unmatched MPN cases in either chronic phase or blast crisis. The *JAK2V617F*$^-$ patient 132 had 11q CNN-LOH with a homozygous *c-CBL* missense mutation (Y371H) in the MPN-blast phase sample. Both the CNN-LOH and the mutation were absent in the corresponding chronic phase, 2 years before disease progression (Figure 5A-Biv).

Besides these already well-known targets, SNP-array analysis detected commonly altered regions on chromosomes 1, 7, 16, 19, and 21 encompassing potentially new candidate genes involved in MPN transformation. These imbalances were either absent or at least very infrequent in the chronic phase of the disease (Figures

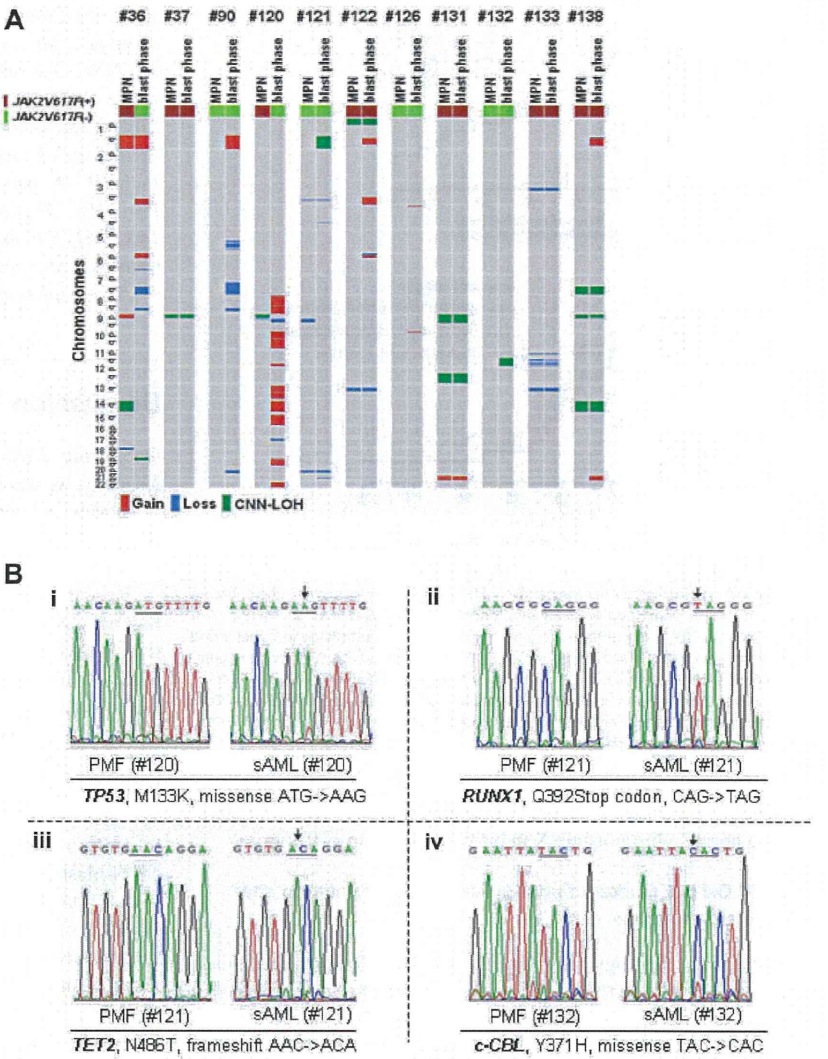


Figure 5. Gains and losses in matched MPN samples and mutational analysis. (A) Most commonly altered genomic regions in MPN samples (left sample column) compared with matched blast phase samples (right sample column) evolved from 11 patients (2 PV, 1 ET, 8 PMF). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). (Bi) Hemizygous *TP53* mutation detected in MPN-blast phase sample of case 120 associated with acquired 17q deletion, which was not present in the MPN phase of case 120. (ii) Hemizygous *RUNX1* mutation detected in MPN-blast phase sample (case 121) associated with acquired deletion at 22q22.1, which was not present in the MPN phase of case 121. (iii) Hemizygous *TET2* mutation detected in MPN-blast phase sample of case 121 associated with acquired cryptic deletion on 4q24, which was not present in the MPN phase of case 121. (iv) Homozygous *c-CBL* mutation detected in MPN-blast phase sample of case 132 associated with acquired 11q CNN-LOH, which was not present in the MPN phase of case 132.

4 and 5A, supplemental Figure 4). Ten percent of unmatched and 18% of matched MPN-blast phase cases had either duplication/amplification or CNN-LOH on 19p. The commonly involved region spanned a small locus (2 Mbp) at 19p13.2, where, among others, the genes *PIN1, ICAM1,* and *CDC37,* which have been associated with carcinogenesis, are located.[24-26] In addition, the minimal region (1.8 Mbp) of amplifications/duplications/trisomy on chromosome 21 detected in 8% of unmatched and 9% matched MPN-blast phase samples harbored the oncogenic transcription regulator *ERG* (q22.2).

Table 2. Pretreatment in 47 MPN-blast phase cases and frequency of 17p and 7q deletions

Pretreatment				
Hydroxyurea	−	+	−	+
Alkylating agents	−	−	+	+
SNP-array alteration (17p vs 7q)				
No *del(17)(p)*, no *del(7(q)*	17	16	0	1
del(7)(q)	2	2	2	2
del(17)(p)	0	4*	0	1*
del(17)(p) and *del(7)(q)*	0	0	0	0

Numbers of blast-phase patients are presented.

MPN indicates myeloproliferative neoplasm; and SNP, single nucleotide polymorphism.

*A total of 5 cases with *del(17)(p)* pretreated with hydroxyurea ($P = .035$).

Complete or partial deletion (−7/7q−), as well as CNN-LOH of the long arm of chromosome 7, was one of the most common abnormalities detected by SNP-array analysis in up to 25% of unmatched and 27% matched samples evolved in the blast phase. SNP-array also revealed 3 unmatched cases (32, 87, and 116) with a heterozygous microdeletion encompassing the 7q22.1 locus, which was not detectable by cytogenetic analysis. Moreover, case 138 with 7qCNN-LOH had a homozygous deletion on 7q22.1 in both the matched MPN and MPN-blast phase samples (supplemental Figure 2A). The minimally deleted region spanned a small region of 0.88 Mbp at 7q22.1 covering only 2 target genes, *CUTL1* and *SH2B2*. The homozygous deletion of these genes in patient 138 was confirmed by QG RT-PCR (supplemental Figure 2B). Deletions of the long arm of chromosome 7 were found more often in MPN-blast phase patients pretreated with hydroxyurea and/or alkylating agents, but the findings were not statistically significant ($P = .2$; Table 2).

Also worth mentioning, 1 microdeletion encompassing the chromosome X–linked *PIG-A* gene occurred in male patient 121 at

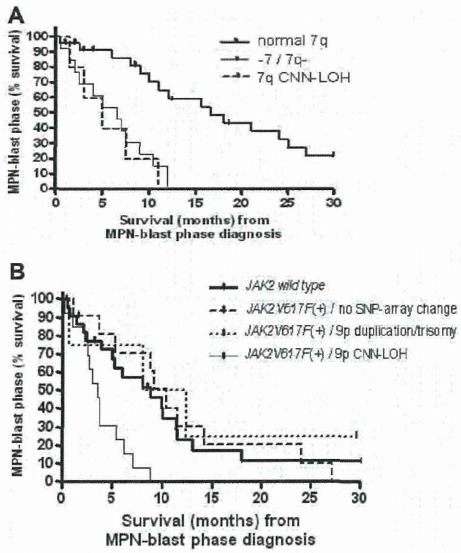


Figure 6. Survival analysis in MPN-blast phase. (A) Survival from the time of diagnosis of blast phase in transformed MPN patients with normal chromosome 7 (normal 7q) compared with either monosomy 7 (−7)/deletion of 7q (7q−) or 7qCNN-LOH. (B) Survival from the time of diagnosis of blast phase in transformed MPN patients with homozygous *JAK2V617F*+ associated with 9pCNN-LOH compared with either heterozygous *JAK2V617F*+ with 9p duplication/trisomy 9 or no abnormality, or patients without the mutation (*JAK2 wild type*). Median survival (months) and the case numbers for each group (transformed PV, ET, or PMF) are listed in supplemental Table 5.

leukemic transformation (supplemental Figure 2C). This patient had a normal chromosome X in his chronic phase of PMF.

CNN-LOH is a marker of poor survival in MPN patients after leukemic evolution

SNP-array technology provides efficient and effective detection of segmental CNN-LOH. In the present study, the most prominent regions for CNN-LOH besides chromosome 9p *(JAK2)* were on 7q and 17p *(TP53)* in patients with MPN-blast phase. In marked contrast to CNN-LOH on 9p, CNN-LOH on 7q or 17p almost never occurred in the chronic phase of the disorder in matched and unmatched samples. As mentioned previously, cases with CNN-LOH and/or deletion of 17p were associated with either complex karyotype or isochromosome 17 and decreased survival.

As also expected, survival in the MPN-blast phase was significantly decreased in patients with −7/7q− (median, 3.75 months) compared with those without chromosome 7 alterations (median, 9 months; $P = .008$). In addition, the unbalanced translocation, der(1;7)(q10;p10), a nonrandom chromosomal abnormality rarely found in AML, was detected by SNP-chip and FISH in 7% of unmatched samples after leukemic evolution and was also associated with an inferior outcome compared with patients without chromosome 7 imbalances ($P = .014$). Strikingly, survival continued to be significantly decreased in MPN-blast phase, when cases with only 7qCNN-LOH were compared with those with a normal 7q ($P = .01$; Figure 6A; supplemental Table 5).

The *JAK2V617F* mutational status in terms of heterozygosity or homozygosity appeared to have no influence on the duration to leukemic evolution. Regardless of the underlying MPN subgroup, no statistical difference in the time to leukemic transformation was found comparing *JAK2V617F*+ patients with normal chromosome 9 to mutant positive blast phase patients with either 9p duplication/trisomy 9 ($P = .28$) or 9pCNN-LOH ($P = .21$). Instead, we found that homozygous *JAK2V617F* had an impact on survival after MPN transformation. Blast phase patients with 9pCNN-LOH resulting in a homozygous *JAK2* mutation had a worse outcome (median, 4 months) compared with *JAK2V617F*+ MPN-blast phase patients with either 9p duplication/trisomy 9 (median, 7.5 months) or no abnormality on 9p (median, 9 months), as well as patients without *JAK2V617F* (median, 7 months, $P = .016$; Figure 6B; supplemental Table 5). Homozygous *JAK2V617F* in association with CNN-LOH diagnosed at leukemic transformation was independent of known risk factors such as 5q−, −7/7q−, or complex karyotype ($P > .05$).

Discussion

Oncogenic *JAK2* signaling is an important event in MPN.[1,2] Recently, we and others showed that homozygosity for *JAK2V617F* is closely related to chromosome 9pCNN-LOH in MPN patients.[1,6,8,9] However, the transformation process of MPN to MPN-blast phase is not well understood.

Recent findings suggested that transition from heterozygosity to homozygosity for *JAK2V617F* is associated with a hyperproliferative disease profile and may be important for disease progression, at least from PV to secondary myelofibrosis.[27] Moreover, Barosi et al showed in a longitudinal prospective study that the presence of a *JAK2V617F* hematopoietic clone was significantly associated with leukemic transformation in PMF.[28] This is in contrast to our present findings showing that not only the mutational status of *JAK2V617F,* but also 9pCNN-LOH with homozygous *JAK2V617F,* had no impact on the time to leukemic transformation in patients with MPN-blast phase. In addition, 2 of the 11 matched MPN samples, initially positive for *JAK2V617F* with either trisomy 9 or 9pCNN-LOH, became negative for these abnormalities after leukemic transformation. Although only tested in unpaired samples, PMF and PV samples also showed a significantly smaller number of both *JAK2V617F*+ and 9pCNN-LOH in the blast phase compared with the chronic phase. Interestingly, and also contrary to the previously cited studies, Tefferi et al noted a significant association between a low *JAK2V617F* allelic burden and evolution to blast phase in a large cohort of PMF patients.[29] Even though these data are not completely congruent with our findings, the results of Tefferi et al and our results point to the coexistence of a more dominant *JAK2V617F*-negative clone with a higher propensity to undergo clonal evolution. This is congruent with recent studies indicating that *JAK2V617F*+ MPN can result in *JAK2V617F*− MPN-blast phase.[30,31] But still, some of our matched cases with *JAK2V617F*+ had no change in abnormalities including *JAK2* mutational status as well as 9pCNN-LOH, allowing the existence of a common pre-*JAK2V617F* clone. Taken together, the presence of *JAK2V617F* appears not to be a prerequisite for leukemic transformation of MPN, suggesting that additional genetic events are required for full transformation.

SNP-array analysis was able to capture practically all cytogenetic abnormalities and to uncover additional lesions with potentially important clinical implications. The number of genomic alterations was more than 2 to 3 times greater in the blast phase as in the chronic phase of matched and unmatched cases with MPN. Noticeably, ET patients had fewer alterations in their chronic phase samples compared with the PV and PMF cases, whereas the number was comparable in all 3 MPN subgroups after their transformation. Being aware of the increased number of new

genomic changes enables investigators to focus on the identification of causative genes associated with the evolution of MPN to leukemia.

Commonly altered regions in blast crisis samples were detected on chromosomes 8, 12, 17, and 21 encompassing *MYC, ETV6, TP53,* and *RUNX1,* respectively, which are already known to be involved in the development of de novo and secondary AML.[18-22] Gain of chromosomal material at 8q24.21 was almost exclusively found in *JAK2V617F*$^-$ samples, suggesting that increased activity of *MYC* might allow selection of clones that do not require the *JAK2* gain-of-function mutation. Furthermore, deletion of 17p *(TP53)* was significantly associated with prior exposure to hydroxyurea as well as a complex karyotype in samples with MPN-blast crisis, which is in accordance with recent results.[32,33] Interestingly, not only deletion, but also 17pCNN-LOH, was associated with a complex karyotype, a poor prognostic marker in myeloid malignancies.

In addition, regions on chromosomes 1q, 7q, 16q, 19p, and 21q were frequently altered in the evolution to the leukemic phase and may harbor promising new candidate genes. Abnormalities involving chromosome 7 are frequently detectable in de novo and secondary AML,[34-37] and preceding studies have found a critical breakpoint region involving a locus at centromeric band 7q22, whereas the telomeric breakpoint varies from q32 to q36. Interestingly, the minimal deleted region in our cohort was located at 7q22.1 encompassing only 2 promising target genes, *SH2B2* (previously named *APS*) and *CUTL1. SH2B2* regulates and enhances *JAK2*-mediated cellular responses,[38] and the *CUTL1* gene encodes for a *CUT* family member of the homeodomain proteins that can repress the expression of developmentally regulated myeloid genes.[39] Moreover, genome-wide inspection for minimal regions of duplications/amplifications and CNN-LOH revealed several interesting genes, such as *PIN1, ICAM1,* and *CDC37* on 19p as well as *ERG* on 21q. Whereas the latter 3 targets have been shown to possess potential progrowth activity in de novo AML and/or MDS,[25,26,40] *PIN1* is known to be overexpressed in a variety of cancers and may act as an oncogene via promotion of cell cycle progression and proliferation.[24]

Mutations of the *c-CBL* gene are tightly associated with 11qCNN-LOH and are commonly diagnosed in patients with chronic myelomonocytic leukemia.[23,41,42] Although MPN shares clinical as well as hematologic features with chronic myelomonocytic leukemia, we detected 11qCNN-LOH only in a minority of our study population, suggesting that *c-CBL* mutations are rare events leading to transformation of chronic MPN to leukemic blast phase.

In contrast to recent findings showing frequent LOH on 4q associated with *TET2* mutations in patients diagnosed with MDS/MPN,[43] we detected CNN-LOH or deletions at 4q24 *(TET2)* only in a minority of our patients in the chronic as well as blast phase of MPN. Nevertheless, our study was not sufficient to explore these findings in more detail and make conclusions on tumor suppressor *TET2* and its potential role in leukemic transformation.

However, with regard to the variety of detected allelic imbalances, we suggest that no single candidate gene or molecular pathway is sufficient and necessary to cause transformation of chronic MPN to blast phase. Like de novo AML, MPN-blast phase appears to be a heterogeneous disease prone to have evolved multiple mechanisms to provide a proliferative advantage to the abnormal leukemic clone.

CNN-LOH involving chromosomal regions that are also frequently affected by deletions may have prognostic implications similar to the deletions visible by karyotyping. In our study, prognostic evaluation was based mainly on SNP-array data from blast phase samples without the incorporation of SNP-array results from the matched chronic phase. Moreover, we implied the survival and clinical outcome only of MPN patients who underwent leukemic transformation, without comparison with survival and outcome in untransformed chronic phase. However, as expected, blast phase patients with loss of chromosomal material on 7q showed poor survival, because this is known to be predictive for rapid progression and poor response in AML therapy.[35-37] MPN-blast phase patients with cytogenetically undetectable 7qCNN-LOH had comparable survival rates to those with −7/7q− in their leukemic cells, which is in accordance with previously published data.[44]

In addition, 9pCNN-LOH with homozygous *JAK2* mutation was also linked to an inferior outcome in MPN-blast crisis in comparison with patients with either heterozygous *JAK2V617F* or wild-type *JAK2*. In contrast to LOH on 17p, the prognostic impact of 9pCNN-LOH was independent of established risk factors such as −7/7q−, 5q−, or complex karyotype. Although *JAK2V617F* in association with 9pCNN-LOH appeared to have no impact on the time to MPN transformation, we suggest that the homozygous driver mutation in combination with additional newly acquired aberrations in terms of a second hit may have an implication on the clinical course of MPN-blast phase patients.

In conclusion, high-density SNP-array technology allowed precise identification of chromosomal aberrations, including CNN-LOH, and complemented conventional cytogenetic techniques in patients with chronic and transformed MPN. Our analysis provided prognostic details to further improve clinical prognosis, as well as novel interesting candidate genes potentially involved in the transformation of MPN.

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Authorship

Contribution: N.H.T and U.O.K. performed the research, analyzed the data, and wrote the paper; D.H.T.L., N.K., G.B.I., T.L., T.W., D.N., M.K.-M., M.K., M.S., L.-Y.S., A.N., and S.D.R. assisted with the research; C.M.-T., R.M., T.H., D.G.G., and A.T. designed and performed the research; and S.O. and H.P.K. directed the overall study.

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of departure from Hardy–Weinberg equilibrium ($P=0.06$), because the variant G allele is significantly more prevalent among Whites than non-Whites with the allele frequency of 0.24 versus 0.073, respectively ($P=0.0003$). Still, the observed associations retained significance in analyses restricted only to Whites. The G allele was associated with better EFS and OS in univariate analyses ($P=0.0173$ and 0.035, respectively, data not shown) and in multivariable analyses ($P=0.023$ and 0.005, respectively, data not shown).

We also observed that the variant A allele of FKBP5 SNP rs7755289 (T>A; intron 8) was significantly associated with worse EFS ($P=0.014$, hazard ratio = 3.193, 95% CI = 1.258–8.104, Figure 1c) and OS ($P=0.0036$, hazard ratio = 4.846, 95% CI = 1.68–14, Figure 1d). In addition, A allele was associated with increased day 22 MRD ($P=0.017$), increased cumulative incidence of relapse ($P=0.045$, hazard ratio = 3.4, 95% CI = 1.03–11.22) and an increased cumulative incidence of treatment-related mortality ($P=0.012$, hazard ratio = 5.57, 95% CI = 1.44–21.47). However, as this SNP occurred with the allele frequency of only ~0.2, the low sample size restricted us from performing further analysis. Although the above mentioned SNPs were the most interesting SNPs, we also observed association of SNP rs16878591 ($P=0.011$) with day 22 MRD levels and SNPs within LD block-2 with *in vitro* ara-C LC_{50} values ($P=0.03$; Table 1).

In previous reports, FKBP5 expression has been shown to positively influence response to cytarabine and gemcitabine. More recently, FKBP5 has been identified as scaffolding protein that facilitates PHLPP-mediated dephosphorylation of AKT-Ser473, thus indicating that higher expression of FKBP5 might contribute to enhanced chemosensitivity.[3–5] siRNA-mediated FKBP5 knockdown increases the resistance to cytarabine and other agents as etoposide, paclitaxel and doxetaxel.[1,3–5] Thus, FKBP5 SNPs may also be associated with response to other agents used in combination with cytarabine in AML patients. In conclusion, our preliminary results suggest that the FKBP5 polymorphisms mentioned above may also be relevant for AML treatment response. These results should be confirmed with functional studies and independent clinical studies. Identification of pharmacogenetic markers of response, such as FKBP5 SNP such as rs3798346, might help in further understanding inter-patient variation in response to chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

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CBL mutation in childhood therapy-related leukemia

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Therapy-related leukemia and myelodysplastic syndrome (t-Leuk/MDS) are mainly caused by topoisomerase II inhibitors that cause acute myeloid leukemia (AML) with an 11q23 translocation or by alkylating agents that induce MDS/AML with an *AML1* mutation and monosomy 7.[1,2] Two types of t-Leuk/MDS can be distinguished, one of which has a long latency (⩾5–7 years) and is seen following alkylating agents, frequently with an preleukemic phase.[1] The other has a short latency period (1–3 years), no preleukemic phase, and is strongly associated with the administration of topoisomerase II inhibitors and chromosomal abnormalities involving 11q23 translocation/*MLL* rearrangement (*MLL*-R).[2] Repair of etoposide (VP-16)-stabilized DNA topoisomerase II covalent complexes may initiate *MLL*-R observed in patients.[3]

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because

with t(8;21) are still unclear, further data accumulation is necessary. Although uncommon in pediatric myeloid malignancies, *IDH1* and *IDH2* mutations, particularly *IDH2* mutations, could contribute to the advanced phenotype of AML. Our findings provide additional impetus for investigating the role of *IDH1* and *IDH2* in the pathophysiology of errors of metabolism and in neoplastic disorders.

Conflict of interest

The authors declare no conflict of interest.

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bjh research paper

Clinical significance of early T-cell precursor acute lymphoblastic leukaemia: results of the Tokyo Children's Cancer Study Group Study L99-15

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Summary

Early T-cell precursor acute lymphoblastic leukaemia (ETP-ALL) is a recently identified subtype of T-ALL with distinctive gene expression and cell marker profiles, poor response to chemotherapy and a very high risk of relapse. We determined the reliability of restricted panel of cell markers to identify EPT-ALL using a previously classified cohort. Then, we applied the cell marker profile that best discriminated ETP-ALL to a cohort of 91 patients with T-ALL enrolled in the Tokyo Children's Cancer Study Group L99-15 study, which included allogeneic stem cell transplantation (allo-SCT) for patients with poor prednisone response. Five of the 91 patients (5·5%) met the ETP-ALL criteria. There were no significant differences in presenting clinical features between these and the remaining 86 patients. Response to early remission induction therapy was inferior in ETP-ALL as compared with T-ALL. The ETP-ALL subgroup showed a significantly poorer event-free survival (4-year rate; 40%) than the T-ALL subgroup (70%, $P = 0{\cdot}014$). Of note, three of four relapsed ETP-ALL patients survived after allo-SCT, indicating that allo-SCT can be effective for this drug-resistant subtype of T-ALL.

Keywords: acute lymphoblastic leukaemia, childhood, Early T-cell precursor, cell marker profile, allogeneic stem cell transplantation.

In approximately 15% of patients with childhood acute lymphoblastic leukaemia (ALL) leukaemic lymphoblasts have an immunophenotype corresponding to immature T cells (Pullen *et al*, 1999). Although the prognosis of childhood T-cell acute lymphoblastic leukaemia (T-ALL) has dramatically improved (Goldberg *et al*, 2003; Pui *et al*, 2009), patients with

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T-ALL continue to have an increased risk of relapse compared to those with B-precursor ALL (Pullen *et al*, 1999; Pui & Evans, 2006; Pui *et al*, 2008). In childhood B-precursor ALL patients, clinical presenting features (age and leucocyte count at diagnosis) and chromosomal translocations predict therapeutic outcome, and have been used for risk-specific adjustments in therapeutic intensity (Pui & Evans, 2006; Pui *et al*, 2008). Much effort has been put into identifying prognostically relevant clinical and biological features for childhood T-ALL (Schneider *et al*, 2000; Weng *et al*, 2004; Gottardo *et al*, 2007; Winter *et al*, 2007; Dalmazzo *et al*, 2009; Karrman *et al*, 2009; Attarbaschi *et al*, 2010; Cleaver *et al*, 2010; Zuurbier *et al*, 2010), but none is sufficiently discriminatory to be used for treatment stratification in contemporary protocols.

A recent study identified a distinct biological subtype of T-ALL, early T-cell precursor ALL (ETP-ALL) (Coustan-Smith *et al*, 2009), characterized by a gene expression profile recapitulating that of normal ETP cells, a subpopulation of thymocytes that retain multi-lineage differentiation potential (Bell & Bhandoola, 2008). ETP-ALL can be recognized by a distinctive cell surface antigen profile: lack of CD1a and CD8, weak CD5, and expression of one or more myeloid- or stem cell-related antigens. Notably, ETP-ALL was associated with an inferior clearance of leukaemia cells after the first phase of remission induction therapy and extremely poor event-free and overall survival in patients treated on intensified chemotherapeutic protocols both at the St Jude Children's Research Hospital and the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) (Coustan-Smith *et al*, 2009).

To verify the impact of prognostic significance of ETP-ALL, we studied patients with T-ALL in the Tokyo Children's Cancer Study Group (TCCSG) L99-15 study (Manabe *et al*, 2008). Because the cell marker panel used in this multicentre protocol did not include all of the markers required for diagnosis of ETP-ALL, we first establish a scoring system based on a more limited panel which could effectively differentiate ETP-ALL from T-ALL in a previously reported cohort (Coustan-Smith *et al*, 2009). Using this scoring system, we retrospectively identified patients with ETP-ALL in the TCCSG L99-15 study, and determined their presenting features, response to chemotherapy, and rates of relapse.

Materials and methods

Patients

Seven hundred and seventy patients (1–18 years of age) diagnosed with ALL were consecutively enrolled in the TCCSG L99-15 study from February 1999 to July 2003 (Manabe *et al*, 2008). The diagnosis of ALL was based on morphological, biochemical, and flow cytometric features of leukaemia cells. Flow cytometric analysis was performed in institutional or commercial laboratories, and the results were reviewed by members of the TCCSG diagnostic committee. Among 754 eligible ALL patients, 91 patients were diagnosed as T-ALL based on the expression of CD7 and at least one other T-cell marker on leukaemia cells with negative myeloperoxidase reaction (<3%); 90 patients were initially enrolled as T-ALL (Manabe *et al*, 2008) and one patient whose initial diagnosis was unclassified leukaemia was retrospectively diagnosed as T-ALL. The study was approved by the institutional review boards of the participating institutions or the equivalent organization with written informed consent from the parents or guardians of the patients.

For testing the usefulness of the scoring system, previously reported data of flow cytometric analyses of T-ALL patients from the St Jude Children's Research Hospital (St Jude cohort) were studied (Coustan-Smith *et al*, 2009). In the St Jude cohort, based on the findings of the flow cytometric analysis and gene expression profile, 17 patients were diagnosed as having ETP-ALL and 122 patients as having typical T-ALL (Coustan-Smith *et al*, 2009).

Treatment protocol

Details of the treatment regimen have been previously reported (Manabe *et al*, 2008). After 1 week oral administration of prednisolone (60 mg/m^2), patients were stratified into three treatment subgroups: those with $<0{\cdot}001 \times 10^9$ blasts/l in peripheral blood were categorized as intermediate risk (IR), those with $0{\cdot}001$–$0{\cdot}999 \times 10^9$ blasts/l as high risk (HR), and those with $\geq 1{\cdot}0 \times 10^9$ blasts/l were categorized as HR-SCT, and regarded as candidates for allogeneic stem cell transplantation (allo-SCT) in first remission. The determination of blasts in peripheral blood and bone marrow was done by microscopic evaluation at each institution. Patients in all three of the treatment subgroups underwent identical induction therapy composed of prednisolone, vincristine, cyclophosphamide, daunorubicin and asparaginase with triple intrathecal injection therapy. IR patients were randomized to receive high-dose cytarabine (2 g/m^2) or cytarabine (75 g/m^2) plus cyclophosphamide and 6-mercaptopurine in the post-remission induction intensification phase. HR and HR-SCT patients were treated with high-dose cytarabine in the post-remission induction intensification phase. Patients with an initial white blood cell (WBC) count $\geq 100 \times 10^9$ blasts/l in the IR and HR subgroups received prophylactic cranial irradiation (12 Gy for patients aged 1–6 years, and 18 Gy for patients aged 7 years and older). HR-SCT patients underwent allo-SCT in first remission; the recommended timing for SCT was after four or five courses of intensification therapy (corresponding to 7–8 months after diagnosis).

Statistical analysis

Clinical features of patients were compared using the chi-square test, and blast counts of bone marrow and peripheral blood were compared using the Mann–Whitney test. To compare cell surface antigen expression levels between patients with ETP-ALL and patients with typical T-ALL in

the St Jude cohort, we performed either the student's *t*-test or the *t*-test for unequal variances based on the *F* value for sample variances. The duration of event-free survival was defined as the time from the initiation of therapy to either treatment failure (relapse, death, or diagnosis of secondary cancer) or to the last day when the patient was confirmed to be in remission. The probabilities of event-free survival and overall survival were estimated by the Kaplan–Meier analysis, and were tested for significance using log-rank test. For univariate and multivariate analysis, the Cox proportional hazards model was employed to assess risk factors on even-free survival. *P* values <0·05 were considered statistically significant.

Results

Development of an ETP-ALL scoring system

ETP-ALL shows a distinctive immature immunophenotype characterized by lack of CD1a and CD8 expression, weak CD5 expression with <75% positive blasts, and expression of one or more of the following myeloid or stem cell antigens on at least 25% of lymphoblasts: CD117, CD34, HLA-DR, CD13, CD33, CD11b and/or CD65 (Coustan-Smith *et al*, 2009). Among the T-ALL patients enrolled in the TCCSGL99-15 study, data for some of the markers were only available in a limited group of patients (e.g. CD1a and CD11b were available for 67% and 7%

(A)

Scoring system based on 6 marker expression		
Score	–1	+1
CD5	≥5%	<75%
CD8	≥5%	<5%
CD13		≥25%
CD33		≥25%
CD34		≥25%
HLA-DR		≥25%

(C)

Scoring system based on 11 marker expression				
Score	–2	–1	+1	+2
CD5	≥75%			<75%
CD8	≥5%			<5%
CD13			≥25%	≥75%
CD33			≥25%	≥75%
CD34			≥25%	≥75%
HLA-DR			≥25%	≥75%
CD2		≥75%	<20%	
CD3		≥75%	<20%	
CD4		≥75%	<20%	
CD10		≥75%	<20%	
CD56			≥20%	

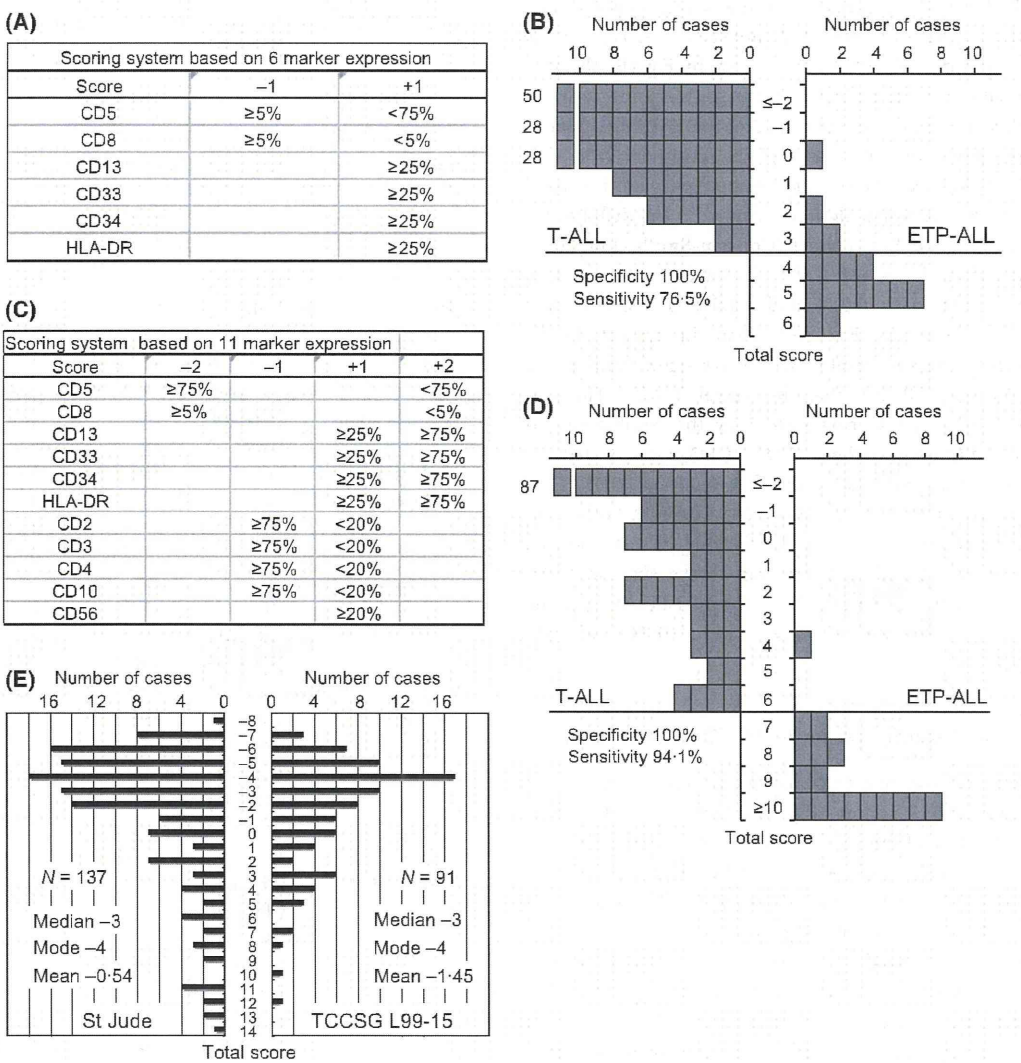

Fig 1. Establishment of a scoring system for immunophenotypical diagnosis of early T-cell precursor acute lymphoblastic leukaemia (ETP-ALL). (A) Scoring system based on the expression of six cell surface markers. (B) Distribution of total score of 6-marker expression in 17 ETP-ALL cases (right) and 122 T-ALL cases (left) of the St Jude cohort. (C) Scoring system based on the expression of 11 markers. (D) Distribution of total score of 11-marker expression in ETP-ALL patients (right) and T-ALL patients (left) of the St Jude cohort. (E) Distribution of total score of 11-marker expression in 139 T-ALL cases of the St Jude cohort (left) and 91 T-ALL cases of the TCCSG L99-15 study (right).

