Table III. Comparison of several studies regarding the outcome of relapsed patients with acute lymphoblastic leukaemia (ALL).

	Patients (*n*)	Rate of CR2 (%)	5-year OS	Prognostic factors for improved OS
LALA-94 (Tavernier *et al*, 2007)	421	44%	8%	Allo-HSCT after relapse CR1 > 1 year Platelet count at relapse >100 × 10^9/l
MRC UKALL12/ECOG2993 (Fielding *et al*, 2007)	609	N.M.	7%	Age at relapse <20 years CR1 > 2 years
PETHEMA trials (Oriol *et al*, 2010)	263	45%	10%	Age at relapse <30 years CR1 > 2 years
GMALL trials (Gokbuget *et al*, 2012)*	547	42% after CTx alone† 23% after Allo-HSCT‡	28% after CTx alone† 15% after Allo-HSCT‡	No Allo-HSCT in CR1 Extramedullary relapse (other than CNS) CR1 > 18 months§ Age at relapse§ (15–25 years vs. 26–45 years vs. 46–55 years) CR after 1st salvage CTx§ Allo-HSCT at any stage§
This study	332	53% after CTx alone† 54% after Allo-HSCT‡	16% after CTx alone† 11% after Allo-HSCT‡	HSCT in CR2¶ WBC count at relapse <10 × 10^9/l¶ Age at relapse <35 years¶

*Patients with Philadelphia chromosome-positive ALL were not included in this report from GMALL trials.
†The rate is among patients who relapsed after CTx alone in CR1.
‡The rate is among patients who relapsed after Allo-HSCT in CR1.
§These factors were associated with better OS in patients who relapsed after CTx alone in CR1.
¶These factors were associated with better OS in patients who achieved CR2 following relapse after CTx alone in CR1.
CR1, first complete remission; CR2, second complete remission; OS, overall survival; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; CTx, chemotherapy; CNS, central nervous system; WBC, white blood cell count; LALA-94, Leucémies Aiguës Lymphoblastiques de l'Adulte 94 trial; MRC UKALL12/ECOG2993, Medical Research Council, United Kingdom ALL 12/Eastern Cooperative Oncology Group 2993 trial; PETHEMA, Programme for the Study and Treatment of Haematological Malignancies; GMALL, German Multicentre Study Group for Adult ALL, N. M., not mentioned.

at 5 years). These findings suggested that we should consider Allo-HSCT as much as possible for relapsed patients.

Prognostic factors that were associated with a better OS from CR2 were younger age at relapse, lower WBC count at relapse, and Allo-HSCT in CR2 among patients who relapsed after chemotherapy alone in CR1 and achieved CR2 following salvage chemotherapy. None of the factors at diagnosis was associated with OS after relapse. Age and Allo-HSCT were the common factors observed in other studies (Table III). The duration of CR1 was not associated with better OS in the multivariate analysis in our study. In other studies, the duration of CR1 was associated with not only better OS but also a high rate of achieving CR2 (Thomas *et al*, 1999; Tavernier *et al*, 2007; Oriol *et al*, 2010). Given that our analysis was limited to patients who achieved CR2, the duration of CR1 might not be a significant factor for OS. Although the WBC count at relapse was not analysed in the other studies, it should be considered as an important factor.

We should note that there might be selection bias regarding the performance of Allo-HSCT in CR2, because it depended on each institution's decision. By a multivariate analysis using logistic regression for all covariates, relapse year (after 2003) and younger age at relapse (younger than 36 years) were significantly associated with the performance of Allo-HSCT in CR2. However, a multivariate analysis for OS including relapse year, age at relapse, and Allo-HSCT in CR2 (treated as a time-dependent covariate) as covariates demonstrated that Allo-HSCT in CR2, as well as age at relapse, was still significantly associated with better OS. In addition, there was no significant interactions between Allo-HSCT in CR2 and relapse year and between Allo-HSCT in CR2 and age at relapse ($P = 0{\cdot}36$ and $P = 0{\cdot}97$, respectively).

Comparisons of different salvage chemotherapy regimens have been limited (Thomas *et al*, 1999; Tavernier *et al*, 2007; Oriol *et al*, 2010). The selection of salvage regimens often depends on the condition of the relapsed patient (Garcia-Manero & Thomas, 2001). In our analyses, the duration of CR1, intensity of chemotherapy at diagnosis, and relapse year were factors that were associated with the selection of salvage regimens. AdVP-type salvage chemotherapy was related to a better OS in patients who had a longer duration of CR1 and a worse OS in those who had a shorter duration of CR1. If we consider that many patients had received induction chemotherapy including doxorubicin, vincristine, and steroids, the duration of CR1 might reflect the sensitivity of ALL to the AdVP-type regimen. Patients who had a longer duration

of CR1 might have had ALL that was sensitive to an AdVP-type regimen, and those who had a shorter duration of CR1 might have had ALL that was refractory to an AdVP-type regimen. In patients who relapsed late, the toxicity of moderate-intensity regimens, such as those including high-dose cytarabine, used as the first salvage chemotherapy might offset their effectiveness. Unlike in a previous study (Thomas *et al*, 1999), the type of chemotherapy at diagnosis did not influence OS following each salvage regimen.

In conclusion, the prognosis of adult patients with relapsed Ph-negative ALL is poor. However, Allo-HSCT after the first relapse could improve the prognosis, especially if performed in CR2. The efficacy of different types of salvage chemotherapy might depend on the duration of CR1, and this should be considered in the selection of the salvage regimen.

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Authorship

Author contributions: S.K. wrote the paper. All authors contributed to writing the paper and checked the final version. S.K. and Y.K. designed the study and analysed data. All authors participated in data collection.

Conflict of interest disclosures

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Participating Centres

References

Amadori, S., Arcese, W., Isacchi, G., Meloni, G., Petti, M.C., Monarca, B., Testi, A.M. & Mandelli, F. (1991) Mitoxantrone, etoposide, and intermediate-dose cytarabine: an effective and tolerable regimen for the treatment of refractory acute myeloid leukemia. *Journal of Clinical Oncology*, **9**, 1210–1214.

Camera, A., Annino, L., Chiurazzi, F., Fazi, P., Cascavilla, N., Fabbiano, F., Marmont, F., Di Raimondo, F., Recchia, A., Vignetti, M., Rotoli, B. & Mandelli, F. (2004) GIMEMA ALL - Rescue 97: a salvage strategy for primary refractory or relapsed adult acute lymphoblastic leukemia. *Haematologica*, **89**, 145–153.

Cornelissen, J.J., van der Holt, B., Verhoef, G.E., van't Veer, M.B., van Oers, M.H., Schouten, H. C., Ossenkoppele, G., Sonneveld, P., Maertens, J., van Marwijk Kooy, M., Schaafsma, M.R., Wijermans, P.W., Biesma, D.H., Wittebol, S., Voogt, P.J., Baars, J.W., Zachee, P., Verdonck, L.F., Lowenberg, B. & Dekker, A.W. (2009) Myeloablative allogeneic versus autologous stem cell transplantation in adult patients with acute lymphoblastic leukemia in first remission: a prospective sibling donor versus no-donor comparison. *Blood*, **113**, 1375–1382.

Fielding, A.K., Richards, S.M., Chopra, R., Lazarus, H.M., Litzow, M.R., Buck, G., Durrant, I.J., Luger, S.M., Marks, D.I., Franklin, I.M., McMillan, A.K., Tallman, M.S., Rowe, J.M. & Goldstone, A.H. (2007) Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. *Blood*, **109**, 944–950.

Garcia-Manero, G. & Thomas, D.A. (2001) Salvage therapy for refractory or relapsed acute lymphocytic leukemia. *Hematology/oncology Clinics of North America*, **15**, 163–205.

Giona, F., Annino, L., Rondelli, R., Arcese, W., Meloni, G., Testi, A.M., Moleti, M.L., Amadori, S., Resegotti, L., Tabilio, A., Ladogana, S., Fioritoni, G., Camera, A., Liso, V., Leoni, P. & Mandelli, F. (1997) Treatment of adults with acute lymphoblastic leukaemia in first bone marrow relapse: results of the ALL R-87 protocol. *British Journal of Haematology*, **97**, 896–903.

Gokbuget, N., Stanze, D., Beck, J., Diedrich, H., Horst, H.A., Huttmann, A., Kobbe, G., Kreuzer, K.A., Leimer, , L., Reichle, A., Schaich, M., Schwartz, S., Serve, H., Starck, M., Stelljes, M., Viardot, A., Wendelin, K., Freund, M., Hoelzer, D. (2012) Outcome of relapsed adult lymphoblastic leukemia depends on response to salvage chemotherapy, prognostic factors, and performance of stem cell transplantation. *Blood*, **120**, 2032–2041.

Jinnai, I., Sakura, T., Tsuzuki, M., Maeda, Y., Usui, N., Kato, M., Okumura, H., Kyo, T., Ueda, Y., Kishimoto, Y., Yagasaki, F., Tsuboi, K., Horiike, S., Takeuchi, J., Iwanaga, M., Miyazaki, Y., Miyawaki, S., Ohnishi, K., Naoe, T. & Ohno, R. (2010) Intensified consolidation therapy with dose-escalated doxorubicin did not improve the prognosis of adults with acute lymphoblastic leukemia: the JALSG-ALL97 study. *International Journal of Hematology*, **92**, 490–502.

Kanda, Y. (2012) Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplantation*, Epub ahead of Print, December 3, 2012; doi:10.1038/bmt.2012.244

Kantarjian, H.M., Estey, E.H., Plunkett, W., Keating, M.J., Walters, R.S., Iacoboni, S., McCredie, K.B. & Freireich, E.J. (1986) Phase I-II clinical and pharmacologic studies of high-dose cytosine arabinoside in refractory leukemia. *American Journal of Medicine*, **81**, 387–394.

Kantarjian, H.M., O'Brien, S., Smith, T.L., Cortes, J., Giles, F.J., Beran, M., Pierce, S., Huh, Y., Andreeff, M., Koller, C., Ha, C.S., Keating, M.J., Murphy, S. & Freireich, E.J. (2000) Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. *Journal of Clinical Oncology*, **18**, 547–561.

Koller, C.A., Kantarjian, H.M., Thomas, D., O'Brien, S., Rios, M.B., Kornblau, S., Murphy, S. & Keating, M. (1997) The hyper-CVAD regimen improves outcome in relapsed acute lymphoblastic leukemia. *Leukemia*, **11**, 2039–2044.

Lejeune, C., Tubiana, N., Gastaut, J.A., Maraninchi, D., Richard, B., Launay, M.C., Sainty, D., Sebahoun, G. & Carcassonne, Y. (1990) High-dose cytosine arabinoside and mitoxantrone in previously-treated acute leukemia patients. *European Journal of Haematology*, **44**, 240–243.

Litzow, M.R. (2009) Evolving paradigms in the therapy of Philadelphia chromosome-negative acute lymphoblastic leukemia in adults. *Hematology/ the Education Program of the American Society of Hematology*, 362–370.

Montillo, M., Tedeschi, A., Centurioni, R. & Leoni, P. (1997) Treatment of relapsed adult acute lymphoblastic leukemia with fludarabine and cytosine arabinoside followed by granulocyte colony-stimulating factor (FLAG-GCSF). *Leukaemia & Lymphoma*, **25**, 579–583.

Oriol, A., Vives, S., Hernandez-Rivas, J.M., Tormo, M., Heras, I., Rivas, C., Bethencourt, C., Moscardo, F., Bueno, J., Grande, C., del Potro, E., Guardia, R., Brunet, S., Bergua, J., Bernal, T., Moreno, M.J., Calvo, C., Bastida, P., Feliu, E. & Ribera, J.M. (2010) Outcome after relapse of acute lymphoblastic leukemia in adult patients included in four consecutive risk-adapted trials by the PETHEMA Study Group. *Haematologica*, **95**, 589–596.

Ottmann, O.G. & Pfeifer, H. (2009) Management of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL). *Hematology/ the Education Program of the American Society of Hematology*, 371–381.

Sakura, T., Hayakawa, F., Yujiri, T., Aoyama, Y., Kondo, E., Fujimaki, K., Ueda, Y., Ohtake, S., Miyazaki, Y., Miyawaki, S., Ohnishi, K. & Naoe, T. (2012) Outcome of Pediatric-Type Therapy for Philadelphia Chromosome-Negative Acute Lymphoblastic Leukemia (ALL) in Adolescents and Young Adults (AYA): a Study by the Japan Adult Leukemia Study Group (JALSG ALL202-U study). *Blood*, **120**, 1464.

Specchia, G., Pastore, D., Carluccio, P., Liso, A., Mestice, A., Rizzi, R., Ciuffreda, L., Pietrantuono, G. & Liso, V. (2005) FLAG-IDA in the treatment of refractory/relapsed adult acute lymphoblastic leukemia. *Annals of Hematology*, **84**, 792–795.

Takeuchi, J., Kyo, T., Naito, K., Sao, H., Takahashi, M., Miyawaki, S., Kuriyama, K., Ohtake, S., Yagasaki, F., Murakami, H., Asou, N., Ino, T., Okamoto, T., Usui, N., Nishimura, M., Shinagawa, K., Fukushima, T., Taguchi, H., Morii, T., Mizuta, S., Akiyama, H., Nakamura, Y., Ohshima, T. & Ohno, R. (2002) Induction therapy by frequent administration of doxorubicin with four other drugs, followed by intensive consolidation and maintenance therapy for adult acute lymphoblastic leukemia: the JALSG-ALL93 study. *Leukemia*, **16**, 1259–1266.

Tavernier, E., Boiron, J.M., Huguet, F., Bradstock, K., Vey, N., Kovacsovics, T., Delannoy, A., Fegueux, N., Fenaux, P., Stamatoullas, A., Tournilhac, O., Buzyn, A., Reman, O., Charrin, C., Boucheix, C., Gabert, J., Lheritier, V., Vernant, J.P., Dombret, H. & Thomas, X. (2007) Outcome of treatment after first relapse in adults with acute lymphoblastic leukemia initially treated by the LALA-94 trial. *Leukemia*, **21**, 1907–1914.

Thomas, D.A., Kantarjian, H., Smith, T.L., Koller, C., Cortes, J., O'Brien, S., Giles, F.J., Gajewski, J., Pierce, S. & Keating, M.J. (1999) Primary refractory and relapsed adult acute lymphoblastic leukemia: characteristics, treatment results, and prognosis with salvage therapy. *Cancer*, **86**, 1216–1230.

Weiss, M.A., Aliff, T.B., Tallman, M.S., Frankel, S. R., Kalaycio, M.E., Maslak, P.G., Jurcic, J.G., Scheinberg, D.A. & Roma, T.E. (2002) A single, high dose of idarubicin combined with cytarabine as induction therapy for adult patients with recurrent or refractory acute lymphoblastic leukemia. *Cancer*, **95**, 581–587.

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ORIGINAL ARTICLE: CLINICAL

Clinical evaluation of WT1 mRNA expression levels in peripheral blood and bone marrow in patients with myelodysplastic syndromes

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Abstract

A study to evaluate WT1 mRNA expression levels in peripheral blood (PB) and bone marrow aspirate (BM) was conducted in 172 patients, including 115 with myelodysplastic syndromes (MDS), in Japan. The level of WT1 mRNA expression was evaluated according to the French–American–British (FAB) and World Health Organization (WHO) classifications (2001, 2008) and using the International Prognostic Scoring System and the WHO Prognostic Scoring System scales. WT1 mRNA expression levels in PB and BM were well correlated ($r = 0.85$), and they tended to increase with disease stage progression and in those at higher risk of leukemic transformation. WT1 mRNA expression can be a useful marker for the diagnosis and risk evaluation of MDS.

Keywords: Myelodysplastic syndromes, WT1 mRNA expression, classification system, peripheral blood, bone marrow

Introduction

Myelodysplastic syndrome (MDS), a clonal disorder of pluripotent hematopoietic stem cells, is a blood disease characterized by dysplasia and ineffective hemopoiesis. Approximately 20–30% of cases of MDS undergo transformation to acute myeloid leukemia (AML) [1].

The expression of Wilms' tumor gene (WT1) has been found to be a new prognostic factor and marker for the detection of minimal residual disease (MRD) in acute leukemia, including AML and acute lymphocytic leukemia (ALL) [2]. A recent study has revealed the clinical relevance of measuring WT1 mRNA for monitoring MRD in AML, primarily due to its high rate of expression (93.9%) in the peripheral blood (PB) of incipient untreated patients with AML, secondarily due to its ability to predict relapse after complete remission (CR), and finally because its levels after consolidation therapy

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show a significant correlation between disease-free survival, overall survival and early relapse [3]. WT1 mRNA expression occurs not only in AML but also in the PB and bone marrow (BM) of patients with MDS [4–9].

Tamaki *et al.* [4] examined the level of WT1 mRNA expression in PB and BM from 57 patients with MDS grouped by the French–American–British (FAB) classification, and 12 patients experienced AML-MDS progression. The results revealed that WT1 mRNA expression in both PB and BM progressively increased with disease stage progression, from refractory anemia (RA), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t), and to AML, suggesting the possibility that the WT1 mRNA expression level reflects the disease stage progression of MDS. Particularly, the patient group who developed leukemia from RAEB or RAEB-t within 6 months showed significantly higher WT1 mRNA expression in PB compared with the group who did not [4].

In accordance with that study, Cilloni *et al.* [6] measured WT1 mRNA expression levels in PB and BM from 131 patients with MDS, and found that: (1) WT1 mRNA expression in PB and BM was confirmed in 78% and 65% of patients with RA, respectively; (2) WT1 mRNA expression in PB and BM was confirmed in all patients with RAEB and secondary AML; (3) the level of WT1 mRNA expression increased with disease stage progression; and (4) the WT1 mRNA expression level was well correlated with the International Prognostic Scoring System (IPSS) scores established by Greenberg *et al.* [10].

In addition to the IPSS, the World Health Organization (WHO) Classification-Based Prognostic Scoring System (WPSS) has been proposed as a prognostic scoring system for MDS [11]. The WPSS consists of three characteristics: WHO subtype classification, considered to be important as a prognostic factor; IPSS-based karyotype abnormalities; and transfusion dependency.

Both the IPSS and WPSS require a chromosomal test as a primary parameter. However, because there are cases in which chromosomal abnormalities cannot be determined [12–14], it is necessary to establish molecular- and genetic-based methods to diagnose and determine the prognosis of MDS. The relatively rapid quantitation of WT1 mRNA is considered to be a useful test to determine the prognosis of MDS and has potential for clinical application, to become a novel marker to complement the current IPSS and WPSS criteria. We performed a clinical study in patients with MDS to demonstrate the usefulness of measuring the WT1 mRNA expression level in PB and BM in the diagnosis and treatment of MDS.

Patients and methods

This study was conducted in accordance with the Declaration of Helsinki, and preliminary approval was obtained from the Institutional Review Board or equivalent organization of each participating institution. Explanations of the study protocol were provided to all patients, and written informed consent was obtained from them before study enrollment.

Patients

From December 2008 to September 2009, 175 patients with MDS, suspected MDS and AML-MDS examined at 17 Japanese medical institutions were enrolled in the study. The subjects were 20 years of age or older and entered in the study regardless of gender, inpatient/outpatient status, or presence or absence of treatment. The 175 patients comprised 106 men (age range 27–88 years, average 65.5 years) and 69 women (age range 22–85 years, average 64.5 years). PB and BM samples from each patient were collected on the same day and used for WT1 mRNA measurement. Three of the 175 enrolled patients were excluded because BM could not be collected due to a dry tap or because the subtype could not be diagnosed. A total of 172 patients were therefore included in the final analysis set.

Diagnosis

Diagnosis of MDS was carried out using a central review format based on the FAB classification [15], the 2001 WHO classification [16] and the 2008 WHO classification [17]. Central review of the bone marrow smear-stained specimens, blood smear-stained specimens, iron-stained specimens, and clot hematoxylin and eosin-stained specimens was carried out by two individuals, one each in the Department of Hemato-Oncology, Saitama International Medical Center, Saitama Medical University, and the Department of Laboratory Medicine, Kawasaki Medical School.

WT1 mRNA measurement method

mRNA was extracted from PB leukocytes and BM nucleated cells at SRL, Inc., Tokyo, Japan using the RNeasy Mini-Kit (Qiagen, Valencia, CA), and the amount containing WT1 mRNA was measured at the Research Laboratory, Diagnostic Division, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). cDNA was synthesized from 1 μg of extracted RNA in a reverse-transcription reaction using random hexamer primers. The amounts of WT1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA were quantitated using real-time polymerase chain reaction (PCR) with a COBAS TaqMan48 analyzer (Roche Diagnostics, Pleasanton, CA), and the respective amounts of WT1 and GAPDH RNA in the sample were calculated by simultaneous reaction with standards of known concentrations.

Method for calculating WT1 mRNA expression

mRNA of the universally expressed housekeeping gene GAPDH was used for correction of variations in the efficiencies of RNA extraction and reverse transcription. As shown in the following formula, the level of WT1 mRNA expression was calculated by dividing the measured amount of WT1 mRNA by the measured amount of GAPDH mRNA and multiplying that value by the average number of copies of GAPDH mRNA found in 1 μg of RNA from PB leukocytes of healthy adults (GAPDH mRNA expression). The average GAPDH mRNA expression in PB leukocytes of healthy adults was reported to be 2.7×10^7 copies/μg RNA based on independent tests in healthy adults [3].

WT1 mRNA expression (copies/μg RNA) = (measured WT1 mRNA [copies/mL]/measured GAPDH mRNA [copies/mL]) × 2.7 × 10^7 (copies/μg RNA)

PB cut-off value

The lower limit of the WT1 mRNA measurement range in the WT1 assay kit is 2500 copies/mL, or 50 copies/μg RNA when converted to copies per microgram of RNA. In this study, a value of 50 copies/μg RNA was set as the cut-off value for WT1 mRNA expression, and a value of 50 or more copies/μg RNA was judged as positive according to the instruction manual of the WT1 mRNA assay kit.

Statistical analysis

The mean ± SD for the log-transformed values of WT1 mRNA expression (copies/μg RNA) was calculated, and then converted back to base 10 and used as the geometric mean. All data below the detection limit were shown as 49 copies/μg RNA. For intergroup comparison of WT1 mRNA expression, a Tukey-Kramer honestly significant difference (HSD) test was performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). For comparison of WT1 mRNA expression between the aplastic anemia (AA) and RA groups, a Wilcoxon rank-sum test and Steel test were performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). The Pearson correlation coefficient was used for analysis of each correlation.

Results

As a result of the central review conducted on all 172 patients, 115 were classified as patients with MDS in the FAB classification, excluding chronic myelomonocytic leukemia (CMML). Similarly, 98 patients in the 2001 WHO classification and 97 in the 2008 WHO classification were classified as patients with MDS (Figure 1).

Analytical results based on FAB classification

WT1 mRNA expression in PB and BM

The 172 patients eligible for analysis were categorized by disease type, and their WT1 mRNA expression levels in PB and BM are shown in Table I. The mean WT1 mRNA expression level in the 115 patients with MDS (excluding CMML) was 360 copies/μg RNA in PB and 2240 copies/μg RNA in BM, and these values were the second highest after the values obtained in patients with AML-MDS (PB: 12 600 copies/μg RNA; BM: 33 100 copies/μg RNA). On the other hand, the WT1 mRNA expression level was less than 50 copies/μg RNA in PB and 90–630 copies/μg RNA in BM in patients with AA, idiopathic cytopenia of unknown significance (ICUS), idiopathic thrombocytopenic purpura (ITP), paroxysmal nocturnal hemoglobinuria (PNH), pure red-cell aplasia (PRCA) and erythroid hypoplasia, which were all lower compared with the level in MDS.

The relationship between WT1 mRNA expression in PB and BM was evaluated in all patients. The regression line formula $y = 0.7329x + 1.4407$ was obtained, indicating a strong correlation ($r = 0.85$) (Figure 2).

WT1 mRNA expression in PB and BM for each MDS disease stage

When the WT1 mRNA expression levels in PB and BM were compared for each MDS subtype based on the FAB classification [Figure 3(a)], the level in both increased proportionally with each MDS classification as the disease

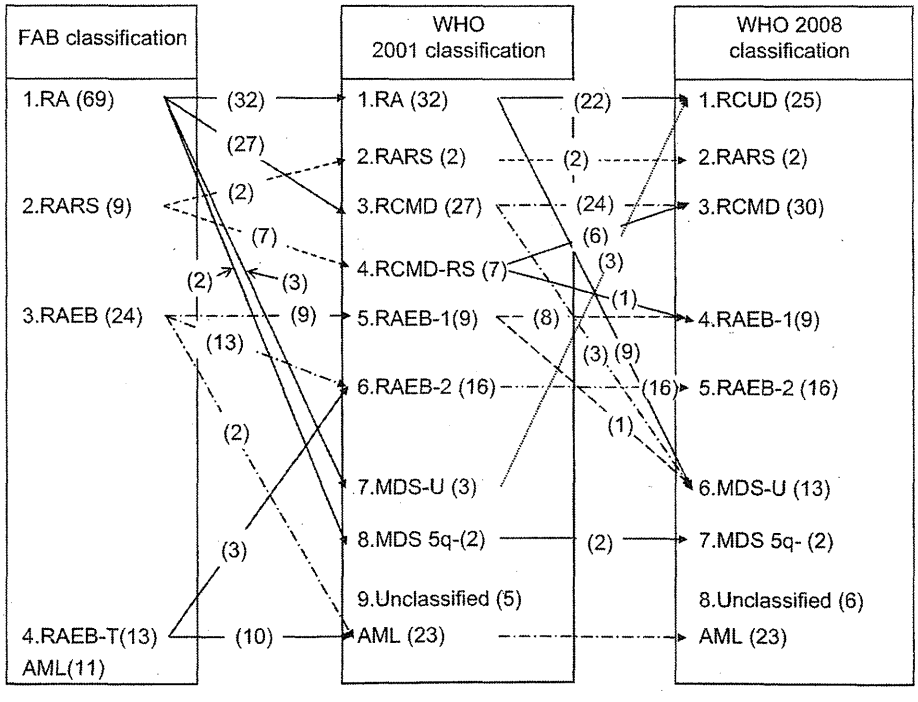

Figure 1. FAB and WHO classification of myelodysplastic syndromes in this study. FAB classification-based MDS subtypes (four subtypes: RA, RARS, RAEB and RAEB-t), 2001 WHO-based MDS subtypes (eight subtypes: RA, RARS, RCMD, RCMD-RS, RAEB-1, RAEB-2, MDS-U and MDS 5q−), 2008 WHO-based MDS subtypes (seven subtypes: RCUD, RARS, RCMD, RAEB-1, RAEB-2, MDS-U and MDS 5q−). Numbers in parentheses represent numbers of patients.

Table I. WT1 mRNA expression levels in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

		WT1 mRNA expression level			
		Peripheral blood		Bone marrow	
Disease	No. of patients	Log (mean ± SD)	Geometric mean (copies/μg RNA)	Log (mean ± SD)	Geometric mean (copies/μg RNA)
MDS	115	2.56 ± 1.05	360	3.35 ± 0.87	2240
AML-MDS	11	4.10 ± 0.96	12 600	4.52 ± 0.77	33 100
AML-MDS (CR)	2	1.89 ± 0.20	80	2.98 ± 0.39	1000
CMML	3	2.17 ± 0.54	150	3.04 ± 0.54	1100
CLL	1	1.92	80	3.33	2140
Atypical CML	1	—	<50	1.95	90
AA	8	—	<50	2.64 ± 0.37	440
ICUS	3	—	<50	2.16 ± 0.36	140
ITP	1	—	<50	2.13	130
PNH	1	—	<50	2.8	630
PRCA	2	—	<50	2.17 ± 0.12	150
Erythroid hypoplasia	1	—	<50	1.94	90
Unclassified	23	2.14 ± 0.56	140	2.96 ± 0.61	910
Total	172	2.50 ± 1.05	320	3.27 ± 0.90	1860

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; CR, complete remission; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; AA, aplastic anemia; ICUS, idiopathic cytopenia of unknown significance; ITP, idiopathic thrombocytopenic purpura; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red-cell aplasia.

stage progressed. Significant differences in both PB and BM expression were seen between RA and RAEB, RA and RAEB-t, refractory anemia with ringed sideroblasts (RARS) and RAEB, and RARS and RAEB-t ($p < 0.05$).

WT1 mRNA expression in PB and BM for each IPSS risk group

WT1 mRNA expression levels in PB and BM for each IPSS risk group were compared in the 115 patients with MDS. A tendency for WT1 mRNA expression to increase in both PB and BM was observed in each IPSS risk group as the risk of transformation to AML increased from low to high. Significant differences ($p < 0.05$) in WT1 mRNA expression were observed in risk groups between low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and high in PB samples; and between low and intermediate-1, low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and higher in BM samples [Figure 3(b)]. The correlation between IPSS score and WT1 mRNA expression was evaluated, and a correlation of $r = 0.57$ was found for both PB and BM samples.

Next, the WT1 mRNA expression levels in PB and BM between IPSS risk groups were compared in the 69 patients with RA [Figure 3(c)]. As the risk increased from low to intermediate-2, the level of WT1 mRNA expression in both PB and BM increased. Moreover, when the distribution of WT1 mRNA expression between each risk group was evaluated, a significant difference ($p < 0.05$) was found in PB between low and intermediate-2; in BM, significant differences were found between low and intermediate-1, and low and intermediate-2.

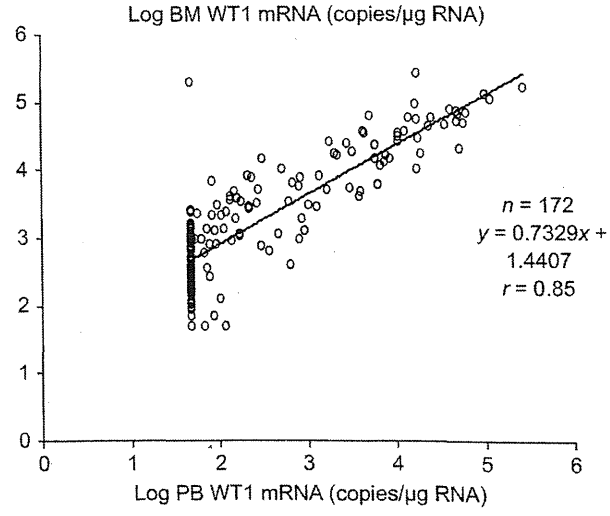

Figure 2. Correlation of WT1 mRNA expression in PB and WT1 mRNA expression in BM.

Correlation between IPSS karyotype and WT1 mRNA expression

A total of 114 patients with MDS were categorized into the three prognostic groups of good, intermediate and poor in accordance with their IPSS karyotype, and the levels of WT1 mRNA expression in their PB and BM samples were compared. One patient with MDS was excluded from this analysis because chromosome testing was not performed. The WT1 mRNA expression level increased in both PB and BM samples as the karyotype indicated a poorer prognosis. Among karyotypes, significant differences ($p < 0.05$) in WT1 mRNA expression were found between the good and intermediate and between the good and poor groups [Figure 3(d)].

Correlation between WT1 mRNA expression and percentage of blasts in BM

The correlation between blast ratio and WT1 mRNA expression in PB and BM was investigated in 114 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlation between blast ratio and PB WT1 mRNA expression was $r = 0.51$, and the correlation between blast ratio and BM WT1 mRNA expression was $r = 0.48$.

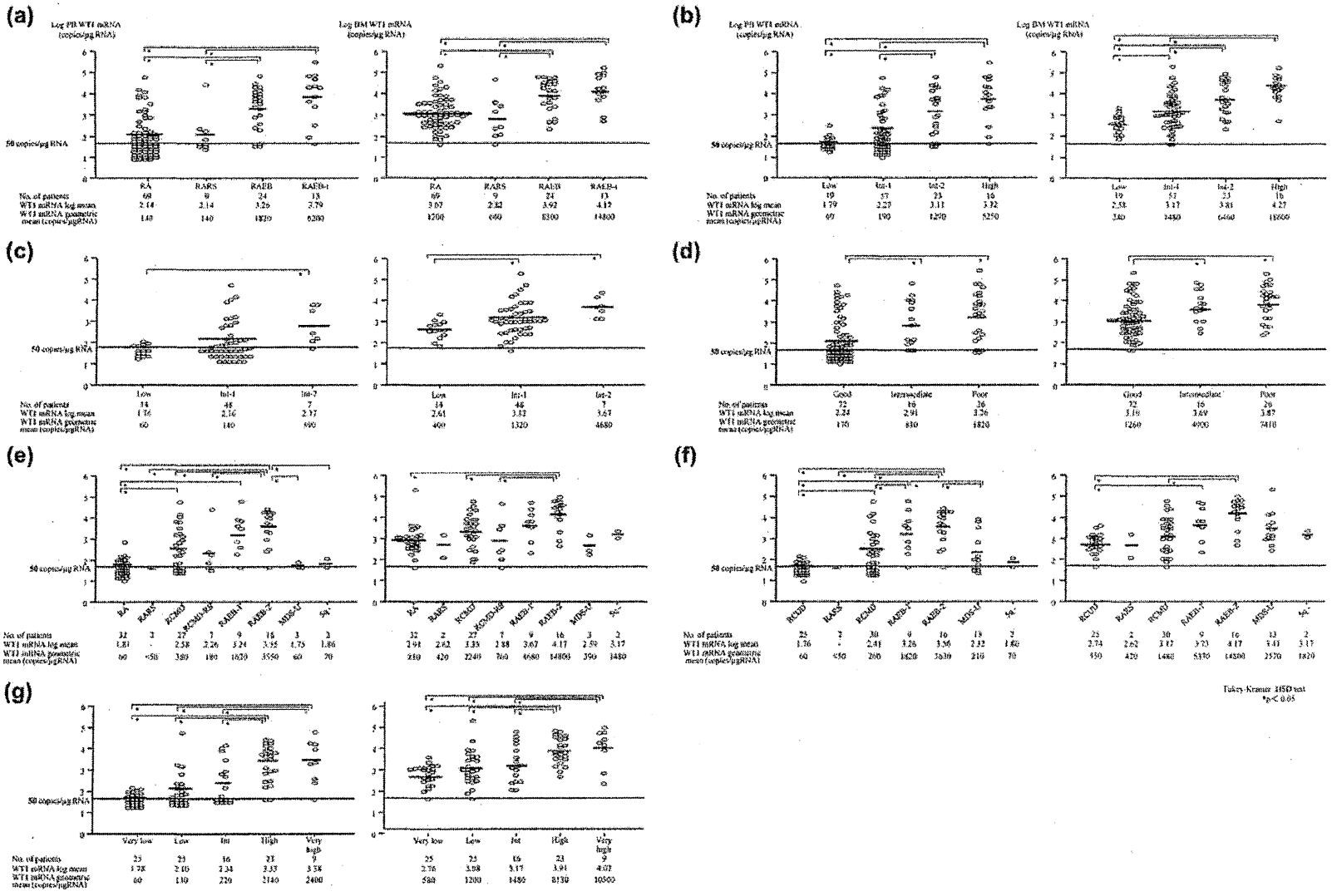

Figure 3. WT1 mRNA expression levels in PB and BM from patients with MDS (a) according to FAB classification, (b) according to IPSS category, (c) patients with RA according to IPSS category, (d) according to chromosomal karyotype, (e) according to WHO 2001 classification, (f) according to WHO 2008 classification, (g) according to WPSS category. In intergroup comparison of WT1 mRNA expression, the Tukey-Kramer HSD test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

Analytical results based on 2001 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2001 WHO classification

Figure 3(e) shows the assay results for WT1 mRNA expression in PB and BM in 98 patients in various MDS disease stages categorized on the basis of the 2001 WHO classification. The WT1 mRNA expression levels in both PB and BM tended to increase with the progression to each MDS subtype. When the levels of WT1 mRNA expression in each disease stage were investigated, significant differences ($p < 0.05$) were found in PB between RA and refractory cytopenia with multilineage dysplasia (RCMD), RA and RAEB-1, RA and RAEB-2, RARS and RAEB-2, RCMD and RAEB-2, RCMD with ringed sideroblasts (RCMD-RS) and RAEB-2, RAEB-2 and unclassified MDS (MDS-U), and RAEB-2 and 5q− syndrome; in BM, significant differences were found between RA and RAEB-2, RCMD and RAEB-2, and RCMD-RS and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2001 WHO Classification

The correlation between the blast ratio and WT1 mRNA expression in PB and BM was investigated in 97 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlations between the blast ratio and WT1 mRNA expression were $r = 0.50$ in PB and $r = 0.46$ in BM.

Analytical results based on 2008 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2008 WHO classification

Figure 3(f) shows the assay results for WT1 mRNA expression in PB and BM in a total of 97 patients in various MDS disease stages categorized on the basis of the 2008 WHO classification. WT1 mRNA expression in both PB and BM tended to increase with the progression to each MDS subtype.

When the distribution of WT1 mRNA expression for each disease stage was examined, significant differences ($p < 0.05$) were found in PB between refractory cytopenia with unilineage dysplasia (RCUD) and RCMD, RCUD and RAEB-1, RCUD and RAEB-2, RARS and RAEB-2, RCMD and RAEB-1, RCMD and RAEB-2, RAEB-1 and MDS-U, and RAEB-2 and MDS-U; in BM, significant differences were found between RCUD and RAEB-1, RCUD and RAEB-2, and RCMD and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2008 WHO classification

The correlations between blast ratio and WT1 mRNA expression in 96 patients (excluding one patient with MDS whose blast ratio could not be measured) were $r = 0.50$ in PB and $r = 0.46$ in BM.

WT1 mRNA expression in PB and BM for each WPSS risk group

WT1 mRNA expression in PB and BM was compared in 98 patients with MDS classified according to WPSS risk group [Figure 3(g)]. As the risk increased from very low to very high, WT1 mRNA expression in both PB and BM also tended to rise. When the distribution of WT1 mRNA for each risk group was evaluated, significant differences ($p < 0.05$) were found in both PB and BM between very low and high, very low and very high, low and high, low and very high, intermediate and high, and intermediate and very high. Moreover, when the correlation between the WPSS score and WT1 mRNA expression was investigated, the values were $r = 0.61$ in PB and $r = 0.55$ in BM.

Differential diagnosis between RA and AA

Differential diagnosis based on WT1 mRNA expression in PB samples

The WT1 mRNA expression level in PB was less than 50 copies/μg RNA in all eight patients with AA, whereas it was less than 50 copies/μg RNA in 34 patients with RA and 50–52 100 copies/μg RNA in 35 of 69 patients with RA. The statistical analysis by Wilcoxon rank-sum test revealed a statistical difference between eight patients with AA and 65 patients with RA ($p = 0.01$). Sixty-nine patients with RA were further categorized into three groups by bone marrow findings: hypoplastic RA ($n = 20$), hyperplastic RA ($n = 15$) and normoplastic RA ($n = 30$), excluding the non-categorized RA ($n = 4$). Significant differences were observed between AA and each of hypoplastic ($p = 0.04$) or normoplastic RA ($p = 0.02$), whereas no difference was shown between the AA and hyperplastic RA group ($p = 0.10$) by Steel test (Figure 4). From these findings, a differential diagnostic cut-off value between RA and AA of 50 copies/μg RNA for WT1 mRNA expression in PB is considered appropriate, for which the sensitivity was 50.7% (35/69) and the specificity was 100% (8/8).

Differential diagnosis based on WT1 mRNA expression in BM samples

The WT1 mRNA expression level in BM was 251–2600 copies/μg RNA in eight patients with AA, whereas it was less than 50 copies/μg RNA in one of 69 patients with RA and 69–196 000 copies/μg RNA in the others. The statistical analysis by Wilcoxon rank-sum test revealed no statistical difference between eight patients with AA and 65 patients with RA. Sixty-nine patients with RA were similarly categorized into three groups: hypoplastic, hyperplastic and normoplastic RA, excluding the non-categorized RA. Statistical analysis by Steel test revealed a significant difference between AA and normoplastic RA groups ($p = 0.04$), whereas there were no significant differences between the AA and each of hypoplastic RA and hyperplastic RA groups (Figure 4).

When receiver operating characteristic (ROC) analysis was performed to evaluate the performance of BM WT1 mRNA expression as an indicator to differentiate between RA and AA, the area under the curve was 0.713, and the Youden index [18] showed 432 copies/μg RNA. Moreover, the sensitivity was 69.6% (48/69), and the specificity was 75.0% (6/8) (Supplementary Figure to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.745074).

When the PB cut-off value of 50 copies/μg RNA was inserted into the regression line formula obtained

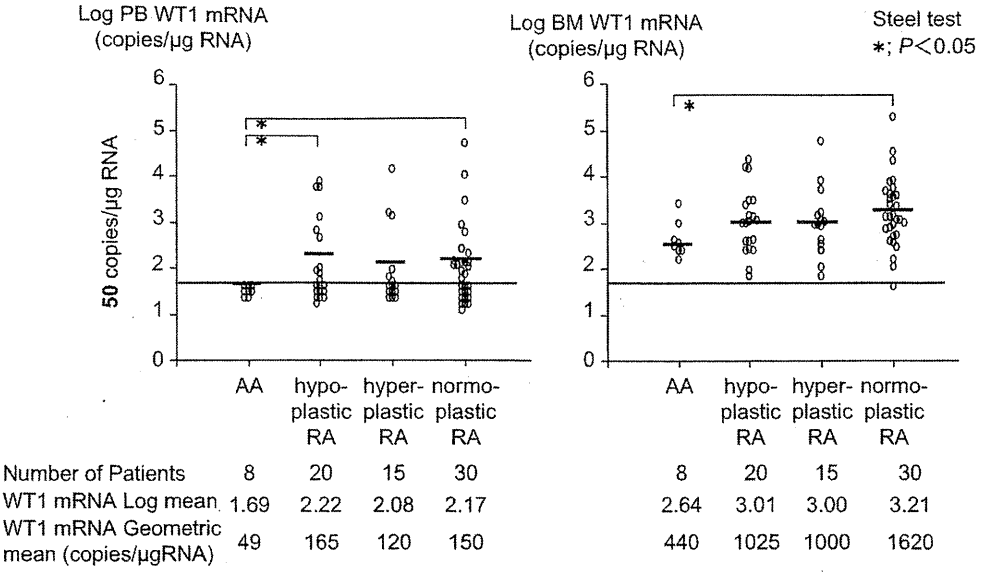

Figure 4. Comparison of WT1 mRNA expression between AA and RA groups (hypoplastic, hyperplastic and normoplastic RA). In intergroup comparison of WT1 mRNA expression, Steel test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

from the correlation between WT1 mRNA expression in PB and BM (Figure 2), BM WT1 mRNA expression became 480 copies/μg RNA. When 500 copies/μg was evaluated as the cut-off value for BM WT1 mRNA expression, the sensitivity was 68.1% (47/69) and the specificity was 75.0% (6/8). Based on these results, 500 copies/μg RNA was considered to be an appropriate cut-off value for the differential diagnosis between RA and AA using WT1 mRNA expression in BM.

Comprehensive analysis using cut-off values

The PB and BM samples in each disease and MDS subtype were further evaluated for their WT1-positive rates, using the WT1 mRNA expression cut-off values determined above (PB: 50 copies/μg RNA; BM: 500 copies/μg RNA) (Table II). For AML-MDS (11 patients), the WT1 mRNA-positive rates were a high 100% (11/11) for PB and 90.9% (10/11) for BM, and in MDS (115 patients), the WT1 mRNA-positive rates were 61.7% (71/115) for PB and 73.0% (84/115) for BM, which were the second highest after AML-MDS. In contrast, all patients with AA, ICUS, ITP, PNH, PRCA and erythroid hypoplasia had low positive rates of 0% for PB and 18.8% (3/16) for BM. The WT1 mRNA-positive rates for PB and BM increased with MDS disease stage progression (Table II).

Table II. WT1 mRNA-positive rate in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

Subtype	No. of patients	WT1 mRNA-positive rate (%)	
		Peripheral blood	Bone marrow
RA	69	50.7 (35/69)	68.1 (47/69)
RARS	9	44.4 (4/9)	44.4 (4/9)
RAEB	24	83.3 (20/24)	87.5 (21/24)
RAEB-t	13	92.3 (12/13)	92.3 (12/13)
AML-MDS	11	100.0 (11/11)	90.9 (10/11)
Total	126	65.1 (82/126)	74.6 (94/126)

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RAEB-t, refractory anemia with excess of blasts in transformation.

Discussion

In this study, the clinical usefulness of the measurement of WT1 mRNA expression in risk assessment of MDS was evaluated using a WT1 assay kit. Recently, a steady stream of reports has indicated the usefulness of WT1 mRNA measurement. The group of Cilloni [6] confirmed that WT1 mRNA expression potentially fulfills all the requirements for an additional marker for risk assessment in MDS, compared with the conventional methods. The measurement of WT1 can be effective, particularly in cases in which BM aspiration and/or cytogenetic analysis fail or are not informative [6].

Furthermore, in their findings in a long-term prospective study, Tamura *et al.* [19] reported that a significant correlation ($p = 0.0186$) was seen between WT1 mRNA expression and survival time when WT1 mRNA expression in PB was categorized into three groups of less than 10^2, 10^2–10^4, and greater than 10^4 copies/μg RNA, that the median survival time for each group was 62.7 months, 29.9 months and 11.6 months, respectively; and that the time until transformation to leukemia was the shortest in the group with the highest WT1 mRNA expression. In addition, they reported that in univariate analysis, WT1 mRNA expression was a predictive parameter for transformation to leukemia, and in multivariate analysis, it was a significant predictive parameter along with the IPSS score [19]. As described above, Tamaki *et al.* reported similar findings [4].

This study was conducted using not only the FAB classification system but also the 2001 and 2008 WHO classification systems. It was confirmed that in all three classification systems, WT1 mRNA expression in both PB and BM increases significantly in MDS subtypes with disease stage

progression. In addition, both PB and BM WT1 mRNA expression increased significantly as the risk of transformation to AML rose in the IPSS and WPSS risk groups. Furthermore, a correlation of $r = 0.57$ between the IPSS score and WT1 mRNA expression was seen in both PB and BM. The correlations between the WPSS score and WT1 mRNA expression were $r = 0.61$ in PB and $r = 0.55$ in BM. In comparison with the IPSS, the WPSS allows the assessment of survival time and progression of leukemic transformation at all time periods during the clinical course, leading to continued prognostic evaluation while reviewing the risk. WT1 mRNA expression correlates with the WPSS prognosis, and despite the single-point quantitation, the results in this study indicate that WT1 mRNA is useful as a time-course prognostic marker in the same manner as the WPSS.

At present, allogeneic hematopoietic stem cell transplant is the only curative treatment for MDS. However, determination of the timing of allogeneic transplant is very difficult because many patients are older, treatment-related deaths frequently occur, and there are large individual differences in the rate of disease progression. Allogeneic transplant is selected as the therapeutic regimen for MDS when no increase in blast cells is confirmed, taking into consideration the development of transfusion dependency and frequency of infections [20]. In addition, allogeneic transplant is selected when a future increase in blast cells is predicted by karyotypic analysis even though no increase is currently observed. It is recommended that transplant be performed before the progression to cytopenia caused by an increase in blast cell clones and before the progression to acute leukemia, although induction chemotherapy may be required when an increase in blast cells is observed [21]. On the other hand, another study suggested that delaying transplant until the advanced stage of disease results in a longer survival time for low and intermediate-1 IPSS risk groups, while early transplant was recommended for the intermediate-2 and high groups [22]. The period after CR is achieved is considered to be the standard timing to perform transplant for acute leukemia, but determining CR is extremely challenging. Our results revealed that periodic monitoring of WT1 mRNA expression in patients with MDS provided useful information for predicting the timing of transplant.

RA, a subtype in the early MDS disease stage, is often difficult to differentiate from AA [23]. In a previous study by Iwasaki *et al.*, no difference in WT1 mRNA expression was observed between RA and AA [9]. However, our data revealed the possibility of WT1 expression level to differentiate AA and RA groups using both peripheral blood and bone marrow samples (Figure 4). In the present statistical analysis, significant differences were observed between AA and hypoplastic RA ($p = 0.04$) in PB. The number of subjects was limited, and further trial is required for more detailed analysis. Moreover, tentative cut-off values for WT1 mRNA expression were set at 50 copies/μg RNA in PB and 500 copies/μg RNA in BM. Although the number of patients was small, the results showed that the level of WT1 mRNA expression could differentiate between RA and AA, with specificity in PB and BM of 100% (8/8) and 75.0% (6/8), respectively. This provides evidence that the measurement of WT1 mRNA expression can play a role in the differential diagnosis of RA and AA.

The WT1 assay kit is used clinically in Japan as a marker to monitor MRD in patients wth AML. In MDS, a clonal disorder of pluripotent hematopoietic stem cells, WT1 mRNA expression increases depending on the MDS subtype and disease stage. In contrast, the mechanism by which WT1 mRNA expression increases in MDS is not considered to correlate simply with the fluctuation in leukemic clones, as seen in AML. In normal hematopoiesis, WT1 mRNA is expressed mainly in CD34-positive cells. In contrast, in patients with MDS, WT1 mRNA is also expressed in CD34-negative cells, particularly in lineages exhibiting abnormalities [24]. In our study, the level of WT1 mRNA expression within the RA group was shown to increase with the increase in IPSS risk [Figure 3(c)]. Moreover, a similar trend of increasing WT1 expression was found in the RCUD and RCMD groups according to the 2008 WHO classification, although no significant increase in blast cells in BM was observed in these groups. Taken together, these findings indicate that the increase in WT1 mRNA expression in patients with MDS may reflect the divergence of MDS clones from normal clones and preleukemic changes.

In patients with MDS, evaluating the changes in WT1 mRNA levels simultaneously in PB and BM samples provides useful information on disease stage progression or risk assessment in individual patients. In addition, the WT1 mRNA-positive rate in each subtype of MDS was high (50–90%) in both PB and BM in this study, suggesting that a single measurement of WT1 mRNA is sufficient for MDS diagnosis, particularly for differentiating RA from AA.

Overall, this study provides evidence that the measurement of the level of WT1 mRNA expression in PB and BM serves as a supplemental marker for MDS diagnosis and prognostic assessment. This assay has great potential to contribute to more appropriate diagnoses and therapeutic decisions in patients with MDS and to evaluate the timing of allogeneic transplant.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References

[1] Locatelli F, Zecca M, Pession A, et al. Myelodysplastic syndromes: the pediatric point of view. Haematologica 1995;80:268-279.
[2] Inoue K, Sugiyama H, Ogawa H, et al. WT1 as a new prognostic factor and new marker for the detection of minimal residual disease in acute leukemia. Blood 1994;84:3071-3079.
[3] Miyawaki S, Hatsumi N, Tamaki T, et al. Prognostic potential of detection of WT1 mRNA level in peripheral blood in adult acute myeloid leukemia. Leuk Lymphoma 2010;51:1855-1861.
[4] Tamaki H, Ogawa H, Ohyashiki K, et al. The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. Leukemia 1999;13:393-399.
[5] Patmasiriwat P, Fraizer G, Kantarjian H, et al. WT1 and GATA1 expression in myelodysplastic syndrome and acute leukemia. Leukemia 1999;13:891-900.
[6] Cilloni D, Gottardi E, Messa F, et al. Significant correlation between the degree of WT1 expression and the International Prognostic

Scoring System score in patients with myelodysplastic syndromes. J Clin Oncol 2003;21:1988-1995.
[7] Bader P, Niemeyer C, Weber G, et al. WT1 gene expression: useful marker for minimal residual diseases in childhood myelodysplastic syndromes and juvenile myelo-monocytic leukemia. Eur J Haematol 2004;73:25-28.
[8] Tamura K, Kanazawa T, Suzuki M, et al. Successful rapid discontinuation of immunosuppressive therapy at molecular relapse after allogeneic bone marrow transplantation in a pediatric patient with myelodysplastic syndrome. Am J Hematol 2006;81:139-141.
[9] Iwasaki T, Sugisaki C, Nagata K, et al. Wilms' tumor 1 message and protein expression in bone marrow failure syndrome and acute leukemia. Pathol Int 2007;57:645-651.
[10] Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079-2088.
[11] Malcovati L, Germing U, Kuendgen A, et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. J Clin Oncol 2007;25:3503-3510.
[12] Heim S, Mitelman F. Chromosome abnormalities in the myelodysplastic syndromes. Clin Haematol 1986;15:1003-1021.
[13] Yunis JJ, Lobell M, Arnesen MA, et al. Refined chromosome study helps define prognostic subtypes in most patients with primary myelodysplastic syndromes and acute myelogenous leukemia. Br J Haematol 1988;68:189-194.
[14] Nowell PC. Chromosome abnormalities in myelodysplastic syndromes. Semin Oncol 1992;19:25-33.
[15] Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 1982;51:189-199.
[16] Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292-2302.
[17] Brunning RD, Porwit A, Orazi A, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2008. pp 88-93.
[18] Perkins NJ, Schisterman EF. The inconsistency of "optimal" cutpoints obtained using two criteria based on the receiver operating characteristic curve. Am J Epidemiol 2006;163:670-675.
[19] Tamura H, Dan K, Yokose N, et al. Prognostic significance of WT1 mRNA and anti-WT1 antibody levels in peripheral blood in patients with myelodysplastic syndromes. Leuk Res 2010;34: 986-990.
[20] Appelbaum FR, Barrall J, Storb R, et al. Bone marrow transplantation for patients with myelodysplasia. Pretreatment variables and outcome. Ann Intern Med 1990;112:590-597.
[21] Nakai K, Kanda Y, Fukuhara S, et al. Value of chemotherapy before allogeneic hematopoietic stem cell transplantation from an HLA-identical sibling donor for myelodysplastic syndrome. Leukemia 2005;19:396-401.
[22] Cutler CS, Lee SJ, Greenberg P, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. Blood 2004;104:579-585.
[23] Nakao S, Deeg HJ, Ishikawa T, et al. Myelodysplastic syndrome. Int J Hematol 2005;82:412-416.
[24] Van Dijk JP, Knops GH, Van De Locht LT, et al. Abnormal WT1 expression in the CD34-negative compartment in myelodysplastic bone marrow. Br J Haematol 2002;118:1027-1033.

Supplementary material available online

Supplementary figure showing ROC analysis of WT1 mRNA expression in BM in RA and AA groups

The price of drugs for chronic myeloid leukemia (CML) is a reflection of the unsustainable prices of cancer drugs: from the perspective of a large group of CML experts

Experts in Chronic Myeloid Leukemia

As a group of more than 100 experts in chronic myeloid leukemia (CML), we draw attention to the high prices of cancer drugs, with the particular focus on the prices of approved tyrosine kinase inhibitors for the treatment of CML. This editorial addresses the multiple factors involved in cancer drug pricing and their impact on individual patients and health care policies, and argues for the need to (1) lower the prices of cancer drugs to allow more patients to afford them and (2) maintain sound long-term health care policies. (*Blood.* 2013;121(22):4439-4442)

The doctrine of *justum pretium*, or just price, refers to the "fair value" of commodities. In deciding the relationship between price and worth (or value), it advocates that, by moral necessity, price must reflect worth. This doctrine may be different from the doctrine of free market economies where prices reflect "what the market bears," or what one is willing to pay for a product. Which doctrine is better? One could argue that when a commodity affects the lives or health of individuals, just price should prevail because of the moral implications. Examples include the price of bread during famines, polio vaccine, ivermectin for river blindness (provided for free by Merck and estimated to have saved the vision of 30 million individuals), and treatments of chronic medical conditions (cardiovascular, hypertension, diabetes, tuberculosis, multiple sclerosis, etc). When commodities are not essential to life or suffering, what the market will bear is appropriate (competition will take care of price) because it is not restrained by ethical considerations. Examples include the price of a Picasso painting, a luxury cruise, a 2-week vacation in New York (or 4 weeks in Houston), a Bentley car, a Brioni suit, etc.

Through positive collaborations with Pharma, experts in chronic myelogenous leukemia (CML) have been fortunate to have 3 drugs approved by the US Food and Drug Administration (FDA) in 2012 for the treatment of CML: bosutinib, ponatinib, and omacetaxine. This is in addition to 3 others approved in the last decade: imatinib, dasatinib, and nilotinib. The 3 new drugs, however, have been priced at astronomical levels: ponatinib at $138 000 per year, omacetaxine at $28 000 for induction and $14 000 per maintenance course, and bosutinib at ~$118 000 per year.[1]

Cancer drug prices have been discussed recently by some financial analysts and tend to be discussed whenever new cancer drugs are approved. This Forum reflects the views of a large group of CML experts who believe that the current prices of CML drugs (1) are too high, (2) are unsustainable, (3) may compromise access of needy patients to highly effective therapy, and (4) are harmful to the sustainability of our national health care systems. These concerns reflect the spiraling prices of cancer drugs in general. Of the 12 drugs approved by the FDA for various cancer indications in 2012, 11 were priced above $100 000 per year. Cancer drug prices have almost doubled from a decade ago, from an average of $5000 per month to >$10 000 per month.[2]

Innovation and discoveries must be rewarded. Pharmaceutical companies that invest in research and development and discover new lifesaving drugs should benefit from healthy revenues. The cost for bringing a new cancer drug to market is reported to be ~$1 billion.[3] This much-argued-about figure, which some independent experts put as low as $60 to 90 million,[4] includes the cost of development of the new (successful) drug and all other drugs that failed during development, and ancillary expenses including the cost of conducting the clinical trials required for approval, bonuses, salaries, infrastructures, and advertising among others. In other words, once a company sells about a billion dollars of a drug, most of the rest is profit.

How are the prices of cancer drugs decided? Of the many complex factors involved, price often seems to follow a simple formula: start with the price for the most recent similar drug on the market and price the new one within 10% to 20% of that price (usually higher). This is what happened with imatinib, priced in 2001 at $2200 per month, based on the price of interferon, which was then the standard treatment.[5]

If drug price reflects value, then it should be proportional to the benefit to patients in objective measures, such as survival prolongation, degree of tumor shrinkage, or improved quality of life. For many tumors, drug prices do not reflect these end points because most anticancer drugs provide minor survival benefits, if at all. For example, in pancreatic cancer, where the median survival is 6 months, a new drug that may prolong survival by 2 months and is priced at $100 000 per year will cost $67 000 over 8 months survived, or $33 500 per additional month lived, equivalent to $400 000 per additional year lived. Similar calculations can be made for other cancers depending on the expected median survival, additional time lived, and therefore the price of an additional year lived. By these measures, the price of cetuximab was valued at ~$800 000 per

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Experts in Chronic Myeloid Leukemia contributed equally to this study and are cited in "Appendix."

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year of increased survival.[2] In many countries, an additional year lived is judged to be "worth" ~$50 000 to $100 000.[6,7] In England, the National Institute for Health and Clinical Excellence values a year lived at about 30 000 British pounds, or ~$50 000.

The situation in CML is different. When imatinib was approved in 2001, its potential benefit in prolonging life was unknown. Considering a median survival of ~5 to 6 years in the pre-imatinib era, a 50% improvement in survival would have extended life by 3 years, which was then a very optimistic outlook. Therefore, the original imatinib price of $30 000 in 2001 may have reflected the cost of development and a projection of anticipated survival, using the price of interferon, the approved commercial drug for CML, as a starting point. In his book, Daniel Vasella, then Chairman and Chief Executive Officer of Novartis, discussed the development of imatinib, the moral imperatives and pressures exerted by oncologists and patients, the need for healthy profit margins, and the decision to price imatinib at a world average of $2200 per month, or $26 000 per year ($30 000 per year in the United States).[5] This, he explained, was considered at the time a high but worthwhile and profitable price. With a prevalence of 30 000 patients in the United States (the effect of imatinib on the prevalence of CML was then difficult to estimate) and full market penetration (ie, most patients with CML receiving imatinib), the annual revenue from imatinib sales in the United States would be ~$900 million, which would have more than recouped the cost of development within 2 years. The revenues over the subsequent years of the patent would represent generous profits to the company.

Imatinib and the new Bcr-Abl tyrosine kinase inhibitors (TKIs) became the most successful class of targeted therapies ever developed in cancer, exceeding all projected survival expectations. With TKI therapy, the annual all-cause mortality in CML declined to 2%, vs a historical rate of 10% to 20%, and the estimated 10-year survival increased from <20% to >80%.[8] Patients with CML now live close to normal lifespans,[9] as long as they receive the appropriate TKIs and adhere to treatment. Their CML condition has become very different from solid cancers, and more similar to indolent disorders like diabetes, hypertension, and cardiovascular disorders, where daily therapy is required indefinitely to produce the anticipated benefit of long-term survival. Grateful patients may have become the "financial victims" of the treatment success, having to pay the high price annually to stay alive.

In Europe and many developed countries, universal health coverage shields patients from the direct economic anxieties of illness. Not so in the United States where patients may pay an average of 20% of drug prices out of pocket (~$20 000-$30 000 per year, a quarter to a third of an average household budget), and where medical illnesses and drug prices are the single most frequent cause of personal bankruptcies.[10] High drug prices may be the single most common reason for poor compliance and drug discontinuation, and the reason behind different treatment recommendations in different countries.

Cancer drug prices vary widely in different geographic regions (Table 1[11,12]). This supports the notion that drug prices reflect geopolitical and socioeconomics dynamics unrelated to the cost of drug development. In the United States, prices represent the extreme end of high prices, a reflection of a "free market economy" and the notion that "one cannot put a price on a human life," as well as a failure of government and insurers to more actively negotiate pricing for anticancer and other pharmaceuticals, in contrast to practices in other parts of the world. This contributes to the very high cost of health care in the United States, estimated at $2.7 trillion in 2011, or 18% of the US gross domestic product, compared with 6% to 9% in Europe.[13] This increased expenditure does not add demonstrable benefit to US patients.[14] At the other extreme are more modest prices in the Middle East, Africa, Latin America, and other emerging nations, where only a minority of patients can afford, as individuals or through government subsidies, to access the CML drugs. In many emerging nations where governments cannot afford to budget for such drugs, CML experts are advocating frontline allogeneic stem cell transplantation because it costs an average of $30 000 to $80 000 as a one-time procedure.[15] This may harm patients because only a fraction may be eligible for transplantation (and may suffer from early mortality and lifelong complications); a smaller fraction are rich enough to pay individually for the price of the drugs, and most are treated intermittently or not at all. The effects of these financial pressures on the long-term survival of patients with CML in national follow-up studies are as yet unknown.

Table 1. Annual price estimates, by region, of drugs approved for the treatment of CML

Country	Price in thousands of US dollars (rounded to nearest $0.5 thousand)		
	Imatinib	Nilotinib	Dasatinib
United States	92	115.5	123.5
Germany*	54	60	90
United Kingdom	33.5	33.5	48.5
Canada	46.5	48	62.5
Norway	50.5	61	82.5
France	40	51.5	71
Italy	31	43	54
South Korea	28.5	26	22
Mexico	29	39	49.5
Argentina	52	73.5	80
Australia	46.5	53.5	60
Japan	43	55	72
China	46.5	75	61.5
Russia	24	48.5	56.5
South Africa	43	28	54.5

Prices in the United States from the Red Book online.[1] Other prices provided by CML experts from their countries.

*In Germany, a new rule, the "Pharmaceutical Market Restructuring Act" or AMNOG (arzneimittelneuordnungsgesetz), took effect in January 2011, by which the prices of new drugs are negotiated according to their benefit in comparison with other drugs on the market for the same indication. Similar rules or laws are also in effect in other European countries.[11] Prices of drugs in Germany may directly or indirectly influence drug prices in 31 countries.[12]

Imatinib was developed as a "goodwill gesture" by Novartis and became a blockbuster, with annual revenues of ~$4.7 billion in 2012. Being one of the most successful cancer targeted therapies, imatinib may have set the pace for the rising cost of cancer drugs. Initially priced at nearly $30 000 per year when it was released in 2001, its price has now increased to $92 000 in 2012,[1] despite the fact that (1) all research costs were accounted for in the original proposed price,[5] (2) new indications were developed and FDA approved, and (3) the prevalence of the CML population continuing to take imatinib was dramatically increasing.[16] This resulted in numerous appeals by patients and advocates to lower the price of imatinib, to no avail so far.[17,18]

What determines a morally justifiable "just price" for a cancer drug? A reasonable drug price should maintain healthy pharmaceutical company profits without being viewed as "profiteering" (making profit by unethical methods, like raising commodity prices after natural disasters). Hillner and Smith suggested this term may apply to the trend of high drug prices, where a life-threatening medical condition is the disaster.[19] Hopes that the fundamentals of a free market economy and market competition will settle cancer

drug prices at lower levels have not been fulfilled. All 5 TKIs approved for CML have annual price ranges of $92 000 to $138 000 in the United States, twice the prices in Europe where governments bargain for bulk prices (Table 1). A new branch of economics, called game theory, details how collusive behavior can tacitly maintain high prices over extended periods of time, despite competitive markets, thus representing a form of "collective monopoly."[20] Interestingly, in South Korea, where annual prices for TKIs range from $21 000 to $28 000, market competition may have worked well, perhaps because of the approval by the Korean health authorities of radotinib (annual prices $21 500), a locally discovered and developed TKI.

The patent expiration date of imatinib, originally set in the United States for May 28, 2013, was later extended by the US Patent Office to January 2015. Patent expiration dates may be different in different countries/regions. Two years is still a long time for patients with CML, the prevalence of which is estimated today worldwide at ~1.2 million to 1.5 million patients. Based on sales, it is estimated that about 235 000 to 250 000 patients (<20%-25%) are receiving imatinib. Support programs like the Glivec International Patient Assistance Program, a joint effort of Novartis and The Max Foundation, provide access to about 60 000 patients, perhaps ~30 000 to 40 000 of whom have CML (Glivec International Patient Assistance Program providing TKIs to 1%-3% of the world's CML population).[21] Thus, treatment penetration of TKIs in CML may be ~25% to 30% globally. When treatment penetration and compliance rates are high (such as in single institutional studies, in cooperative group trials, and in Sweden), the estimated 10-year survival rates are >80%.[8,9,22] When treatment penetration may be lower, outcome may be worse. In the United States, ~10% of patients fail to take prescribed drugs, largely because of cost.[23] Trends of CML survival in the United States show an improvement since 2001, but the estimated 5-year survival rate is still ~60%, suggesting lower treatment penetration rates in the United States compared with Sweden.[22,24] Unaffordable CML drug prices may be preventing many patients from accessing these lifesaving drugs. Lowering the prices of TKIs will improve treatment penetration, increase compliance and adherence to treatment, expand the population of patients with CML who live longer and continue on TKI therapy, and (paradoxically) increase revenues to pharmaceutical companies from sales of TKIs.

Early introduction of generics has been estimated to have saved the US health care budget about $1.1 trillion over 10 years.[25] In leveraging drug prices, companies may engage in "pay-for-delay" strategies that delay generic drugs from being available. Arrangements by pharmaceutical companies that pay generic companies to delay entering the market with a generic version profit both companies, but financially hurt the national health care system and patients. The Hatch-Waxman Act provides a 6-month market exclusivity for the first FDA-approved generic version of a branded drug. The intent of the act is to encourage the rapid launch of low-cost generics and reduce health care costs. Other generics can be marketed afterward. By launching their own generics (called "authorized generics") at low prices, branded drug companies have diminished generic company profits, resulting in delays of access of generics and reduced competition.[26] Delays of generic TKIs through "pay-for-delay" or "authorized generic" approaches may harm patients with CML and should be avoided at all cost.

As physicians, we follow the Hippocratic Oath of "*Primum non nocere*," first (or above all) do no harm. We believe the unsustainable drug prices in CML and cancer may be causing harm to patients. Advocating for lower drug prices is a necessity to save the lives of patients who cannot afford them. Pricing of cancer and other drugs involves complex societal and political issues which (1) demand immediate attention and (2) will need to consider many factors and involve many constituencies including FDA and governmental regulators; legislation changes; patent laws; multitudes of US and international regulatory agencies; offices of human research protection; impediments by lawyers and contract research organizations, which increase the cost of clinical research; patient advocacy groups; excessive regulation and bureaucracy; profits of physicians and hospitals/pharmacies; insurance companies; pharmaceutical companies; etc.

We propose to begin the dialogue by organizing regular meetings, involving all parties concerned, to address the reasons behind high cancer drug prices and offer solutions to reduce them. For CML, and for other cancers, we believe drug prices should reflect objective measures of benefit, but also should not exceed values that harm our patients and societies.

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Authorship

Contribution: All authors contributed equally to the creation of this manuscript.

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A complete list of the Experts in Chronic Myeloid Leukemia appears in "Appendix."

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Appendix

The Experts in Chronic Myeloid Leukemia are listed by region below.

North America

Camille Abboud; Ellin Berman; Adam Cohen; Jorge Cortes; Daniel DeAngelo; Michael Deininger; Steven Devine; Brian Druker; Amir Fathi; Elias Jabbour; Madan Jagasia; Hagop Kantarjian; Jean Khoury; Pierre Laneuville; Richard Larson; Jeffrey Lipton; Joseph O. Moore; Tariq Mughal; Susan O'Brien; Javier Pinilla-Ibarz; Alfonso Quintas-Cardama; Jerald Radich; Vishnu Reddy; Charles Schiffer; Neil Shah; Paul Shami; Richard T. Silver; David Snyder; Richard Stone; Moshe Talpaz; Ayalew Tefferi; Richard A. Van Etten; Meir Wetzler.

Europe and Russia

Elisabetta Abruzzese; Jane Apperley; Massimo Breccia; Jenny Byrne; Francisco Cervantes; Ekaterina Chelysheva; R. E. Clark; Hugues de Lavallade; Iryna Dyagil; Carlo Gambacorti-Passerini;

John Goldman; Ibrahim Haznedaroglu; Henrik Hjorth-Hansen; Tessa Holyoake; Brian Huntly; Philipp le Coutre; Elza Lomaia; Francois-Xavier Mahon; David Marin-Costa; Giovanni Martinelli; Jiri Mayer; Dragana Milojkovic; Eduardo Olavarria; Kimmo Porkka; Johan Richter; Philippe Rousselot; Giuseppe Saglio; Guray Saydam; Jesper Stentoft; Anna Turkina; Paolo Vigneri; Andrey Zaritskey.

Latin America

Alvaro Aguayo; Manuel Ayala; Israel Bendit; Raquel Maria Bengio; Carlos Best; Eduardo Bullorsky; Eduardo Cervera; Carmino DeSouza; Ernesto Fanilla; David Gomez-Almaguer; Nelson Hamerschlak; Jose Lopez; Alicia Magarinos; Luis Meillon; Jorge Milone; Beatriz Moiraghi; Ricardo Pasquini; Carolina Pavlovsky; Guillermo J. Ruiz-Arguelles; Nelson Spector.

Australia and Asia

Christopher Arthur; Peter Browett; Andrew Grigg; Jianda Hu; Xiao-jun Huang; Tim Hughes; Qian Jiang; Saengsuree Jootar; Dong-Wook Kim; Hemant Malhotra; Pankaj Malhotra; Itaru Matsumura; Junia Melo; Kazunori Ohnishi; Ryuzo Ohno; Tapan Saikia; Anthony P. Schwarer; Naoto Takahashi; Constantine Tam; Tetsuzo Tauchi; Kensuke Usuki; Jianxiang Wang.

Middle East and Africa

Fawzi Abdel-Rahman; Mahmoud Deeb Saeed Aljurf; Ali Bazarbachi; Dina Ben Yehuda; Naeem Chaudhri; Muheez Durosinmi; Hossam Kamel; Vernon Louw; Bassam Francis Matti; Arnon Nagler; Pia Raanani; Ziad Salem.

References

1. Red Book online database. Accessed February 20, 2013.
2. Fojo T, Grady C. How much is life worth: cetuximab, non-small cell lung cancer, and the $440 billion question. *J Natl Cancer Inst.* 2009; 101(15):1044-1048.
3. Goozer M. The $800 Million Pill: The Truth Behind the Cost of New Drugs. Berkley, CA: University of California Press; 2004.
4. Light DW, Lexchin JR. Pharmaceutical research and development: what do we get for all that money? BMJ. 2012;345:e4348.
5. Vasella D. Magic Cancer Bullet: How a Tiny Orange Pill Is Rewriting Medical History. New York, NY: Harper Collins Publishers; 2003: 15-18,126,160-163,171-181.
6. Lee CP, Chertow GM, Zenios SA. An empiric estimate of the value of life: updating the renal dialysis cost-effectiveness standard. *Value Health.* 2009;12(1):80-87.
7. Rascati KL. The $64,000 question—what is a quality-adjusted life-year worth? *Clin Ther.* 2006;28(7):1042-1043.
8. Kantarjian H, O'Brien S. The chronic leukemias. In: Goldman L, Schafer A, Arend W, et al, eds. Cecil Medicine. 24 ed. Philadelphia, PA: Elsevier Saunders; 2012:1209-1218.
9. Gambacorti-Passerini C, Antolini L, Mahon FX, et al. Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. *J Natl Cancer Inst.* 2011; 103(7):553-561.
10. Himmelstein DU, Thorne D, Warren E, Woolhandler S. Medical bankruptcy in the United States, 2007: results of a national study. *Am J Med.* 2009;122(8):741-746.
11. Bundesministerium für Gesundheit. The act on the reform on the market for medicinal products (Gesetz zur Neuordnung des Arzneimittelmarktes - AMNOG). http://www.bmg.bund.de/ministerium/english-version/amnog.html. Accessed February 20, 2013.
12. The Pharma Letter. German pharma criticizes new AMNOG vetting procedure. March 2012. http://www.thepharmaletter.com/file/111771/german-pharma-criticizes-new-amnog-vetting-procedure.html. Accessed February 20, 2013.
13. Centers for Medicare & Medicaid Services. National Health Expenditure Projections 2011-2021. http://www.cms.gov/Research-Statistics-Data-and-Systems/Statistics-Trends-and-Reports/NationalHealthExpendData/Downloads/Proj2011PDF.pdf. Accessed October 2012.
14. Himmelstein DU, Woolhandler S. Cost control in a parallel universe: Medicare spending in the United States and Canada. *Arch Intern Med.* 2012;172(22):1764-1766.
15. Ruiz-Argüelles GJ, Tarin-Arzaga LC, Gonzalez-Carrillo ML, et al. Therapeutic choices in patients with Ph-positive CML living in Mexico in the tyrosine kinase inhibitor era: SCT or TKIs? *Bone Marrow Transplant.* 2008;42(1):23-28.
16. Huang X, Cortes J, Kantarjian H. Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. *Cancer.* 2012;118(12): 3123-3127.
17. Silverman E. Petition demands Novartis lower US Gleevec price. http://www.pharmalot.com/2012/05/petition-demands-novartis-lower-us-gleevec-price/. Accessed February 20, 2013.
18. Health Watched. US patient petition calling for reduction of Gleevec price. http://healthwatched.org/2012/05/09/us-patient-petition-calling-for-reduction-of-gleevec-price/. Accessed February 20, 2013.
19. Hillner BE, Smith TJ. Efficacy does not necessarily translate to cost effectiveness: a case study in the challenges associated with 21st-century cancer drug pricing. *J Clin Oncol.* 2009; 27(13):2111-2113.
20. Stiglitz J. The Price of Inequality. New York, NY: W.W. Norton and Company, Inc.; 2012:45.
21. The Max Foundation. Glivec International Patient Assistance Program (GIPAP). http://www.themaxfoundation.org/gipap/Default.aspx. Accessed February 20, 2013.
22. Björkholm M, Ohm L, Eloranta S, et al. Success story of targeted therapy in chronic myeloid leukemia: a population-based study of patients diagnosed in Sweden from 1973 to 2008. *J Clin Oncol.* 2011;29(18): 2514-2520.
23. The Economist. The costly war on cancer. New cancer drugs are technically impressive. But must they cost so much? May 26, 2011. http://www.economist.com/node/18743951. Accessed February 20, 2013.
24. Chen Y, Wang H, Kantarjian H, Cortes J. Trends in chronic myeloid leukemia incidence and survival in the United States from 1975 to 2009 [published online ahead of print December 5, 2012]. Leuk Lymphoma. doi: 10.3109/10428194.2012.745525.
25. Wyatt E. Justices to take up generic drug case. The New York Times. December 8, 2012;B1.
26. Federal Trade Commission. Authorized generic drugs: short-term effects and long-term impact. http://www.ftc.gov/os/2011/08/2011genericdrugreport.pdf. Accessed February 20, 2013.

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ORIGINAL ARTICLE

Normal karyotype acute myeloid leukemia with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically distinct entity with a favorable outcome

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Abstract Recently, the presence of *CEBPA* mutation was identified as an important prognostic factor for normal karyotype (NK) acute myeloid leukemia (AML). Because AML with *CEBPA* mutation is closely associated with CD7, CD15, CD34, and HLA-DR expression, we investigated the prognostic implications of CD7+ CD15+ CD34+ HLA-DR + immunophenotype in NK-AML. We analyzed the immunophenotype of 329 patients with NK-AML from the Japan Adult Leukemia Study Group (JALSG) AML97 population. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype was classified as the CEBPA type, and NK-AML that did not meet this criterion was considered as

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the non-CEBPA type. The influence of the CEBPA status on event-free survival (EFS) and overall survival (OS) was assessed using log-rank test and a multivariate Cox proportional hazard regression model. Furthermore, the surface antigen expression profile in AML according to the *CEBPA* mutation status (monoallelic or biallelic) was also investigated. Of the 329 NK-AML patients that were studied, 39 and 243 were classified as having CEBPA and non-CEBPA type NK-AML, respectively. Patients with CEBPA type NK-AML had significantly better EFS and OS than those with non-CEBPA type NK-AML. Multivariate analysis showed that the CEBPA type and white blood cell (WBC) counts of $>20 \times 10^9$/L were independent prognostic factors for EFS and OS. Moreover, NK-AML with the biallelic *CEBPA* mutation was more closely associated with CD34 positivity than that with the monoallelic *CEBPA* mutation. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically discrete entity, and this may have a possible role in risk stratification.

Keywords Normal karyotype acute myeloid leukemia · CD7 · CD15 · CD34 · HLA-DR · CEBPA · Prognostic factor

Introduction

In recent years, immunophenotyping of hematologic neoplasms has become standard practice to establish a diagnosis and define the origin of the malignant cell lineage. Patients with acute myeloid leukemia (AML) often show aberrant cellular antigen expression as well as chromosomal abnormalities. The clinical significance of surface antigen expression has been studied for more than 20 years, but thus far, it has yielded inconsistent results [1]. Nevertheless, if the evaluation of antigen expression is limited to a subtype of AML, we would be more likely to find a significant relationship between surface antigen expression and prognosis. For example, the significance of CD56 expression as an adverse prognostic factor in both acute promyelocytic leukemia (APL) and AML with t(8;21) is widely accepted [2, 3].

Normal karyotype (NK)-AML is the most common subtype of AML, accounting for 40–50 % of cases [4-6]. Patients with this subtype are considered to have an intermediate risk, and upfront hematopoietic stem cell transplantation (HSCT) is commonly recommended [7-9]. However, even though treatment strategies seem promising, the prognosis of NK-AML is variable when molecular evaluation of the *FLT3*, *NPM1*, and *CEBPA* mutations is taken into account [10-14]. Schlenk et al. [10] reported that NK-AML patients with the *CEBPA* mutation or *NPM1* mutation, but without the *FLT3* mutation, had a favorable prognosis and that upfront HSCT in these patients did not contribute to the overall survival (OS). Although the detection of subgroups is necessary for decisions on the most appropriate treatment strategy, routine molecular diagnoses are often difficult in clinical practice.

AML with the *CEBPA* mutation has a homologous surface antigen expression that is closely associated with CD7, CD15, CD34, and HLA-DR positivity [15, 16]. We speculated that immunophenotyping for CD7, CD15, CD34, and HLA-DR in patients with NK-AML could identify a distinct subtype of AML that clinically mimics AML with the *CEBPA* mutation. In the Japan Adult Leukemia Study Group (JALSG) AML97 study, almost 42 % of the patients with AML were diagnosed with NK-AML. For this study, results of surface antigen expression were obtained at the time of enrollment. Further, we used data from the JALSG AML97 study to investigate the clinical significance of these surface antigens for the prognosis of patients with NK-AML.

Patients and methods

Patients

We conducted a retrospective review of patient data from the multicenter JALSG AML97 study. Detailed information of this study and its results has previously been reported [17, 18]. Briefly, between December 1997 and July 2001, patients aged 15–64 years, with newly diagnosed de novo AML, excluding those with APL, were consecutively enrolled to the JALSG AML97 study. In total, 789 of the 809 AML patients were eligible for the study, and informed consent was obtained from all patients or their guardians before enrollment. The study protocol was approved by the research ethics boards of all participating institutions, and the study was conducted in accordance with the Declaration of Helsinki.

Cytogenetic studies

The results from the cytogenetic studies, which were performed at each of the institutions, were reported to the JALSG Statistical Center. Routinely, 20 metaphases were counted for each patient and analyzed according to the recommendations of the International System for Human Cytogenetic Nomenclature.

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Flow cytometry

Immunophenotyping was performed at each institution, primarily on freshly collected bone marrow or peripheral blood samples that were collected at the time of diagnosis. Leukemic cell analysis was performed at local or reference laboratories by standard immunofluorescence methods using monoclonal antibodies directed against the CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD13, CD15, CD19, CD33, CD34, CD41a, CD56, and HLA-DR surface antigens. Samples were considered positive if at least 20 % of blasts expressed the antigen.

Treatment regimen used in the JALSG AML97 study

Induction therapy consisted of Ara-C at a dose of 100 mg/m^2 per day as a continuous infusion on days 1–7 and idarubicin (IDR) at a dose of 12 mg/m^2 per day as a 30-min infusion on days 1–3. Patients who did not achieve remission after the first induction cycle were given the same therapy again. Patients who obtained complete remission (CR) within two courses of induction therapy were randomly assigned to a group that received either four courses of standard dose consolidation therapy without maintenance (arm A) or three courses of standard dose consolidation along with six courses of maintenance therapy (arm B). In the JALSG AML97 study, the 5-year overall survival rate and the 5-year disease-free survival (DFS) rate between the arms were not statistical different [17].

Surface antigen expression profile according to the CEBPA mutant pattern

We also investigated the surface antigen expression profiles according to *CEBPA* mutant pattern in 318 AML patients based on the data records of AML patients enrolled at the Kumamoto and Nagasaki Universities. High molecular weight genomic DNA was extracted from the bone marrow or peripheral blood samples after Ficoll separation of mononuclear cells. Mutations of the *CEBPA* gene was detected by genomic DNA PCR, and direct sequencing was performed at each institution, as described previously [19, 20].

Statistical analysis

OS for all patients was defined as the period from the date of diagnosis to the date of death. Event-free survival (EFS) was defined as the period from the date of diagnosis to the date of the first recurrence after CR or any cause of death. All patients who underwent HSCT were censored from the EFS analysis on the date of HSCT treatment. The Kaplan-Meier method was used to estimate the EFS and OS. The log-rank test was used to compare the EFS or OS of the two groups. Factors that could potentially affect clinical outcome, including age, sex, WBC count, performance status at diagnosis, and the expression of each surface antigen were analyzed by the multivariate Cox proportional hazard regression model. Fisher's exact test and Student's *t* test were used to compare factor differences between the two groups. Statistical analysis was performed with the JMP software version 8.0.1 (SAS Institute Inc., Cray, NC, USA).

Results

Definition of the CEBPA type

For this study, CEBPA type NK-AML was defined as NK-AML that showed the CD7+ CD15+ CD34+ HLA-DR + immunophenotype because these antigens are commonly expressed in AML with the *CEBPA* mutation [15, 16]. Non-CEBPA type NK-AML was defined as NK-AML that did not have the CD7+ CD15+ CD34+ HLA-DR + immunophenotype.

In total, 329 patients were diagnosed with NK-AML. The expression of CD7, CD15, CD34, and HLA-DR was examined in 303, 201, 306, and 302 patients, respectively. Of the

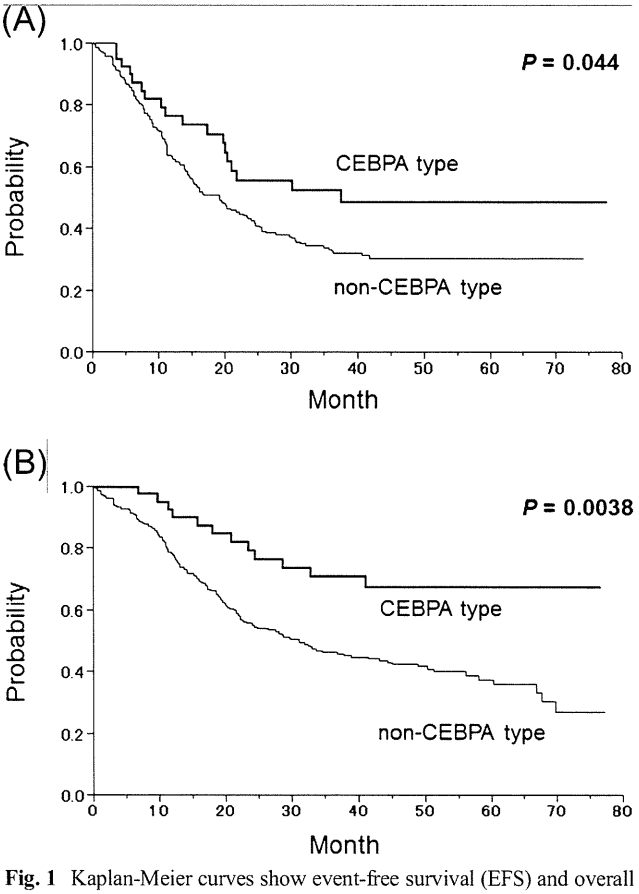

Fig. 1 Kaplan-Meier curves show event-free survival (EFS) and overall survival (OS) according to the CEBPA status. EFS and OS for each group are shown in **a** and **b**, respectively. Log-rank test revealed the 5-year EFS to be 48.5 and 30.5 % for patients with CEBPA and non-CEBPA type NK-AML, respectively, which was significantly different (P=0.044). The 5-year OS of patients with CEBPA and non-CEBPA type NK-AML was also significant (67.4 and 35.7 %, respectively; P=0.0038)

Table 1 Analysis of prognostic factors for event-free survival in the NK-AML population (n=329)

Factors	Number (positive/negative)	Univariate analysis		Multivariate analysis	
		HR (95 % CI)	P value	HR (95 % CI)	P value
Age > 50 years	131/198	1.04 (0.77–1.39)	0.812		
Female sex	141/188	0.81 (0.60–1.09)	0.168		
WBC count of $>20\times10^9$/L	165/164	1.74 (1.30–2.34)	0.0002	1.56 (1.14–2.14)	0.0052
Performance status ≥2	29/300	1.21 (0.72–1.91)	0.461		
CD7	108/195	0.96 (0.70–1.31)	0.808		
CD15	113/88	0.77 (0.52–1.13)	0.174		
CD34	166/140	1.26 (0.94–1.71)	0.127		
HLA-DR	255/47	1.16 (0.77–1.82)	0.483		
CEBPA type	39/243	0.61 (0.36–0.96)	0.034	0.59 (0.35–0.94)	0.026

329 NK-AML patients, 39 were classified as having the CEBPA type and 243 were classified as having the non-CEBPA type NK-AML. We excluded 47 patients whose of immunophenotype could not be determined.

EFS and OS

The 5-year EFS rate for patients with CEBPA type NK-AML was 48.5 %, which was significantly higher than the 30.5 % for patients with non-CEBPA type NK-AML (P=0.044, Fig. 1a). Furthermore, the 5-year OS rate for patients with CEBPA type NK-AML was also significantly higher than that for patients with non-CEBPA type NK-AML (67.4 vs 35.7 %, P=0.0038, Fig. 1b).

Univariate analysis showed that the outcome of patients with increased WBC counts at diagnosis was significantly worse (Tables 1 and 2), in agreement with previous reports [1]. Furthermore, CEBPA type was also a significant factor for better EFS and OS (Tables 1 and 2). Multivariate analysis showed CEBPA type and increased WBC counts to be independent prognostic factors (Tables 1 and 2). Other factors such as age, performance status, sex, or the expression of each of the single surface antigens did not affect the EFS and OS rates.

Our study included 12 patients with CEPBA type NK-AML and 77 patients with non-CEBPA type NK-AML who received HSCT. There was no significant difference among patients who received HSCT in these two groups (Table 3). The 2-year OS after HSCT in these groups were 61 and 41 %, respectively, which did not reach significance (P=0.467).

Clinical profiles in CEBPA type

The CEBPA type was identified as an independent prognostic factor for EFS and OS. Therefore, we analyzed the characteristics of CEBPA type (Table 3). Sex, WBC count, or performance status was not associated with CEBPA or non-CEBPA type NK-AML. In contrast, compared with non-CEBPA type NK-AML, CEBPA type NK-AML was associated with younger age, higher myeloperoxidase (MPO)-positive rates, frequent presentation with Auer rods, and a French-American-British (FAB) classification of M1 or M2.

Table 2 Analysis of prognostic factors for overall survival in the NK-AML population (n=329)

Factors	Number (positive/negative)	Univariate analysis		Multivariate analysis	
		HR (95 % CI)	P value	HR (95 % CI)	P value
Age > 50 years	131/198	1.19 (0.89–1.60)	0.240		
Female sex	141/188	0.80 (0.59–1.08)	0.144		
WBC count of $>20\times10^9$/L	165/164	1.51 (1.13–2.03)	0.0059	1.44 (1.05–1.97)	0.023
Performance status ≥2	29/300	1.23 (0.72–1.96)	0.437		
CD7	108/195	0.79 (0.57–1.09)	0.150		
CD15	113/88	0.72 (0.49–1.07)	0.101		
CD34	166/140	1.10 (0.81–1.50)	0.526		
HLA-DR	255/47	0.95 (0.64–1.47)	0.807		
CEBPA type	39/243	0.41 (0.22–0.71)	0.0008	0.40 (0.21–0.69)	0.0005