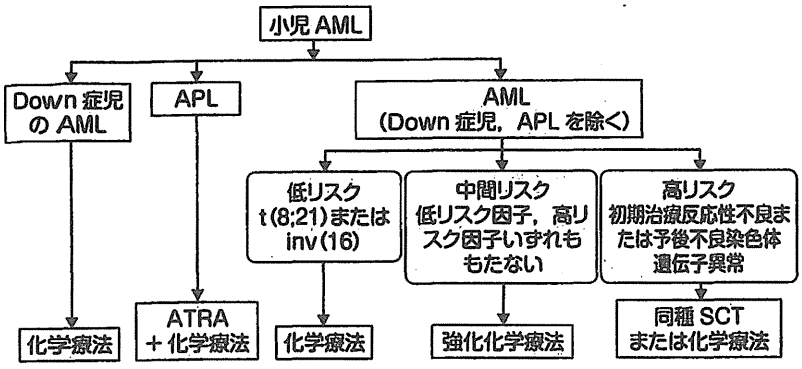


図　小児 AML の診療アルゴリズム
(日本小児血液学会編：小児白血病・リンパ腫の診療ガイドライン 2011 年版，金原出版，p3，2011)

けて記載する(図)。

1. 狭義の新規診断 AML(*de novo* AML)

1) 予後因子と治療層別化：もっとも重要な予後因子は，AML 細胞の染色体核型と寛解導入療法における治療反応性であり，同種造血幹細胞移植(Stem cell transplantation：SCT)の適応を含めた AML の寛解導入後治療は，これらの予後因子に基づいたリスク層別化により決定される。現在のわが国の治療プロトコール(臨床試験)では，t(8；21)(q22；q229)陽性，inv(16)(p13；q22)あるいは t(16；16)(p13；q22)陽性の場合は低リスク群として化学療法のみの治療が行われ，モノソミー 7 や 5q−，t(16；21)(p11；q22)，t(9；22)(q34；q11.2)陽性など予後不良な染色体異常がある場合や，初回寛解導入療法後の非寛解例は，高リスク群として第一寛解期における同種 SCT の適応となる。低リスク群にも高リスク群にも当てはまらない場合には中間リスク群とされる。

さらに，新規の AML 関連遺伝子異常の一つである FMS-like tyrosine kinase 3(FLT3)の internal tandem duplication (FLT3-ITD)が強力な予後不良因子であることがわかってきたため，初の全国統一小児 AML プロトコールである日本小児白血病リンパ腫研究グループ(JPLSG)の AML-05 試験では，FLT3-ITD 陽性例は高リスク群に分類された。しかし，他の新規遺伝子変異の意義は必ずしも確立しているわけではなく，今後さらなる検討が必要である。

初回寛解導入療法後の治療反応性は重要な予後因子であるが，従来の形態学的評価では検出困難であった微小残存病変(MRD)を，フローサイトメトリー法あるいは PCR 法で検出する方法が検討されており，近い将来リスク層別に用いられる可能性が高い。

2) 化学療法：小児 AML の治療の主体は多剤併用化学療法であり，その中心はシタラビン(Ara-C)とアントラサイクリン系薬剤である。これらの 2 剤を中心とした寛解導入療法と大量 Ara-C 療法を含む強化療法を，計 4〜5 コース程度行うのが標準的である。

AML の寛解導入療法は，Ara-C 100 mg/m$^2$の 7 日間およびダウノマイシン 45 mg/m$^2$の 3 日間投与を行う“7 and 3”療法が基本であるが，小児では第 3 の薬剤としてエトポシドなどを加えた治療レジメンが寛解導入療法として一般的に使用されており，主な研究グループの完全寛解率は 85〜90％に達している(表 2)。

寛解導入後治療は，Ara-C とアントラサイクリン系薬剤を用いた多剤併用化学療法であり，化学療法による治療成績の向上に伴い，最近のリスク層別化治療のもとで

表 2　小児 *de novo* AML の主な治療成績

| 試験名 | 期間 | N | CR | コース数 | 同種移植 | EFS(年) | OS(年) |
|---|---|---|---|---|---|---|---|
| AML99* | 2000-2002 | 240 | 94% | 6 | 19% | 61%(5) | 75%(5) |
| CCLSG AML9805 | 1998-2002 | 101 | 90% | 8 | 19% | 53%(5) | 74%(5) |
| SJCRH AML02 | 2002-2008 | 230 | 94% | 5 | 27% | 63%(3) | 71%(3) |
| MRC AML12 | 1995-2002 | 455 | 92% | 4〜5 | 7% | 56%(5) | 66%(5) |
| AML-BFM98 | 1998-2003 | 473 | 88% | 5＋維持療法 | — | 49%(5) | 62%(5) |
| NOPHO93 | 1993-2000 | 243 | 92% | 6〜7 | 23% | 48%(5) | 65%(5) |
| CCG 2961 | 1996-2002 | 901 | 88% | 3 | 18% | 42%(5) | 52%(5) |

*小児 AML 共同治療研究会　(日本造血細胞移植学会ガイドライン：小児急性骨髄性白血病，2012)
CCLSG：小児癌白血病研究グループ，SJCRH：St. Jude 小児がん研究病院，MRC：Medical Research Council，NOPHO：Nordic Society of Pediatric Hematology and Oncology，CCG：Children's Cancer Group，CR：完全寛解，EFS：無イベント生存率，OS：全生存率

は同種SCTは高リスク群に限られてきている。維持療法は一部の国でまだ行われているが，その意義は一般的に否定的であり，わが国では現在小児AMLに対して維持療法を行っていない。

小児AMLで用いられているアントラサイクリン系薬剤としては，ダウノルビシン，イダルビシン，ミトキサントロンがあるが，その優劣については結論が出ていない。そのほかの薬剤ではエトポシドなどが併用されることが多い。抗CD33モノクローナル抗体に抗腫瘍性抗生物質カリケアマイシンを結合させた製剤ゲムツズマブ・オゾガマイシン(GO)については，現時点では再発例や難治例を対象に単剤で使用することが推奨されている。

3)第一寛解期のSCT：1980年代～1990年代前半にかけて，第一寛解期小児AMLに対して自家SCTが試みられたが，少なくとも生存率の比較において自家SCTと通常の化学療法との差はなく，現在小児では自家SCTの適応はないと考えられている。

1990年代後半以降，リスク層別化治療が導入されるようになり，t(8；21)やinv(16)陽性例などの低リスク群に対しては，第一寛解期における同種SCTの適応はない，とするコンセンサスが得られた。第一寛解期における同種SCTの適応については，わが国では，AML99試験の結果，高リスク群の5年OSが57%と，中間リスク群，低リスク群の72～87%と比較して明らかに不良であることから，高リスク群のみが同種SCTの適応としている。第二寛解期の場合は，わが国の16歳未満例の移植後5年生存率が61.4%とSCTによる救済が期待できるため，同種SCTを行うことが推奨されている。化学療法による治療成績の向上，晩期合併症を含めた同種SCTのリスクおよび第二寛解期の移植成績が比較的良好であることなどから，第一寛解期における同種SCTの適応は縮小される傾向にある。

2. APL：DICを合併することが多く，Auer小体を有する特徴的な細胞(ファゴット細胞)がほとんどの症例で認められるため，臨床診断は比較的容易であるが，染色体検査あるいはFISH法あるいはサザンブロット法で，t(15；17)(q22；q12)(染色体検査)，PML-RARα融合遺伝子(FISH法)，RARA遺伝子再構成(サザンブロット法)を確認する必要がある。

APLは，APL細胞により惹起されるDICのため，従来は寛解導入中に死亡する症例が多く，きわめて予後不良の疾患であった。しかし1988年に全トランスレチノイン酸(ATRA)による分化誘導療法が報告され，以後ATRAを従来の化学療法に導入することにより寛解導入療法中の死亡が減少し，寛解導入率が向上して劇的に治療成績が向上した。その後，わが国でも小児AML99-M3研究などATRA併用の治療が登場し，AML99-M3研究では寛解導入率96.2%，7年EFS 91.4%と非常に良好な結果を残した。2005年末には，AML99-M3研究を基本骨格としたJPLSG AML-P05が全国の臨床試験として始まった。これはATRAと抗癌剤の同時併用による寛解導入療法，強化療法および，ATRA単独による維持療法からなる。以上より，現時点でわが国では①ATRAと抗癌剤併用による寛解導入療法，②アントラサイクリン系薬剤を中心とした強化療法，③ATRA単独あるいは抗癌剤併用による維持療法が，小児APL治療の標準的骨格として確立されつつある。

1997年に報告された三酸化ヒ酸(ATO)は，ATRA使用後再発例において優れた再寛解導入率を示し，その後成人を中心に再発例，初発例に対する治療報告が増えている。そして①ATRA耐性例にも有効である，②アントラサイクリン系薬剤の総投与量を減らせる，③無イベント生存率(EFS)をさらに向上する可能性があるなどの理由から，近年は成人初発APL例に対してATOを導入した大規模な臨床研究が増加している。小児APLに関しては，米国のグループが再発APL13症例をATO単剤で治療し再寛解導入率85%と良好な結果を報告し，小児初発APLに対してもATRAと抗癌剤の併用療法に匹敵する治療成績が示されてきている。しかしATRAやATOによる治療はいわゆる化学療法の副作用，合併症が少ない反面，APL分化症候群やATOによるQT延長などの独特な合併症対策が必要である。

3. ダウン症候群(DS)に伴うAML(ML-DS)：新生児期に発症するtransient abnormal myelopoiesis(TAM)との鑑別は，臨床症状や白血病細胞の特徴からは困難である。また，骨髄線維症の合併により骨髄スメア標本での診断が困難な場合もある。DSに発症したAML(ML-DS)は，非DSに発症したAMLに比べ，ほとんどが4歳以下と発症年齢が低く，急性巨核芽球性白血病(FAB分類M7)が多い。ML-DSは非DS AMLに比べ，治療合併症が多い一方で，治療反応性がよいことが知られるようになり，現在は独立した治療が行われている。治療は非DS AMLと同様，Ara-Cとアントラサイクリン系薬剤を基本とし，欧米のようにML-DSと同じ骨格を用いて投与量の減量を行っているものと，わが国のようにML-DSに特化した治療を行っているものとがあるが，いずれも70～80%のEFSが得られている。治療強度が低い化学療法プロトコールにより良好な成績が得られている

ため，乳幼児のAMLではDSを診断，確認することは非常に重要である。

わが国では，中等量Ara-C(100 mg/m$^2$/日×7日)を主体とした欧米の治療よりも減弱した治療を行ってきた。AML99のDSプロトコールでは，Ara-Cにピラルビシンとエトポシドを組み合わせたレジメンを5回繰り返すというシンプルな治療を行って，4年EFSが83%と良好な成績を残している。JPLSGでは2008年からAML-D05の臨床試験を行って，標準危険群ではAML99DSよりさらに治療の軽減を図っており，現在結果の解析が待たれるところである。

最後に，本稿の内容は日本小児血液学会の「小児白血病・リンパ腫の診療ガイドライン2011年版」と造血細胞移植学会の「疾患別ガイドライン」の，おのおの小児AMLの項から多くの引用を行っている。詳細は各ガイドラインを参照されたい。

Key Words: WHO分類，リスク層別化治療，急性前骨髄球性白血病，ダウン症候群，日本小児白血病リンパ腫研究グループ

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Pediatr Blood Cancer

# PBSCT Is Associated With Poorer Survival and Increased Chronic GvHD Than BMT in Japanese Paediatric Patients With Acute Leukaemia and an HLA-Matched Sibling Donor

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***Background.*** Peripheral blood stem cells (PBSC) may be used as an alternative to bone marrow (BM) for allogeneic transplantation. Since peripheral blood stem cell bank from unrelated volunteer donor has been started in Japan, use of PBSC allografts may be increased. Therefore we surveyed the outcomes of Japanese leukemia children after PBSC and BM transplantation. ***Procedure.*** This retrospective study compared the outcomes of 661 children (0–18 years) with acute lymphoblastic leukaemia (ALL) or acute myeloid leukaemia (AML) who received their first allogeneic peripheral blood stem cell transplantation (PBSCT; n = 90) or bone marrow transplantation (BMT; n = 571) from HLA-matched siblings between January 1996 and December 2007. ***Result.*** Neutrophil recovery was faster after PBSCT than after BMT (ALL: $P < 0.0001$; AML: $P = 0.0002$), as was platelet recovery (ALL: $P = 0.0008$; AML: $P = 0.0848$). However, the cumulative incidence of chronic graft-versus-host disease (GvHD) was higher after PBSCT than after BMT (ALL: 26.0% vs. 9.9%, $P = 0.0066$; AML: 41.6% vs. 11.1%, $P < 0.0001$). The 5-year disease-free survival (DFS) was lower after PBSCT than after BMT for ALL (40.6% vs. 57.1%, $P = 0.0257$). The 5-year overall survival (OS) was lower after PBSCT than after BMT for ALL (42.4% vs. 63.7%, $P = 0.0032$) and AML (49.8% vs. 71.8%, $P = 0.0163$). Multivariate analysis revealed the use of PBSC was a significant risk factor for DFS and OS. PBSCT and BMT did not differ in relapse rate, acute GvHD for ALL and AML, or in DFS for AML. ***Conclusion.*** PBSC allografts in Japanese children engraft faster but are associated with poorer survival and increased chronic GvHD. Pediatr Blood Cancer © 2013 Wiley Periodicals, Inc.

**Key words:** acute leukaemia; bone marrow transplantation; children; chronic graft-versus-host disease; peripheral blood stem cell transplantation

## INTRODUCTION

Allogeneic peripheral blood stem cell (PBSC) transplantation (PBSCT) was established along with allogeneic bone marrow (BM) transplantation (BMT) in the last decade [1–7]. In October 2010, a bank that stores PBSC from unrelated volunteer donors was established in Japan. Other progress in this area in Japan relates to the registration of haematopoietic stem cell transplantation (HSCT), which, until five years ago, involved four separate registry organisations. However, in 2006, the registers of these organisations were computerised and unified under the Transplantation Registry Unified Management Program (TRUMP) [8]. In 2008, the HSCT data of paediatric patients that had been stored on paper in the four registries were entered into TRUMP electronically. TRUMP has thus made it possible to analyse the paediatric HSCT data with greater accuracy.

While several prospective and retrospective randomised controlled trials (RCTs) and meta-analyses that compared BMT and PBSCT have been published [3,4,6,9–15], most have focused on adult patients only. To survey the outcomes of Japanese children after allogeneic HSCT from related donors, TRUMP data were used to conduct a retrospective, multi-centre study that compared the outcomes of 661 paediatric patients with leukaemia after their transplantation with allogeneic PBSC or BM from HLA-matched siblings. The impact of chronic graft-versus-host disease (GvHD) after transplantation was also examined.

## PATIENTS AND METHODS

### Study Population

The Japan Society for Haematopoietic Cell Transplantation uses a standardised reporting form to collect the data of individual transplant


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Conflict of interest: Nothing to declare.

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patients from each transplant centre. Follow-up reports are also submitted annually after transplantation. Between January 1996 and December 2007, the data of 1,048 paediatric patients with acute lymphoblastic leukaemia (ALL) or acute myeloid leukaemia (AML) who underwent a myeloablative preparative regimen and allogeneic BMT or PBSCT from a family donor were reported to the Japan Society for Haematopoietic Cell Transplantation. Patients were excluded from the study if their data were incomplete (n = 20), if they had received BM together with PBSC (n = 15), if they received grafts from father or mother (n = 322), or if they received grafts from anyone other than an HLA-identical sibling or a sibling whose HLA matched in all but one antigens (n = 30). As a result, 661 patients were included in this study. This study was approved by the Data Management Committee for the Nationwide Survey of the Japan Society for Haematopoietic Cell Transplantation.

### Methods of HLA Typing

The method of HLA typing varied from time to time among participated institutes. In general, serological or low-resolution HLA typing was performed by lymphocyte cytotoxicity test until 2003 and reversed SSO after 2003. High-resolution HLA typing was done for class II alleles by PCR-RFLP from 1992 to 2000, and done by SBT for class I and II after 2000.

### Engraftment Evaluation

Neutrophil engraftment was defined as an absolute neutrophil count greater than $0.5 \times 10^9$/L occurring on the first of three consecutive days. Platelet engraftment was defined as the first day with a platelet count greater than $50 \times 10^9$/L without further need for platelet transfusions.

### End Points and Definition

End points were assessed on the date of last patient contact and were analysed on the 31 October 2009. The study end points were relapse rate (RR), disease-free survival (DFS), overall survival (OS), acute and chronic GvHD and transplantation-related mortality (TRM). Acute GvHD was graded according to standard criteria by the attending physicians of each hospital [16]. Chronic GvHD was graded as limited (localised skin or single organ involvement) or clinically extensive [17]. The reported causes of death were reviewed and categorised and TRM was defined as death with no evidence of disease. DFS was defined as survival without evidence of relapse, the event under study being death or relapse. Patients who died as a result of relapse or disease progression after transplantation were considered to have died of their original disease.

Risk status at transplantation was categorised as either standard or high. Standard-risk (SR) disease included ALL in first or second complete remission (CR1, CR2) except when it was Philadelphia chromosome-positive [Ph (+)] or exhibited mixed-lineage leukaemia gene (MLL) rearrangement. SR disease also included AML in CR1 or CR2. All other diseases status were categorised as high-risk (HR) disease.

### Statistical Analysis

The cumulative incidences of neutrophil and platelet recovery, acute and chronic GvHD, relapse, survival and TRM of the PBSCT and BMT groups were compared by using the log-rank test to compare Kaplan and Meier curves. The 95% confidence intervals (CIs) were calculated by using the Greenwood formula [18]. The significance of all other differences between both groups was estimated by using the Chi-square test for categorical variables and the Kruskal–Wallis test for continuous variables. Multivariate Cox analysis [19] was used to study the potential effect of the following factors on OS and DFS: age, diagnosis, disease risk, stem cell source and donor-recipient HLA compatibility.

## RESULTS

### Patient Characteristics

The data of 661 paediatric patients with acute leukaemia who underwent their first stem cell transplantation were analysed. The patients were divided according to whether they had ALL (n = 411) or AML (n = 250). The characteristics of these two groups are shown in Table I. Of the ALL group, 60 (15%) received PBSCT and 351 (85%) received BMT. Of the AML group, 30 (12%) received PBSCT and 220 (88%) received BMT. The ages of both the ALL and AML groups ranged from 0 to 18 years and the median ages were both 9.0 years. For the ALL group, 247 (60%) and 164 (40%) had SR and HR disease respectively, while 202 (81%) and 48 (19%) of the AML group had SR and HR disease, respectively. The PBSC and BM recipients in the ALL and AML groups did not differ in terms of gender (ALL, $P = 0.28$; AML, $P = 0.23$) or conditioning regimen (ALL, $P = 0.37$; AML, $P = 0.42$). But for the ALL group, the PBSC recipients were significantly older than the BM recipients (ALL, $P < 0.01$; AML, $P = 0.20$).

These ALL and AML groups were also divided further according to the disease risk into SR and HR subgroups. For all four subgroups, the gender ratios of the PBSC and BM recipients did not differ significantly (ALL SR, $P = 0.29$; ALL HR, $P = 0.93$; AML SR, $P = 0.64$; AML HR, $P = 0.23$). The ages of the PBSC and BM recipients did not differ significantly either except for in the ALL HR subgroup, where PBSCT was associated with a significantly higher median age than BMT (ALL SR, $P = 0.13$; ALL HR, $P < 0.01$; AML SR, $P = 0.12$; AML HR, $P = 0.89$).

### Engraftment Associated With PBSCT and BMT in ALL and AML

In the ALL group, 59/60 and 350/351 patients after PBSCT and BMT survived more than 28 days, while in the AML group, 30/30 and 220/220 patients after PBSCT and BMT survived more than 28 days. Of these surviving patients in ALL, engraftment occurred in 58 (98.3%) patients after PBSCT and 346 (98.9%) patients after BMT. Of the surviving patients in AML, 29 (96.7%) patients after PBSCT and 219 (99.5%) patients after BMT exhibited engraftment.

The median times to recovering a neutrophil count of $>0.5 \times 10^9$/L for the ALL and AML groups and their SR and HR subgroups are shown in Figure 1. For the ALL group, PBSCT and BMT were associated with median times of 13 and 16 days ($P < 0.0001$). Similarly, the median times after PBSCT and BMT in the ALL SR subgroup were 13 and 16 days ($P < 0.0001$), respectively. For the ALL HR subgroup, these times were 13 and 16 days ($P = 0.0003$), respectively. The median times after PBSCT and BMT in the AML group were 12.5 and 17 days, respectively ($P = 0.0002$), while for the AML SR subgroup, they were 12 and 17 days, respectively ($P < 0.0001$). However, in the AML HR subgroup, PBSCT (14.5 days), did not differ significantly from BMT (18 days) in terms of neutrophil recovery ($P = 0.1795$). Thus, PBSCT was associated with

**TABLE I. Patient Characteristics**

| | ALL (n=411) | | | | AML (n=250) | | | |
|---|---|---|---|---|---|---|---|---|
| | PBSC | BM | *P*-value | Total | PBSC | BM | *P*-value | Total |
| Number of patients | 60 (15) | 351 (85) | | 411 | 30 (12) | 220 (88) | | 250 |
| Median age, years [range] | 11.0 [4–18] | 8.0 [0–18] | <0.01 | 9.0 [0–18] | 11.5 [0–17] | 9.0 [0–18] | 0.20 | 9.0 [0–18] |
| Sex (male/female) | 33/27 | 222/129 | 0.28 | 255/156 | 14/16 | 132/88 | 0.23 | 146/104 |
| Risk group | | | 0.03 | | | | <0.01 | |
| Standard risk | 28 (47) | 219 (62) | | 247 (60) | 18 (60) | 184 (84) | | 202 (81) |
| High risk | 32 (53) | 132 (38) | | 164 (40) | 12 (40) | 36 (16) | | 48 (19) |
| Conditioning regimen | | | 0.37 | | | | 0.42 | |
| TBI-based | 44 (73) | 279 (79) | | 323 (79) | 11 (37) | 102 (46) | | 113 (45) |
| Chemotherapy-based | 16 (27) | 72 (21) | | 88 (21) | 19 (63) | 118 (54) | | 137 (55) |
| GVHD prophylaxis | | | | | | | | |
| CSA+short MTX | 32 (53) | 167 (48) | | 199 (48) | 18 (60) | 108 (49) | | 126 (50) |
| CSA alone | 9 (15) | 49 (14) | | 58 (14) | 5 (17) | 23 (10) | | 28 (11) |
| Short MTX alone | 7 (12) | 94 (27) | | 101 (25) | 1 (3) | 65 (30) | | 66 (26) |
| FK506+short MTX | 5 (8) | 15 (4) | | 20 (5) | 0 (0) | 8 (4) | | 8 (3) |
| Standard risk | | | | | | | | |
| Median age, years [range] | 10.5 [4–17] | 8.0 [1–18] | 0.13 | 8.0 [1–18] | 12.0 [0–17] | 9.0 [0–18] | 0.12 | 9.0 [0–18] |
| Sex (male/female) | 15/13 | 144/75 | 0.29 | 159/88 | 9/9 | 108/76 | 0.64 | 117/85 |
| High risk | | | | | | | | |
| Median age, years [range] | 11.0 [5–18] | 8.0 [0–18] | <0.01 | 9.0 [0–18] | 9.0 [1–16] | 10.0 [0–17] | 0.89 | 10.0 [0–17] |
| Sex (male/female) | 18/14 | 78/54 | 0.93 | 96/68 | 5/7 | 24/12 | 0.23 | 29/19 |

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BM, bone marrow; PBSC, peripheral blood stem cell; TBI, total body irradiation; CSA, cyclosporine; MTX, methotrexate. Standard-risk disease includes ALL in first or second complete remission (unless Philadelphia chromosome positivity or mixed-lineage leukemia gene rearrangement was present) and AML in first or second complete remission. High-risk disease includes all other diseases statuses. Values are given as n (%).

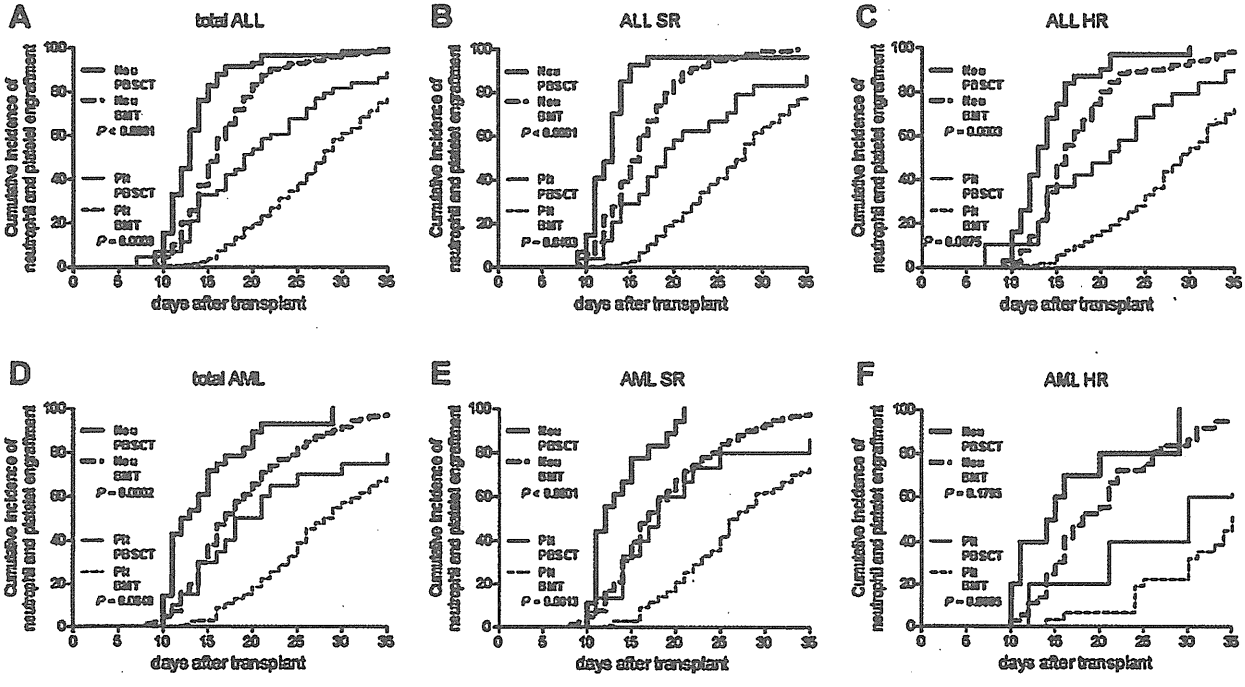


Fig. 1. Engraftment to neutrophil count $>0.5 \times 10^9$/L and engraftment to platelet count $>50 \times 10^9$/L. A: total ALL; (B) ALL standard-risk; (C) ALL high-risk; (D) total AML; (E) AML standard-risk; and (F) AML high-risk.

significantly faster neutrophil recovery than BMT in total ALL, ALL SR, ALL HR, total AML and AML SR.

As shown in Figure 1, for the ALL group, the median times to recovering a platelet count of $>50 \times 10^9$/L after PBSCT and BMT were 19 and 28 days, respectively ($P = 0.0008$). For the ALL SR subgroup, the times after PBSCT and BMT were 19 and 28 days, respectively ($P = 0.0403$), while for the ALL HR subgroup, the times were 21 and 29 days, respectively ($P = 0.0075$). For the AML group, these times after PBSCT and BMT were 19.5 and 28 days, respectively ($P = 0.0848$), while the times for the AML SR subgroup were 18 and 26.5 days, respectively ($P = 0.0013$). For the AML HR subgroup, these times were 30 and 35 days, respectively ($P = 0.8885$). Thus, PBSCT was associated with significantly faster platelet recovery than BMT in the ALL group, ALL SR, ALL HR and AML SR.

## Relapse Rate, Disease-Free Survival and Overall Survival Associated With PBSCT and BMT in ALL and AML

As shown in Supplementary Figure 1, the RR after PBSCT and BMT did not differ significantly for ALL and AML or their SR and HR subgroups (total ALL, $P = 0.0663$; ALL SR, $P = 0.0977$; ALL HR, $P = 0.7708$; total AML, $P = 0.1549$; AML SR, $P = 0.4334$; AML HR, $P = 0.9871$).

PBSCT and BMT in the ALL group were associated with 5-year DFS values of 40.6% and 57.1%, respectively ($P = 0.0257$; Fig. 2A) and with OS values of 42.4% and 63.7%, respectively ($P = 0.0032$; Fig. 2A). In the AML group, PBSCT and BMT were associated with 5-year DFS values of 52.9% and 67.4%, respectively ($P = 0.0677$; Fig. 2D) and OS values of 49.8% and 71.1%, respectively ($P = 0.0163$; Fig. 2D). In ALL SR, PBSCT and BMT were associated with 5-year DFS values of 59.1% and 70.5%, respectively ($P = 0.2584$; Fig. 2B) and OS values of 58.2% and 76.9%, respectively ($P = 0.0579$; Fig. 2B). In ALL HR, PBSCT and BMT were associated with 5-year DFS values of 23.0% and 34.0%, respectively ($P = 0.2930$; Fig. 2C) and OS values of 28.8% and 40.1%, respectively ($P = 0.2507$; Fig. 2C). In AML SR, PBSCT and BMT were associated with 5-year DFS values of 71.8% and 72.5%, respectively ($P = 0.8519$; Fig. 2E) and OS values of 68.6% and 77.3%, respectively ($P = 0.6297$; Fig. 2E). In AML HR, PBSCT and BMT were associated with 5-year DFS values of 25.0% and 40.0%, respectively ($P = 0.4275$; Fig. 2F) and OS values of 22.2% and 43.2%, respectively ($P = 0.1339$; Fig. 2F).

As shown in Table II, multivariate Cox analysis revealed that PBSCT was significant risk factor for a poorer DFS (HR = 1.37; 95% CI, 1.01–1.88; $P = 0.044$) and OS (HR = 1.51; 95% CI, 1.09–2.09; $P = 0.013$). In addition, having HR disease was found to be a significant adverse risk factor for DFS (HR = 3.32; 95% CI, 2.59–4.26; $P < 0.001$) and OS (HR = 3.58; 95% CI, 2.73–4.70; $P < 0.001$).

## Acute GvHD Associated With PBSCT and BMT in ALL and AML

As shown in Supplementary Figure 2, PBSCT was associated with higher cumulative incidences of grades II–IV acute GvHD than BMT only in AML HR (57.6% vs. 23.2%, $P = 0.0264$) but not in the ALL group (31.2% vs. 21.8%, $P = 0.0826$), ALL SR (26.0% vs. 19.7%, $P = 0.4255$), ALL HR (34.9% vs. 25.4%, $P = 0.1784$), the AML group (33.9% vs. 18.0%, $P = 0.0506$) and AML SR (15.4% vs. 17.1%, $P = 0.8503$). Six patients died of grade IV acute GvHD: one patient with ALL after PBSCT, two patients with ALL after BMT and three patients with AML after BMT.

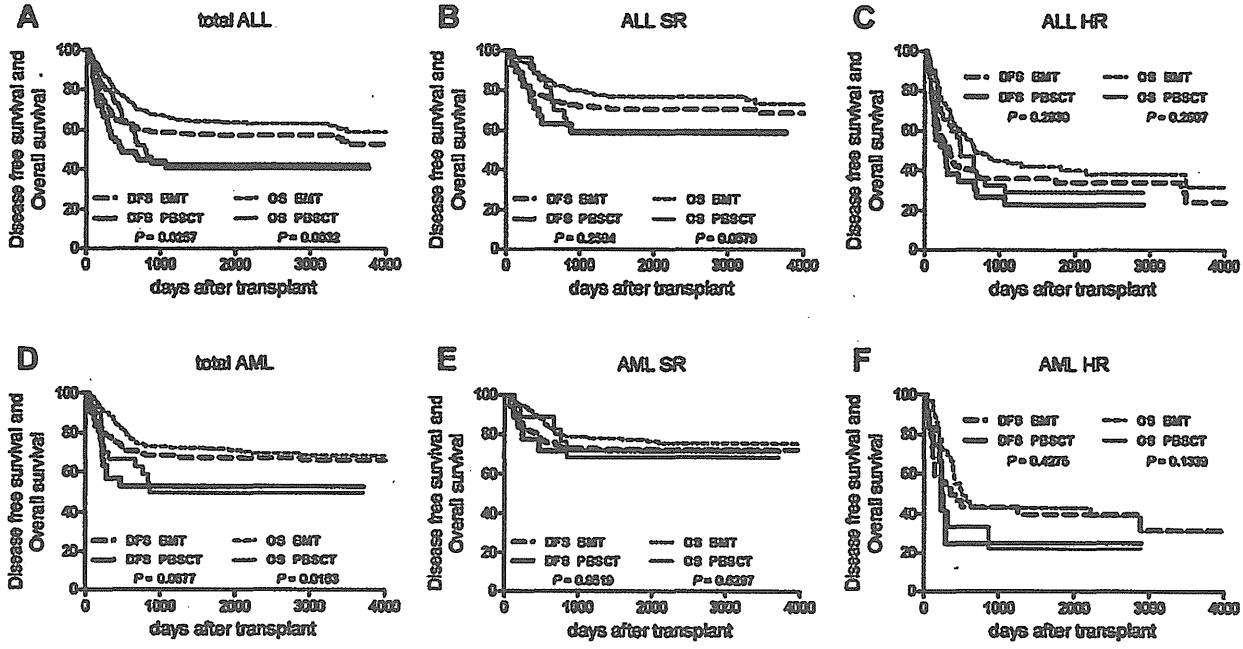


Fig. 2. Disease free survival and overall survival. A: total ALL; (B) ALL standard-risk; (C) ALL high-risk; (D) total AML; (E) AML standard-risk; and (F) AML high-risk.

TABLE II. Multivariate Model of Prognostic Risk Factors for Disease Free Survival and Overall Survival

| | Disease free survival | | | Overall survival | | |
|---|---|---|---|---|---|---|
| Variable | Hazard ratio | 95% conf. interval | *P*-value | Hazard ratio | 95% conf. interval | *P*-value |
| Diagnosis: AML vs. ALL | 0.91 | 0.70–1.18 | 0.470 | 1.03 | 0.78–1.36 | 0.849 |
| Risk group: HR vs. SR | 3.32 | 2.59–4.26 | <0.001 | 3.60 | 2.74–4.72 | <0.001 |
| Stem cell source: peripheral blood vs. bone marrow | 1.38 | 1.01–1.88 | 0.044 | 1.51 | 1.09–2.09 | 0.013 |

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; HR, high-risk; SR, standard-risk; HLA, human leucocyte antigen.

As shown in Supplementary Figure 2, the cumulative incidences of grades III–IV acute GvHD was similar for PBSCT and BMT in the ALL group (9.1% vs. 7.7%, $P=0.7741$), ALL SR (0% vs. 4.8%, $P=0.3027$), ALL HR (16.2% vs. 12.6%, $P=0.5896$), the AML group (15.7% vs. 8.5%, $P=0.3268$), AML SR (9.1% vs. 8.3%, $P=0.9534$) and AML HR (27.1% vs. 9.8%, $P=0.1729$).

## Chronic GvHD Associated With PBSCT and BMT in ALL and AML

Shown in Figure 3, PBSCT was associated with a higher cumulative incidence of any grade of chronic GvHD than BMT for the ALL group (52.6% vs. 19.6%, $P=0.0002$), ALL SR (39.7% vs. 18.3%, $P=0.0007$), ALL HR (48.8% vs. 21.6%, $P=0.0949$), the AML group (56.3% vs. 23.1%, $P=0.0002$), AML SR (40.2% vs. 21.0%, $P=0.0905$) and AML HR (81.8% vs. 39.7%, $P=0.0027$). Similarly, as shown in Figure 3, the cumulative incidence of extensive chronic GvHD was significantly higher in PBSCT than BMT for the ALL group (26.0% vs. 9.9%, $P=0.0066$), ALL SR (24.3% vs. 8.3%, $P=0.0059$), the AML group (41.6% vs. 11.1%, $P<0.0001$), AML SR (30.6% vs. 9.9%, $P=0.0215$) and AML HR (56.4% vs. 23.5%, $P=0.0046$). However, the difference observed in ALL HR did not achieve statistical significance (36.4% vs. 12.7%, $P=0.3225$). In addition, as shown in Figure 3, the cumulative incidence of limited chronic GvHD was higher after PBSCT than after BMT for the ALL group (34.6% vs. 10.6%, $P=0.0172$) and ALL SR (39.0% vs. 10.8%, $P=0.0419$), but it was similar for ALL HR (18.1% vs. 9.9%, $P=0.1812$), the AML group (23.1% vs. 13.2%, $P=0.3273$), AML SR (12.8% vs. 12.0%, $P=0.9658$) and AML HR (54.5% vs. 20.6%, $P=0.2462$).

## Transplantation-Related Mortality Associated With PBSCT and BMT in ALL and AML

As indicated by Figure 4, for the ALL group, cumulative incidences of TRM in PBSCT and in BMT were 5.1% and 4.0% at

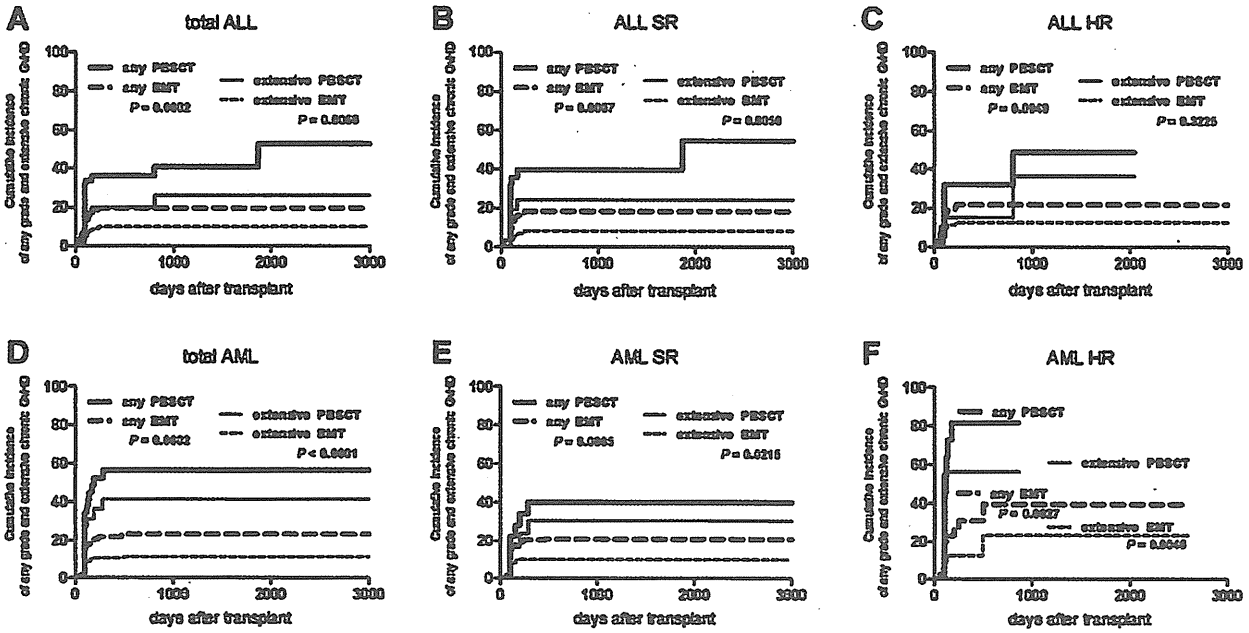


Fig. 3. Cumulative incidence of any grade and extensive chronic GvHD. A: total ALL; (B) ALL standard-risk; (C) ALL high-risk; (D) total AML; (E) AML standard-risk; and (F) AML high-risk.

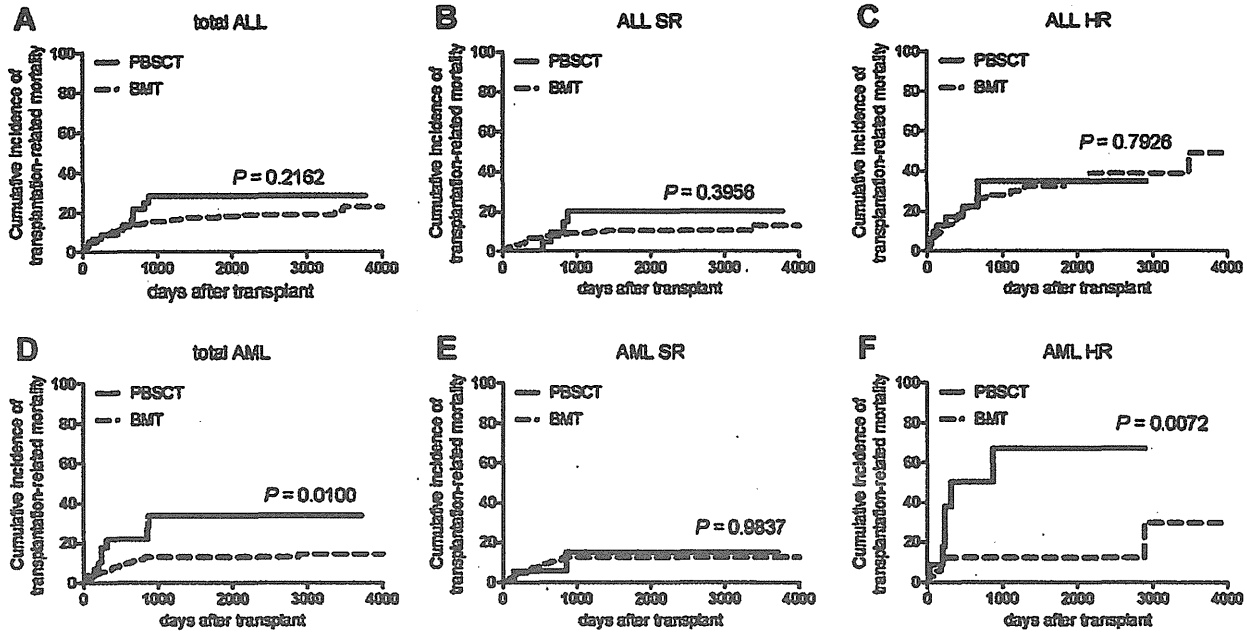


Fig. 4. Cumulative incidence of transplantation-related mortality. A: total ALL; (B) ALL standard-risk; (C) ALL high-risk; (D) total AML; (E) AML standard-risk; and (F) AML high-risk.

100 days, 8.8% and 9.6% at 1 year and 28.2% and 15.3% at 3 years, respectively ($P = 0.2162$). For the AML group, cumulative incidences of TRM in PBSCT and in BMT were 3.3% and 1.8% at 100 days, 21.6% and 6.0% at 1 year and 33.7% and 12.7% at 3 years, respectively ($P = 0.0100$). Thus, for both ALL and AML, PBSCT and BMT were associated with similar TRM incidences early after transplantation, PBSCT is associated with higher TRM incidences later after transplantation, only for the AML group and AML HR (ALL SR, $P = 0.3956$; (ALL SR, $P = 0.3956$; ALL HR, $P = 0.7926$; AML SR, $P = 0.9837$; AML HR, $P = 0.0072$; Supplementary Table I).

## DISCUSSION

This retrospective study compared the outcomes of allogeneic HSCT using two stem cell sources, namely BM or PBSC, in 661 Japanese children with acute leukaemia. The stem cell donors were HLA-matched sibling donors and the study was based on data acquired between January 1996 and December 2007. It was observed that PBSCT was associated with more rapid haematopoietic recovery than BMT, as has also been shown in most previous studies with adult patients [3,4,9,11–15] and paediatric patients [20]. The OS and DFS were significantly lower after PBSCT than after BMT.

Multivariate analysis revealed that the use of peripheral blood allografts was an adverse risk factor for OS and DFS. Similarly, while previous studies of adults with acute leukaemia suggest that PBSCT and BMT were associated with equivalent survival rates [9,11,15], the IBMRT study of children and adolescents reported that PBSCT was associated with lower survival and higher TRM [21]. The higher mortality observed in children after PBSCT is likely to be due to the higher incidences of chronic GvHD. This has been reported in younger patients with acquired severe aplastic anemia [22]. Thus, peripheral blood allografts in younger recipients may be associated with a higher risk of mortality and chronic GvHD, which suggests that caution should be exercised when considering the use of PBSC in paediatric patients.

It should be noted that this is a retrospective study, which has a number of limitations. In particular, we could not exclude the possibility that unidentified confounding variables could affect the transplant outcomes and could not adjust the data for unknown or unmeasured factors. Thus, the results presented here should be interpreted with caution. Nevertheless, these observations suggest that BM should be the preferred stem cell source for children with acute leukaemia who have HLA-matched sibling donors. It remains to be seen whether these conclusions can be extrapolated to alternative donors, namely HLA-mismatched related donors and HLA-identical unrelated donors. At present, there are no guidelines regarding the preferred source of stem cells (PBSC or BM) from unrelated volunteer donors. However, the biggest risk associated with selecting PBSC over BM concerns the fact that higher numbers of T cells are infused, which increases the risk of chronic GvHD and transplantation-related mortality. This risk may be greater with unrelated volunteer donors than with sibling donors. Indeed, it has been shown that when using unrelated donors, a single HLA mismatch increases the risk of GvHD more significantly if the transplant involves PBSC than if it involves BM [23]. Since a bank that stores PBSC from unrelated volunteer donors is now available in Japan, it is likely that peripheral blood allografts will be used more frequently for allogeneic transplantation in children. However, before such widespread clinical changes take place, the risks and benefits of the various allografts that are available should be considered. In cases of PBSC will be used more frequently such as active infections at SCT, mycophenolate

mofeti of GvHD prophylaxisis. More detailed analyses and future trials may reveal that BM stem cells and PBSC are suitable for different situations.

PBSCT did not differ from BMT in terms of the incidence of grades III–IV acute GvHD for the total ALL and total AML groups (and all of their SR and HR subgroups). This has also been observed in several studies with adult patients [3,12–14] and paediatric patients [20], even though there are 10-fold more T cells in the peripheral blood than in the BM. This may relate to the use of G-CSF [24,25]. However, the AML HR subgroup was observed to develop grades II–IV acute GvHD significantly more often after PBSCT than after BMT. It is possible that the acute GvHD experienced by the PBSC recipients in the AML HR subgroup reflects the induction of a graft-versus-leukemia effect. However, arguing against this is that the RR after PBSCT and BMT in the AML HR subgroup was similar. The observation that allogeneic PBSCT is not associated with a higher incidence of acute GvHD does not appear to extend to chronic GvHD. As observed in other studies with adult patients [3,9,11,13,26,27], PBSCT was associated with a significantly higher incidence of chronic GvHD than BMT in the total ALL and total AML groups (and the ALL SR and AML HR subgroups). In particular, PBSCT was associated with a significantly higher incidence of extensive chronic GvHD in the total ALL and total AML groups along with all SR and HR subgroups apart from ALL HR. Although we were not able to investigate organ injury in detail, another study has shown that chronic GvHD after PBSCT involves higher numbers of organs and requires longer and multiple courses of immunosuppressive therapy [28].

While a meta-analysis has demonstrated that allogeneic PBSCT is associated with a significant decrease in relapse in both early and late stage patients [29], our study found that the RR after PBSCT and BMT did not differ significantly for ALL and AML. This was also observed by a study analysing the IBMTR/EBRT registry data of adult patients with leukaemia [10], as well as by the IBMTR study of children and adolescents with acute leukaemia [21]. The allogeneic graft-versus-leukaemia effect varies from one disease to another and depends on the stage of the disease and donor histocompatibility. In our study, which only assessed paediatric patients with acute leukaemia, increases in the incidence of extensive chronic GvHD did not lead to a concomitant decrease in the RR. It will be necessary to examine the relationship between GvHD and relapse in the future.

In summary, our study demonstrates that while the use of peripheral blood allografts from HLA-matched sibling donors in Japanese paediatric patients with ALL or AML leads to faster engraftment, it is also associated with poorer survival and quality of life due to chronic GvHD.

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## REFERENCES

1. Kessinger A, Smith DM, Strandjord SE, et al. Allogeneic transplantation of blood-derived T cell-depleted hemopoietic stem cells after myeloablative treatment in a patient with acute lymphoblastic leukemia. Bone Marrow Transplant 1989;4:643–646 [PubMed: 2573397].
2. Russel NH, Hunter AE, Rogers S, et al. Peripheral blood stem cells as an alternative to marrow for allogeneic transplantation (letter). Lancet 1993;341:1482 [PubMed: 8099182].
3. Heldal D, Tjønnfjord G, Brinch L, et al. A randomised study of allogeneic transplantation with stem cells from blood or bone marrow. Bone Marrow Transplant 2000;25:1129–1136 [PubMed: 10849524].
4. Schmitz N, Bacigalupo A, Hasenclever D, et al. Allogeneic bone marrow transplantation vs filgrastim-mobilised peripheral blood progenitor cell transplantation in patients with early leukaemia: First results of a randomised multicentre trial of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant 1998;21:995–1003 [PubMed: 9632272].
5. Vigorito AC, Azevedo WM, Marques JFC, et al. A randomized prospective comparison of allogeneic bone marrow and peripheral blood progenitor cell transplantation in the treatment of haematological malignancies. Bone Marrow Transplant 1998;22:1145–1151 [PubMed: 9894716].
6. Blaise D, Kuentz M, Fortanier C, et al. Randomized trial of bone marrow versus lenograstim-primed blood cell allogeneic transplantation in patients with early-stage leukemia: A report from the Societe Francaise de Greffe de Moelle. J Clin Oncol 2000;18:537–546 [PubMed: 10653869].
7. Gratwohl A, Baldomero H, Horisberger B, et al. Current trends in hematopoietic stem cell transplantation in Europe. Blood 2002;100:2374–2386 [PubMed: 12239145].
8. Atsuta Y, Suzuki R, Yoshimi A, et al. Unification of hematopoietic stem cell transplantation registries in Japan and establishment of the TRUMP System. Int J hematol 2007;86:269–274 [PubMed: 17988995].
9. Schmitz N, Beksac M, Hasenclever D, et al. Transplantation of mobilized peripheral blood cells to HLA-identical siblings with standard-risk leukemia. Blood 2002;100:761–767 [PubMed: 12130483].
10. Schmitz N, Eapen M, Horowitz MM, et al. Long-term outcome of patients given transplants of mobilized blood or bone marrow: A report from the International Bone Marrow Transplant Registry and the European Group for Blood and Marrow Transplantation. Blood 2006;108:4288–4290 [PubMed: 16946302].
11. Champlin RE, Schmitz N, Horowitz MM, et al. Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). Blood 2000;95:3702–3709 [PubMed: 10845900].
12. Powles R, Mehta J, Kulkarni S, et al. Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: A randomized trial. Lancet 2000;355:1231–1237 [PubMed: 10770306].
13. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med 2001;344:175–181 [PubMed: 11172139].
14. Couban S, Simpson DR, Barnett MJ, et al. A randomized multicenter comparison of bone marrow and peripheral blood in recipients of matched sibling allogeneic transplants for myeloid malignancies. Blood 2002;100:1525–1531 [PubMed: 12176866].
15. Ringden O, Labopin M, Bacigalupo A, et al. Transplantation of peripheral blood stem cells as compared with bone marrow from HLAidentical siblings in adult patients with acute myeloid leukemia and acute lymphoblastic leukemia. J Clin Oncol 2002;20:4655–4664 [PubMed: 12488410].
16. Przepiorka D, Weisdorf D, Martin P, et al. 1994; Consensus conference on acute GVHD grading. Bone Marrow Transplant 1995:825–828 [PubMed: 7581076].
17. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. Am J Med 1980;69:204–217 [PubMed: 6996481].
18. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat 1958;53:457–481.
19. Cox DR. Regression models and life tables (with discussion). J Royal Stat, Series B 1972;34:187–220.
20. Lin YF, Lairson DR, Chan W, et al. Children with acute leukemia: A comparison of outcomes from allogeneic blood stem cell and bone marrow transplantation. Pediatr Blood Cancer 2011;56:143–151 [PubMed: 21108446].
21. Eapen M, Horowitz MM, Klein JP, et al. Higher mortality after allogeneic peripheral-blood transplantation compared with bone marrow in children and adolescents: The Histocompatibility and Alternate Stem Cell Source Working Committee of the International Bone Marrow Transplant Registry. J Clil Oncol 2004;22:4872–4880 [PubMed: 15520055].
22. Schrezenmeier H, Passweg JR, Marsh JC, et al. Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe acquired aplastic anemia. Blood. 2007;110:1397–1400 [PubMed: 17475907].
23. Garderet L, Labopin M, Gorin NC, et al. Patients with acute lymphoblastic leukaemia allografted with a matched unrelated donor may have a lower survival with a peripheral blood stem cell graft compared to bone marrow. Bone Marrow Transplant 2003;31:23–29 [PubMed: 12621503].
24. Pan L, Delmonte JJ, Jalonen CK, Ferrara JL. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. Blood 1995;86:4422–4429 [PubMed: 8541530].
25. Zeng D, Dejbakhsh JS, Strober S. Granulocyte colony-stimulating factor reduces the capacity of blood mononuclear cells to induce graft-versus-host disease: Impact on blood progenitor cell transplantation. Blood 1997;90:453–463 [PubMed: 9207483].
26. Cutler C, Giri S, Jeyapalan S, et al. Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: A meta-analysis. J Clin Oncol 2001;19:3685–3691 [PubMed: 11504750].
27. Tanimoto TE, Yamaguchi T, Tanaka Y, et al. Comparative analysis of clinical outcomes after allogeneic bone marrow transplantation versus peripheral blood stem cell transplantation from a related donor in Japanese patients. Br J Haematol 2004;125:480–493 [PubMed: 15142119].
28. Mohty M, Kuentz M, Michallet M, et al. Chronic graft-versus-host disease after allogeneic blood stem cell transplantation: Long-term results of a randomized study. Blood 2002;100:3128–3134 [PubMed: 12384409].
29. Stem Cell Trialists' Collaborative Group. Allogeneic peripheral blood stem-cell compared with bone marrow transplantation in the management of hematologic malignancies: An individual patient data meta-analysis of nine randomized trials. J Clin Oncol 2005;23:5074–5087 [PubMed: 16051954].

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# Naturally occurring oncogenic GATA1 mutants with internal deletions in transient abnormal myelopoiesis in Down syndrome

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Brief Report

MYELOID NEOPLASIA

# Naturally occurring oncogenic GATA1 mutants with internal deletions in transient abnormal myelopoiesis in Down syndrome

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**Key Points**

- Naturally occurring oncogenic GATA1 mutants with internal deletions contribute to transient abnormal myelopoiesis in Down syndrome.

**Children with Down syndrome have an increased incidence of transient abnormal myelopoiesis (TAM) and acute megakaryoblastic leukemia. The majority of these cases harbor somatic mutations in the *GATA1* gene, which results in the loss of full-length GATA1. Only a truncated isoform of GATA1 that lacks the *N*-terminal 83 amino acids (GATA1-S) remains. We found through genetic studies of 106 patients with TAM that internally deleted GATA1 proteins (GATA1-IDs) lacking amino acid residues 77-119 or 74-88 (created by splicing mutations) contributed to the genesis of TAM in 6 patients. Analyses of GATA1-deficient embryonic megakaryocytic progenitors revealed that the GATA1 function in growth restriction was disrupted in GATA1-IDs. In contrast, GATA1-S promoted megakaryocyte proliferation more profoundly than that induced by GATA1 deficiency. These results indicate that the internally deleted regions play important roles in megakaryocyte proliferation and that perturbation of this mechanism is involved in the pathogenesis of TAM. (*Blood.* 2013;121(16):3181-3184)**

## Introduction

Children with Down syndrome (DS) are known to have a high risk of developing transient abnormal myelopoiesis (TAM) and subsequent acute megakaryoblastic leukemia (DS-AMKL).[1-4] Blast cells in the majority of patients with TAM and DS-AMKL have mutations in the second exon of the *GATA1* gene.[5,6] The mutations turn off the production of full-length GATA1. Instead, *N*-terminally truncated GATA1 protein (GATA1-S) was translated from the second methionine at codon 84, which is identical to the truncated GATA1 isoform found in the healthy human.[7] In contrast, only a few patients with AMKL have been reported to harbor 21-disomy blasts with the GATA1 mutation.[8,9] Therefore, GATA1-S is believed to be a prerequisite for the pathogenesis of TAM and DS-AMKL in children with DS, and unrestricted proliferation of megakaryocytic progenitors in DS-AMKL is thought to be provoked by a mechanism involving GATA1-S. However, the molecular mechanism of how GATA1-S contributes to the genesis of TAM and DS-AMKL remains elusive.

GATA1 regulates the proliferation of immature megakaryocytic progenitors. Indeed, active proliferation of immature megakaryocytic progenitors derived from GATA1-deficient mouse embryos is restricted by introduction of wild-type GATA1, but not by GATA-S.[10] GATA1-deficient mice rescued with transgenic expression of GATA1-S (or GATA1-ΔNT) are found to exhibit hyper-megakaryopoiesis in a limited embryonic and postnatal period, resembling the phenotype in human TAM cases.[11] In contrast, another report indicates that targeting mice expressing GATA1 protein with a deletion of 64 *N*-terminal amino acids, but retaining the 65th to 83rd amino acid residues intact, has demonstrated that the embryos display a transient megakaryocytic phenotype only during the early embryonic stage, not in the late-embryonic and postnatal stages.[12] We surmise that this difference simply may be a result of missing the region corresponding to the 65th to 83rd amino acids.

Here, we have identified novel GATA1 mutants with internal deletions (IDs) of either amino acid residues 77-119 or 74-88 (GATA1-IDs) in 6 patients. We found that the GATA1-IDs lost their activity in the regulation of megakaryocyte growth. These results demonstrate that disruption of ID regions is implicated in the pathogenesis of TAM.

## Study design

This study was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine. All animal experiments

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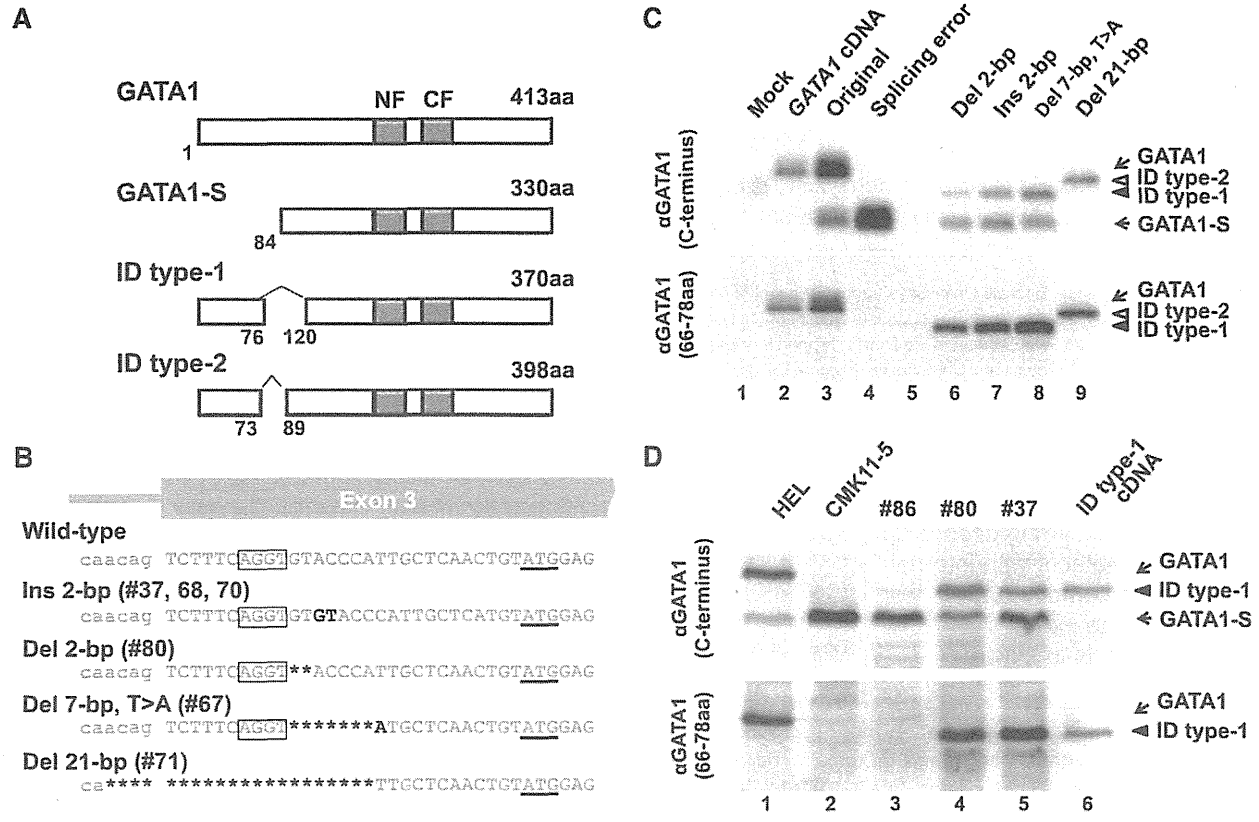


**Figure 1. GATA1 mutant proteins with internal deletions.** (A) A schema of mutant GATA1 proteins observed in patients with TAM. The amino acid sequence of GATA1-ID proteins was deduced from the sequence of *GATA1* cDNA obtained from patients with TAM. Dark boxes indicate *N*-finger (NF) and C-finger (CF) domains. ID indicates internal deletion. (B) Somatic mutations of the *GATA1* gene found in ID type 1 and type 2 patients. Missing, inserted, or substituted nucleotides are highlighted with dark color. A second translation initiation codon located in the third exon is underlined. The AGGT sequence functioning as an alternative splice donor site in mutant *GATA1* genes of ID type 1 patients is circled. Note that a mutant *GATA1* gene found in TAM patient 71 (ID type 2) lost a splice acceptor site in exon 3 because of the 21-nucleotide deletion. (C) Expression of GATA1 proteins in cells transfected with minigenes using anti-GATA1 antibodies recognizing the C terminus (upper) and residues between the 66th and 78th amino acids (lower) of the GATA1 protein. GATA1-ID proteins are recognized by the antibody against amino acid residues 66-78 of GATA1, whereas GATA1-S is not (lanes 6-9). Cells transfected with mock pcDNA3.1 (lane 1), pcDNA3.1-*GATA1* cDNA (lane 2), original minigene (lane 3), and *GATA1* minigene harboring a splicing error mutant in the 3′ boundary of intron 1[13] (lane 4) are used as positive and negative controls for GATA1 and GATA1-S, respectively. (D) GATA1 ID type 1 protein and GATA1-S are detected in the TAM blast cells from patients 80 (lane 4) and 37 (lane 5), whereas only GATA1-S is expressed in the blast cells from patient 86 harboring a conventional type of *GATA1* gene mutation in TAM cases (lane 3). Note that relatively abundant GATA1-S is recognized in patient 37 because of the intermixing of genetically distinct clone of cells expressing only GATA1-S (supplemental Table 1). Human erythroleukemia cells (HEL, lane 1) were used as a control for GATA1 and GATA1-S. DS-AMKL cells (CMK11-5, lane 2) and BHK-21 cells transfected with cDNA encoding GATA1 ID type 1 protein (lane 6) were used as controls for GATA1-S and GATA1 ID type 1, respectively.

were approved by the Institutional Animal Experiment Committee of Tohoku University. All clinical samples were obtained with informed consent from the parents of all patients with TAM in accordance with the Declaration of Helsinki. Additional information can be found in the supplemental text on the *Blood* website.

## Results and discussion

Between 2003 and 2010, we screened *GATA1* mutations by direct sequencing, using cDNAs prepared from TAM blasts provided by 106 patients with DS on request from referring hospitals. Acquired *GATA1* mutations were detected in 99 (93.4%) patients (supplemental Table 1). The majority of the mutations resulted in the GATA1-S mutant protein, which lacks the entire *N*-terminal transactivation domain. Importantly, we found new mutations harboring IDs of 43 and 15 amino acids in 5 patients (patients 37, 67, 68, 70, and 80) and in 1 patient (patient 71), respectively. We refer to these mutants as GATA1-ID type 1 and GATA1-ID type 2, respectively (Figure 1A). Clinical features in patients with TAM who have GATA1-ID mutations were shown in supplemental Table 2. All of these patients showed high white blood cell counts in the peripheral blood, which is known to be a risk factor for early death.[13]

We determined the genomic DNA sequences of these cases. As shown in Figure 1B, the mutations in GATA1-ID type 1 were located in a site immediately 3′ of the consensus motif for a splice donor site AGGT[14] (Ins 2-bp in patients 37, 68, and 70; Del 2-bp in patient 80; and Del 7-bp T>A in patient 67), whereas 21 bp containing a splice acceptor site in front of exon 3 was deleted in GATA1-ID type 2 (Del 21-bp). To verify the transcripts achieved through the putative splice donor site created by mutations in GATA1-ID type 1, we introduced identified mutations into *GATA1* minigene expression vectors[13] and transduced them into hamster fibroblast cell line BHK-21. We found 3 variant transcripts in the cases of GATA1-ID type 1 mutations (supplementary Figure 1A-B): a full-length transcript with deletion or insertion of nucleotides [Ex-2 (+) (PTC)], a short transcript lacking exon 2 by alternative splice variant skipping of exon 2 for GATA1-S [Ex-2 (−)], and an aberrant transcript in which 129 nucleotides were spliced out from exon 3 (Del 129-bp). In contrast, 2 disparate transcripts with deletions of 45 or 137 nucleotides were created by

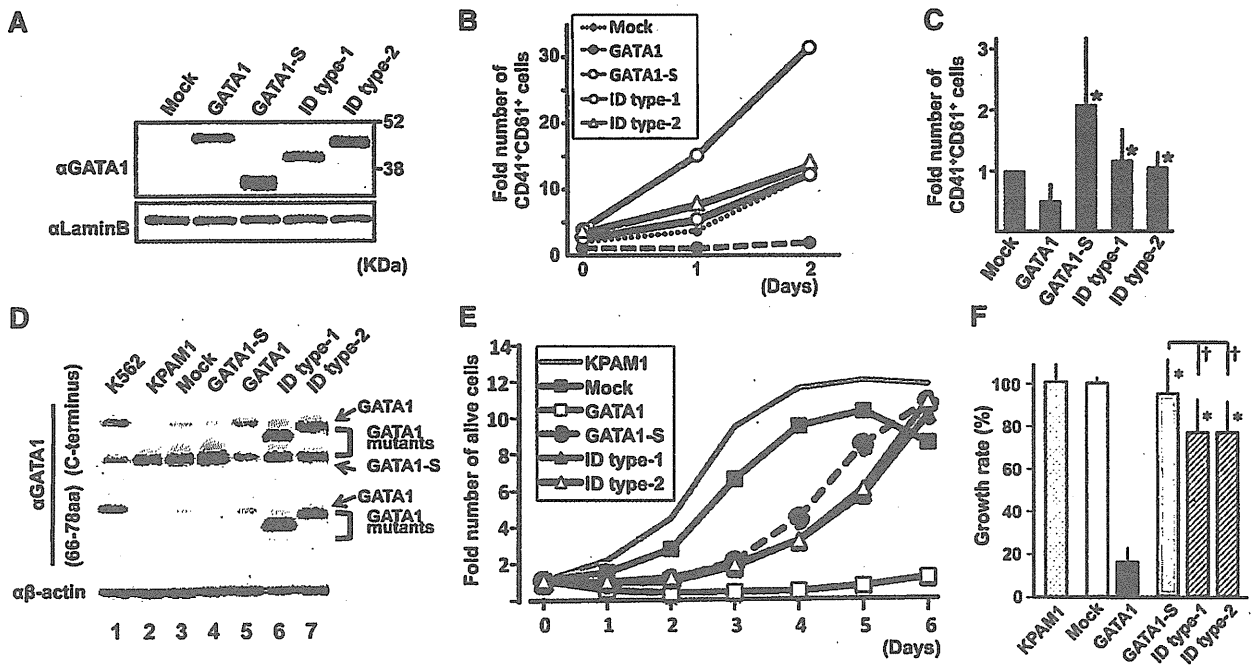


**Figure 2. GATA1 ID proteins showed restricted antiproliferative activity.** (A) Expression of GATA1 and GATA1 mutant proteins in cultured megakaryocytes at day 0, using an antibody against the C terminus of GATA1. The amount of protein loaded was quantified using an anti-Lamin B antibody on the same membrane. (B) Time-course change in the number of $CD41^+CD61^+$ cells. The value in the mock case at day 0 is set to 1. The result is representative of 4 independent experiments. (C) Comparison of the number of $CD41^+CD61^+$ cells at day 2. The value in the mock case is set to 1 in every experiment. The mean values and standard deviations from 4 independent experiments are presented. Asterisks indicate a significant difference compared with wild-type GATA1 ($P < .05$). (D) Immunoblot analysis of ectopic expression of GATA1 proteins in KPAM1 cells using anti-GATA1 antibodies against C terminus (upper) and residues between amino acids 66 and 78 (middle). The loading volume was quantified using anti-β-actin antibody (lower). (E) Growth curves of KPAM1 cells after ectopic expression of GATA1 proteins. Average values obtained from 6 wells are shown. The value at day zero is set to 1 for each. The growth curve of the original KPAM1 cells was analyzed as a control. Representative data from 3 independent experiments are shown. (F) Relative growth rate of KPAM1 cells at 5 days after ectopic expression of GATA1 mutant proteins. The average value of growth rate in the mock case is set to 100% in every experiment. The mean values and standard deviations from 18 wells obtained in 3 independent experiments (6 wells in each) are presented. Asterisks and daggers indicate significant differences compared with wild-type GATA1 and GATA-S, respectively ($P < .01$).

mutation in GATA1-ID type 2, using alternative acceptor sites in exon 3.

To examine whether the GATA1-ID proteins were produced from the mutant alleles, we performed immunoblotting analysis with 2 distinct antibodies recognizing the C terminus and amino acids 66-78, respectively. We detected GATA1-ID type 1 protein in addition to GATA1-S in the cells transfected with the minigenes harboring Ins 2-bp, Del 2-bp, or Del 7-bp T>A mutations, whereas only GATA1-ID type 2 protein was expressed on transfection of the minigene with a Del 21-bp mutation (Figure 1C). Consistent with the minigene results, a significant amount of GATA1-ID type 1 protein and GATA1-S had accumulated in patients 80 and 37, whereas only GATA1-S was detected in the TAM blasts of patient 86, who had only a short transcript skipping exon 2 because of a point mutation in the exon 2–intron 2 boundary (Figure 1D). Thus, splicing errors were occurred in GATA1-ID type 1 and type 2 patients, leading to the production of GATA1-ID proteins.

We next examined how GATA1-ID proteins affect the proliferation of embryonic megakaryocytic progenitors. We retrovirally transduced GATA1-S and GATA1-ID mutants into lineage-negative cells derived from megakaryocyte-specific *Gata1*-deficient ($Gata1^{\Delta neo\Delta HS}$) embryos[15] and induced differentiation toward the megakaryocytic lineage. The number of $CD41^+CD61^+$ megakaryocytes was significantly higher in cases transduced with GATA1-ID proteins than with wild-type GATA1, despite almost equivalent expression levels of GATA1 proteins (Figure 2A-C). GATA1-S-transduced cells unexpectedly acquired a hyperproliferative potential compared with mock cells, probably because of an unknown function that resides in the GATA1 *N*-terminal region (Figure 2B-C).

We next analyzed cell proliferation using the DS-AMKL cell line KPAM1, in which GATA1-S was predominantly expressed with a very low level of full-length GATA1 (Figure 2D).[16] On transduction with full-length GATA1 retrovirus, proliferation of KPAM1 cells was markedly reduced. In contrast, GATA1-ID type 1 and type 2 moderately restricted the proliferation of KPAM1 cells, but the restriction activity was significantly stronger than that of GATA1-S (Figure 2E-F). These results thus demonstrate that the ID regions indeed contribute to the regulation of AMKL cell proliferation.

Our newly identified GATA1-ID mutants have highlighted a much narrower set of sequences responsible for the pathogenesis of TAM than has previously been suggested by the loss of the *N*-terminal sequence, as in GATA1-S. The missing region identified by the GATA1-ID proteins contains a consensus motif (LxCxE, amino acids 81-85) essential for the interaction with pRb,[17] which is also lost in GATA1-S. Interaction with hypophosphorylated pRb-E2F complex has been reported to be important for GATA1 to support the normal proliferation and differentiation of erythroid progenitors.[17] Consistent with this notion, GATA1-S failed to repress E2F activation, which was followed by activation of mTOR signaling in the GATA1-S fetal megakaryocytes and DS-AMKL cells.[18] Because the protein levels of cyclin D1 and $p27^{Kip}$ are reciprocally regulated by the

mTOR pathway, and thereby cause pRb to be phosphorylated,[19] cell-cycle progression in response to the mTOR pathway may be potentiated by the enfeebled function of LxCxE motif of GATA1-S. Thus, we are one step closer to a molecular understanding of GATA1-related leukemias.

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## Authorship

Contribution: T.T., R.K., E.K., H. Kaneko, R.W., and K.T. contributed to the experiments; T.T., M.S., R.S., M.Y., and E.I. contributed to the study design, funding, project conception, and manuscript writing; and H. Kanegane, M.M., M.E., T.M., S.A., and Y.H. contributed to the clinical sample collection and phenotype analyses.

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## References

1. Zipursky A, Poon A, Doyle J. Leukemia in Down syndrome: a review. *Pediatr Hematol Oncol.* 1992;9(2):139-149.
2. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, Head DR. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia.* 2003;17(2):277-282.
3. Hitzler JK. Acute megakaryoblastic leukemia in Down syndrome. *Pediatr Blood Cancer.* 2007;49 (7 Suppl):1066-1069.
4. Malinge S, Izraeli S, Crispino JD. Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome. *Blood.* 2009; 113(12):2619-2628.
5. Wechsler J, Greene M, McDevitt MA, Anastasi J, Karp JE, Le Beau MM, Crispino JD. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet.* 2002; 32(1):148-152.
6. Xu G, Nagano M, Kanezaki R, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. *Blood.* 2003;102(8):2960-2968.
7. Rainis L, Bercovich D, Strehl S, et al. Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. *Blood.* 2003;102(3):981-986.
8. Harigae H, Xu G, Sugawara T, Ishikawa I, Toki T, Ito E. The GATA1 mutation in an adult patient with acute megakaryoblastic leukemia not accompanying Down syndrome. *Blood.* 2004; 103(8):3242-3243.
9. Hama A, Yagasaki H, Takahashi Y, et al. Acute megakaryoblastic leukaemia (AMKL) in children: a comparison of AMKL with and without Down syndrome. *Br J Haematol.* 2008;140(5):552-561.
10. Muntean AG, Crispino JD. Differential requirements for the activation domain and FOG-interaction surface of GATA-1 in megakaryocyte gene expression and development. *Blood.* 2005; 106(4):1223-1231.
11. Shimizu R, Kobayashi E, Engel JD, Yamamoto M. Induction of hyperproliferative fetal megakaryopoiesis by an N-terminally truncated GATA1 mutant. *Genes Cells.* 2009;14(9): 1119-1131.
12. Li Z, Godinho FJ, Klusmann JH, Garriga-Canut M, Yu C, Orkin SH. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. *Nat Genet.* 2005; 37(6):613-619.
13. Kanezaki R, Toki T, Terui K, et al. Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. *Blood.* 2010;116(22):4631-4638.
14. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* 1987;15(17): 7155-7174.
15. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J.* 1997;16(13):3965-3973.
16. Toki T, Kanezaki R, Adachi S, et al. The key role of stem cell factor/KIT signaling in the proliferation of blast cells from Down syndrome-related leukemia. *Leukemia.* 2009;23(1):95-103.
17. Kadri Z, Shimizu R, Ohneda O, et al. Direct binding of pRb/E2F-2 to GATA-1 regulates maturation and terminal cell division during erythropoiesis. *PLoS Biol.* 2009;7(6):e1000123.
18. Klusmann JH, Godinho FJ, Heitmann K, et al. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. *Genes Dev.* 2010;24(15): 1659-1672.
19. Gera JF, Mellinghoff IK, Shi Y, et al. AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. *J Biol Chem.* 2004; 279(4):2737-2746.

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ORIGINAL ARTICLE

# High *WT1* mRNA expression after induction chemotherapy and *FLT3*-ITD have prognostic impact in pediatric acute myeloid leukemia: a study of the Japanese Childhood AML Cooperative Study Group

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**Abstract** The prognostic value of *WT1* mRNA expression in pediatric acute myeloid leukemia (AML) remains controversial. A sample of newly diagnosed ($n = 158$) AML patients from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, were simultaneously analyzed for *WT1* expression, cytogenetic abnormalities and gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*). *WT1* expression (including more than 2,500 copies/μgRNA) was detected in 122 of the 158 (77.8 %) initial diagnostic AML bone marrow samples (median 45,500 copies/μgRNA). Higher *WT1* expression was detected in French American British (FAB)-M0, M3, M7 and lower expression in M4 and M5. Higher *WT1* expression was detected in AML with inv(16), t(15;17) and Down syndrome and lower in AML with 11q23 abnormalities. Multivariate analyses demonstrated that *FLT3*-internal tandem duplication (ITD), *KIT* mutation, *MLL*-partial tandem duplication were correlated with poor prognosis; however, higher *WT1* expression was not. *FLT3*-ITD was correlated with *WT1* expression and prognosis. Furthermore, 74 *WT1* expression after induction

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chemotherapy was analyzed. Higher *WT1* expression after induction chemotherapy was significantly correlated with M1 or M2/M3 marrow, *FLT3*-ITD and poor prognosis. Multivariate analyses in 74 AML patients revealed that *FLT3*-ITD, *MLL*-PTD, and *KIT* mutations were associated with poor prognosis; however, *NRAS* Mutation, *KRAS* mutation and high *WT1* expression (>10,000 copies/μgRNA) did not show poor prognosis. Our findings suggest that higher *WT1* expression at diagnosis does not correlate with poor prognosis, but that *WT1* expression after induction chemotherapy is considered to be a useful predictor of clinical outcome in pediatric AML.

**Keywords** *WT1* · *FLT3* · AML · Pediatric

## Introduction

The risk classification for acute myeloid leukemia (AML) patients according to cytogenetic abnormalities has been widely accepted in pediatric and adult AML [1–4]. AML patients with t(8;21), inv(16) and t(15;17) have been classified into the low risk (LR) group, while those with 5q- and -7 have been categorized into the high risk (HR) group, and others into the intermediate risk (IR) group [2–4]. Detecting chimeric transcripts such as, *PML-RARA* appears to be a good method for detecting minimal residual disease (MRD); however, approximately half of AML patients lack a suitable leukemia-specific marker; thus, there has been considerable interest in developing alternative approaches for MRD detection. One strategy involves the use of flow cytometer to identify and monitor leukemia-associated aberrant phenotypes [5]. Another approach is to detect Wilms tumor 1 (*WT1*) mRNA, which is highly expressed in AML blasts relative to normal PB and BM using a quantitative reverse transcribed-polymerase chain reaction (qRT-PCR) [6–12]. Several researchers have suggested that *WT1* expression is a good prognostic marker; thus, monitoring of the *WT1* mRNA expression is useful for MRD [7–12]. However, the prognostic relevance of *WT1* expression in AML at diagnosis is still controversial. Previous research has suggested that high *WT1* expression at diagnosis correlated with poor prognosis [6–9], while some others did not [10, 11, 15]. Recent work suggests that high *WT1* expression after chemotherapy correlates with poor prognosis [13–15]. This may depend on the uncertain mechanism of *WT1* mRNA expression in AML blasts due to the fact that *WT1* has a role not only for the suppression of tumors, but also for oncogenic potential [16, 17]. Recently, *FLT3*-internal tandem duplication (ITD) [18–21], *MLL*-partial tandem duplication (PTD) [20, 21] and *KIT* gene mutations [22, 23] have been strongly associated with a poor prognosis of adult and pediatric AML. A recent study suggested that *NRAS* gene alterations were associated with poor prognosis, while others did not find the same results [24]. High *WT1* expression was suggested to correlate with *FLT3*-ITD [25], however, the association between *WT1* expression and several gene alterations remains unknown in adult and pediatric AML. Recently, *WT1* gene mutations were frequently found in AML patients with normal karyotypes who showed poor clinical outcome [26, 27].

Thus, the present study aimed to simultaneously investigate *WT1* mRNA expression and examine the association of *WT1* mRNA expression with several gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*) in a sample of 158 newly diagnosed AML patients. We found that *FLT3*-ITD patients showed higher *WT1* mRNA expression at time of diagnosis as well as after the induction of chemotherapy; however, the prognostic significance of *WT1* mRNA expression was found only after induction chemotherapy. We discuss the usefulness of *WT1* mRNA expression associated with several gene alterations in pediatric AML patients.

## Patients and methods

### Patients

Patients included 318 newly diagnosed pediatric AML patients [0–15 years old, (240 de novo AML patients, 32 t(15;17)-AML, and 46 Down syndrome related myeloid leukemia (DS-ML)] from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, recruited between January 2000 and December 2002 [23, 28, 29]. Diagnosis of AML was made according to French–American–British (FAB) classification. Cytogenetic analysis was performed using the G-banding method. Among them, 158 samples at diagnosis were available for molecular analysis including, 13 patients with FAB-M3 and 10 patients with Down syndrome (DS) who were treated with different treatment protocols (see Table 1). Excluding FAB-M3 and DS, there were no significant differences between the analyzed 135 patients and the unanalyzed 105 patients regarding age (median, 6 years [range: 0–15 years] versus 6 years [range: 0–15 years], respectively) and initial WBC count (median, $24.8 \times 10^9$/L [range: $1.65–621.0 \times 10^9$/L] versus $13.8 \times 10^9$/L [range: $1.0–489.0 \times 10^9$/L, $p = .0764$], respectively). Also, no significant difference between the analyzed and not-analyzed patients in FAB-M3 and DS was found. The treatment protocol and risk classification were previously reported and described elsewhere [23, 28, 29]. We analyzed *WT1* expression in 85 paired bone marrow patient samples after 1st induction chemotherapy and also tested the blast counts for M1 (blast counts <5 %), M2 (5 % ≤ blast counts <25 %), M3 marrow (>25 %) at 22–74 days (median, 44 days) after the

Table 1 Genetic alterations and *WT1* mRNA expression in AML subgroup according to FAB classification

| FAB classification | No. of patients | *FLT3*-ITD | *FLT3*-D835Mt | *MLL*-PTD | *KIT* -Mt | *NRAS* -Mt | *KRAS* -Mt | *WT1* expression (median) (copies/μgRNA)* | |
|---|---|---|---|---|---|---|---|---|---|
| M0 | 6 | 1 | 0 | 1 | 0 | 2 | 0 | 64,750 | (2,400–190,000) |
| M1 | 24 | 4 | 2 | 7 | 0 | 1 | 2 | 23,000 | (0–390,000) |
| M2 | 46 | 4 | 2 | 5 | 8 | 4 | 4 | 23,000 | (85–680,000) |
| M3 | 13 | 3 | 3 | 0 | 0 | 0 | 0 | 140,000 | (450–290,000) |
| M4 | 22 | 1 | 1 | 4 | 3 | 2 | 3 | 9,200 | (50–150,000) |
| M5 | 25 | 5 | 3 | 3 | 0 | 2 | 5 | 9,600 | (0–440,000) |
| M6 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 670 | – |
| M7 | 9 | 1 | 0 | 1 | 0 | 0 | 0 | 36,000 | (0–980,000) |
| M7 with DS | 10 | 0 | 0 | 0 | 1 | 0 | 1 | 63,500 | (140–630,000) |
| Unclassified | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 27,000 | (14,000–40,000) |
| Total | 158 | 20 | 11 | 21 | 12 | 11 | 15 | 23,500 | (0–980,000) |
| | (%) | 12.7 | 7.0 | 13.3 | 7.6 | 7.0 | 9.5 | | |

*ITD* internal tandem duplication, *PTD* partial tandem duplication, *Mt* mutation, *DS* Down syndrome

* The difference of median *WT1* mRNA expression in each subgroups according to FAB classification was statistically significant ($p = 0.0011$)

initiation of induction chemotherapy. The evaluation for complete remission (CR) was conducted after two consecutive induction chemotherapy cycles. We also analyzed *WT1* expression at the time point after three consecutive chemotherapy cycles ($n = 56$) and the completion of therapy ($n = 47$).

Next, the 28 bone marrow samples from the healthy individuals who were the donors for bone marrow transplantation aged 7–52 years old from Nagoya University Hospital were also analyzed. Informed consent was obtained from the patients or their care-givers, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center and Nagoya University Hospital approved the present study.

## Detection of *WT1* mRNA expression by qRT-PCR

Total RNA extracted from the bone marrow samples was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *WT1* expression was measured using real time PCR system (ABI 7700, Applied Biosystems). A set of primers used in the present study were as follows, *WT1* forward primer located in exon 6 (5′-GAT AAC CAC ACA ACG CCC ATC-3′) and reverse primer located in exon 6 and 7 (5′-CAC ACG TCG CAC ATC CTG AAT-3′) [12]. The TaqMan probe (5′-ACA CCG TGC GTG TGT ATT CTG TAT TGG-3′) was designed to hybridize at the sense strand of exon 6 and labeled with FAM as reporter dye at the 5′ end and with the quencher dye carboxy-teramethyl-rhodamin (TAMRA) at the 3′ end terminus. As endogenous mRNA control ubiquitous gene *GAPDH* mRNA was simultaneously quantified using a set of primers [forward primer (5′-GAA GGT GAA GGT CGG AGT C-3′), reverse primer (5′-GAA GAT GGT GAT GGG ATT TC-3′)] and TaqMan probe (5′-CAA GCT TCC CGT TCT CAG CC-3′). The *WT1* expression was corrected by the each *GAPDH* mRNA expression and multiplied by $2.7 \times 10^7$ (copies/μgRNA), because 1.0 μg RNA contains $2.7 \times 10^7$ GAPDH. *WT1* mRNA expression was examined in 158 individuals from the diagnostic bone marrow samples, 85 paired patient samples after the 1st induction chemotherapy, 56 samples after three consecutive chemotherapy sessions, and 47 samples during the finalization of therapy.

## Analyses of *FLT3*, *KIT*, *MLL* and *RAS* genes

Mutational analysis for internal tandem duplication (ITD) within the JM domain and D835 mutation (D835Mt) within the TK2 domain of *FLT3* were performed as previously described [30, 31]. The mutation analysis of kinase domain, extracellular domain and transmembrane domain for the *KIT* gene was performed with RT-PCR followed by direct sequencing as previously reported [23]. *MLL*-partial tandem duplication (PTD) was examined by simple first round RT-PCR with 35 cycles using the primer pair 6.1 (located on exon 9) and E3AS (located on exon 4) as previously described [21, 32, 33]. The amplified products were purified and directly sequenced to confirm the *MLL*-PTD.

Exons 2 and 3 of the *NRAS* and *KRAS* genes were amplified by RT-PCR and directly sequenced using primer pairs for *NRAS* as previously reported. [34]

## Statistical analysis

Estimates of the survival distributions were performed using the Kaplan–Meier method. Differences were

compared using a log-rank test. Overall survival (OS) was defined as the time from diagnosis to death (owing to any cause), or to the last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to the date of relapse and death. Patients with Down syndrome ($n = 10$) or FAB-M3 ($n = 13$) were treated using different protocols, thus, we analyzed the 5-year OS and EFS in 135 patients except for patients with DS and FAB-M3. Mann–Whitney $U$ tests were performed to detect the statistical differences between two groups and the Krusukal–Wallis test was performed to detect differences among several groups. The prognostic significance of the clinical variables was assessed using a Cox proportional hazards model. These statistical analyses were performed with the statistical software StatView (Abacus Concepts, Inc.). For all analyses, the $p$ values were two-tailed, and a $p$ of less than 0.05 ($p < .05$) was considered to be statistically significant.

## Results

### *WT1* mRNA expression in normal bone marrow samples

We analyzed *WT1* mRNA expression in 28 RNA samples extracted from mononuclear cells of normal bone marrow samples and found that it ranges from 123 to 3,725 copies/μgRNA (median, 1,200 copies/μgRNA, see supplemental Figure 1). We determined the cut-off value to be 2,500 copies/μgRNA, because the value for the 90th percentile was 2,519 copies/ugRNA. Alternatively, the cut-off value for peripheral blood was 50 copies/μgRNA while using the same method [12]. Additionally, we set the *WT1* mRNA expression for bone marrow samples more than 2,500 copies/μgRNA as *WT1* expression positive.

### *WT1* mRNA expression at diagnosis, gene alterations and clinical outcomes

The *WT1* expression at time of diagnosis in 122 (77.8 %) of the 158 AML patients demonstrated to have more than the cut-off value (2,500 copies/ugRNA, see supplemental Figure 1) and was markedly elevated (median, 45,500 copies/μgRNA). No association was found between *WT1* expression and sex, age or initial WBC count. Complete remission (CR) was obtained in 149 of the 158 patients (94.3 %) after two consecutive induction chemotherapy cycles; however, the median *WT1* expression at time of diagnosis was not statistically different in patients with or without CR. The *WT1* expression in each FAB subgroup was statistically different (p=0.0011), (see Table 1). High *WT1* mRNA expression was found in FAB-M0, M3, M7, whereas low levels of expression were found in M4 and M5 ($p < 0.0001$). The median *WT1* mRNA expression in each karyotypic subgroup is presented in Table 2. Higher *WT1* mRNA expression was found in t(15;17), inv(16) and in patients with Down syndrome, whereas lower expression of *WT1* was found in AML patients with an 11q23 abnormality. In particular, more than 90 % of t(15;17), inv(16) and Down syndrome samples presented with a *WT1*-positive (>2,500 copies/μgRNA); whereas more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative.

*WT1* expression and prognosis were not different between patients with and without *FLT3*-ITD in 13 t(15;17) AML patients. In FAB-M7, there was no difference in *WT1* expression in DS and non-DS patients.

Excluding 13 FAB-M3 and 10 DS patients, 102 out of 135 patients (75.6 %) were *WT1* positive at the time of diagnosis. Multivariate analyses in 135 AML patients revealed that *FLT3*-ITD, *MLL*-PTD and *KIT* mutations were associated with poor prognosis, however, *FLT3*-D835 mutation (Mt), *N-RAS* Mt, *K-RAS* Mt and high *WT1* expression (>100,000 copies/μgRNA) did not show poor prognosis in patients in 5-year OS and EFS (see Tables 3, 4). Specifically, *FLT3*-ITD was found in 20 (12.7 %) and *FLT3*-D835Mt was found in 11 (7.0 %) of the 158 patients. The median *WT1* mRNA expression of patients with *FLT3*-ITD, D835Mt and wild type *FLT3* was 89,500 copies/μgRNA (330–330,000 copies/μgRNA), 85,000 copies/μgRNA (0–250,000 copies/μgRNA) and 18,500 copies/μgRNA (0-980,000 copies/μgRNA), respectively. This difference was statistically significant ($p = 0.0025$); however, our data suggested that only *FLT3*-ITD indicated poor prognosis [5-year overall survival for *FLT3*-ITD, D835Mt and wild type were 35.3, 100 and 84.3 %, respectively ($p < 0.0000001$)] [21]. For additional genetic alterations, *MLL*-PTD was found in 21 (13.3 %) out of 158 patients, the median *WT1* expression was not different in patients with (median, 21,000 copies/μgRNA) or without *MLL*-PTD (median, 28,000 copies/μgRNA), however, the prognosis was quite different within both subgroups [5y OS 56.3% in *MLL*-PTD (+) and 83.2 % in *MLL*-PTD(-)] [21]. *KIT* gene mutation (Mt) was found in 12 (7.6 %) out of 158 patients. The median *WT1* expression was not different in patients with (13,500 copies/μgRNA) or without *KIT*-Mt (32,500 copies/μgRNA). Interestingly, the median *WT1* expression in t(8;21)-AML patients with or without *KIT*-Mt was statistically different [3,300 copies/μgRNA (85-55,000 copies/μgRNA) when compared with 30,000 copies/μgRNA (200-680,000 copies/μgRNA)] ($p = 0.020$). Mutation in codons 12, 13 or 61 of the *NRAS* gene was found in 11 (7.0 %) out of 158 patients, the median *WT1* expression was not different in patients with (36,000 copies/μgRNA) or without *NRAS*-Mt (23,000 copies/μgRNA). Mutation in codons 12, 13 or 61 of the *KRAS* gene in 15 (9.5 %) out of

Table 2 Genetic alterations and *WT1* mRNA expression in AML subgroup according to karyotypic abnormalities

| Karyotypic abnormalities | Number of patients | *FLT3*-ITD | *FLT3*-D835Mt | *MLL*-PTD | *KIT* | *NRAS* | *KRAS* | *WT1* mRNA expression (copies/μgRNA) | |
|---|---|---|---|---|---|---|---|---|---|
| Normal | 33 | 9 | 2 | 8 | 2 | 2 | 3 | 18,000 | (73–280,000) |
| t(8;21) | 46 | 2 | 1 | 4 | 8 | 4 | 5 | 23,500 | (85–680,000) |
| inv(16) | 7 | 0 | 2 | 0 | 1 | 2 | 0 | 56,000 | (8,700–220,000) |
| 11q23 abnormalities | 20 | 0 | 1 | 5 | 0 | 1 | 4 | 720 | (0–150,000) |
| t(15;17) | 13 | 3 | 3 | 0 | 0 | 0 | 0 | 140,000 | (450–290,000) |
| Down syndrome | 10 | 0 | 0 | 0 | 1 | 0 | 1 | 63,500 | (140–360,000) |
| Others[a] | 29 | 6 | 2 | 4 | 0 | 2 | 2 | 23,000 | (0–980,000) |
| Total | 158 | 20 | 11 | 21 | 12 | 11 | 15 | 32,000 | (0–980,000) |
| | (%) | 12.7 | 7.0 | 13.3 | 7.6 | 7.0 | 9.5 | | |

The median value of *WT1* mRNA expression is shown and the difference was statistically significant ($p = 0.0003$)

[a] The subgroup of other karyotypic abnormalities included 2 patients with unknown karyotype

Table 3 Prognostic factors for overall survival in 135 AML patients except for FAB-M3 and Down syndrome

| Variable | *P* value | Odds ratio | 95% CI |
|---|---|---|---|
| *FLT3*-ITD | <0.0001 | 6.767 | 2.844–16.101 |
| *FLT3*-D835Mt | n.d. | n.d. | n.d. |
| *MLL*-PTD | 0.0443 | 2.229 | 1.021–4.870 |
| *KIT*-Mt | 0.0148 | 3.661 | 1.290–10.395 |
| *NRAS*-Mt | 0.3538 | 1.789 | 0.523–6.122 |
| *KRAS*-Mt | 0.1313 | 2.407 | 0.769–7.537 |
| *WT1*>100,000 copies/μgRNA | 0.8733 | 0.931 | 0.386–2.244 |

Table 4 Prognostic factors for Event Free Survival in 135 AML patients except for FAB-M3 or Down syndrome

| Variable | *P* value | Odds ratio | 95% CI |
|---|---|---|---|
| *FLT3*-ITD | 0.0015 | 3.455 | 1.607–7.430 |
| *FLT3*-D835Mt | 0.3770 | 0.404 | 0.054–3.019 |
| *MLL*-PTD | 0.0213 | 2.177 | 1.123–4.221 |
| *KIT*-Mt | 0.0013 | 3.816 | 1.686–8.635 |
| *NRAS*-Mt | 0.7613 | 0.832 | 0.254–2.726 |
| *KRAS*-Mt | 0.1849 | 1.852 | 0.745–4.607 |
| *WT1*>100,000 copies/μgRNA | 0.9169 | 0.960 | 0.447–2.063 |

158 patients, the median *WT1* expression was not different with (23,000 copies/μgRNA) or without *KRAS*-Mt (24,000 copies/μgRNA).

In patients with normal karyotype, the median *WT1* expression in patients with and without *FLT3*-ITD were 65,000 copies/μgRNA (73–280,000 copies/μgRNA 9 *FLT3*-ITD and 2 *FLT3*-D835Mt) and 7,150 copies/μgRNA (330–240,000 copies/μgRNA, 22 *FLT3* wild type), respectively, which was a statistically significant ($p = 0.023$).

In AML with 11q23 abnormalities, the median *WT1* expression was quite low (median, 720 copies/μgRNA) and more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative. There were significant differences among each of the subgroups with t(9;11) ($n = 8$; median = 35 copies/μgRNA); t(6;11) ($n = 3$; 81,000 copies/μgRNA); and other variants ($n = 9$; 780 copies/μgRNA) ($p = 0.0139$). Furthermore, there were significant differences in their clinical outcomes, with 5-year OS rates nearly to 100 % in t(9;11), 0 % in t(6;11) and 77.8 % in other variants, respectively.

*WT1* mRNA expression after 1st induction therapy

We also evaluated the *WT1* expression and blast counts in 85 bone marrow samples including 5 t(15;17) and 6 DS patients after 1st induction chemotherapy. There were no significant differences between the analyzed 85 patients and unanalyzed 73 patients regarding age (median 6 vs. 7-years old, respectively), WBC count (median 20,200 vs. 21,400/μl, respectively), or initial *WT1* expression level (median 21,000 vs. 32,000 copies/μg RNA, respectively).

In 5 t(15;17)-AML patients examined, *WT1* expression decreased by a log of 1–3 after induction chemotherapy except for one patient who showed 32,000 copies/μgRNA after induction chemotherapy. This patient died after relapse. In all six DS patients analyzed, *WT1* expression decreased by a log of 2–3 and all were cut-off value after induction chemotherapy. All these patients were alive without relapse.

Excluding 5 t(15;17) and 6 DS patients, 58 out of 74 patients (78.4 %) were *WT1* positive at the time of diagnosis (median, 18,000 copies/μgRNA) and 11 out of 74 patients (14.9 %) remained *WT1* positive after induction chemotherapy (median, 215 copies/μgRNA) (see