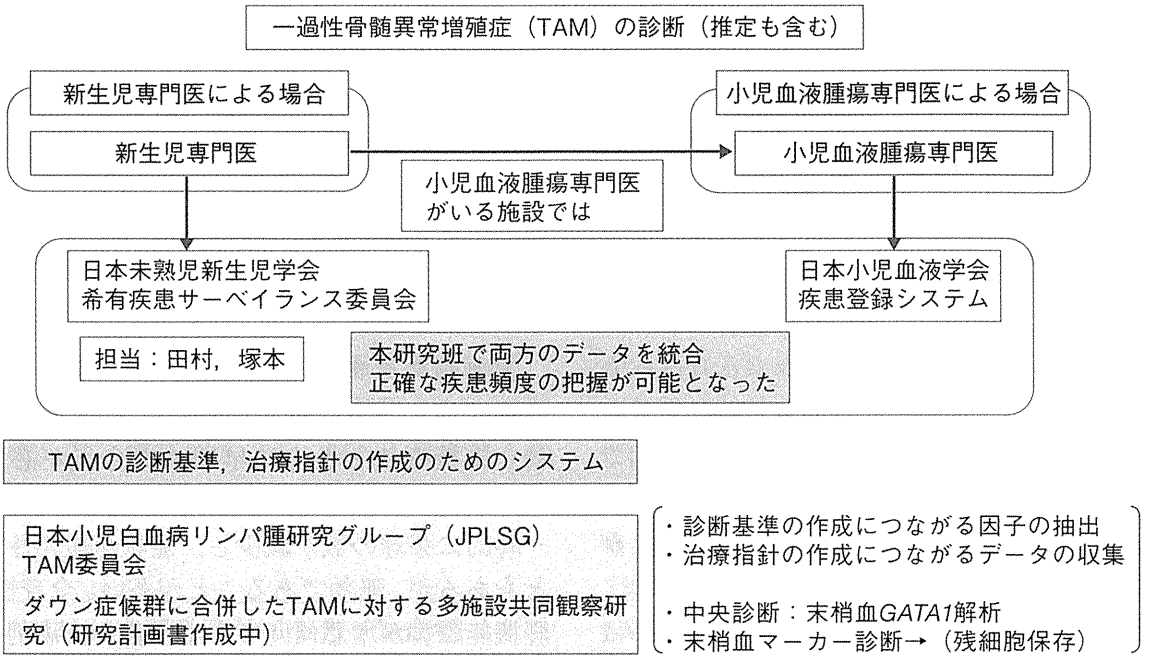

図　TAMの正確な患者数を把握するためのシステム

療指針の作成に関する研究」班(TAM班)ができ，今後診断と治療のガイドライン作成と科学的な臨床研究にもとづく治療成績の改善が期待される。また小児血液学会のシステムに登録されていない新生児側の症例については，日本未熟児新生児学会の希有疾患サーベイランス委員会の対象疾患として承認されたので，これまで把握ができなかった新生児施設のみに入院したTAM症例も把握できるようになった(図)。

おわりに

TAMの中でも約20%の予後不良群の臨床的特徴や生物学的特徴を明らかにし，これらに対する有効な治療法を確立することは重要である。そのためにも今後各科で連携して厚生労働省のTAM班による治療指針を確立し，多数例に対する前方視的研究を行う必要がある。

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特集：新 WHO 分類—MDS 委員会/白血病委員会

ダウン症候群に関連した骨髄増殖症

——2008 WHO 分類より——

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Myeloid Proliferation Related to Down Syndrome in the Updated 2008 WHO Classification

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Abstract Approximately 10% of Down syndrome (DS) neonates manifest a hematologic disorder referred to as transient abnormal myelopoiesis (TAM)/transient myeloproliferative disorder (TMD). Because this disorder resolves spontaneously, it is considered to have a good prognosis. In fact, 10-20% of these cases are life threatening or fatal. In 20% of the affected cases, acute megakaryoblastic leukemia (AMKL) subsequently develops before 4 years of age. The morphologic, immunophenotypic and genetic features of TAM are biologically identical to those of DS-AML. On the other hand, DS-AMKL has unique morphologic, immunophenotypic, clinical and molecular features, including *GATA1* mutation, that justify its separation from other AMKL. In Japan, more than 90% of cases with DS-AMKL achieved a complete remission, and the estimated 4-year event-free survival rate was 80%. Therefore this disorder is unique to children with DS. For the first time "myeloid proliferations related to DS" was classified as a distinct entity which includes (1) TAM and (2) myeloid leukemia associated with DS in the updated 2008 WHO Classification 4th edition. We introduce these new criteria and add a supplementary explanation.

要　旨　ダウン症候群（DS）では約 10%が，新生児期に一過性骨髄異常増殖症（TAM）あるいは一過性骨髄増殖性疾患（TMD）と呼ばれる一過性の類白血病反応を発症する．TAM は自然寛解するものが多く予後良好と考えられているが，実際は 10〜20%に致死的なものがある．TAM を発症した症例の約 20%が 4 歳までに骨髄異形成症候群（MDS）や急性巨核芽球性白血病（AMKL）に移行する．TAM の芽球は形態学，免疫表現型，遺伝子発現からも AMKL と区別ができない．一方 DS-AMKL は，特異な形態と免疫表現型，*GATA1* 遺伝子変異を含めた分子遺伝学的・臨床的特徴から他の AMKL とは区別される．日本では DS-AMKL は，治療強度を弱めた化学療法でも寛解導入率が 90%以上，4 年無病生存率も 80%を超えている．こうした特徴のため，2008 年改訂の WHO 分類第 4 版で，DS 関連骨髄増殖症がはじめて独立した病型として（1）TAM，（2）DS 関連骨髄性白血病に分類された．この新分類を抜粋し若干の解説を加えた．

Key words: Down syndrome, transient abnormal myelopoiesis, acute megakaryoblastic leukemia, GATA1, WHO classification

I. はじめに

急性白血病については，1976 年以来提唱されている細胞形態を基本とした FAB 分類が世界的に活用されてきた．染色体や遺伝子異常の情報が明らかとなり，治療選択にも分子レベルを基盤とした分類の必要性が高まっている．それを受け WHO 分類第 3 版が 2000 年に出されたが，小児に関しては成人の分類をそのまま当てはめることの問題点が指摘された[1]．

ダウン症候群（DS）では，約 10%が新生児期に一過性骨髄異常増殖症（transient abnormal myelopoiesis, TAM）あるいは一過性骨髄増殖性疾患（transient myelo-

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proliferative disorder, TMD）と呼ばれる一過性の類白血病反応を発症する．TAMは自然寛解するものが多く予後良好と考えられているが，実際は致死的なものがあり，どの時期にどのような治療を行うかを明らかにすることが重要な課題となっている．TAMの発症症例の約20%が，4歳までに骨髄異形成症候群（MDS）や急性巨核芽球性白血病（AMKL）に移行する．TAMの芽球は形態学，免疫組織学的，遺伝子発現からもAMKLと区別ができない．TAMでは通常は21トリソミー以外に核型に異常はないが，MDSやAMKLでは高率に付加的核型異常が認められる[2]．DS-AMKLは抗腫瘍薬に対する薬剤感受性が高く治療成績が向上している[3,4]．

近年，DSの白血病の分子機構についてさまざまなことが明らかになり，DS-MDS/AMLは特徴的で，他のAMLとは異なっていることが示され，2008年改訂のWHO分類第4版に「DSに関連した骨髄増殖症 Myeloid proliferation related to Down syndrome」がはじめて独立した病型として分類され加わった．新分類を紹介し若干の解説を加えた．

II. 「DSに関連した骨髄増殖症　Myeloid proliferation related to Down syndrome」—抜粋[5]

DSでは，non-DSと比べて白血病発症のリスクが10～100倍上昇する[6,7]．そのリスクは成人になっても同様である．4歳未満のnon-DSでは急性リンパ性白血病（ALL）と急性骨髄性白血病（AML）の比率は4:1であり，同じ年齢群のDSでは1.0:1.2でほぼ同等である．

5歳未満のDSではAMKLがnon-DSと比べおよそ150倍の頻度で発症する．4歳未満ではDSのAMLの70%はAMKLであるのに対して，non-DSでのその割合は3～6%である．DS-AMKLは，特異な形態と免疫表現型，*GATA1*遺伝子変異を含めた分子遺伝学的，臨床的特徴から，他の型のAMKLとは区別されている[8]．これらのさまざまな特徴はDS関連白血病が，WHO分類の中で特別な型の白血病として扱われる根拠となっている．4歳未満のDS-AMLには，独特な性質をもつAMKLが多く，それに加えてDSの新生児の約10%にはTAM/TMDと呼ばれる，形態的にDS-AMKLとは区別できない病態が存在する[9,10]．

この異常は数週間から3カ月で自然に軽快するが，異常があった症例の20～30%は，1～3年以内に自然軽快しないAMKLを発症する．DS児では上述した病態が注目されるが，これらの病態には年齢特異性があり，DS児にはALLとAMLのどちらも発症することを認識することが重要である．DS児でもnon-DS児と同様，詳細な形態学的，免疫組織学的，細胞遺伝学的または分子生物学的評価が必要であり，それに基づいた適切な治療が行われるべきである[11]．

1. 一過性骨髄異常増殖症：Transient abnormal myelopoiesis (TAM)/transient myeloproliferative disorder (TMD)

定義：TAMは，AMLとは臨床的，形態的に区別できないDSの新生児に特有な病態である．芽球は巨核球系の特徴をもっている．

疫学：TAMはDSの新生児の約10%に発症する．モザイク型21トリソミーで表現型が正常の新生児にもまれに発症する．

臨床的特徴：初診時には，血小板減少症がもっとも一般的な症状で，他の血球減少症は比較的少ない．著明な白血球増多（3～5万/μl，しばしば10万/μl以上）を呈し，芽球比率は骨髄よりも末梢血のほうがしばしば高い．合併症として肝脾腫があり，まれに，心不全，過粘稠度症候群，脾臓壊死，進行性肝線維症がある[12]．患者の約80%は生後3カ月以内に自然寛解するが，一部は生命を脅かし，または致死的な臨床経過をとる．

形態学的，免疫表現型：TAMの芽球形態，免疫表現型の特徴は，多くのDS-AMLと同じである．末梢血および骨髄中の芽球は，細胞質は好塩基性で，粗造な好塩基性顆粒とブレブという細胞突起を有し（Fig. 1），これらの芽球が巨核芽球であることを示唆している．末梢血で好塩基球増多症を示すものもあり，骨髄では赤芽球系，巨核球系細胞にしばしば異形成を認める[9]．TAMの芽球は特徴的な免疫表現型を示す[13]．表面マーカーは多くの症例でCD34, CD56, CD117, CD13, CD33, CD7, CD4 dim, CD42, TPO-R, IL-3R, CD36, CD61, CD71が陽性となるが，myeloperoxidase, CD15, CD14, glycophorin Aが陰性である．約30%の症例でHLA-DRが陽性である．巨核球系の診断には抗CD41抗体と抗CD61抗体の診断的価値が高い．

遺伝学：21トリソミーに加えて*GATA1*遺伝子体細胞変異がTAMの芽球に存在する[8,14,15]．発現アレイやゲノムアレイでDS-AMLとTAMの間に発現の相違があるという報告があるが，まだ追認されていない[16-18]．

予後と予後因子：高率に自然治癒する特徴があるが，1～3年後に，これらの20～30%がAMLに進行する[19]．TAMに対する化学療法はいまだ確立されていない．

2. DS関連骨髄性白血病：Myeloid leukemia associated with Down syndrome (ML-DS)

定義：5歳未満では，DS児はnon-DS児と比べて50倍の頻度で急性白血病になりやすい．DS-AMLは，通

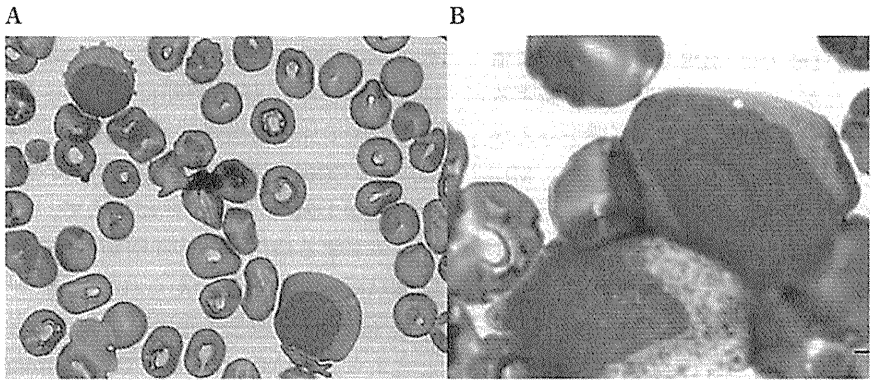

Fig. 1 Peripheral blood smear from 18-day-old infant with DS and TAM
A blood test revealed a white blood cell count of 11,300/μl (blast 11%), a hemoglobin concentration of 9.2 g/dl and a platelet count of $3.0\times10^4/\mu$l. Immunophenotypic analysis showed the presence of CD7, CD13, CD33, CD34, CD41, CD56, and HLA-DR. Cytogenetic study showed trisomy 21 as the sole abnormality. *GATA1* mutation was detected. The process resolved spontaneously over a period of 22 days. A: Two blasts showing basophilic cytoplasm. The intermediately differentiated blast has a cytoplasmic bleb (right). B: More undifferentiated blasts without blebs are also shown (right). (These pictures were provided from Hasegawa and Manabe)

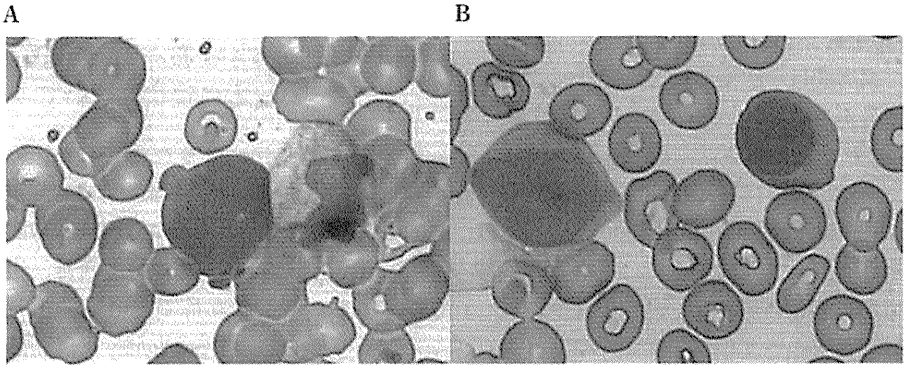

Fig. 2 Bone marrow smear from ML associated with DS in a 2-year-old boy who has no history of TAM
The bone marrow aspiration was dry tap. Immunophenotypic analysis showed that blasts were positive for CD7, CD13, CD33, and CD56, but negative for CD34 and CD41. In addition to trisomy 21, another chromosomal abnormality was detected. The *GATA1* mutation was detected. A: An intermediately differentiated blast showing basophilic cytoplasm, distinct nucleoi and cytoplasmic blebbing as show here (left). B: The undifferentiated blast doesn't have a cytoplasmic bleb (left). The erythroblast has coarse chromatin (right). (These pictures were provided from Hasegawa and Manabe)

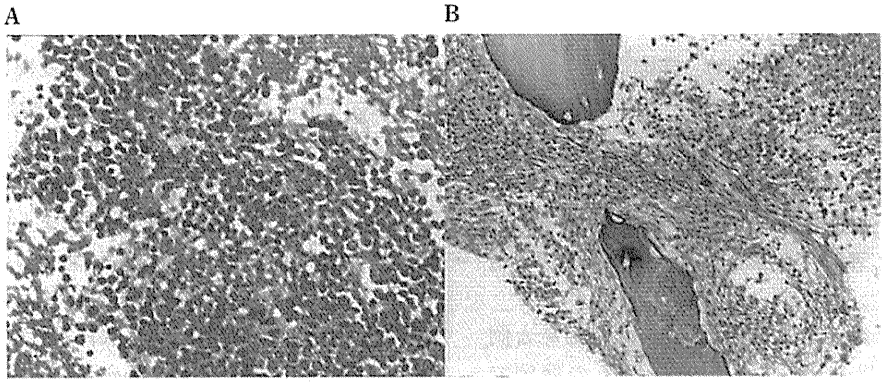

Fig. 3 Bone marrow biopsy in a patient with ML associated with DS (reproduced from Pediatric MDS CD Atlas 2002 edited by Committee for MDS in The Japanese Society of Pediatric Hematology)
A: A biopsy showed proliferation of leukemic blasts. B: On a biopsy specimen by silver stain, myelofibrosis, ranging from reticulin to collagenous is seen.

常AMKLであり，新生児期を超えたDSにおける急性白血病の50%にも上る．DSではMDSとAMLの間に生物学的相違がない．したがって両者を区別して診断することに意味はなく，予後にも治療にも何も影響を与えない．このような病型はDS児に特異なものであり，ML-DSには，MDSとAMLの両方を含んでいる．

疫学：DSにおけるMLの大部分は5歳までに発症する．DSの約1〜2%が5歳までにAMLを発症し，小児のAML/MDSの約20%はDS児である[1,9,12]．ML-DSはTAMの既往のある児の20〜30%にTAM発症後1〜3年の間に発症する．

臨床的特徴：この疾患の多くは，3歳までに発症する．骨髄の芽球が20%未満の場合は通常無症状で，血小板減少が初発症状となる．一般に，小児不応性血球減少（RCC）に相当する前白血病期が芽球の過剰なMDSまたは明らかな白血病に先行する．

形態学：数カ月続く前白血病期には，この疾患はRCCの特徴をもち有意な芽球の増加がない．赤血球細胞は大球性である．異形成は一次性の不応性血球減少症よりも顕著となる場合がある．

AMLの場合，芽球とときに赤芽球系前駆細胞が通常末梢血に存在する．赤血球はしばしば著明な細胞大小不均等，ときに涙滴赤血球を示す．血小板数は通常減少し，巨大血小板が観察される．

骨髄穿刺では，白血病芽球の形態は，円形から少し不規則な核をもち，細胞質は中等度の塩基性で，細胞質突起が存在する（Fig. 2）．細胞質に好塩基性顆粒に似た粗い顆粒を含んだ芽球がさまざまな程度でみられる．顆粒は通常myeloperoxidase陰性である．赤血球前駆細胞は，しばしば巨核球性変化や，二核，三核，核の断片化を含む異形成を示す．顆粒球系細胞の異形成がみられることもある．

骨髄中心部は網状線維の濃密なネットワークで構成され，骨髄は吸引困難となる（Fig. 3）．赤血球造血は芽球比率が低い症例で増加し，病気の進展とともに減少する．好中球系の成熟過程にある細胞は減少する．芽球が濃厚に浸潤している場合は，巨核球の異形成がまれにみられる．AMKLでは，巨核球が異形成の強い小型の小巨核球として集塊をなし著明に増加し，ときに前巨核球の増加をみる．

免疫表現型：DS-AMKLの細胞は，TAMの芽球と同様の表現型を示す[13]．表面マーカーは多くの症例でCD117, CD13, CD33, CD7, CD4, CD42, TPO-R, IL-3R, CD36, CD41, CD61 CD71が陽性となるが，myeloperoxidase, CD15, CD14, glycophorin Aは陰性である．しかしTAMと異なり，CD34は50%に，CD56とCD41はおよそ30%の症例で陰性である．DSの他の型のAMLの細胞は，おのおのAML分類に一致する表現型を示す．白血病細胞の免疫組織学的診断に際しては，TAM同様，CD41とCD61が巨核芽球系の診断にとくに有用である．

遺伝学：21トリソミーに加えて，転写因子GATA1をコードする遺伝子の体細胞変異はDSのTAMまたはMDS/AMLの病因と考えられている[8,14,20]．5歳以上のAMLは*GATA1*遺伝子変異がなく“通常の”MDS/AMLと考えられている．ML-DSでは，trisomy 8は13〜44%にみられ[1,21]，monosomy 7はたいへんまれである．

予後と予後因子：4歳未満の*GATA1*遺伝子変異のあるML-DSは，化学療法に対する反応がよく，その予後はnon-DSのAMLと比較してとても良好である[22]．ML-DSは，ML-DSに適したプロトコールで治療されるべきである．*GATA1*遺伝子変異のある年長児のML-DSは，non-DSのAMLと比較して予後が不良である[23]．

III. DSのTAMおよびAMKLの治療と日本の現状について

米国，日本，ヨーロッパのTAM 264人の報告がある[10,24,25]．通常TAMは自然軽快するが，10〜20%が死亡する．治療を要するTAMの症例は，未熟児，白血球数10万/μl以上のほか，重度の凝固異常，出血，進行性肝障害，腹水および3カ月で完全寛解しない症例等で，cytarabine少量療法（1 mg/kg/day 7日間）の治療の有効性が示されている[10,25-27]．

日本では小児血液学会MDS委員会が全国212の施設にアンケート調査を行い，2003〜2005年に発症したDSのTAM 73例について後方視的調査を行った．73例中19例（26%）が死亡し，16例（22%）はTAMの病態に直接起因する早期死亡症例であった．TAMが自然軽快したうちの2例がMDS，11例がAMKLを発症し，解析した10例中9例に*GATA1*遺伝子変異がみられた．予後不良因子は，在胎週数37週未満，初診時白血球数10万/μl以上，直接ビリルビン5 mg/dl以上，全身浮腫であった．TAMの重症例の治療成績を改善するため，適正な治療選択のために，前方視的登録による症例把握と標準化された推奨治療の確立が日本における課題である[24]．

一方，DS-AMKLでは芽球の薬剤感受性が高く，治療強度を弱めた日本小児AML共同研究グループの治療（pirarubicin 25 mg/m^2 2日，cytarabine 100 mg/m^2 7日，etoposide 150 mg/m^2 3日）で，寛解導入率は97.2%，無病生存率（EFS）も80%以上と治療成績は向上している．予後不良因子はmonosomy 7であった[4]．WHO分類では，

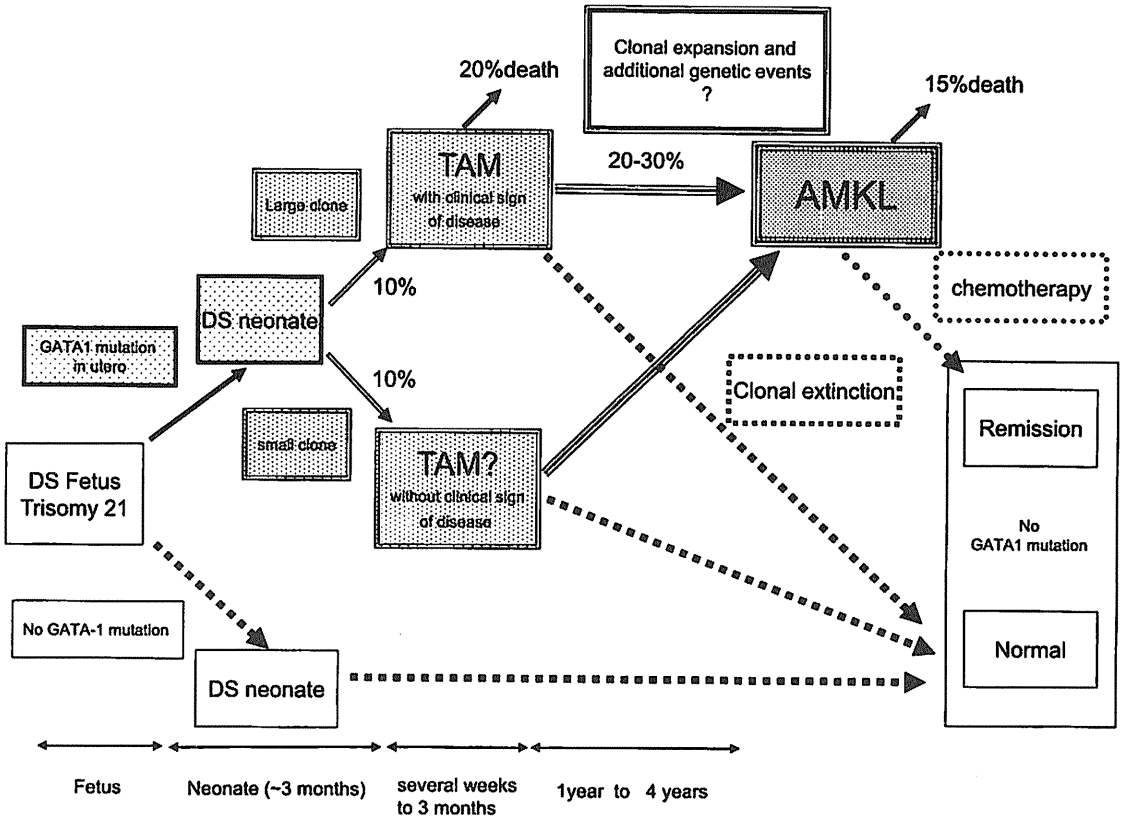

Fig. 4 A modified model for the progression of TAM and AMKL in Down syndrome[28]
Trisomy 21 is the first event of leukemogenesis of DS. The subsequent mutation of *GATA1* in the fetus period is the second hit, and contributes to the development of TAM.

DS-ML で monosomy 7 は非常にまれと記されているが，本邦の報告[4]では AMKL に限ると，72 例中 6 例（8%）と比較的頻度は高く，人種差によるものかもしれないが，予後不良因子でもあり注意が必要であると思われた．

また TAM の診断がなされていないものがあり，すべての AMKL において TAM の既往があるかどうかは明らかではない．最近の報告では，TAM の既往があるほうが，*de novo* の AMKL よりも治療成績がよいとされている[25]．

AMKL では血小板減少を伴った MDS の期間がしばしば認められる．したがって，TAM を cytarabine 少量療法で治療し MDS/AMKL への進展を予防できるかもしれない．また，TAM から自然回復した *GATA1* 変異のある臨床的に無症状な状態における治療が AMKL への進展を予防できるかどうかについても明らかではない．これについて I-BFM のグループでは *GATA1* 遺伝子変異をモニターし，cytarabine 治療で MDS/AMKL への進展を制御できないか臨床研究を開始しており，予防法の発見につながるかもしれない．

IV. 発症のメカニズムに関する最新の知見と考察

現在考えられている TAM から AMKL に至る経路を示した（Fig. 4）[28]．

Wechsler らは，*GATA1* 遺伝子変異は後天的に AMKL に認められ，DS でも AMKL 以外の白血病では検出されなかったことを報告した[29]．一方，non-DS の AMKL で *GATA1* 遺伝子変異がみつかった症例では後天的 21 トリソミーが確認されている[16]．*GATA1* 遺伝子は X 染色体上に存在し，赤血球，巨核球の分化に必須の転写因子である．*GATA1* 遺伝子変異は，AMKL だけでなく TAM や MDS でも検出されることが報告され，とくに TAM では，ほぼすべての症例に検出され白血病の早期の遺伝子変異であると考えられた．とくに，在胎 29 週で出生した一卵性双生児に発症した TAM の症例から同一の *GATA1* 変異が検出されたことから，*GATA1* 変異は 29 週以前の胎児期にすでに出現していたことが示された[30]．

21トリソミーによる遺伝子過剰な状態下に，胎児期に*GATA1*遺伝子に変異が起こり，その後いくつかの遺伝子の変異が蓄積してMDS，AMKLに進展すると考えられている．TAMからDS-AMKLへの進展に関与する遺伝子変異としては，TAMでは*JAK3*が5/40例，*TP53*が1/13例に，DS-AMKLでは*JAK3*が7/53例，*FLT3*が2/35例，*TP53*が6/28例，*JAK2*が2/32例に検出されているが，いずれも頻度は高くなかった[27]．

臨床的に無症状のDS児の10%に*GATA1*遺伝子変異が検出されるが，軽症のTAMは見逃されている可能性がある．また，異なる*GATA1*変異をもつ複数のクローンが存在することも報告されている[31]．*GATA1*遺伝子の転写活性ドメインはN末端に存在し，約50-kDの完全長の*GATA1*蛋白ではなく，N末端を欠く40-kDのshort form変異GATA1タンパク質（GATA1s）が発現している．

一方，DS-AMKLより高頻度に発症するDS-ALLでは化学療法の反応性が劣る．21トリソミーに加えて染色体数の異常，とくにX染色体の付加が28/130例にみられる．また，遺伝子の欠失，*JAK2* R683G変異による遺伝子の活性化が示されている[32]．この発症には*GATA1*変異の関与はなく，骨髄増殖性疾患で高頻度にみられる*JAK2* V671Fとも異なる部位の変異であり，むしろ頻度は低いがDS-AMKLに*JAK2* V671F変異が報告されており興味深い[27]．

V. おわりに

発癌は，遺伝子変異の集積—多段階発癌というメカニズムで進展していくことが知られている．DSにおけるTAMの発症からMDS/AMKLへの進展は，白血病発症の分子機構を知る非常によいモデルと考えられている．近年マイクロアレイや動物モデルから，21番上の4つの遺伝子*AML1*，*ERG*，*ETS2*，*GABPA*がTAMからMDS/AMKLへの進展に関わる候補遺伝子とする報告があり[27]，さらに研究が進むと考えられる．WHO分類は，日々新たに明らかになる臨床試験，基礎研究による知見を評価し，病理医，臨床医，科学研究者が協力し，常に更新されていくことが期待されている．

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High frequencies of simultaneous *FLT3*-ITD, *WT1* and *KIT* mutations in hematological malignancies with *NUP98*-fusion genes

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Acute myeloid leukemia (AML) is heterogeneous in clinical features and molecular pathogenesis. Cooperating alterations of several genes, including oncogenes or tumor suppressor genes, lead to AML development.[1] AML leukemogenesis is thought to require at least two different types of genetic change: class I mutations, which confer a proliferative or survival advantage; and class II mutations, which block myeloid differentiation and provide self-renewability.[1] In hematological malignancies with 11p15 translocations, the nucleoporin (*NUP*) *98* gene is reportedly fused to various partner genes, often including homeobox genes, such as *HOXA9, A11, A13, C11, C13, D11, D13* and *PMX1*.[2] With respect to the oncogenic mechanism of NUP98-HOX fusion proteins, a previous study using a murine bone marrow transplantation assay revealed that NUP98-HOXA9, -HOXD13 and -PMX1 fusion proteins induce myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN), which progress to AML.[2] This latency period indicates that additional genetic events might be required for leukemic transformation. Therefore, we examined somatic mutations of the *FLT3, KIT, WT1, RUNX1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes, which are prevalent in AML, in leukemia patients with *NUP98* fusion genes. This study was approved by local ethical committee.

Sixteen patients with chromosomal 11p15 translocations included nine with *NUP98-HOXA9*, two with *NUP98-HOXA13*, two with *NUP98-HOXA11* and one each with *NUP98-HOXC11, NUP98-HOXD11, NUP98-HOXD13* or *NUP98-NSD3* (Table 1). The partner gene fused to *NUP98* could not be detected in one patient with t(4;11)(q21;p15); however, fluorescent *in situ* hybridization analysis using a probe containing *NUP98* showed split signals (data not shown). No patients had any additional chromosomal abnormality except for chromosomal 11p15 translocations (Supplementary data). Two patients with t(7;11)(p15;p15) had double *NUP98* fusion transcripts: patient (PN) 13 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA13* fusions, and PN14 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA11* fusions. In all, 15 of the 16 patients with *NUP98*-related hematological malignancies were diagnosed as having myeloid malignancies, and the other patient (PN16) were initially diagnosed as having T-cell non-Hodgkin's lymphoma with t(4;11)(q21;p15), and transformed into acute myelomonocytic leukemia with the same t(4;11) (lineage switch). Patients with myeloid malignancies consisted of 10 patients with AML, 2 patients with MDS and 3 patients with MPN.

We examined the internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations of the *FLT3* gene in 16 patients, and detected ITDs in nine (56.3%) patients, and TKD mutations in none (Table 1, Figure 1a). The incidence of *FLT3*-ITD in our study was much higher than that in an AML cohort reported previously (12–35%).[1] A high frequency of *FLT3*-ITD was previously reported in 30–35% of AML patients with either normal karyotype or with t(15;17)(q21;q11) resulting in *PML-RARA*, and in 70% of AML patients with t(6;9)(p23;q34) resulting in *DEK-CAN/NUP214*.[1] Interestingly, both *NUP98* and *NUP214* encode a part of the nucleoporin complex. The general activation effects on reporters of the DEK-CAN/NUP214 fusion protein are specific for myeloid cells.[3] Moreover, in murine bone marrow transplantation assays, NUP98-related fusion proteins such as NUP98-HOXA9, -HOXD13 and -PMX1 induced MDS or MPN, which progressed to AML.[2] These results demonstrate that the nucleoporin-related proteins share a common ability for myeloid differentiation. Furthermore, the very tight correlation between *nucleoporin*-related fusion genes and *FLT3*-ITD suggest that *FLT3*-ITD may contribute to the myeloid leukemogenesis involved in *nucleoporin*-related fusions.

We further examined mutations of the *KIT, WT1, AML1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes,[4] which are prevalent in AML. *KIT, NRAS* and *KRAS* mutations were found in four (25.0%), three (18.8%) and two (12.5%) patients, respectively (Table 1, Figure 1b). *WT1* aberrations were found in eight patients (50.0%; Table 1, Figure 1c). No mutations were found in the other four genes (*RUNX1, CEBPA, NPM1* and *MLL*). The mutations in *KIT* were all missense mutations including Val399Ile, Met541Leu and Asp816Val, and all mutations of *NRAS* and *KRAS* were Gly13Asp. All of *KIT, NRAS* and *KRAS* mutations were heterozygous. The aberrations in *WT1* comprised a frameshift insertion of exon 7 in four patients, missense mutation of exon 9 in one, deletion of exon 5 in one and deletion of the whole cording region in two. Frameshift and

Table 1 Clinical features and additional mutations of patients with *NUP98*-related leukemias

PN	Age	Sex	Disease	WBC at diagnosis	Karyotype	Fusion partner gene of NUP98	CR	Relapse	Therapy	Prognosis	FLT3	KIT	WT1	NRAS	KRAS
PN1	14	M	AML-M1	12500	t(11;12)	*HOXC11*	yes	yes	Chemo+SCT	Death	ITD	Val399Ile	del	WT	WT
PN2	12	F	AML-M2	133100	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT
PN3	13	M	AML-M2	460000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN4	13	F	AML-M2	147000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Alive	WT	WT	WT	WT	WT
PN5	15	M	AML-M2	22700	t(7;11)	*HOXA9*	yes	no	Chemo+SCT	Alive	WT	WT	WT	WT	Gly13Asp
PN6	57	M	AML-M2	252000	t(7;11)	*HOXA13*	yes	yes	Chemo	Death	ITD	WT	WT	WT	WT
PN7	38	M	AML-M2	6400	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Asp816Val	ins4bpfsX	WT	WT
PN8	15	M	AML-M4	187900	t(2;11)	*HOXD11*	yes	no	Chemo+SCT	Alive	WT	WT	ins4bpfsX	WT	Gly13Asp
PN9	56	M	AML-M4	204500	t(7;11)	*HOXA9*	yes	yes	Chemo	Lost to follow-up	ITD	WT	WT	WT	WT
PN10	62	M	AML-M4	6500	t(2;11)	*HOXD13*	yes	no	Chemo	Alive	ITD	WT	WT	WT	WT
PN11	60	M	RA	6250	t(8;11)	*NSD3*	no	ND	Chemo	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN12	69	F	RAEB	2500	t(7;11)	*HOXA9*	no	ND	Chemo	Death	WT	WT	WT	WT	WT
PN13	45	M	CMML	29800	t(7;11)	*HOXA9/HOXA13*	yes	yes	Chemo	Death	ITD	WT	Arg250Trp	WT	WT
PN14	58	F	CML(Ph-)	11200	t(7;11)	*HOXA9/HOXA11*	yes	no	Chemo	Alive	ITD	WT	del	WT	WT
PN15	3	F	JMML	39400	t(7;11)	*HOXA11*	yes	no	Chemo+SCT	Alive	WT	WT	del exon5	Gly13Asp	WT
PN16	51	F	T-NHL	2600	t(4;11)	undetermined	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT

Abbreviations: AML, acute myeloid leukemia; Chemo, chemotherapy; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; del, deletion; F, female; JMML, Juvenile myelomonocystic leukemia; M, male; ND, not determined; Ph-, Philadelphia chromosome; PN, patient number; RA, refractory anemia; RAEB-t, refractory anemia with excess of blasts in transformation; SCT, stem cell transplantation; T-NHL, T-cell non-Hodgkin's lymphoma; WBC, white blood cell; WT, wild type.
t(11;12), t(11;12)(p15;q13); t(2;11), t(2;11)(q31;p15); t(4;11), t(4;11)(q21;p15); t(7;11), t(7;11)(p15;p15); t(8;11), t(8;11; p11; p15).

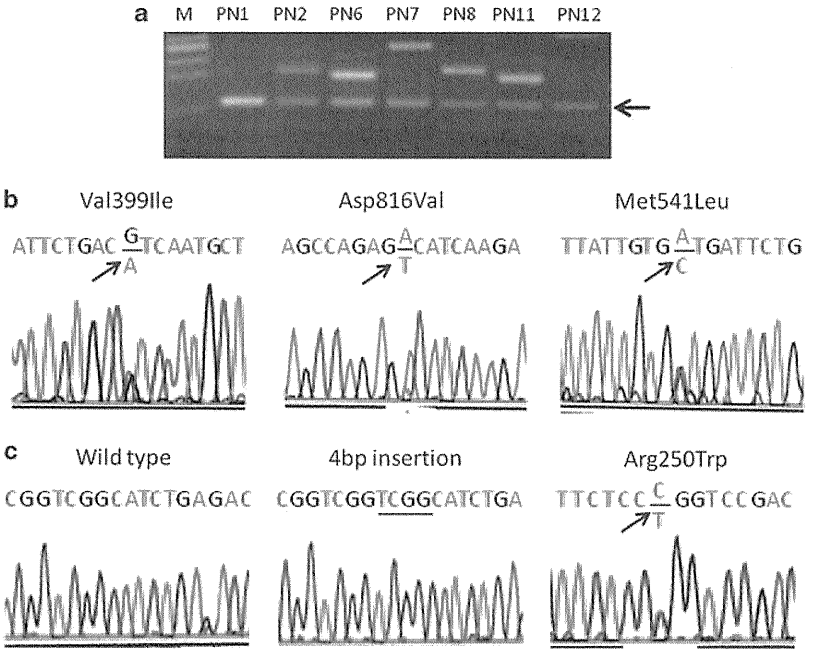

Figure 1 *FLT3*-ITD, *KIT* and *RAS* mutations, and *WT1* aberrations. (**a**) Identification of *FLT3*-ITD by reverse transcription PCR. M, size marker; arrow indicates wild-type allele. (**b**) *KIT* mutations. All figures show the sequence of PCR products. (**c**) *WT1* aberrations. Left panel shows wild type of *WT1* exon 7. Middle panel shows 4-bp insertion in exon 7 of *WT1*. Right panel shows *WT1* missense mutation. Left and middle panels show the sequence of each plasmid subclone, and right panel shows that of PCR products.

missense mutation of *WT1* are heterozygous, whereas deletion was homozygous. *FLT3*-ITD, *KIT* and *RAS* mutations reportedly confer cellular proliferative abilities.[1] In our study, 14 patients (88%) had at least one mutation involved in cellular proliferation (*FLT3, KIT* or *RAS*). Recently, Chou *et al.*[5] reported that the *NUP98-HOXA9* fusion is strongly associated with *KRAS* and *WT1* mutations. *Nras* and *Kras* mutations were frequently found in AML developed in transgenic mice expressing *NUP98-HOXD13*.[6] These results indicate that *NUP98*-related leukemias have a high frequency of mutations involved in growth advantage.

Interestingly, five of the six patients with *WT1* aberrations had *FLT3*-ITD, and three of the five patients with both *FLT3*-ITD and *WT1* aberrations had a *KIT* mutation, although the simultaneous *FLT3*-ITD and *KIT* mutations are reportedly very rare[1]. These results suggest that the *NUP98*-related leukemias share a distinct molecular subgroup in leukemias. In addition, all four patients with *KIT* mutations had both *FLT3*-ITD ($P=0.04$) and *WT1* aberrations ($P=0.03$), whereas all five patients with *RAS* mutations did not have *FLT3*-ITD. In all, 14 (88%) of the 16 patients had either *FLT3*-ITD or *RAS* mutations, but they were mutually exclusive as described in previous papers.[1] These

suggest the distinct molecular basis between *NUP98*-related leukemias having *FLT3*-ITD and those having *RAS* mutations.

The relationships between clinical features and gene mutations were described in Table 1. In our study, male patients were more likely than female patients to have *FLT3*-ITD ($P=0.01$) and patients with *FLT3*-ITD have leukocytosis ($P=0.08$) more than those without *FLT3*-ITD. Patients with *RAS* mutations were significantly younger than those without the mutations (median age of 15 vs 56 years; $P=0.04$). In total, 9 (64.3%) of the 14 patients who achieved complete remission relapsed, and 9 (60.0%) of the 15 patients whose data were available died, although they were treated by different protocols (Table 1). All three patients who had both *FLT3*-ITD and *KIT* mutations, and five (83.3%) of the six patients who had both *FLT3*-ITD and *WT1* aberrations, died. Many studies have shown that *FLT3*-ITD is related to a poor prognosis in AML patients,[1] and that *KIT* mutations are associated with a worse outcome in CBF-leukemia patients.[1] *WT1* mutations are also reported to be a poor prognostic factor in adult AML patients with normal karyotypes.[7] These results suggest that simultaneous occurrence of *FLT3*-ITD, *KIT* mutations and *WT1* aberrations in *NUP98*-related leukemia may be associated with poor prognosis.

FLT3-ITD, *KIT* and *RAS* mutations lead to constitutive activation of downstream pathway, resulting in acquirement of a proliferative advantage.[1] In a mouse model, *FLT3*-ITD alone does not induce AML, and *RAS* mutations can induce myeloid leukemia with distinct leukemogenic strengths and phenotypes.[1] *NUP98*-related fusions alone require long periods of time to induce AML, although these fusions induce MDS or MPN by impaired myeloid differentiation.[2] Cooperation between BCR-ABL (which enhances proliferation) and NUP98-fusion (which inhibits differentiation) lead to CML blast crisis.[2] Moreover, the *WT1* mutations were clustered within the DNA binding domain, and were subsequently considered to impair the ability of DNA to bind to target genes associated with apoptosis, cell cycle or cellular proliferation.[8] These results suggest that a high frequency of cell proliferation gene mutations may contribute to leukemogenesis in *NUP98*-related leukemia, and that simultaneous occurrence of *FLT3-ITD* and *WT1* aberrations may have an important role in the clinical outcome of *NUP98*-related leukemia.

Conflict of interest

The authors declare no conflict of interest.

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ONCOGENOMICS

Array-based genomic resequencing of human leukemia

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To identify oncogenes in leukemias, we performed large-scale resequencing of the leukemia genome using DNA sequence arrays that determine ~9 Mbp of sequence corresponding to the exons or exon–intron boundaries of 5648 protein-coding genes. Hybridization of genomic DNA from CD34-positive blasts of acute myeloid leukemia ($n = 19$) or myeloproliferative disorder ($n = 1$) with the arrays identified 9148 nonsynonymous nucleotide changes. Subsequent analysis showed that most of these changes were also present in the genomic DNA of the paired controls, with 11 somatic changes identified only in the leukemic blasts. One of these latter changes results in a Met-to-Ile substitution at amino-acid position 511 of Janus kinase 3 (JAK3), and the JAK3(M511I) protein exhibited transforming potential both *in vitro* and *in vivo*. Further screening for *JAK3* mutations showed novel and known transforming changes in a total of 9 out of 286 cases of leukemia. Our experiments also showed a somatic change responsible for an Arg-to-His substitution at amino-acid position 882 of DNA methyltransferase 3A, which resulted in a loss of DNA methylation activity of >50%. Our data have thus shown a unique profile of gene mutations in human leukemia.

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Keywords: resequencing; AML; JAK3; DNMT3A

Introduction

Leukemias are clonal disorders of hematopoietic stem cells or immature progenitors. Several subtypes of leukemia are associated with disease-specific karyotype anomalies in the malignant blasts. Most cases of acute promyelocytic leukemia a subtype of acute myeloid leukemia (AML), for instance, are associated with a t(15;17) chromosomal rearrangement that results in the production of the PML-RARA fusion-type oncoprotein (Tallman and Altman, 2008). Similarly, another subtype of AML is associated with a t(8;21) rearrangement, resulting in the production of the oncogenic RUNX1-CBFA2T1 protein (Nimer and Moore, 2004).

The karyotype of leukemic blasts is an important determinant of the long-term prognosis of affected individuals. AML with t(15;17), t(8;21) or inv(16) rearrangements thus constitutes a subgroup of leukemias with a 'favorable' karyotype, with a 5-year survival rate of >60%, whereas AML with an 'adverse' karyotype (monosomy 7, monosomy 5 or complex anomalies) has a 5-year survival rate of only <15% (Grimwade *et al.*, 1998). The prognosis of AML with a normal karyotype (constituting ~50% of all AML cases) is substantially worse than that with a favorable karyotype, with a 5-year survival rate of 24% (Byrd *et al.*, 2002), indicating that blasts with a normal karyotype may contain transforming genes generated as a result of (1) sequence alterations, (2) epigenetic abnormalities or (3) small chromosomal rearrangements not detectable by the G-banding technique. Indeed, several genes, including *NPM1* and *KIT*, have been found to be mutated and activated in AML blasts with a normal karyotype (Schlenk *et al.*, 2008).

The identification of transforming genes in AML will require large-scale resequencing of the blast genome. Although a new generation of sequencing technologies is now available, whole-genome resequencing of many samples remains a demanding task (Bentley *et al.*, 2008; Wheeler *et al.*, 2008). Although DNA microarray-based sequencing is suitable for analysis of multiple samples, currently available platforms are limited in the number of nucleotides that each array is able to probe. To overcome such limitations, we have now applied the extra-large arrays ('wafers') manufactured by Perlegen Sciences (Mountain View, CA, USA) (originally developed for typing of

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single-nucleotide polymorphisms) (Patil *et al.*, 2001) to resequencing of the human genome. Our two-step analysis of human leukemia specimens ($n=20$) has identified a novel transforming mutation in the gene for Janus kinase 3 (JAK3) and a hypomorphic mutation in that for DNA methyltransferase 3A (DNMT3A).

Results

Sequencing strategy

Oligonucleotide probes on the sequencing wafer for the first phase of our study were designed to detect nonsynonymous nucleotide changes in the coding exons of the genome. Intronic sequences (GT in the splicing donor sequence AG-GT and AG in the splicing acceptor sequence AG-G) adjacent to coding exons were also interrogated with the wafer to capture splicing anomalies. Genes examined by the wafer included those known to be mutated in cancer and reported in the catalog of somatic mutations in cancer (COSMIC, http://www.sanger.ac.uk/genetics/cgp/cosmic) as of September 2006 ($n=338$) and those related to the regulation of DNA repair ($n=419$), chromatin structure ($n=299$), redox regulation ($n=102$), epigenetic regulation ($n=44$), cell signaling ($n=2490$), protein kinases ($n=314$), gene transcription ($n=797$), cell cycle ($n=297$), apoptosis ($n=312$), DNA replication ($n=144$) or other functions ($n=92$) (Figure 1a). A total of 5648 genes were thus analyzed with the wafer.

To efficiently isolate oncogenes generated by point mutation using our sequencing array, we selected leukemic blasts with a karyotype characterized by few chromosome anomalies and by few copy number variations of chromosomes, as determined by comparative genomic hybridization with single-nucleotide polymorphism-typing arrays (Supplementary Figure S1). We isolated 15 cases of *de novo* AML, 4 cases of AML that developed from myelodysplastic syndrome, and 1 case of myeloproliferative disorder negative for the JAK2(V617F) and MPL(W515L) mutations (Kralovics *et al.*, 2005; Pikman *et al.*, 2006) (Supplementary Table S1).

From each of these 20 individuals enrolled in the study, we purified immature blasts positive for the surface expression of CD34 (leukemic fraction) as well as a paired control fraction of mature T cells positive for the surface expression of CD4. Although monocytes-macrophages may also express a low level of CD4 at the cell surface, our magnetic bead-based purification system preferentially enriched mature T cells with a high level of CD4 expression; contamination of the mature T-cell fraction with monocytes-macrophages was judged to be <9% by flow cytometry (Supplementary Figure S2).

Given the potential presence of substantial numbers of unreported single-nucleotide polymorphisms in the human genome, we adopted a two-step analysis to select somatic changes (Figure 1b). In phase I, genomic DNA was isolated from the $CD34^+$ fraction, subjected to mid-range PCR amplification and hybridized with the wafer to examine ~9 Mbp of nucleotide sequence. In phase II, we constructed a smaller wafer to investigate only the

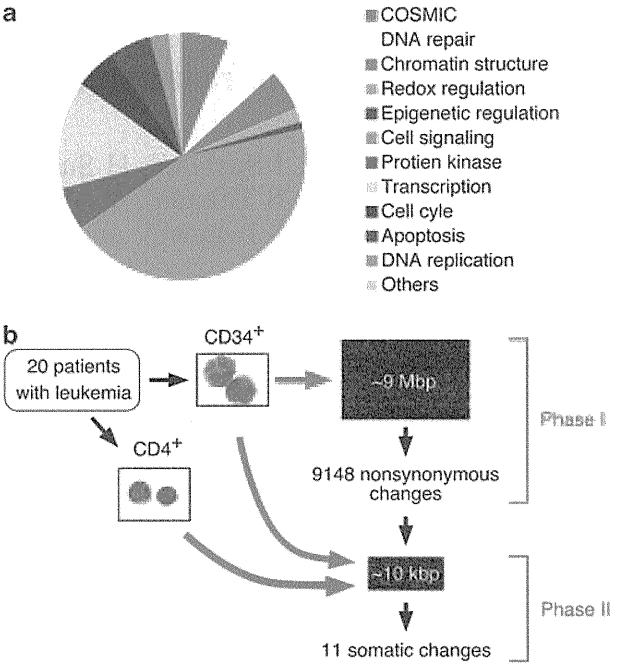

Figure 1 Resequencing of the leukemia genome with wafers. (**a**) Genes interrogated by the phase I wafer ($n=5648$) included those listed in the COSMIC database and those categorized on the basis of function of the encoded protein as indicated. (**b**) $CD34^+$ and $CD4^+$ cell fractions were purified from individuals with leukemia ($n=20$). Genomic DNA of the former fractions was assayed with the phase I wafer including ~9 Mbp of sequence, resulting in the isolation of 9148 nonsynonymous nucleotide changes in 3403 independent genes. The phase II wafer was then constructed to analyze these 9148 changes and was hybridized with genomic DNA from both $CD34^+$ and $CD4^+$ fractions separately. Only 11 mutations were found to be present in the former fraction but not in the latter.

nucleotides shown to be changed in phase I relative to the human reference sequence. Genomic DNA isolated from leukemic blasts and paired control fractions was then analyzed individually with the phase II wafer. We assumed that a nucleotide change was a germline polymorphism if it was observed in both leukemic and control fractions of the same individual, and that it was a somatic mutation if it was observed in the former fraction but not in the latter.

Identification of the JAK3(M511I) mutation

Screening of the leukemic blasts of the 20 individuals for point mutations in phase I yielded 9148 nonsynonymous changes among 3403 independent genes, a frequency similar to that observed in other large-scale resequencing studies performed with capillary sequencers (Sjoblom *et al.*, 2006; Greenman *et al.*, 2007). However, analysis of $CD4^+$ fractions showed that most of these sequence changes were also present in the paired control genome, leaving only 11 nonsynonymous somatic mutations in 11 genes (Supplementary Table S2). Such small number of somatic mutations is in a good agreement with the eight somatic mutations found in AML through whole-genome resequencing using the

Illumina Genome Analyser (Illumina, San Diego, CA, USA) (Ley *et al.*, 2008). All of our 11 somatic changes were confirmed by analysis of both genomic DNA and cDNA of the corresponding specimens with a capillary sequencer (data not shown). These data thus support the necessity of examining paired noncancerous specimens to pinpoint somatic changes in the cancer genome.

One of the gene mutations found only in the $CD34^+$ fractions results in a Met-to-Ile change at amino-acid position 511 of JAK3. A heterozygous *JAK3* mutation responsible for the amino-acid change was confirmed in both genomic DNA and cDNA from the $CD34^+$ fraction, but not in those from the corresponding $CD4^+$ fraction of patient ID JM07 (Supplementary Figure S3), who had *de novo* AML (M1 subtype) and a normal karyotype (Supplementary Table S1). In contrast to JAK2, activating mutations in which are preferentially associated with myeloproliferative disorder, several gain-of-function mutations (such as I87T, P132T, Q501H, A572V, R657Q and V722I) of JAK3 have recently been associated with acute megakaryoblastic leukemia of children (Walters *et al.*, 2006; Sato *et al.*, 2008). Other JAK3 mutations (such as A573V and A593T) were also identified in the same disorder, and an M576L substitution was detected in an adult with acute megakaryocytic leukemia (AML, M7 subtype) (Kiyoi *et al.*, 2007), although the transforming potential of these changes remains unknown.

Given that the M511I mutant of JAK3 has not previously been described and that the relevance of JAK3 to the pathogenesis of adult AML has not been extensively investigated, we first focused on the function of JAK3(M511I). The M511 residue is located in the linker region between the Src homology 2 (SH2) domain and the pseudokinase domain of JAK3 (Figure 2a). The transforming mutation Q501H that is associated with juvenile acute megakaryoblastic leukemia (Sato *et al.*, 2008) is also located in this region. Given that JAK3 is abundant in and has an essential role in the development of lymphocytes (Russell *et al.*, 1995), we examined the expression level of *JAK3* in AML blasts. The gene was expressed at a high level in most AML specimens ($n = 52$), with its expression level being greater than that of *JAK2* in all but three cases (Supplementary Figure S3).

To examine the transforming potential of JAK3(M511I), we introduced the mutant or wild-type protein into the interleukin-3 (IL-3)-dependent mouse cell line 32D (Greenberger *et al.*, 1983). Although 32D cells forced to express wild-type JAK3 underwent rapid apoptosis after withdrawal of IL-3, those expressing JAK3(M511I) continued to grow even in the absence of IL-3, although at a reduced rate compared with that of cells expressing the artificially generated, highly transforming mutant JAK3(V674A) (Choi *et al.*, 2007) (Supplementary Figure S3). 32D cells differentiate into terminal granulocytes in the presence of granulocyte colony-stimulating factor. However, cells expressing the M511I or V674A mutant of JAK3 maintained an exponential rate of growth, without any sign of differentiation, in the presence of granulocyte colony-stimulating factor (Figure 2b, Supplementary Figure S3), supporting the notion that the M511I mutant has transforming potential.

To directly examine the leukemogenic activity of JAK3(M511I), we generated a recombinant retrovirus encoding this mutant and used it to infect murine hematopoietic stem cells. Reconstitution of the bone marrow of lethally irradiated mice with such infected cells resulted in marked lymphocytosis in peripheral blood and enlargement of the spleen in the recipient animals (Figure 2c). The cells in the peripheral blood, spleen and bone marrow of the recipients responsible for these phenotypes manifested a medium-sized, blastic morphology, and flow cytometric analysis revealed them to be $CD8^+$ T cells (Figure 2c). The clonal nature of these proliferating T cells was further confirmed by Southern blot analysis (Supplementary Figure S4), indicative of the development of T-cell acute lymphoblastic leukemia in the recipient mice.

To assess the prevalence of *JAK3* mutations in adult leukemia, we further examined the nucleotide sequence of the entire coding region of *JAK3* cDNA in an additional 266 specimens of leukemic blasts. The coding region of *JAK3* cDNA was successfully amplified by PCR from 83 specimens. We could further identify 4 distinct *JAK3* sequence changes in 8 of these 83 samples: 1 case with G62S, 4 cases with Q501H, 2 cases with R657Q and 1 case with R918C (Figure 2a). Taking into account the 20 cases evaluated in the phase I analysis, we thus identified a total of 9 cases with a mutant form of JAK3 (3.1%) among 286 cases of leukemia (Supplementary Table S3). Our identification of known transforming JAK3 mutants (Q501H and R657Q) originally associated with acute megakaryoblastic leukemia prompted us to determine the prevalence of these two changes in another cohort of AML ($n = 148$), revealing two cases with JAK3(Q501H) and one case with JAK3(R657Q). In addition, analysis of a hematopoietic cell line (KCL22) (Kubonishi and Miyoshi, 1983) established from a patient with chronic myeloid leukemia in BC revealed yet another mutation (L1017M) of JAK3 (Figure 2a).

To directly compare the transforming potential of these various JAK3 mutants, we introduced each protein into the IL-3-dependent mouse B-cell line BA/F3 and examined the growth properties of the resulting transfectants. Whereas all cells expressing the JAK3 mutants proliferated in a similar manner in the presence of IL-3 (data not shown), culture without IL-3 revealed marked differences in the transforming potential among the mutants. JAK3(M511I) was the most efficient oncokinase, with a transforming activity similar to that of JAK3(V674A). The frequent mutants JAK3(Q501H) and JAK3(R657Q) exhibited weaker but still pronounced transforming potential, whereas the remaining mutants (G62S, R918C and L1017M) showed an even lower potential (Figure 2d).

Somatic mutations of DNMT3A

Another somatic mutation identified in the phase II data set was a heterozygous change in *DNMT3A* that results

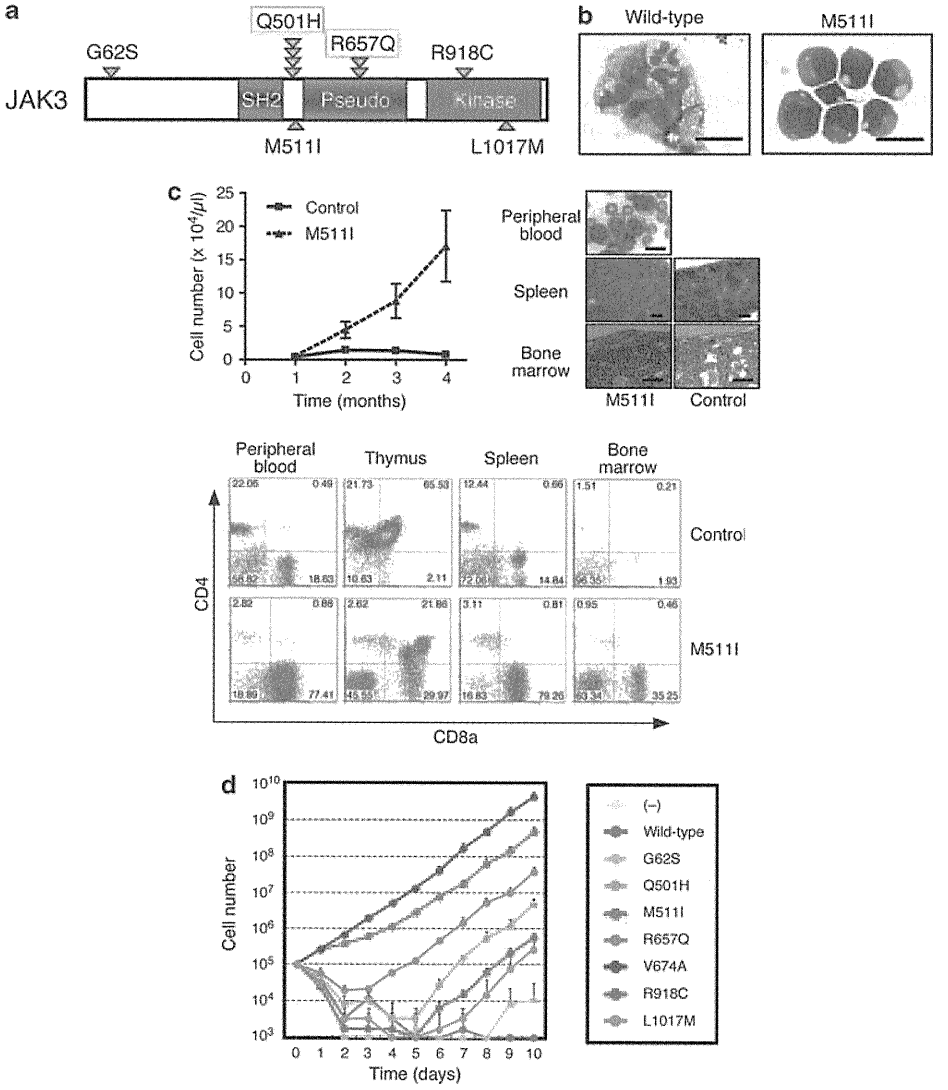

Figure 2 Identification of JAK3 mutants in leukemia. (**a**) Amino-acid substitutions detected in this study are shown relative to the domain organization of JAK3. The mutations M511I (one case) and Q501H (four cases) are located in the linker region between the SH2 and pseudokinase domains of JAK3, whereas G62S (one case), R657Q (two cases) and R918C (one case) are located in the amino-terminal region, the pseudokinase domain and the kinase domain, respectively. The KCL22 cell line also harbors an L1017M mutation within the kinase domain of JAK3. Previously known activating mutations of JAK3 (Q501H and R657Q) are indicated by red rectangles. (**b**) Mouse 32D cells expressing wild-type human JAK3 or the JAK3(M511I) mutant were incubated with G-CSF (0.5 ng/ml) for 14 days, stained with Wright–Giemsa solution and examined by light microscopy. Scale bars, 20 μm. (**c**) C57BL/6 mice were irradiated and then injected intravenously with syngeneic CD34$^-$KSL hematopoietic stem cells infected with a retrovirus encoding JAK3(M511I) or the corresponding empty virus (control). The number of white blood cells in peripheral blood was counted at the indicated times thereafter; data are means ± s.d. for 10 mice in each group (upper left panel). Peripheral blood, spleen and bone marrow isolated from recipient mice 3 months after cell injection were stained with the Wright–Giemsa solution (peripheral blood) or hematoxylin–eosin (spleen and bone marrow) and were then examined by light microscopy (upper right panel); scale bars represent 10, 200 and 50 μm, respectively. Mononuclear cells isolated from peripheral blood, thymus, spleen and bone marrow of recipient mice 3 months after cell injection were subjected to flow cytometric analysis of surface expression of CD4 and CD8a (lower panel). (**d**) Control BA/F3 cells (–) or those expressing the indicated JAK3 mutants were cultured without IL-3 for the indicated times, after which the cell number was determined. Data are means + s.d. of triplicates from a representative experiment.

in an R882H substitution in the encoded protein (Figure 3a, Supplementary Figure S5). DNMT3A, together with DNMT3B, has an essential role in *de novo* methylation of the human genome (Okano *et al.*, 1999), and an aberrant methylation profile (hypermethylation of CpG islands and hypomethylation of other regions) is a hallmark of cancer cells (El-Osta, 2004). Despite a direct linkage between such methylation changes and silencing of tumor-suppressor genes in cancer, the molecular mechanism responsible for such abnormal methylation remains unknown. Our data thus provide the first evidence of somatic mutation of a DNA methyltransferase gene in cancer cells. Mutations in the catalytic domain of DNMT3B have been shown to be

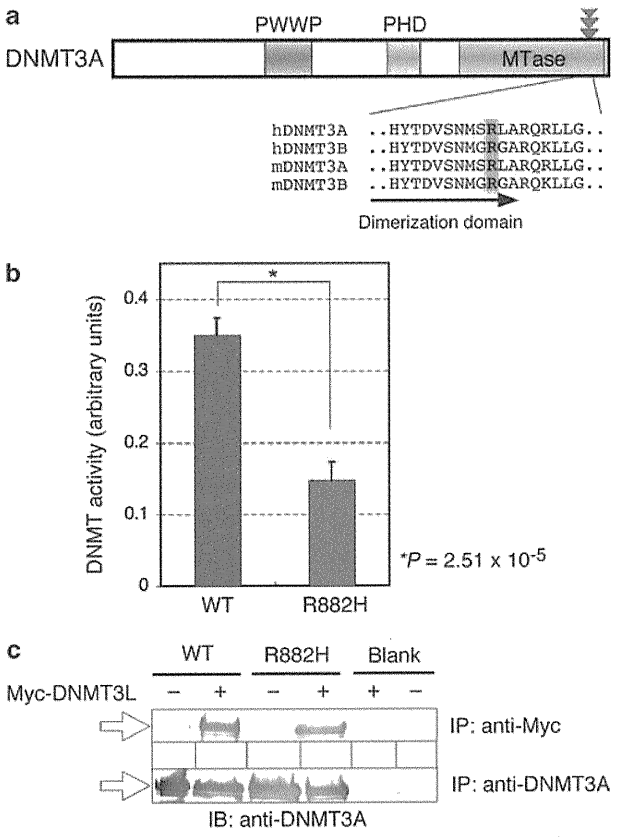

Figure 3 Identification of a DNMT3A mutant in leukemia. (**a**) Domain organization of human DNMT3A showing that the R882 residue found to be mutated in leukemia is conserved among human (h) and mouse (m) members of the DNMT3 family. DNMT3A contains a tetrapeptide PWWP domain, polybromo homology domain (PHD) and methyltransferase (MTase) domain. The R882 residue is located in the homodimerization region present within the MTase domain. (**b**) Wild-type (WT) and R882H forms of DNMT3A were expressed in and purified from insect cells and then subjected to an *in vitro* assay of methyltransferase activity. Data are means + s.d. of triplicates from a representative experiment. The *P*-value was determined by Student's *t*-test. (**c**) Lysates of HEK293 cells expressing Myc epitope-tagged DNMT3L and wild-type or R882H forms of DNMT3A, as indicated, were subjected to immunoprecipitation (IP) with antibodies to Myc or to DNMT3A, and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to DNMT3A. The position of DNMT3A (wild-type or mutant) is indicated by an open arrow.

responsible for a hereditary syndrome characterized by ICF (immunodeficiency, instability of the centromeric region of chromosomes and facial anomalies) in humans (Ehrlich, 2003). One of the mutation sites of DNMT3B (R823) associated with the ICF syndrome corresponds to the residue of DNMT3A (R882) shown to be mutated in this study.

The R882 residue of DNMT3A is considered to participate in the homodimerization and activation of the protein (Jia *et al.*, 2007) (Figure 3a). To determine whether the R882H mutation affects the catalytic activity of DNMT3A, we expressed mutant and wild-type proteins separately in insect cells, purified them to near homogeneity and subjected them to an *in vitro* assay of methyltransferase activity with a synthetic substrate (Suetake *et al.*, 2003). The catalytic activity of DNMT3A(R882H) was $<50\%$ of that of the wild-type protein (Figure 3b). DNMT3L acts as a coactivator for the methyltransferase activity of DNMT3A or DNMT3B through its association with the latter proteins (Jia *et al.*, 2007). The R882H mutation did not affect the interaction of DNMT3A with DNMT3L in transfected mammalian cells (Figure 3c) or its sensitivity to DNMT3L as examined by the *in vitro* assay of methyltransferase activity (data not shown). These data thus suggested that the R882H mutation directly inhibits the enzymatic activity of DNMT3A.

Screening of another cohort of leukemia cases ($n=54$) for mutant forms of *DNMT3A* revealed another two patients with a mutation of the same amino acid (R882H in one patient and R882C in the other) (Supplementary Table S4). Therefore, we identified a total of 3 cases with an R882 mutation (4.1%) among 74 cases of leukemia. Screening for mutations of *DNMT3B* failed to detect any somatic changes in the same individuals (data not shown), suggesting that *DNMT3A* is a preferential target in leukemia.

Multistep transformation in leukemia

Although $>99\%$ of nucleotide changes in the phase I data were also observed in the paired $CD4^+$ cells, it is unlikely that all of these changes are actually germline polymorphisms because they include established oncogenic mutations. They thus include 190 nucleotide changes previously described in cancer cells (Supplementary Table S5), such as those giving rise to NRAS(Q61H) in patient ID JM17 and to FLT3(D835Y) in patient ID JM08 (Figure 4a). Given that both NRAS(Q61H) and FLT3(D835Y) are well-characterized oncoproteins (Yamamoto *et al.*, 2001), it is unlikely that these individuals harbored such nucleotide changes in the germ line. There are at least two possible explanations for these findings. First, it is possible that purification of the $CD4^+$ fraction was not efficient, with the result that this fraction was contaminated by $CD34^+$ cells. However, the *CD4* expression ratio for the $CD4^+$ and $CD34^+$ fractions of each individual was $\geqslant 17.1$ (median $=40.1$) (Figure 4b), and contamination of the $CD4^+$ fraction with $CD34^+$ cells at such a level would not likely produce detectable changes in Sanger sequencing outputs (compare, for instance, the signal intensities of the normal and mutant alleles in Figure 4a).

Furthermore, although CD4 expression has been occasionally observed in AML blasts (Schwonzen *et al.*, 2007), quantitation of *CD4* and *CD34* mRNA within our purified $CD34^+$ fractions failed to detect a significant level of the former message in the blasts (Supplementary Figure S6). Therefore, it is unlikely that contamination of $CD4^+$ leukemic blasts within the purified, control $CD4^+$ fraction substantially affected the sequencing results in our phase II experiment.

Rather, it is more likely that leukemia may develop in a stepwise manner with a substantial time interval

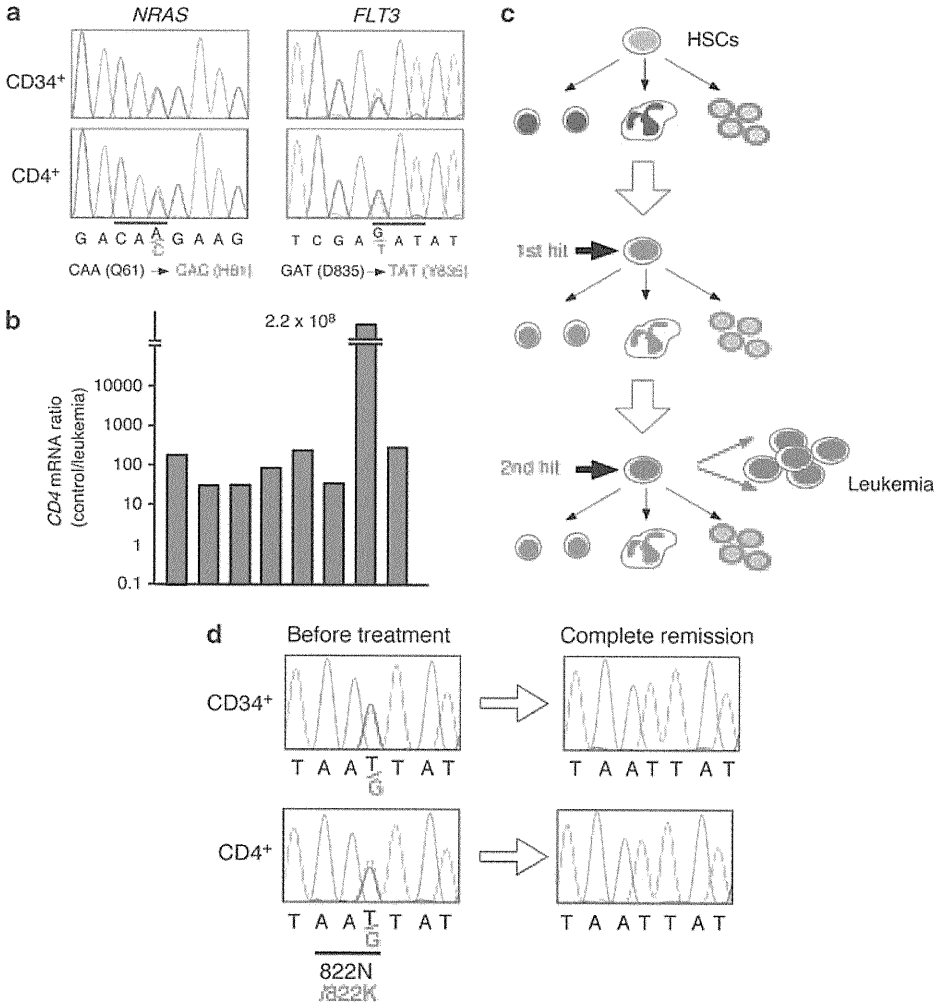

Figure 4 Proposed stepwise nature of leukemogenesis. (**a**) Sequencing electrophoretograms for the regions surrounding codon 61 of *NRAS* or codon 835 of *FLT3* in genomic DNA from the CD34$^+$ and CD4$^+$ fractions of patient IDs JM17 and JM08, respectively. Heterozygous nucleotide changes that give rise to NRAS(Q61H) or FLT3(D835Y) were detected in both fractions of the corresponding patients. (**b**) The amount of *CD4* mRNA in the CD4$^+$ (control) and CD34$^+$ (leukemia) fractions of leukemia patients (with a substantial amount of control *GAPDH* mRNA) was quantitated by reverse transcription and real-time PCR analysis and expressed as the control/leukemia ratio. (**c**) Hematopoietic stem cells (HSCs) give rise to a wide range of mature blood cells. Even after the first hit (mutation) of the genome, HSCs retain their full differentiation capacity, and therefore produce differentiated cells harboring this first hit. After the second hit, the affected cell fraction undergoes full transformation to leukemia. (**d**) Sequencing electrophoretograms for the genome of CD34$^+$ and CD4$^+$ fractions from patient ID JM03 showing a heterozygous mutation for KIT(N822K) before chemotherapy but not after.

between steps (Figure 4c). If a first hit occurs in the genome of hematopoietic stem (or progenitor) cells and if such a somatic change does not result directly in the generation of full-blown leukemia, the preleukemic clones may give rise to terminally differentiated blood cells (including CD4$^+$ cells). After a certain period, a second (or possibly a third) hit occurs in the immature cells and triggers the rapid growth of leukemic clones without differentiation. In such a scenario, terminally differentiated 'normal' cells may still harbor the first hit in their genome.

Support for this latter possibility was provided by patient ID JM03, who had AML (M2 subtype) with a t(8;21) chromosome anomaly. Before chemotherapy, the genomic DNA of both CD34$^+$ and CD4$^+$ fractions from this patient harbored a heterozygous mutation of *KIT* that results in the production of a constitutively activated mutant protein, KIT(N822K) (Shimada *et al.*, 2006) (Figure 4d). The same change was also detected in cDNA prepared from the CD34$^+$ fraction (data not shown). Leukemic blasts in this patient were sensitive to standard chemotherapeutic regimens, and the patient underwent complete remission. Examination of CD34$^+$ and CD4$^+$ fractions obtained during the remission period revealed that the N822K codon change was no longer detectable not only in the CD34$^+$ fraction but also in the CD4$^+$ fraction (Figure 4d). These data thus support the scenario shown in Figure 4c: The N822K

change represents the first hit and was present in differentiated blood cells, and the corresponding pre-leukemic clones were simultaneously eradicated together with the leukemic clones by chemotherapy.

On the other hand, as shown in Supplementary Tables S1 and S2, a heterozygous mutation for NRAS(G12S) was found only in the $CD34^+$ fraction, but not in the $CD4^+$ fraction of the patient ID JM16. Conventional chemotherapy for this patient eradicated the leukemic blasts carrying the mutation (Supplementary Figure S7), also confirming that a successful treatment results in the disappearance of cells with a (possible) 'second hit'.

Our hypothesis of the stepwise leukemogenesis is also consistent with the previous detection of the *RUNX1-CBFA2T1* oncogene in differentiated blood cells (Kwong *et al.*, 1996; Miyamoto *et al.*, 1996, 2000).

Discussion

Our large-scale genomic resequencing of human leukemia specimens with DNA microarrays has identified recurrent nucleotide changes responsible for the generation of JAK3 and DNMT3A mutants. Whereas JAK3 mutants were unexpectedly found in adult AML, their transforming ability, and possibly their contribution to leukemogenesis, varied substantially. However, our bone marrow transplantation experiments showed that at least one of these JAK3 mutants (M511I) directly participates in the development of leukemia. Identification of the M511I mutation of JAK3 in the leukemic fraction but not in the control fraction of patient ID JM07 suggests that this mutation may be the second hit triggering AML. Given that the blasts of this patient had a normal karyotype, it is likely that the first hit is present in the genome of both fractions. Karyotyping of other patients with *JAK3* mutations showed a total of three cases with a normal karyotype, one case with t(8;21), and one case with a numerical anomaly of several chromosomes (Supplementary Table S3), suggesting that *JAK3* mutations may be preferentially associated with leukemia with a normal karyotype.

Although JAK3(M511I) was identified in AML, our bone marrow transplantation experiments with hematopoietic stem cells expressing this mutant yielded T-cell acute lymphoblastic leukemia. In contrast to human leukemia, in which JAK3 changes may constitute a second hit (probably in progenitor cells), JAK3(M511I) may have been expressed in all hematopoietic cells of the recipient mice. JAK3(M511I) thus likely triggered leukemia within a T-cell fraction the intracellular context of which is optimized for JAK3 signaling.

It has been frequently observed that transgenic mouse or bone marrow transplantation experiments for leukemic oncogenes do not accurately recapitulate the original leukemia subtypes (Wong and Witte, 2001). Transgenic mice expressing $p210^{BCR\text{-}ABL1}$, for instance, usually develop T-cell lymphoma or acute lymphoblastic leukemia, not chronic myeloid leukemia. Furthermore, bone marrow transplantation with hematopoietic progenitor cells expressing $p210^{BCR\text{-}ABL1}$ often leads to development of lymphoma, AML, acute lymphoblastic leukemia or macrophage tumors. Generation of malignancy in such systems may, thus, be elaborately influenced by mouse strains, promoter fragments for artificial expression and/or cell types to be used for gene transduction.

Our detection of recurrent DNMT3A hypomorphic mutations in leukemia clones may indicate the presence of an abnormal methylation profile in the genome of such blasts. However, given the limited amount of the specimens available, we were able to investigate microsatellite stability only at certain loci (Koinuma *et al.*, 2005), revealing no apparent microsatellite instability (data not shown). We also generated BA/F3 cells expressing wild-type or R882H forms of DNMT3A to compare the methylation status of some CpG islands in the genome; again, we detected no discernable differences between the two cell preparations (data not shown). However, given that BA/F3 cells contained two copies of wild-type *Dnmt3a* in addition to multiple copies of mutant *DNMT3A*, whereas the leukemic blasts likely harbor one copy each of the wild-type and mutant *DNMT3A* alleles, the clinical relevance of the R882 mutant requires further examination under the latter condition. Cell proliferation/differentiation is indeed influenced substantially by the copy number of *DNMT3* genes (Okano *et al.*, 1999; Ehrlich, 2003).

Our observations indicate the importance of preparing paired normal fractions in large-scale resequencing projects, but they also reveal a difficulty in the preparation of *bona fide* 'normal' fractions in the case of leukemic disorders. Our data thus indicate that nonleukemic blood cells may harbor early genomic hits, rendering them inappropriate as controls. Furthermore, a substantial proportion of fingernail DNA was recently shown to be derived from donor cells among recipients of allogeneic stem cell transplants (Imanishi *et al.*, 2007), indicating that nonblood cells may contain DNA derived from transplanted cells. Therefore, it is possible that buccal, fingernail or even hair cells may not be suitable as normal cell controls. In contrast to solid tumors, for which blood cells are appropriate as paired normal fractions, leukemic disorders require that caution be taken to discriminate somatic nucleotide changes from germline polymorphisms.

Materials and methods

Wafer sequencing

$CD34^+$ and $CD4^+$ fractions were isolated from leukemic individuals using CD34microbeads and CD4microbeads, respectively, and a MidiMACS separator (Miltenyi Biotec, Gladbach, Germany). All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both the Jichi Medical University and the Nagasaki University. DNA sequencing wafers were designed and processed at Perlegen Sciences. Genes to be interrogated on the wafers were selected from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db = gene)

by searching with various keywords characteristic to each subcategory (such as DNA repair, regulation of chromatin structure, etc.), followed by manual inspection. The final gene list for the wafers is shown in Supplementary Table S6. Construction of the wafers, quality control analysis and data processing are described in Supplementary Text.

JAK3 analysis

Complementary DNAs for JAK3 mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and ligated into the pMX retroviral vector (Onishi *et al.*, 1996). Ecotropic recombinant retroviruses encoding each mutant were produced in BOSC23 cells transfected with the corresponding pMX-based plasmid and were used to infect BA/F3 or 32D cells as described previously (Choi *et al.*, 2007). Both types of cell were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Life Technologies, Carlsbad, CA, USA) and mouse IL-3 (Sigma, St Loius, MO, USA) at 10 Units/ml; differentiation of 32D cells was induced by culture in the presence of serum and mouse granulocyte colony-stimulating factor (Sigma) at 0.5 ng/ml. A concentrated preparation of a retrovirus with a VSV-G envelope and encoding both JAK3(M511I) and enhanced green fluorescent protein was used to infect $CD34^-$ c-Kit^+ $Sca\text{-}1^+$ $Lineage\text{-}marker^-$ ($CD34^-KSL$) hematopoietic stem cells isolated from the bone marrow of C57BL/6 mice, and the infected cells were transplanted into lethally irradiated mice congenic for the *Ly5* locus (Iwama *et al.*, 2004). *CD4*, *JAK2* and *JAK3* mRNAs were quantitated by reverse transcription and real-time PCR analysis using an ABI7900HT system (Life Technologies) and with the primers 5′-CTGGAATCCAACATCAAGGTTCTG-3′ and 5′-AATTGTAGAGGAGGCGAACAGGAG-3′ for CD4, 5′-CTCCAGAATCACTGACAGAGAGCA-3′ and 5′-CCAC TCGAAGAGCTAGATCCCTAA-3′ for *JAK2* and 5′-GAGC TCTTCACCTACTGCGACAAA-3′ and 5′-AGCTATGAAA AGGACAGGGAGTGG-3′ for JAK3; the cDNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with the primers 5′-GTCAGTGGTGGACC TGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. The relative abundance of the cDNAs of interest was calculated from the threshold cycle (C_T) for each cDNA and that for *GAPDH* cDNA.

DNMT3A analysis

Recombinant His_6-tagged DNMT3A or DNMT3A(R882H) was expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA), and each protein was purified by stepwise column chromatography as described previously (Suetake *et al.*, 2003). The enzymatic activity of each protein was assayed with -*S*-adenosyl-L-methionine (GE Healthcare, Waukesha, WI, USA) and dIdC or dGdC as substrates (Suetake *et al.*, 2003). The association between Myc epitope-tagged human DNMT3L and wild-type or R882H forms of human DNMT3A in transfected HEK293 cells was examined by immunoprecipitation and immunoblot analyses.

Conflict of interest

The authors declare no conflict of interest.

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