Table 4 Best hematologic response (ITT population)

Disease	Evaluation criteria	*n* (%)
CML-CP ($N = 16$)	Not evaluable	10 (63)
	Evaluable	6 (38)
	Complete hematologic response	6 (100)
	Stable disease	0
	Progression of disease	0
	Not assessable	0
CML-AP ($N = 7$)	Hematologic response	5 (71)
	Complete hematologic response	1 (14)
	Marrow response with no evidence of leukemia	3 (43)
	Return to chronic phase	1 (14)
	Stable disease	1 (14)
	Progression of disease	0
	Not assessable	1 (14)
CML-BC ($N = 4$)	Hematologic response	2 (50)
	Complete hematologic response	1 (25)
	Marrow response with no evidence of leukemia	0
	Return to chronic phase	1 (25)
	Stable disease	2 (50)
	Progression of disease	0
	Not assessable	0
Relapsed/refractory Ph+ ALL ($N = 5$)	Hematologic response	1 (20)
	Complete response	1 (20)
	Partial response	0
	Hematologic improvement	0
	Stable disease	1 (20)
	Progression of disease	3 (60)
	Not assessable	0
Ph+ ALL with minimal residual disease ($N = 2$)	Complete response	2 (100)
	Stable disease	0
	Progression of disease	0
	Not assessable	0

involving 12 amino acids were detected. There were no patients with a T315I mutation, which is known to cause imatinib and nilotinib resistance. HR and CyR could be observed in patients with any of the disease stages who were administered nilotinib, regardless of BCR-ABL mutation status and regardless of their specific mutation (Table 6).

3.3.5 Safety

Non-hematologic adverse events that were suspected to be related to nilotinib are summarized in Table 7. These were

Table 5 Cytogenetic response (Ph+ CML, ITT population)

	n (%)		
	CML-CP $N = 16$	CML-AP $N = 7$	CML-BC $N = 4$
Evaluable	16 (100)	7 (100)	4 (100)
Major CyR	15 (94)	1 (14)	2 (50)
Complete	11 (69)	1 (14)	2 (50)
Partial	4 (25)	0	0
Minor CyR	0	0	1 (25)
Minimal CyR	1 (6)	3 (43)	0
None	0	1 (14)	0
Not assessable	0	2 (29)	1 (25)

mostly mild to moderate in severity. The most commonly reported events were rash (50%), headache (32%), nausea (32%), vomiting (29%) and pyrexia (24%); however, grade 3 or higher events were uncommon.

The numbers of patients with newly occurring or worsening grade 3 or 4 laboratory abnormalities are summarized in Table 8. Grade 3 or 4 abnormalities in neutropenia and thrombocytopenia occurred in 50 and 28% of patients, respectively. These hematologic abnormalities were generally manageable with dose interruptions and reductions, and support with hematopoietic growth factors or transfusions occasionally. Only one patient discontinued from study treatment due to thrombocytopenia.

The majority of biochemistry abnormalities were mild to moderate in severity, resolved spontaneously with continued dosing of nilotinib. Grade 3 or 4 elevations in AST and ALT occurred in 6 and 12% of patients, respectively. Grade 3 or 4 total bilirubin occurred in 3%. Grade 3 or 4 elevations of lipase occurred in 15% of patients. Pancreatitis was reported in 1 patient; however, it was transient and resolved with dose interruption and reduction. No patients discontinued therapy due to serum biochemistry abnormalities. Gastrointestinal and central nervous system hemorrhage of grade 3 or 4 was not reported.

One death was occurred in the study, or within 28 days of discontinuing study. The patient discontinued study treatment because of back pain on study day 14 and died as a result of cardiac failure due to cardiac tamponade and pericardial effusion on day 16. Grade 3 or 4 peripheral edema, pericardial effusion, or pleural effusion was not reported in other patients. Because of a preclinical signal indicating that nilotinib could potentially prolong the QT interval, frequent ECG was performed during the study. One patient experienced a prolongation in the QTcF interval exceeding 500 ms. This event resolved spontaneously with dose reduction of nilotinib. No episodes of torsades de pointes were observed. No tendency was observed for the incidence of adverse events to increase or

Table 6 Hematologic response and cytogenetic response by BCR-ABL mutation at baseline (ITT population)

Disease type	Mutation	Hematologic response		Major cytogenetic response		Major molecular response	
		N^a	*n* (%)	N^b	*n* (%)	N^c	*n* (%)
CML-CP ($N = 16$)	No mutation	4	4 (100)	12	12 (100)	12	6 (50)
	Any mutation	2	2 (100)	4	3 (75)	4	3 (75)
	D276G	1	1 (100)	1	1 (100)	1	1 (100)
	M244V	1	1 (100)	1	0 (0)	1	0 (0)
	F359I	–	–	1	1 (100)	1	1 (100)
	F311I	–	–	1	1 (100)	1	1 (100)
CML-AP ($N = 7$)	No mutation	1	1 (100)	1	0 (0)	1	0 (0)
	Any mutation	6	4 (67)	6	1 (17)	6	1 (17)
	M351T	1	1 (100)	1	0 (0)	1	0 (0)
	Y253H	2	1 (50)	2	0 (0)	2	0 (0)
	F359I/L387 M	1	1 (100)	1	1 (100)	1	1 (100)
	F311I/M244V	1	1 (100)	1	0 (0)	1	0 (0)
	E279K	–	–	–	–	1	0 (0)
CML-BC ($N = 4$)	No mutation	2	1 (50)	2	1 (50)	2	1 (50)
	Any mutation	2	1 (50)	2	1 (50)	2	1 (50)
	F317L	1	1 (100)	1	1 (100)	1	1 (100)
	F359V	1	0 (0)	1	0 (0)	1	0 (0)
Ph+ ALL ($N = 7$)	No mutation	3	2 (67)	–	–	3	1 (33)
	Any mutation	4	1 (25)	–	–	3	0 (0)
	E255K, V/G250E	1	0 (0)	–	–	1	0 (0)
	E459K	1	0 (0)	–	–	1	0 (0)
	E255V	1	0 (0)	–	–	1	0 (0)
	F359V	1	1 (100)	–	–	–	–

[a] Number of patients deemed to be evaluable for hematologic response when an analysis of BCR-ABL mutations was performed post-baseline

[b] Number of patients deemed to be evaluable for cytogenetic response when an analysis of BCR-ABL mutations was performed post-baseline

[c] Number of evaluable patients to be included in the mutation category, i.e., patients who had mutation data and who did not have MMR at baseline

for their onset to be delayed as treatment with the study drug continued.

4 Discussion

Imatinib, the first BCR-ABL TKI approved for the treatment of Ph+ CML and Ph+ ALL, has demonstrated clinical efficacy. However, resistance develops in some patients and treatment options for patients who are resistant to, or intolerant of, imatinib have been very limited. Nilotinib is a more potent and more selective inhibitor of the BCR-ABL protein tyrosine kinase.

The results of this study show the high level of clinical activity of nilotinib in Japanese patients with CML and Ph+ ALL as the overseas Phase II registration study. The rates of CHR and MCyR were relatively higher than that of overseas data. However, due to the limited data in Japanese patients, it is difficult to draw a meaningful conclusion. Overall, imatinib resistance or intolerance, and baseline BCR-ABL mutation status, did not appear to have an impact on response to nilotinib.

The most frequent drug-related adverse events were rash, headache, nausea, vomiting, and pyrexia; however, grade 3 or 4 events were uncommon. Grade 3 or 4 peripheral edema or pleural effusion was not reported. Though neutropenia and thrombocytopenia occurred in 50 and 28% of patients, respectively, these were generally manageable with dose interruptions and reductions, and support with hematopoietic growth factors or transfusions occasionally. Hemorrhage of grade 3 or 4 was not reported. The majority of serum biochemistry abnormalities were infrequent, and mild to moderate in severity, resolved spontaneously with continued dosing of nilotinib.

Although imatinib intolerance constituted 75% (12/16) in CML-CP patients, no patient experienced same serious side effects or side effects lead to discontinuation of administration of nilotinib. Nilotinib and imatinib have some structural features in common, but the minimal occurrence of cross-intolerance between the 2 agents may represent significant therapeutic advantage. It is important to note that no imatinib-intolerant patient on this study had achieved a prior CyR on imatinib at any time.

Table 7 Non-hematologic adverse events suspected to be related to nilotinib (10% or more, SAF population)

	Total ($N = 34$)			
	All grades		Grade 3/4	
	n	%	*n*	%
Rash	17	50	1	3
Headache	11	32	2	6
Nausea	11	32	1	3
Vomiting	10	29	0	0
Pyrexia	8	24	0	0
Malaise	5	15	0	0
Hepatic function abnormal	5	15	0	0
Anorexia	5	15	0	0
Eczema	5	15	0	0
Constipation	4	12	0	0
Stomach discomfort	4	12	0	0
Chest pain	4	12	0	0
Back pain	4	12	1	3
Muscle spasms	4	12	0	0
Erythema	4	12	0	0
Pruritus	4	12	0	0

Consistent with previous findings, the pharmacokinetic profile of nilotinib showed moderate inter-individual variability in this study with Japanese CML patients. The observed variation in nilotinib pharmacokinetics may be partly attributed to the inter-individual variability in CYP3A4, since CYP3A4 activity has been shown to vary among different individuals and nilotinib is mainly metabolized by CYP3A4. Pharmacokinetic parameters obtained in this study were similar with those in non-Japanese patients, indicating that there would be no ethnic difference in pharmacokinetic profile of nilotinib [16]. Thus, the mean steady-state plasma trough level of 1170 mg/mL (2.2 μM) represents a concentration sufficient to inhibit the in vitro proliferation of most cell lines expressing imatinib-resistant mutant forms of BCR-ABL (with the exception of the T315I mutation) [9].

In summary, nilotinib is highly active and safe, and provides an effective treatment for Japanese patients with Ph+ CML whose disease becomes resistant or intolerant to imatinib. Promising activity is also observed in relapsed/refractory Ph+ ALL patients.

Table 8 Newly occurring or worsening grade 3/4 laboratory abnormalities (SAF population)

	n/N (%)				
	CML-CP $N = 16$	CML-AP $N = 7$	CML-BC $N = 4$	Ph+ ALL $N = 7$	Total $N = 34$
Hematology					
WBC	5/16 (31)	3/7 (43)	3/4 (75)	3/6 (50)	14/33 (42)
Neutrophils	6/16 38)	5/7 (71)	2/3 (67)	3/6 (50)	16/32 (50)
Lymphocyte	6/16 (38)	1/7 (14)	2/4 (50)	2/6 (33)	11/33 (33)
PLT	3/16 (19)	2/6 (33)	2/4 (50)	2/6 (33)	9/32 (28)
Hemoglobin	3/16 (19)	4/7 (57)	4/4 (100)	4/7 (57)	15/34 (44)
Biochemistry					
ALP	0/16 (0)	0/7 (0)	0/4 (0)	0/7 (0)	0/34 (0)
AST (GOT)	0/16 (0)	0/7 (0)	1/4 (25)	1/7 (14)	2/34 (6)
ALT (GPT)	2/16 (13)	0/7 (0)	1/4 (25)	1/7 (14)	4/34 (12)
Bilirubin (total)	1/16 (6)	0/7 (0)	0/4 (0)	0/7 (0)	1/34 (3)
Amylase	1/16 (6)	0/7 (0)	0/4 (0)	0/7 (0)	1/34 (3)
Lipase	4/16 (25)	1/7 (14)	0/4 (0)	0/7 (0)	5/34 (15)
Phosphate (hypo)	2/16 (13)	1/7 (14)	1/4 (25)	1/6 (17)	5/33 (15)
Glucose (hyper)	2/16 (13)	1/7 (14)	0/4 (0)	0/7 (0)	3/34 (9)
Glucose (hypo)	0/16 (0)	0/7 (0)	0/4 (0)	0/7 (0)	0/34 (0)

Patients are counted only for the worst grade observed post-baseline

n number of patients who had less than grade *X* at baseline, and worsened to grade *X* post-baseline; *N* total number of patients evaluable post-baseline who had less than grade *X* at baseline

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特集　MDS診療の最近の進歩

第IV版WHO分類によるMDSの分類と診断*

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Key Words：myelodysplastic syndrome(MDS)，WHO classification，diagnosis，karyotype

はじめに

骨髄異形成症候群(myelodysplastic syndromes；MDS)は造血細胞の異常な増殖とアポトーシスによる細胞死によって特徴づけられる造血器腫瘍とされている．骨髄は過形成であるものの，末梢血液では血球減少を呈するというものである．しかし，こうした疾患概念はあるもののWHO分類の中に記されているように，"These disorders, ... remain among the most challenging of the myeloid neoplasms for proper diagnosis and classification."，すなわち骨髄性腫瘍の中で疾患単位の確立がもっとも遅れている造血器腫瘍でもある．

本稿では昨年改訂された第IV版WHO分類においてMDSがどのように取り扱われているのか，新たに取り入れられた病型などを含めて概説する．

MDS診断の際の芽球カウントについて

第IV版WHO分類[1]では，まず概説の部分でMDS診断における芽球カウントの問題を取り上げている．急性骨髄性白血病(acute myeloid leukemia；AML)，なかでも赤白血病(erythroleukemia /acute erythroid leukemia)とMDSの鑑別の際に，赤芽球が全骨髄有核細胞の50%を超えていた場合non-erythroid cell[NEC，非赤芽球細胞(注：この際の「非赤芽球細胞」は赤芽球だけでなく，リンパ球，形質細胞，マクロファージを除いてカウントすることになっている)]における芽球割合が20%以上かどうかをもってAMLとMDSを区別している．この使い分けは今回の改訂でもそのまま用いられているが，MDSの病型分類の際にNECという考えをもち込むかどうかについてはこれまではっきりと規定されていなかった[2]．すなわち，赤芽球系細胞が50%を超えているが芽球はNECの20%未満であるような例においてMDS病型決定の際の芽球割合を全骨髄有核細胞で計算するか，それともNECで計算するかというものである．コンセンサスはないもののWHOコミッティーの多くが全骨髄有核細胞における芽球%を用いることを推奨している(表1)．とくに不応性貧血(refractory anemia；RA)や環状鉄芽球を伴う不応性貧血(RA with ring sideroblasts；RARS)においては赤芽球増加例が多く，NECでの芽球割合を用いた病型判定を行うとこうした例の臨床的グレードを実際の病態より高くつけてしまう恐れがある．一方で，多系統に異形成を有する例で強い赤芽球異形成があり，かつ好中球への分化が乏しいあるいはほとんどない例などは，全骨髄有核細胞を用いて芽球割合を算定すると逆にhigh-grade MDSとならない場合がある．このような例については今後の症例集積と検討が必要としている．

* Diagnosis and classification of MDS by WHO classification 4th edition.

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表1　赤芽球割合が50%以上の際の診断について

骨髄中の赤芽球系細胞%	末梢血・骨髄所見	その他の所見	診　断
50%以上	末梢血または骨髄ANCの20%以上が芽球	AML with MDS-related changesに適合	AML with MDS-related changes
80%以上がほとんど成熟傾向のない未分化な赤芽球	骨髄芽球はほとんどない・あっても少数	顆粒球系成分はあっても少ない	Pure erthroid leukemia
50%以上	骨髄のANC, 末梢血で芽球が20%未満	芽球がNECの20%以上	Acure erythroid/myeloid leukemia
50%以上	骨髄のANC, 末梢血で芽球が20%未満	芽球がNECの20%未満	MDS：MDSの病型は末梢血中または骨髄ANCにおける芽球割合で分類する

ANC：全骨髄有核細胞

表2　WHO分類であげられているそれぞれの血球系統の異形成

赤芽球系細胞の異形成	
核	nuclear budding
	internuclear bridging
	Karyorhexis
	multinuclearity
	megaroblastic change
細胞質	ring sideroblasts
	vacuolisation
	periodic acid-Schiff positivity
顆粒球系細胞の異形成	
核	hypolobulation (pseudo Pelger-Huet)
	hypersegmentation
細胞質	hypogranularity
	pseudo Chédiak-Higashi granules
大きさ	small size
巨核球系細胞の異形成	
	micromegakaryocyte
	hypolobulated nuclei
	non-lobulated nuclei
	multiple, widely-separeted nuclei

MDS診断の概要

MDSはクローン性の造血器疾患で，血球減少，1系統以上の血球異形成，無効造血と白血病進展リスクによって特徴づけられる．血球減少は無効造血によるが，そこにはアポトーシスが関与している．International Prognostic Scoring System (IPSS)によって規定されている血球減少はヘモグロビン値10g/dl，好中球実数1,800/mm³，血小板数10万/mm³を下回るものとされているが[1]，形態的あるいは細胞遺伝学的特徴がはっきりとしている場合にはこれらの数字を満たさないからといってMDSが否定されるものではない．末梢血および骨髄中の芽球は20%未満であり，これがAMLとの境界である．一般に芽球割合が高い例ほどMDSからAMLへの移行頻度が上昇する．形態異形性も疾患の病態と関連しており，たとえば1血球系統にのみ異形成をもつRA, RARSなどは進行性の骨髄不全を示すことが多いものの長期にわたって白血病化は稀である．このように形態学的特徴，すなわち芽球割合，異形成などによっても症例ごとの予後は大きく異なっている[1]．

MDSの診断のためには上記の血球減少に加えて形態的に芽球，血球の異形成と環状鉄芽球を同定する必要がある．芽球割合を求めるためには有核骨髄細胞500個の分類が，末梢血では200個の白血球分類が推奨されている．血球異形成の同定には質の良いスメア標本が必要である．MDS診断のための標本では，標本作成後の乾燥や染色状況を正確に判断した後にはじめて，血球異形成を判定するよう心がける必要がある．異形成の判定はそれぞれの系統で10%以上の細胞が異形成をもつ場合に陽性とされ，巨核球系では30個以上の巨核球を観察して判断するようにと記載されている．表2に，WHO分類であげられているそれぞれの血球系統の異形成を示す．異形成はMDSの生物学的特性を反映すると考えられるが，染色体異常と形態的特性の関連が知られており，両者の関連を裏づけている(後述)．血球減少と各血球系統の異形成の関連を表3に示す．ほとんどの場合は異形成を示す系統の血球で検査上も減少がみられるが，今回のWHO分類では1系統に異形成があり汎血球減少を示す場合はMDS, unclassified (MDS-U)とするようになっている[1]．

表3 芽球増生がない場合の血球減少，異形成と診断の関連

血球減少	異形成	診断カテゴリー
1血球減少， 2血球減少	1系統	Refractory cytopenia with unilineage dysplasia(RCUD) Refractory anemia(RA) Refractory neutropenia(RN) Refractory thrombocytopenia(RT)
1血球減少， 2血球減少	1系統かつ 環状鉄芽球15%以上	RA with ring sideroblasts(RARS)
汎血球減少	1系統	MDS, unclassified(MDS-U)
1血球減少， 2血球減少， 汎血球減少	多系統	Refractory cytopenia with multilineage dysplasia(RCMD)
1血球減少， 2血球減少， 汎血球減少	多系統かつ 環状鉄芽球15%以上	Refractory cytopenia with multilineage dysplasia(RCMD)

MDS診断における染色体核型

MDSにおいてもAMLと同様に細胞遺伝学的，分子生物学的研究は精力的に行われ多くの成果が上がっているが，AMLにおける細胞遺伝学・分子生物学ほどの解析は進んではいない．しかし，MDSの50%程度は染色体異常をもっており，MDSの予後予測，クローン性造血の確定，形態的特徴を含む臨床所見との関連などその所見は大きな臨床的意味をもっている．たとえばdel(5q)単独の異常例はMDS全体が男性に多いのと対照的に女性に多く，非(低)分葉巨核球，大球性貧血，正常あるいはやや増加した血小板数で良好な長期予後を示す(5q-症候群)．17番染色体短腕の欠損したMDSやAMLでは偽ペルゲル異常や空胞をもつ好中球がみられ，骨髄では芽球増加を認めず，単核の巨核球造生と赤芽球系細胞の減少が特徴的とされている．複雑な核型異常を示す例では多くの場合5番，7番の染色体異常を伴っており予後不良を示唆する．また，del(20q)単独例は赤芽球系と巨核球系の異常が多く，3番染色体異常は異常な巨核球造血と関連している．表4に示す染色体異常はいくつかの例外を除いて不応性貧血だが異形成の明らかではない例に同定されれば暫定的にMDSの診断をつける根拠とされる．

表4 MDSにみられる染色体異常

	頻度 MDS	治療関連 MDS
Unbalanced		
+8	10%	
−7 or del(7q)	10%	50%
−5 or del(5q)	10%	40%
del(20q)	5〜8%	
−Y	5%	
i(17q) or t(17p)	3〜5%	
−13 or del(13)	3%	
del(11q)	3%	
del(12p) or t(12p)	3%	
del(9q)	1〜2%	
idic(X)(q13)	1〜2%	
Balanced		
t(11;16)(p23;q13.3)		3%
t(3;21)(q26.2;q22.1)		2%
t(1;3)(p36.3;q21.2)	1%	
t(2;11)(p21;q23)	1%	
inv(3)(p23;q34)	1%	
t(6;9)(p23;q34)	1%	

MDSのカテゴリーについて

第IV版WHO分類によるMDSのカテゴリーを表5にあげる．これまでと異なりrefractory cytopenia with unilineage dysplasia(RCUD)が新たに作られ，RAに加えて，これまでになかったrefractory neutropenia(RN)，refractory thrombocytopenia(RT)が含まれる．また，小児にみられるMDSの特性を考慮して小児を対象にrefractory cytopenia of childhood(RCC)がprovisional entityとして作られた．それらを含めて個々のカテゴリーを概説する．全体の病型診断の要点を表6にまとめて示す．

表5　第IV版WHO分類によるMDSのカテゴリー

Refractory cytopenia with unilineage dysplasia(RCUD)
Refractory anemia(RA)
Refractory neutropenia(RN)
Refractory thrombocytopenia(RT)
Refractory anemia with ring sideroblasts(RARS)
Refractory cytopenia with multilineage dysplasia(RCMD)
Refractory anemia with excess blasts(RAEB)
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome, unclassifiable(MDS, U)
Childhood myelodysplastic syndrome
Provisional entity：Refractory cytopenia of childhood (RCC)

Refractory cytopenia with unilineage dysplasia(RCUD)/1系統に異形成を有する不応性血球減少

このカテゴリーは単一系統の異形成を示すMDSをまとめたもので，赤血球系のみに有意な異形成をもつRA，顆粒球系(好中球系)の異形性だけをもつRN，巨核球系異型性のみのRTを含んでいる．異形成を有する系統にのみ血球減少が観察されることが多いが，時には2系統の血球減少がある．こうした場合も異形成が1血球系であればRCUDのカテゴリーに分類される．しかし，1系統の異形性だが汎血球減少がある症例も存在し，それはRCUDではなくMDS-Uに分類することになっている．異形成の判定，血球減少のレベルについては前述のとおりであるが，非クローン性疾患における異形成を除外する必要がある．異形成そのものが，必ずしもクローン性造血の証拠とはなりえず，とくにRCUD診断では薬物使用，化学物質への曝露歴など臨床情報が重要となる．ビタミンB_{12}欠乏症，葉酸欠乏症，重金属への曝露，亜ヒ酸の投与，抗生剤(cotrimoxazole)，先天性造血障害，ウイルス感染(パルボウイルスB19)，ミコフェノール酸，化学療法剤，G-CSF，PNH(発作性夜間血色素尿症)，銅欠乏，亜鉛過剰などさまざまな原因によって非クローン性の形態的異形性が生じ，なかには血球減少を伴うものも多い．したがって，クローン性を証明するような所見が得られない場合には(たとえば正常核型例)，MDSの診断を下す前に6か月程度の経過観察が必要とされている．

RCUDにおいては原則として芽球増生はなく，稀な場合を除いて芽球が末梢血にはみられない．したがって末梢血中に1％の芽球かつ骨髄中に5％未満の芽球が2回以上の検査で確認される場合には，RCUDではなくMDS-Uとするよう記載され，さらに，末梢血中に2～4％の芽球があり，骨髄中の芽球が5％未満の場合にはrefrac-

表6　MDS病型の末梢血・骨髄所見

病　型	末梢血所見	骨髄所見
Refractory cytopenia with unilineage dysplasia(RCUD)	単血球減少または2血球減少*1 芽球は稀，(<1％)*2	1系統の異形性，芽球<5％，環状鉄芽球<赤芽球の15％
Refractory anemia with ring sideroblasts(RARS)	貧血あり，芽球なし	環状鉄芽球が赤芽球の15％以上 赤芽球系の異形性のみ，芽球は5％未満
Refractory cytopenia with multilineage dysplasia (RCMD)	血球減少，芽球は1％未満*2 アウエル小体なし，単球は1,000未満	2系統以上の血球10％以上で異形性あり 芽球は5％未満，アウエル小体なし，環状鉄芽球は問わない
RA with excess blasts-1 (RAEB-1)	血球減少，芽球は5％未満，アウエル小体なし，単球は1,000未満	1もしくは多血球系統の異形性 芽球は5～9％，アウエル小体なし
RAEB-2	血球減少，芽球は5～19％，アウエル小体は問わない，単球は1,000未満	1もしくは多血球系統の異形性，芽球は10～19％，アウエル小体は問わない*3
MDS-unclassified(MDS-U)	血球減少，芽球は1％未満	異形成はいずれの系統でも10％未満だが，MDSを推定できる染色体異常あり
MDS associated with isolated del(5q)	貧血，血小板数は正常または増加 芽球はない，または稀	低分葉巨核球の増加，芽球は5％未満，染色体でdel(5q)単一異常，アウエル小体なし

*1：2血球系での減少はありうる．汎血球減少の場合はMDS-Uに分類する．
*2：骨髄芽球が5％未満であっても，末梢血中の芽球が2～4％の場合はRAEB-1とする．RCUD，RCMDにおいて末梢血中の芽球が1％のときにはMDS-Uとする．
*3：アウエル小体があり，末梢血芽球が5％未満かつ骨髄芽球が10％未満の場合はRAEB-2とする．

tory anemia with excess blasts(RAEB)-1と診断すべきであるとされている．

RAにおいては正または大球性，正色素性貧血を呈しdimorphismがみられる．末梢血中の芽球は1％未満で好中球，血小板数は正常であり，異形成も認めらない．骨髄の赤芽球系細胞は減少から増加までさまざまで，10％以上にはっきりとした異形性が認められる．環状鉄芽球は赤芽球の15％未満である．好中球，巨核球は正常または軽度の異形性をもつがその割合は10％未満である．

RNは末梢血または骨髄において10％以上の好中球系細胞にのみ低分葉あるいは過分葉といった異形成を示す．好中球減少は薬剤，毒物曝露，感染症，免疫学的機序，などさまざまな原因で生ずるためそれらを除外する必要がある．他の血球系には有意な異形成はない．

RTは10％以上の巨核球(30個以上を観察して判定)に異形成を認めるもので，他の系統には有意な異形成はない．慢性免疫性血小板減少症(chronic autoimmune thrombocytopenia)との鑑別は臨床・形態学的に時にきわめて困難なことがあり，細胞遺伝学的検査が役立つ場合がある[5]．

Refractory anemia with ring sideroblasts (RARS)/環状鉄芽球を伴う不応性貧血

骨髄赤芽球系細胞の15％以上に環状鉄芽球(ring sideroblasts)を伴うMDSのRAをRARSとしている．赤芽球系の異形成と貧血がみられるもので，赤芽球系以外の骨髄細胞には異形成を認めない．芽球は骨髄において5％未満である．診断の際には二次性に環状鉄芽球を生ずる病態を除外する必要がある．

環状鉄芽球は赤芽球においてミトコンドリアにおける鉄代謝が異常となり，ミトコンドリアに鉄が蓄積するものである[6]．正球性から大球性貧血で，典型的には末梢血の赤血球は正色素性赤血球(大部分を占める)と低色素性赤血球のdimorphism(2相性)を示す．末梢血には芽球はなく，骨髄においても5％未満である．骨髄は赤芽球過形成となり異形成は基本的に赤芽球系のみに認められ，好中球系，巨核球系には10％未満にしか認められない．ヘモジデリンを貪食したマクロファージが増加していることもある．鉄染色において15％以上の赤芽球系細胞に環状鉄芽球があるが，環状鉄芽球は5個以上の鉄顆粒が核周の1/3以上にわたって分布しているものと定義されている．環状鉄芽球は他のMDS病型にも観察されるが，芽球％，異形成の認められる血球系統数によって上記以外は他の病型となる．Refractory cytopenia with multilineage dysplasia(RCMD)に環状鉄芽球が認められる場合はRARSより予後が不良である．環状鉄芽球を生ずる非クローン性病態としてアルコール，鉛，ベンゼン中毒，薬剤(イソニアジド)，亜鉛投与，銅欠乏，先天性環状鉄芽球症があり，鑑別を要する[7]．

RCMD/多系統に異形成を伴う不応性貧血

RCMDは2血球系統以上に有意な異形成があるMDSで，末梢血芽球が1％未満，骨髄芽球が5％未満のものを指す．芽球にアウエル小体はなく，末梢血中の単球増加もない．それぞれが認められる場合はRAEB, CMMLの診断となる．末梢血の血球減少(ヘモグロビン値10g/dl，好中球実数1,800/mm^3，血小板数10万/mm^{3}未満)は1血球系以上にあるが，染色体所見，形態所見がはっきりとしていれば，MDSの診断はそれらの数字にとらわれるものではない．末梢血中に2～4％の芽球が認められれば，骨髄中の芽球が5％未満であってもRAEB-1の診断をつけるようになっている．アウエル小体を認める場合はRAEB-2となる．認められる異形成はRCUDよりも強く，時には環状鉄芽球が15％を超えることもある．以前はRCMD-RSというカテゴリーがあったが，この版ではRCMDとひとつになっている．染色体異常はトリソミー8，モノソミー7または5，del(5q)，del(20q)，複雑核型など50％程度の症例にみられる[8]．

RAEB/芽球増加を伴う不応性貧血

RAEBは骨髄に5～19％の，末梢血に2～19％の芽球を認めるMDSで，芽球の割合によってさらにRAEB-1, RAEB-2に分けられる．RAEB-1は骨髄5～9％，末梢血2～4％の芽球を認める場合でこの基準を超えるとREAB-2とされる．この

両者ではAMLへの移行頻度，生存に差が認められる．また，芽球にアウエル小体が認められれば芽球の割合にかかわらず(たとえRAの範疇であっても)RAEB-2となる．

RAEBでは骨髄は過形成，3血球系にさまざまな異形成があり，一般にはその程度も強い．骨髄生検で未分化な前駆細胞の集合がみられることがあり，abnormal localization of immature precursors(ALIPS)と呼ばれ重要な所見である．増加している芽球はCD34, CD117に加えて骨髄系のマーカーが陽性のことが多く，生検標本でのCD34免疫組織染色は幼若細胞増加の判定に役立つ．

MDSの15%程度で骨髄線維化を伴い，MDS with fibrosis(MDS-F)と呼ばれる[9]．治療関連MDSでも時に骨髄線維化がみられ，またHIV関連骨髄症など反応性の線維化もあるため，鑑別が必要である．しかし，線維化が独立して予後と関連するのか明らかになっていない．多くのMDS-FはRAEBに分類され，この場合芽球の増加を正しく判定するには骨髄生検を実施するしかない．AMLの一病型であるacute panmyelosis with myelofibrosis(APMF)との鑑別も必要になる．

Myelodysplastic syndrome with isolated del(5q)/del(5q)単独異常をもつMDS

染色体検査にて単独でdel(5q)をもつMDSがこのカテゴリーに含まれる．末梢血では芽球1%未満で，骨髄でも5%未満である．5q-症候群と呼ばれる．女性に多く，大球性貧血を示す．血小板数は正常ないし，やや増加している．骨髄では巨核球が増加しており，これらは低分葉の核をもっている．一方で赤芽球系，顆粒球系の異形成は明らかではなく，赤芽球成分は減少していることがある．

この一群は無治療であっても予後が良く，生存期間中央値は145か月といわれている．しかし，国内(おそらくはアジア)では欧米と比較して頻度が低い[10]．近年レナリドマイドが著効を示す疾患としても広く知られるようになった[11]．

MDS-U/ 分類不能のMDS

MDSが考えられるものの，これまで述べてきたカテゴリーに含まれないものをMDS-Uとしている．①RCUDで末梢血液に1%以上の芽球が認められる例，②汎血球減少を伴うRCUDカテゴリーに入る例，③芽球増加がなく血球異形成も10%未満だが染色体所見からMDSとされる例などはここに分類することになっている．MDS-Uとされた例についてはその後注意深く経過を追う必要がある．後に別の病型へ変化した際には，病型の変更を行うことになっている．

Childhood MDS/小児のMDS

このカテゴリーは，今回 "provisional" として設定された．小児においてMDSはきわめて稀な疾患で，小児造血器腫瘍のおおよそ5%を占めるとされる．初発MDS(*de novo* MDS)は，先天性あるいは後天性血液疾患に続発する二次性MDS, 化学療法後に発症する治療関連MDSとは区別されるべきである．以前はダウン症候群にみられる造血異常が*de novo* MDSに含まれることもあったが，今回のWHO分類では"Myeloid proliferations related to Down syndrome" としてカテゴリーが作られており，そうした例は小児MDSには含まれない．

小児MDSにおいても成人の病型がみられるが，芽球の増加がみられない病型では頻度に違いがあり，たとえば小児においてはRARS, 5q-症候群は大変稀である．RCUDに分類されるような例は，成人ではRAが多いが小児ではRN, RTが多くみられる点も違っている．骨髄低形成を示す例も多い．そのため小児に対してrefractory cytopenia of children(RCC)カテゴリーをprovisionalとして設定してある．RCCとはMDSの中で末梢血中の芽球が2%未満，骨髄中の芽球は5%未満のもので，赤芽球系，顆粒球系，巨核球系に有意な異形成を示すものである[12]．前述のようにRCCの75%は低形成骨髄であり，骨髄不全症候群，なかでも後天性の再生不良性貧血や先天性の造血不全との鑑別に骨髄生検が欠かせないとされている．小児でも種々の病態で形態的異形成が観察されうるため(表7)，幅広い鑑別が必要なことは成人と同じである．症例も少なくRAEBの意義など今後の検討すべき課題も多々あり，provisional entityとされた．

表7 Refractory cytopenia of childrenと類似の異形成を呈しうる疾患

- 感染症(サイトメガロウイルス，ヘルペスウイルス，パルボウイルスB19，リーシュマニア)
- ビタミン欠乏(ビタミンB_{12}，葉酸，ビタミンE)
- 代謝異常(メバロン酸キナーゼ欠損)
- リウマチ性疾患
- 自己免疫性リンパ増殖性疾患(FAS欠損症)
- ミトコンドリア異常症(ピアソン症候群)
- 先天性骨髄不全症候群(Fanconi貧血，先天性角化不全症，Shwachmann-Diamond症候群，無巨核球性血小板減少症，橈骨欠損を伴う血小板減少症，先天性橈尺骨癒合症，Seckel症候群)
- 発作性夜間血色素尿症
- 造血回復期の後天性再生不良性貧血

おわりに

今回の改訂ではRCUDとしてこれまで触れられていなかったRN，RTが新たに記載された．赤血球系以外の1血球系にのみ異形成をもち，1または2血球減少をもつ症例はMDSの多数を占めるわけではないがこれまでおそらくRAとして分類されていたと思われる．今後はこのカテゴリーに症例が集積されいわゆるRAとの違いが検討されていくであろう．また，小児MDSカテゴリーが作られたことで，この分野の研究が進むことが期待される．

今回の改訂において染色体に関する記載は詳しくなっているが，白血病やリンパ腫と比較すると分子レベルでの疾患解析については記載が少ない．5q-症候群におけるレナリドマイドの著効や脱メチル化薬の効果など治療反応を切り口とした疾患の解析も求められる．

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ORIGINAL ARTICLE

High-resolution melting analysis for a reliable and two-step scanning of mutations in the tyrosine kinase domain of the chimerical *bcr-abl* gene

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Abstract For relevant imatinib therapy against Philadelphia (Ph)-positive leukemias, it is essential to monitor mutations in the chimerical *bcr-abl* tyrosine kinase domain (TKD). However, there is no universally acceptable consensus on how to efficiently identify mutations in the target TKD. Recently, high-resolution melting (HRM) technology was developed, which allows gene scanning using an inexpensive generic heteroduplex-detecting dsDNA-binding dye. This study aimed to validate the introduction of HRM in a practical clinical setting for screening of mutations in sporadic sites of the chimerical *bcr-abl* TKD. All chimerical and wild-type *abl* TKD regions selectively amplified were used for HRM assays and direct sequencing. The HRM test had approximately 5–90% detection sensitivity for mutations. In contrast to mixture samples with mutant and wild-type cells, all mutant cell samples had indeterminate melting curves equivalent to those of the wild-type due to formation of only a homodulex. This issue was improved by the addition of exogenous wild-type DNA after PCR. Subsequently, HRM results gave a high accordance rate of 97.8% (44/45 samples) compared to the sequencing data. The discordant results in one appear to be due to unsuccessful amplification. Thus, HRM may be considered to be suitable for reliable scanning of mutations in the chimerical *abl* TKD in a clinical setting.

Keywords Ph · *bcr-abl* · Mutation · Melting analysis · HRM

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1 Introduction

The chemical agent, imatinib, has a high therapeutic response for diseases carrying the chimerical *bcr-abl* gene. For example, the first imatinib treatment was reported to give approximately 80 and 60% complete molecular response rates in chronic myelogenous leukemia (CML) and Philadelphia (Ph)-positive acute lymphoblastic leukemia (ALL), respectively [1, 2]. However, in many cases, mutations in the chimerical *abl* tyrosine kinase domain (TKD) were revealed to precede resistance to imatinib, resulting in disease relapse and progression to advanced disease. At present, although it is known that there are several causative factors in resistance, such as expression of a rapid drug efflux protein and non-*bcr-abl*-dependent transformation involving the *src* family, TKD mutations in the chimerical gene are thought to play a major role in resistance acquisition [3]. Therefore, detection of mutations becomes essential in cases treated with imatinib. Moreover, mutations associated with imatinib therapy emerge as a Ph-positive subclone from minimal residual leukemia (MRD) even in the hematological remission period [4, 5]. This indicates the need for highly sensitive tests to detect only Ph-positive leukemic clones. To date, several methods to analyze mutations including direct and subcloning sequencing have been employed, but the respective methods have merits and demerits and are not

always sensitive [6]. Recently, to analyze genetic variations (SNPs, mutations and methylations), a novel melting analysis called high-resolution melting (HRM) with an automated instrument and real-time PCR apparatus was used [7, 8]. HRM is used to characterize samples according to their dissociation profile as they transit from double-strand DNA (dsDNA) to single-strand (ssDNA). Therefore, mixture samples with mutant and wild-type cells are easily identified by differences in melting curve shapes. Mutant sequence variants produce a Tm shift compared with the wild-type [9]. In addition, it is a reliable- and closed-tube system without high-cost fluorescence probes [10, 11]. Thus, to introduce HRM assays in clinical settings to detect Ph-positive subclones with *bcr-abl* kinase domain mutations, the relevance and validation of the assay prior to direct sequencing was studied.

2 Materials and methods

2.1 Samples and processing of cDNA

A total of 19 Ph-positive samples were used, consisting of 10 unlinked and already mutation-known specimens, 8 fresh practical samples from 6 patients with CML, 2 patients with ALL and one sample from a Ph-positive K562 cell line. All patients with Ph-positive leukemias were being treated with imatinib at 400–800 mg per day and had hematological remission, but were positive for *bcr-abl* real-time RT PCR. As controls, 16 peripheral blood samples from normal volunteers and 10 cell lines consisting of HTLV-1-associated cell lines (Hut102, KK1, KOB, OMT, MT2, SO4, ST1), T cell lines of Jurkat and MOLT4, and the monocytic line U937 were used.

Total RNA was extracted from total leukocyte guanidinium thiocyanate lysates using ISOGEN (Nippon gene, Toyama, Japan). cDNA was synthesized using oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, Carsland, CA, USA). Practical and stocked samples used in this study were applied under the approval (15040708) of the ethics committee and the condition of the criteria of the Japanese Association of Laboratory Medicine.

2.2 Study design for HRM assay in the chimerical *abl* TKD region

Our study for the detection of mutation was designed in a two-step manner: firstly, genetic alteration screening by HRM analysis (Fig. 1), using the LightCycler 480 (Roche Molecular System, Alameda, CA, USA) for HRM and real-time thermal cycling; secondly, only samples positively screened by HRM were directly sequenced. First of all, each sample was examined for *bcr-abl* chimerical status by

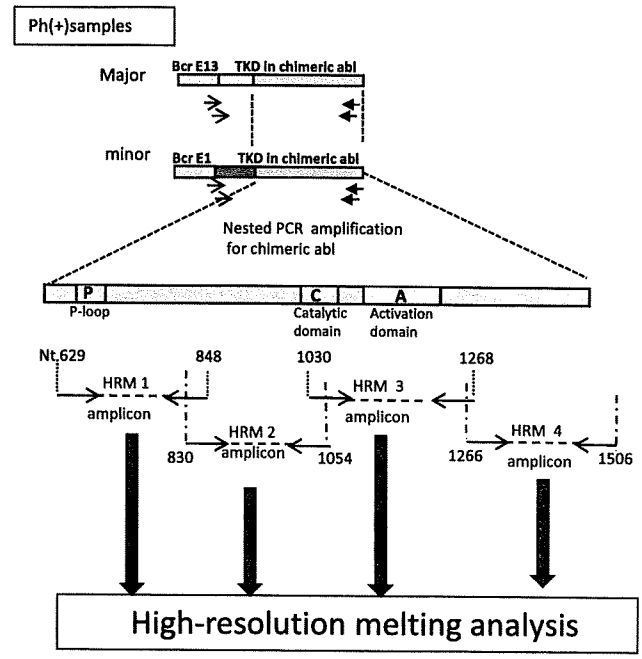

Fig. 1 Study design of high-resolution melting (HRM) and the structure of the tyrosine kinase domain (TKD). Firstly, a part of chimerical *bcr-abl* TKD is selectively amplified by nested PCR. Using amplified chimerical *bcr-abl* products as a template, the second PCR for the HRM assay is performed, generating four intercalating dye amplicons, HRM-1, -2, -3 and -4. Then, HRM analyses were done by using the LightCycler Gene Scanning Application

the conventional method [12]. If positive for major or minor chimerical types, these *bcr-abl* kinase domains were selectively amplified, generating a fragment of 1504 bp for b2/a2 and 1579 bp for b3/a2, using primers previously reported [13].

For HRM analysis, PCR products of 50–250 bp length are recommended for best discrimination. Therefore, we applied a modified method previously reported by Poláková et al. [10], generating 4 amplicons, designated as HRM1, 2, 3 and 4 of 220, 225, 239 and 241 bp, corresponding to nt 629–848, 830–1054, 1030–1268, and 1266–1506 (NM_005157), respectively. For HRM, a PCR reaction was performed in 20 μl reaction volumes containing 1 μl of 1/200 diluted template generated as described above, Master Mix, Taq DNA polymerase, dNTP mix, HRM dye, 3 mM $MgCl_2$, primers [10], and 1 M GC melt, according to the instructions of Roche Applied Science (Manheim, Germany). The PCR was monitored by real-time cycling and a strong fluorescent signal was generated only when bound to dsDNA, that is the touchdown PCR cycling and HRM conditions [14]. HRM melting curve data were obtained by slowly increasing the temperature, from 60 to 90°C at a rate of 100 acquisitions per 1°C. The melting status and changes in Tm value were analyzed using the Roche HRM algorithm (Gene Scanning Software, Roche Supplied Science, Manheim, Germany),

depicting graphs of fluorescence-normalized and temperature-shifted melting curves and difference plots. The cell line K562 was used as a wild-type reference sample.

2.3 Sequencing

In this study, to compare to accuracy of HRM analysis, regardless of the first-step negative samples, all of the samples used were confirmed by sequencing the regions of the selectively amplified chimerical *abl* TKD, as well as *abl* TKD from Ph-negative controls using a Big Dye terminator kit Ver 3.1 (Applied Biosystems, Carsland, CA, USA) and the ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

3 Results

3.1 HRM assay validation

For HRM scoring, Ph(+) K562 was set up as a wild-type genotype and the 2 or 3 normal blood samples were monitored as negative controls. First of all, using three different samples with mutations, HRM analysis in duplicate was performed, generating constant positive melting curves both in terms of shape and peak height with a range of melting temperatures (Tm) from 84 to 86°C (Fig. 2). On the other hand, 16 samples without the Ph-chromosome constantly produced the wild-type scanning profiles according to the Roche HRM program, as shown in Fig. 3 (normalized and temperature-shifted melting curves and difference plots).

Interestingly, as shown in Fig. 2b, no correlation was observed between fluorescence heights and the ratio of the mutant and wild-type: the peak was higher in mixture samples with mutant and wild-type cells than samples with only mutant clones. To address this strange relation, variable mixture samples with mutant and wild-type cells diluted by exogenous control DNA from wild-type cells were subjected to HRM assay. Figure 4 shows that samples containing only mutant cells (bottom graph) produced indeterminate signals with low peaks, but the mixture samples containing variable wild-type cell burden (5–90%) displayed apparently higher peaks, indicating the existence of mutation. This shows that 100% mutant samples may become false negative, indistinguishable from only wild-type patterns. To form only homoduplex, dsDNA in either mutant or wild-type DNA probably accounted for the indeterminate evaluation in all mutant or wild-type samples.

Accordingly, to avoid false negatives in samples with all mutant cells, we organized screen mutations using two divided samples; one was an original and the other mixed with exogenous wild-type cells, as shown in Fig. 5. Using this strategy, the test performance of HRM was examined in this study.

3.2 HRM assay results

The HRM assay was blindly examined in duplicate by a single researcher and then compared to sequence data. As summarized in Table 1, the HRM test was positive for 13 (68.4%) of 19 Ph-positive leukemias, including a Ph-positive K562 cell line. Using the same amplicons as

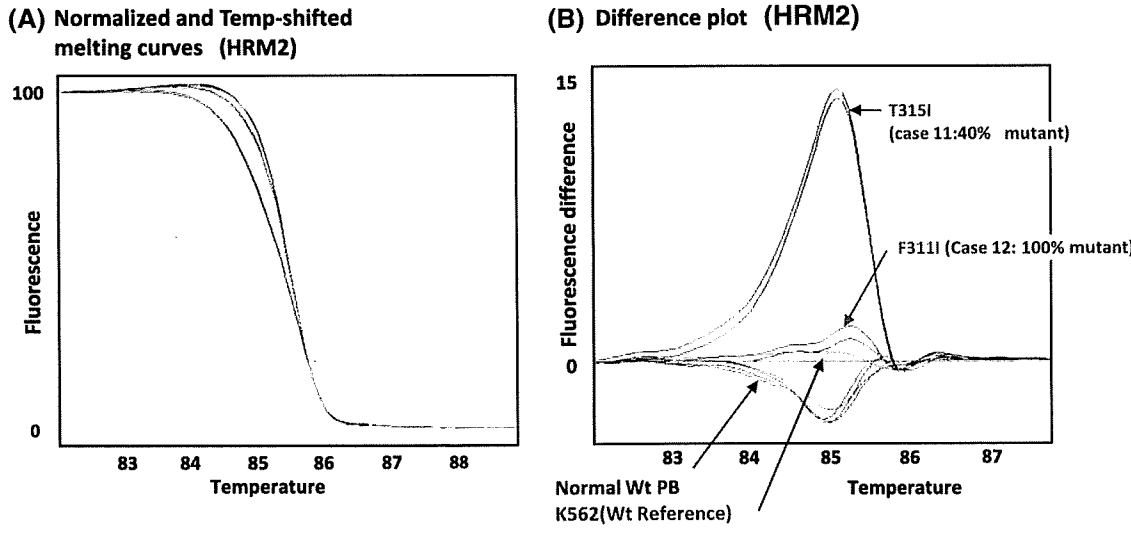

Fig. 2 Validation of the HRM assay using samples with 100% mutant cells and samples with variable % mutant cells. Duplicate assays gave rise to the similar results, indicating the good reproducibility in both normalized and temperature-shifted melting curves and difference plots. Ph-positive K562 cells were used as a wild-type (Wt) reference

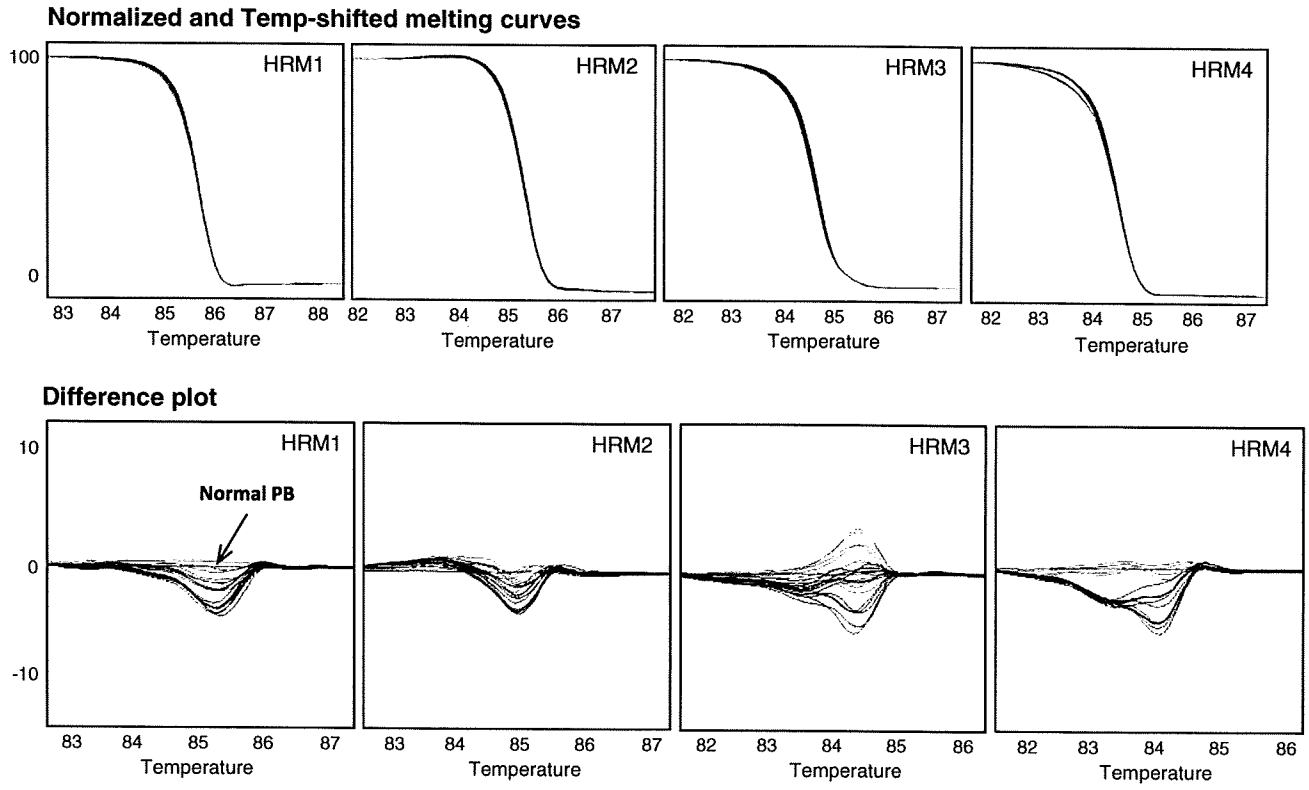

Fig. 3 Validation of HRM test performance using negative controls with wild-type TKD demonstrated by direct sequencing in peripheral blood from 16 healthy persons and 9 hematopoietic cell lines, excluding the U937 cell line. All 4 HRM analyses were evaluated to be wild-type by both normalized-temperature-shifted melting curves according to the Gene Scanning Application Algorithm. Normal peripheral blood from volunteers was used as a wild-type reference

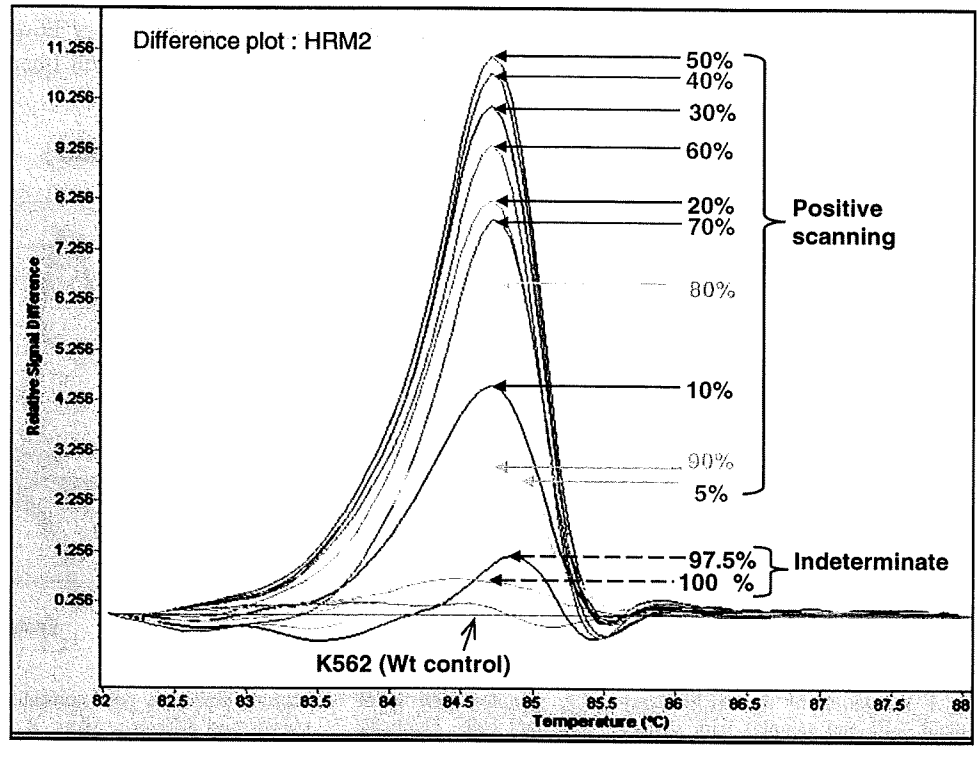

Fig. 4 Changes in the heights of peaks and Tm values depending on the difference in the mixture ratio of mutant and wild-type cells. HRM assay revealed indeterminate signals in samples with only mutant cells (around 100% mutant cell samples). On the other hand, the mixture samples diluted to 5% mutant cells produced typical positive scanning patterns, indicating that the best mixture ratio was 50 versus 50%

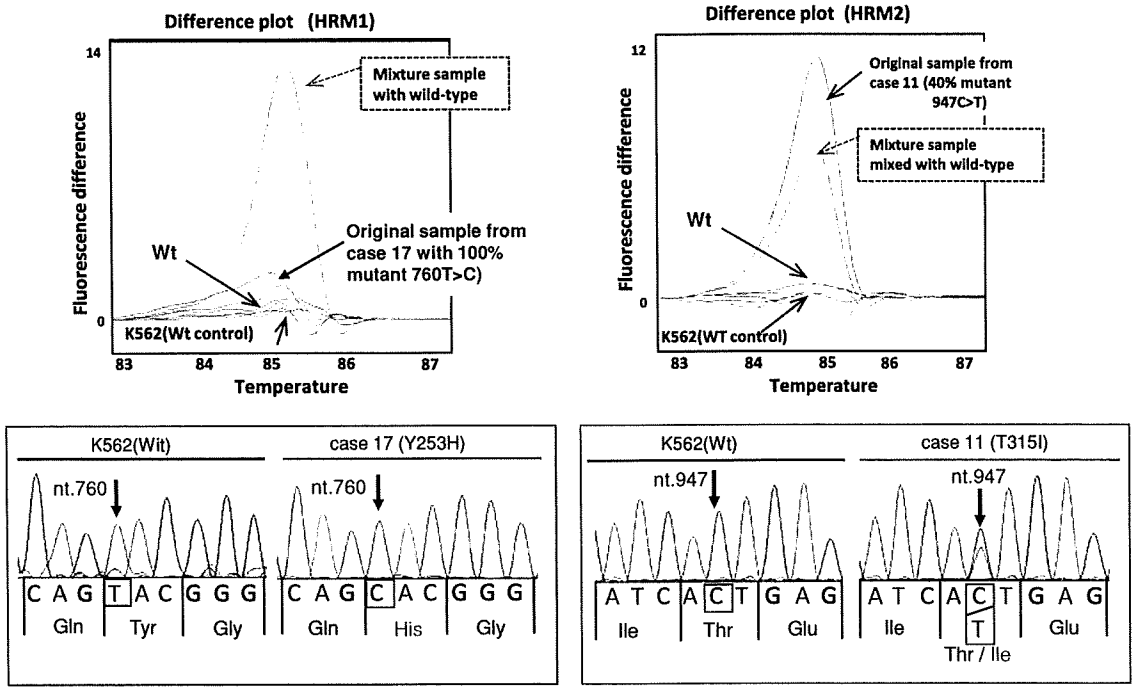

Fig. 5 Representative cases (A and B) of HRM and sequencing analyses. The left panel showed that the addition of wild-type DNA into the 100% mutant (760 T > C) sample made discrimination easy. On the other hand, the right panel showed that the change in the melting curves between the original (947 C > T) and the mix allowed discrimination

above, direct sequencing identified 14 missense mutations (73.6%) out of 19 Ph(+) leukemias. The positive and negative accordance rate of both tests was 94.7% among 19 Ph(+ or −) samples. The discrepancy in sample no. 5, negative in the HRM and positive in the sequencing, was expected before HRM analysis, because the PCR efficiency was not so good. As expected, the direct sequencing disclosed a problematic issue for PCR, in that a mutation (nt 838) existed within the annealing sequence (nt830-849) of the primer.

Next, of 26 Ph-negative samples consisting of 10 cell lines and 16 normal blood controls, HRM assays produced negative findings in all but one. The positive one for HRM was U937 derived from myelomonocytoid leukemic cells. The sequencing revealed a mutation of E308V, which was expected to be somatic as it is one of the oncogenes.

Conclusively, the accordance rate of the two methods was 97.8% in all 45 cases of Ph-positive/-negative leukemias and controls.

4 Discussion

Most patients with Ph-positive leukemias, especially chronic CML, who receive imatinib as first-line therapy achieve good cytogenetic and molecular responses. However, long-term molecular studies suggest that around 25–30% of patients seem not to achieve successful responses and undergo disease progression. Major causes of imatinib resistance include the emergence of leukemic clones with mutations in the tyrosine kinase domain of *bcr-abl*. This indicates that it is necessary to screen for mutations in early phase of the emergence of mutation clones. Unfortunately, there is generally no acceptable consensus on when and by which technology the TKD mutations should be screened. At present, direct sequencing, denaturing high-performance liquid chromatography (D-HPLC), denaturing gradient gel electrophoresis (DGGE), allele-specific oligonucleotide-polymerase chain reaction (ASO-PCR) and pyro-sequencing are available, but the respective methods have merits and demerits for practical clinical settings [6]. A novel technology of HRM with the development of instruments and saturating intercalating dyes is emerging for the detection of nucleic acid sequence variations and is now applied in practical diagnostic settings [7, 15]. The two-step method allows to avoid the direct sequencing for the entire region of all samples.

In this study, the HRM in our system was shown to efficiently and simply differentiate mutations in the chimerical *bcr-abl* TKD region by using LightCycler technology and a software algorithm. In particular, it is noteworthy that mixture samples with mutant and wild-type cells were easily detectable with high sensitivity (approximately 5%). On the other hand, samples with all

Table 1 Summary of the results on mutations examined by both HRM and direct sequencing analyses

Sample	Clinical dx	bcr-abl	HRM results	TKD	nt substitute	Exon
Unlinked						
1	CML	Major	Mut	Y253F	761 A > T	4
2	CML	Major	Mut	Y253H	760 T > C	4
3	CML	Major	Mut	E255 V	767 A > T	4
4	CML	Major	Mut	E255 K	766 G > A	4
5	CML	Major	NE	E279 K	838 G > A	5
6	CML	Major	Mut	T315I	947 C > T	6
7	CML	Major	Mut	F317L	954 C > A	6
8	CML	Major	Mut	M351T	1055 T > C	6
9	CML	Major	Mut	H396R	1190 A > G	7
10	CML	Major	Mut	F486S	1460 T > C	9
Fresh						
11	CML	Major	Mut	T315I	947 C > T	6
12	CML	Major	Mut	F311I	934 T > A	6
13	CML	Major	Mut	L370R	1112 T > G	7
14	CML	Major	Wt	Wt		
15	CML	Major	Wt	Wt		
16	CML	Major	Wt	Wt		
17	ALL	Minor	Mut	Y253H	760 T > C	4
18	ALL	Minor	Wt	Wt		
Cell lines						
19	K562	Major	Wt	Wt		
20	U937	(–)	Mut	E308V	926 A > T	5
21	KOB	(–)	Wt	Wt		
22	KK1	(–)	Wt	Wt		
23	ST1	(–)	Wt	Wt		
24	SO4	(–)	Wt	Wt		
25	OMT	(–)	Wt	Wt		
26	MT2	(–)	Wt	Wt		
27	Hut102	(–)	Wt	Wt		
28	Jurkat	(–)	Wt	Wt		
29	MOLT4	(–)	Wt	Wt		
Normal PB						
30–45		(–)	Wt	Wt		

Mut mutation, *Wt* wild-type, *NE* not evaluated

mutant cells often present with indeterminate low peaks in difference plots, causing confusing interpretations. This is probably the main defect in this HRM technology, resulting from a dependency on heteroduplex formation due to the mixture ratio of the mutant and wild-type. Therefore, to avoid false negatives with samples containing all mutant cells, it was shown that the addition of exogenous control DNA was useful (Figs. 4, 5). Actually, since the ratio of mutant cells in the samples is unknown in practical samples, we adopted an HRM assay system, which measures using a double feature: an original one and mixtures of the mutant and exogenous wild-type DNA in the ratio of 1:1. Practical examples are shown in Fig. 5, indicating that the mixed sample (left panel) makes it easy to discriminate, whereas the change in the positive peak pattern was tolerable if a 40% mutant sample was diluted to 50%.

Finally, our HRM results were in agreement in all but one of 45 samples with an accordance rate of 97.8% compared with sequencing data. The discrepancy in the results in one sample was expected due to an accidental relation between the primer and mutation sites, as described above. The quality of HRM is thought to be highly dependent on real-time amplification, so that we are now revising part of the primer set and appropriate sequence length for HRM.

Clearly, this is a rapid, simple, accurate screening method using HRM technology for chimerical *bcr-abl* TKD mutations involved in resistance to imatinib. Since resistant Ph-subclones emerge from MRD and increase step by step in parallel with the long imatinib therapy duration, the HRM assay system is a suitable and useful method to better manage Ph-positive leukemias, for example, to decide on dose escalation or cessation of imatinib, alternation of new drugs or different therapies with dasatinib and bone marrow transplantation. Actually, we are applying this method in a routine clinical setting prior to sequencing to select only mutation-positive samples.

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ORIGINAL ARTICLE

Comprehensive analysis of cooperative gene mutations between class I and class II in *de novo* acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) has been thought to be the consequence of two broad complementation classes of mutations: class I and class II. However, overlap-mutations between them or within the same class and the position of *TP53* mutation are not fully analyzed. We comprehensively analyzed the *FLT3, cKIT, N-RAS, C/EBPA, AML1, MLL, NPM1,* and *TP53* mutations in 144 newly diagnosed *de novo* AML. We found 103 of 165 identified mutations were overlapped with other mutations, and most overlap-mutations consisted of class I and class II mutations. Although overlap-mutations within the same class were found in seven patients, five of them additionally had the other class mutation. These results suggest that most overlap-mutations within the same class might be the consequence of acquiring an additional mutation after the completion both of class I and class II mutations. However, mutated genes overlapped with the same class were limited in *N-RAS, TP53, MLL*-PTD, and *NPM1*, suggesting the possibility that these irregular overlap-mutations might cooperatively participate in the development of AML. Notably, *TP53* mutation was overlapped with both class I and class II mutations, and associated with morphologic multilineage dysplasia and complex karyotype. The genotype consisting of complex karyotype and *TP53* mutation was an unfavorable prognostic factor in entire AML patients, indicating this genotype generates a disease entity in *de novo* AML. These results collectively suggest that *TP53* mutation might be a functionally distinguishable class of mutation.

Key words acute myeloid leukemia; overlap mutations; TP53; multilineage dysplasia; prognosis

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Acute myeloid leukemia (AML) is a genetically and phenotypically heterogeneous disease (1). In 1999, the third edition of the World Health Organization (WHO) classification of the myeloid neoplasms classified AML into four major categories: AML with recurrent genetic abnormalities (AML-RGA), AML with multilineage dysplasia (AML-MLD), AML, therapy-related, and AML not otherwise categorized (2). The first category included AML with t(8;21)(q22;q22), (*AML1/ETO*), inv(16)(p13q22) or t(16;16)(p13;q22), (*CBFB/MYH11*), t(15;17)(q22;q12), (*PML/RARA*) and 11q23 (*MLL*) abnormalities, which create fusion genes associated with leukemogenesis. Each balanced translocation corresponded to characteristic cytogenetical and clinical

features. These translocations can be detected by reverse transcriptase-mediated PCR (RT-PCR), which becomes a sensitive, rapid and objective method for diagnosis (3). However, the classification of other categories is based on morphology of bone marrow (BM) cells and on the history of patients, although a number of genetic alterations, which are involved in the pathogenesis of AML and associated with the prognosis of patients, have been documented (4). Recently, it has been demonstrated that mutations of *FLT3*, *NPM1*, and *C/EBPA* genes are preferentially found in AML with normal cytogenetics and are highly implicated in the prognosis (5). The fourth edition of the WHO classification have included *NPM1* and *C/EBPA* mutations as provisional entities in AML-RGA, but not *FLT3* mutation because it is associated with a number of other entities (6). Furthermore, the AML-MLD category has been renamed as AML with myelodysplasia-related changes, in which myelodysplastic syndrome (MDS)-related cytogenetic abnormality, as well as previous history of MDS and MLD, has been included as a criteria for the diagnosis. However, it was suggested that AML is the consequence of two broad complementation classes of mutations: those that confer a proliferative and/or survival advantage to hematopoietic progenitors (class I mutation) and those that impair hematopoietic differentiation and confer properties of self-renewal (class II mutation) (7). In addition, clinical significance of genetic alterations in the setting of morphologic MLD remains unclear. Therefore, it is necessary to analyze genetic alterations comprehensively, taking them into account all together rather than individually to elucidate the genetic background and prognostic impact in AML (8).

It has been generally considered that *FLT3*, *cKIT*, and *N-RAS* mutations are class I mutations, and *C/EBPA* and *AML1* mutations, and *AML1/ETO*, *CBFB/MYH11*, *PML/RARA*, and *MLL* abnormalities are class II mutations, while overlap mutations of these mutations between class I and class II or within the same class in a clinical sample are not fully characterized, and the positions of *NPM1* mutation and the partial tandem duplication of the *MLL* gene (*MLL*-PTD) remain unclear. *TP53* mutations are reportedly infrequent but are associated with a poor prognosis in *de novo* AML (9–11). In addition, an association between *TP53* mutations and complex karyotype in therapy-related MDS and AML has been reported (12, 13). However, the position of *TP53* mutation remains unclear.

In this study, we comprehensively analyzed mutations of *FLT3*, *cKIT*, *N-RAS*, *C/EBPA*, *AML1*, *MLL*, *NPM1*, and *TP53* genes as well as cytogenetics in newly diagnosed *de novo* AML to disclose the feature of their overlap mutations. Furthermore, we examined the association of cooperative mutations with clinical characteristics and morphologic MLD of *de novo* AML.

Patients and methods

Patients and samples

The diagnosis of AML was based on the WHO classification. All BM smears from patients were evaluated by the authors according to the WHO criteria and morphological diagnosis was confirmed. The study population included 144 newly diagnosed *de novo* AML patients from January 1990 who were received the remission induction therapy in our institutes. We unselectively included all patients into the present study if their samples were available. The median age and WBC count at the diagnosis of the analyzed patients were 52 yr (range, 15–85 yr) and 10.3×10^9/L (range, $0.6–351 \times 10^9$/L), respectively. Twenty-one patients were of age 65 yr or older. Cytogenetic analysis revealed that a normal karyotype was found in 54 patients and an abnormal karyotype was in 90 patients including 19 t(8;21)(q22;q22), 3 inv(16)(p13q22), 14 t(15;17)(q22;q12) and 2 11q23 abnormalities. AML-MLD was identified in 34 patients, who did not have a history of MDS. BM samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committee of Nagoya university school of medicine.

Therapy

Among the AML patients analyzed, patients younger than 65 yr old were treated with the AML protocols of the Japan Adult Leukemia Study Group or their modifications (14, 15). Briefly, the induction therapy consisted of cytarabine (Ara-C) and idarubicin (IDR) or Ara-C and daunorubicin (DNR). Patients who achieved complete remission (CR) subsequently received three courses of consolidation therapy consisted of high-dose Ara-C or four courses of consolidation consisted of Ara-C and mitoxantrone, Ara-C and DNR, Ara-C and aclarubicin, and Ara-C, etoposide, vincristine and vindesine. Patients aged 65 yr or older received the dose-reduced induction therapy consisted of Ara-C and IDR or Ara-C and DNR. For the consolidation therapy, four courses of the dose-reduced regimen were administered.

Cytogenetics analysis

The cytogenetic G-banding analysis was performed with standard methods. A complex karyotype was defined as