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REVIEW

Current approach to relapsed acute lymphoblastic leukemia in children

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Abstract

Recurrent acute lymphoblastic leukaemia (ALL) is a common disease for pediatric oncologists and accounts for more deaths from cancer in children than any other malignancy. Although most patients achieve a second remission, about 50% of relapsed ALL patients do not respond to salvage therapy or suffer a second relapse and most children with relapse die. Treatment must be tailored after relapse of ALL, since outcome will be influenced by well-established prognostic features, including the timing and site of disease recurrence, the disease immunophenotype, and early response to retrieval therapy in terms of minimal residual disease (MRD). After reinduction chemotherapy, high risk (HR) patients are clear candidates for allogeneic stem cell transplantation (SCT) while standard risk patients do better with conventional chemotherapy and local therapy. Early MRD response assessment is currently applied to identify those patients within the more heterogeneous intermediate risk group who should undergo SCT as consolidation therapy. Recent evidence suggests distinct biological mechanisms for early *vs* late relapse and the recognition of the involvement of certain treatment resistance related genes as well cell cycle regulation and B-cell development genes at relapse, all providing the opportunity to search for novel target therapies.

Key words: Children; Relapse; Acute lymphoblastic leukaemia

Core tip: Selected recent publications regarding the current management of childhood relapsed acute lymophoblastic leukemia have been reviewed. Controversies, current lines of investigation and possible future directions are discussed.

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INTRODUCTION

Despite current cure rates above 75%[1], relapse is the most important obstacle in definite cure of children with acute lymphoblastic leukaemia (ALL)[2]. Depending on certain risk factors, such as age at diagnosis, sex, ethnicity, presenting white blood cell (WBC) count, hematopoietic lineage of the disease, cytogenetic abnormalities, and early response to primary therapy (Table 1)[3], approximately 20% of children diagnosed with ALL will experience relapse after current frontline therapy[4-17]. In a retrospective analysis of 9585 patients registered within 10 consecutive Children's Cancer Group (COG) studies, the relapsed cohort had a higher percentage of patients who fell in the age range < 1 or $\geqslant 10$ years, as well as a higher proportion of males, patients with initial WBC count > 100000/mL, and African American or Hispanic ethnicity. Slow early response was also associated with a higher risk of relapse. By contrast, there was no significant difference in the distribution

Table 1 Features at primary diagnosis of acute lymphoblastic leukaemia associated with an increased risk of relapse[3]

Clinical features		High-risk group stratification[1]
Age	Infants < 1 yr old	Yes
	⩾ 10 yr	Yes[2]
WBC	⩾ 50×10^9/L	Yes[2]
Sex	Male	No
Ethnicity	Blacks	No
	Native American	No
	Alaskan Native	No
	Hispanic	No
CNS status	CNS3	No
Response to therapy		
Morphological response	PPR	Yes
	Induction failure[3]	Yes
MRD ⩾ 0.01%	After induction (day 33)	Yes
	After consolidation (day 78)	
Biology		
Immunophenotype	T-cell	No
	Early T-cell precursor	Accepted by some study groups
Genetic alterations	*BCR-ABL1*	Yes
	MLL translocation	Yes if age < 1 yr
	Hypodiploidy (< 44 chromosomes)	Yes
	TCF3-PBX1 (E2A-PBX1)	No
	TCF3-HLF	Accepted by some study groups
	iAMP21	Accepted by some study groups
	BCR-ABL1-like ALL[4]	No
	IKZF1 mutation or deletion	No

[1]Some features have prognostic importance but are not commonly used in risk stratification; [2]The National Cancer Institute (NCI)/Rome risk criteria categorize as high risk patients all those patients with WBC ⩾ 50×10^9/L and/or age ⩾ 10 yr; [3]Induction failure: failure to achieve morphological remission after 4 to 6 wk of induction therapy; [4]The BCR-ABL1-like or Ph-like ALL with a gene expression profile similar to that of Ph + ALL. ALL: Acute lymphoblastic leukaemia; CNS: Central nervous system; CNS3: ⩾ 5 WBC/μL in cerebrospinal fluid with blasts or cranial nerve palsy; MRD: Minimal residual disease; PPR: Poor prednisone response defined as ⩾ 1×10^9/L leukemic blasts in the peripheral blood after 1 wk prednisone prophase; WBC: White blood cell count.

of the immunophenotype (B-precursor or T-cell) between the patients who relapsed *vs* those who did not[16]. Recurrent ALL is a relatively common disease for pediatric oncologists, and given the relatively high prevalence of newly diagnosed ALL, relapsed ALL still has a higher incidence than the new diagnoses of many of the most common pediatric malignancies and represents one of the most common childhood cancer. The number of children with ALL who experience treatment failure each year is similar to the number of children with newly diagnosed acute myeloid leukemia or rhabdomyosarcoma[2,18]. Moreover, relapsed ALL accounts for more deaths from cancer in children than any other malignancy and represents a major cause of death among children[17,19-23]. In the early 1980s, ALL relapse was regarded as an almost incurable disease[24]. Today, most patients achieve a second remission. However, about 50% of relapsed ALL patients do not respond to salvage therapy or suffer a second relapse[20-22,24-26]. For these patients, prognosis is extremely poor with survival rates below 10%[27]. Despite substantial second remission rates and a wide availability of haematopoietic stem cell transplantation (SCT), most children with relapse die[2].

After remission reinduction, recommendations for continuation therapy include ongoing intensive chemotherapy with or without radiation therapy or SCT. As in newly diagnosed patients, treatment must be tailored after relapse of ALL, since outcome will be influenced by several risk factors. Decisions regarding optimal postremission therapy in relapsed ALL are frequently based on well-established prognostic features, including the timing and site of disease recurrence, the disease immunophenotype, and, more recently, on evaluation of early response in terms of minimal residual disease (MRD) at the end of the reinduction phase[20,26,28-31]. With conventional approaches (intensive chemotherapy and/or SCT), disease free survival (DFS) rates range approximately from 20% to 50% depending on the study, time to end point, and the patient population. Though slightly different variables were measured, results from different study groups showed similar poor outcomes for patients in second complete remission (CR2)[16,20-22,24,25,32-34]. Thus, there is a relative lack of success in the induction of durable second remissions using conventional chemotherapy combinations and the benefits of SCT *vs* aggressive chemotherapy for different patient groups remain unclear. Although the best treatment approach for relapsed ALL remains uncertain, there is agreement that when relapse occurs early, leukemia-free survival remains dismal; most children still die despite aggressive chemoradiotherapy approaches, including transplantation, and novel salvage regimens are needed[18,21,24,35,36]. Relapsed ALL represents the focus of considerable pediatric re-

Table 2 Basic concepts of acute lymphoblastic leukemia relapse

Site of relapse				Ref.
BM	> 25% blasts in the BM (M3 marrow) and/or blasts cell in the PB	Isolated BM	No evidence of ALL in the CNS or any other site	[21,24,26,28,30,36,38]
		Concurrent or combined	⩾ 5% blasts in the BM in combination with EM ALL	[22,24,26,28,30,38,39]
Isolated CNS	⩾ 5 cells/mm^3 with leukemic blasts in a cytocentrifuge preparation of the cerebro-spinal fluid demonstrating leukemic blasts (cytomorphological) without major blood contamination (⩽ 20 erythrocytes/mm^3)[1] OR clinical signs of CNS disease OR a leukaemic mass found on cranial computed tomography or magnetic resonance imaging		< 5% blasts in the BM, no blasts in the PB and absence of leukemic infiltrations elsewhere	[24,25,28,30,36,38,39]
Isolated testicular[2]	Leukemic infiltrations in the testis demonstrated by biopsy (both microscopically and immunologically)		< 5% blasts in the BM, no blasts in the PB and absence of leukemic infiltrations elsewhere	[24]
Other extramedullary	Leukemic infiltrations demonstrated by biopsy (both microscopically and immunologically)		< 5% blasts in the BM, no blasts in the peripheral blood and absence of leukemic infiltrations elsewhere	[24,38]
Length of first CR				
COG classification				[16,26,28,29,36]
Early	Within 36 mo from initial diagnoses	Very early	< 18 mo from initial diagnoses	
		Intermediate	18-36 mo from initial diagnosis	
Late	⩾ 36 mo from initial diagnosis			
BFM classification				
Early	Occurring within 6 mo of the completion of frontline therapy	Very early	Within 18 mo from diagnosis	[42]
Late	More than 6 mo after the completion of frontline therapy			
Response evaluation after relapse				
CR[3]	M1 marrow	(< 5% blasts by bone marrow aspirate) in absence of clinical signs of disease with no evidence of circulating blasts or extramedullary disease and a recovered bone marrow[4]		[19,22,25,28,30,38]
	M2 marrow	presence of 5% to 25% blasts in the BM aspirate by conventional morphology		[28]
	M3 marrow	presence of > 25% blasts in the BM aspirate by conventional morphology		[28]
CNS remission	< 5 WBC cells/mL regardless of cytologic evaluation			[36]
Remission of testicular relapse	defined clinically by return to normal testicular size			[36]
Reinduction failure	Reinduction treatment not resulting in CR			[19]
Refractory patients	Surviving patients after reinduction failure			[19]
Relapse after a new remission	A pathologically confirmed M3 marrow (⩾ 25% leukemic blasts) or the presence of leukemia in any other site (*e.g.*, CNS, PB)			[19]
Treatment failure[5]	All cases of relapse and reinduction failure			[19]
MRD response	positive	Identification of ⩾ 0.01% blasts (1/10000) in the BM using flow cytometry–based assays		[28]
	negative	< 0.01% blasts in the BM using flow cytometry–based assays		[28]

[1]Some studies require the demonstration of the presence of leukemic cells in the CSF in two consecutive CSF samples taken with an interval of at least 24 h (van de Berg, PBC-2011; Barredo, JCO-2006); [2]In some studies, testicular relapse was diagnosed in case of uni- or bilateral painless enlargement of the testicles (Reismüller, BJH-2009; Saarinen-Pihkala, JCO-2006). In case of unilateral testicular relapse, excluding a subclinical involvement of the contralateral testis is recommended (Einsiedel, JCO-2005); [3]Second, third and subsequent remissions are designated as CR2, CR3 ... respectively; [4]Recovery of the BM is assumed in case that white blood cell (WBC) count is > 2.0 × 10^9/L and platelet count > 50 × 10^9/L (van de Berg, PBC-2011). Other studies considered the recovery of peripheral counts if absolute neutrophil count is ⩾ 750-1000/μL with platelet count ⩾ 75000-100000/μL) (Ko, JCO-2010, Raetz, JCO-2008, Raetz, 2012). Some studies consider patients without platelet recovery but fulfilling the remaining criteria for CR as "CR without platelets" (Ko, JCO-2010, Raetz, JCO-2008, Kolb, Leukemia-2003); [5]Treatment failures, development of a second malignant neoplasm, or death from any cause are generally considered events for disease-free survival (DFS) analysis (Ko, JCO-2010). ALL: Acute lymphoblastic leukaemia; BFM: Berlin-frankfurt-münster; BM: Bone marrow; CNS: Central nervous system; COG: Children Oncology Group; CR: Complete remission (complete response); EM: Extramedullary; MRD: Minimal residual disease; PB: Peripheral blood.

search and alternative treatment options exploring distinct mechanisms of action are being pursued[17,37]. New studies clearly need to address how to effectively treat relapsed patients and maintain durable remissions[16].

BASIC CONCEPTS OF ALL RELAPSE

Site of relapse and length of first remission are the major criteria for the classification of patients after first relapse. According to the site of relapse, patients are commonly classified as isolated marrow, concurrent marrow, isolated central nervous system (CNS), isolated testicular and other extramedullary relapses with or without CNS involvement (Table 2)[16].

Marrow relapse is generally defined as a bone marrow (BM) showing greater than 25% blasts (M3) by conventional morphology and/or blasts cells in the peripheral blood[21,24,26,28,30,36,38]. Isolated BM (medullary)

relapse is like marrow relapse but without evidence of ALL in the CNS, testicles or any other site. Combined BM relapse is defined as ≥ 5% blasts in the BM in combination with extramedullary ALL[22,24,26,28,30,38,39]. Accordingly, isolated extramedullary relapses are those with a clinically-overt extramedullary manifestation of leukemia and less than 5% marrow infiltration[24]. Isolated CNS relapse is defined as ≥ 5 cells/mm^3 in a cytocentrifuge preparation of the cerebro-spinal fluid demonstrating leukemic blasts (cytomorphological) without major blood contamination (≤ 20 erythrocytes/mm^3) or clinical signs of CNS disease or a leukemic mass found on cranial computed tomography or magnetic resonance imaging, and < 5% blasts in the bone marrow, no blasts in the peripheral blood and the absence of leukemic infiltrations elsewhere (Table 2)[22,25,28,30,36,38,39]. Some studies require the demonstration of the presence of leukemic cells in the cerebrospinal fluid (CSF) in two consecutive CSF samples taken with an interval of at least 24 h[38,40]. Evidence suggests that submicroscopic involvement of the BM with leukemia is a frequent finding in patients with "apparently" isolated CNS relapse[41].

Isolated relapse elsewhere (testicle, skin, bone, orbita, mediastinum, lymph nodes, and tonsils) can be defined as leukemic infiltrations demonstrated by biopsy (both microscopically and immunologically), with < 5% blasts in the BM, no blasts in the peripheral blood and an absence of leukemic infiltrations elsewhere (Table 2)[38]. In some studies, testicular relapse was diagnosed in case of uni- or bilateral painless enlargement of the testicles[22,33]. In the case of unilateral testicular relapse, it is recommended to rule out a subclinical involvement of the contralateral testis[24].

Although a cut-off point between early and late relapses is often made at 3-6 mo after treatment cessation, the definitions for "early" *vs* "late" relapse differ slightly among different study groups. The COG categorizes relapses as "early" (recurrence occurring within 36 mo after initial diagnoses) or "late" (occurring ≥ 36 mo after initial diagnosis). Early relapses are further classified as "very early", if they occur < 18 mo, or "intermediate", if they occur 18-36 mo after initial diagnosis[16,26,28,29,36]. For the Berlin-Frankfurt-Münster (BFM) group, the time-point of relapse is defined in relation to the date of primary diagnosis and the date of completion of primary therapy (*i.e.*, the end of the antileukemic therapy of the frontline protocol). Although completion of primary therapy often corresponds to the end of the maintenance therapy, in a few patients, it may correspond to the end of a short and intensive first line treatment, or to the end of an inadequately short primary treatment. For the BFM group, the end of frontline therapy is as much or even more important than the duration of the first remission. Therefore, those relapses occurring within 6 mo of the completion of frontline therapy are classified as "early", while "late" relapses are those occurring more than 6 mo after the completion of frontline therapy. The concept of "very early" relapse coincides with that of the COG classification (*i.e.*, relapses occurring within 18 mo after diagnosis)[42]. Thus, assuming that, for most patients, the duration of contemporary frontline treatment is 24 mo, there is a six months gap between the COG and the BFM criteria for the definition of late relapse so that relapses occurring between 30 and 36 mo after initial diagnosis should be considered as "late" within BFM trials, while COG trials should consider them as early relapses.

Patients are considered to have achieved a complete response (CR) if reinduction treatment results in an M1 marrow (< 5% blasts by BM aspirate) in the absence of clinical signs of disease with no evidence of circulating blasts or extramedullary disease and a recovered BM (Table 2)[19,22,25,28,30,38]. M2 and M3 marrow response are defined as the presence of 5% to 25% and > 25% blasts in the BM aspirate by conventional morphology, respectively[28]. Recovery of the BM is assumed in cases where the white blood cell (WBC) count is > 2.0 × 10^9/L and platelet count > 50 × 10^9/L[38]. Other studies considered the recovery of peripheral counts if absolute neutrophil count is ≥ 750-1000/μL with platelet count ≥ 75000-100000/μL)[19,28,29]. Some studies consider patients without platelet recovery but fulfilling the remaining criteria for CR as "CR without platelets"[19,28,43]. CNS remission is commonly defined as < 5 WBC cells/mL regardless of the cytologic evaluation and remission of testicular relapse is defined clinically by a return to normal testicular size[36]. Reinduction treatment not resulting in CR is generally termed reinduction failure and surviving patients are termed refractory[19]. Regarding MRD response, the identification of ≥ 0.01% blasts (1/10000) in the BM using flow cytometry-based assays is generally assumed as MRD positive (negative < 0.01%)[28]. After a new remission is achieved, relapse is defined as a pathologically confirmed M3 marrow (≥ 25% leukemic blasts) or the presence of leukemia in any other site (*e.g.*, CNS, peripheral blood)[19]. Relapses and reinduction failures are collectively termed treatment failures within most studies. Treatment failures, the development of a second malignant neoplasm, or death from any cause are generally considered events for DFS analysis[19].

CONTRIBUTING FACTORS FOR ALL RELAPSE

Clinical

Age and WBC at primary diagnosis of ALL are the most important clinical prognostic factors. Infants < 1 year old and children ≥ 10 years have the worse prognosis (Table 1)[3]. Risk factors predicting CNS relapse after the first CR include T-cell immunophenotype, hyperleukocytosis, high-risk genetic abnormalities, and the presence of leukemic cells in the CSF at the time of diagnosis[44].

Biological

Understanding the biological factors contributing to relapse will probably contribute to identifying new agents

Table 3 Clinical and biological data of early *vs* late relapses

	Clinical data	Biological explanation	Ref.	Biological evidence	Ref.
Early relapse	Patients failing to achieve CR2 with the same agents used at primary diagnosis usually do not respond to different drug combinations	Intrinsic drug resistance: the malignant cells responsible for relapse are present at diagnosis and are selected for during treatment	Yang *et al*[45], 2008	Genome-wide analysis of DNA CNAs and LOH on matched diagnostic and relapse BM samples revealed that the majority (94%) of relapse cases was related to the presenting diagnostic leukemic clone	Mullighan *et al*[46], 2008
	Equivalent post-relapse survival for patients undergoing different intensity regimens at primary diagnosis	The malignant cells responsible for relapse are present at diagnosis and mutate to a resistant phenotype through the acquisition of spontaneous mutations	Freyer *et al*[17], 2011	Primary diagnosis and relapse clones originates from a common ancestral clone and acquire distinct CNAs before emerging as the predominant clone at diagnosis or relapse	
	Decrease in CR rates after subsequent relapses and treatment attempts	Acquisition of resistance-conferring mutations induced by initial treatment	Ko *et al*[19], 2010	Adquisition of new genetic alterations at relapse, often involving cell proliferation and B-cell development pathway	Bhojwani *et al*[48], 2006 Yang *et al*[45], 2008 Mullighan *et al*[46], 2008 Hogan *et al*[49], 2011
Late relapse	The distribution of patients experiencing early and late relapses were highly skewed towards NCI HR in the former group and NCI standard risk in the latter	Late relapse represents de novo development of a second leukaemia from a common premalignant clone	Nguyen *et al*[16], 2008	Distinct patterns of gene expression in pairs of relapsed samples from patients who relapse early from those relapsing later	Bhojwani *et al*[48], 2006
				Pattern of NOTCH1 mutations and genome-wide copy number showed a common clonal origin between diagnosis and early relapse but not for late relapses of T-cell ALL	Szczepanski *et al*[53], 2011
				Distinct pattern of deletions at the non-translocated TEL allele at primary diagnosis and relapse of TEL-AML1-positive ALL	Zuna *et al*[51], 2004

ALL: Acute lymphoblastic leukaemia; CAN: Copy number abnormalities; CR: Complete remission; CR2: Second complete remission; HR: High risk; LOH: Loss-of-heterozygosity; NCI: National Cancer Institute.

able to increase the chances of a sustained second remission and cure. Studying the biology of these diseases at diagnosis, in minimal residual disease states after selection by chemotherapy, and at relapse, provides a unique opportunity to dissect pathways and identify potential therapeutic strategies for relapsed childhood ALL and may improve our understanding of how to use current therapy as well as identifying new targets[16,37,45].

It has generally been assumed that relapse is the consequence of the emergence of a drug-resistant leukemia subclone which was already present at diagnosis and that was selected during frontline therapy. During initial therapy, this minor population would exhibit only moderate reduction relative to the bulk of the diagnostic leukemic cells but would rapidly expand before clinical relapse[45]. Although most relapsed patients achieve a second CR2 with drug combinations involving the same agents used at primary diagnosis, those patients who fail to enter in remission are not likely to be salvaged using different drug combinations, suggesting intrinsic drug resistance[45]. The equivalent post-relapse survival for patients undergoing different intensity regimens as first line therapy, suggests that the malignant cells responsible for relapse are present at diagnosis and mutate to a resistant phenotype through the acquisition of spontaneous mutations that are dependent on intrinsic genomic instability rather than treatment exposures[17]. Lesion specific backtracking studies revealed that in most cases the relapse clone existed as a minor sub-clone within the diagnostic sample prior to the initiation of therapy suggesting that the relapse clone was selected for during treatment. In only a minority (6%) of ALL cases did the relapse clone represent the emergence of a genetically distinct and thus unrelated second leukemia[46]. These findings indicate that the diagnosis and relapse clones originated from a common ancestral clone and acquired distinct copy number abnormalities (CNAs) before emerging as the predominant clones at diagnosis or relapse. In this model, relapse emerges from a drug-resistant subclone present at initial diagnosis that is selected during treatment regardless of the nature of the frontline therapy delivered[17]. This data support the hypothesis that many relapses may be the result of the selection of a relatively resistant clone already present at initial diagnosis rather than the generation of a novel clone by mutation[18,47]. Resistant leukemia subclones are probably present at primary diagnosis in those patients destined for early relapse. Early-relapse mechanisms appear to be more homogeneous and are suggestive of the selection of a resistant, more proliferative clone (Table 3)[48]. Alternatively, the acquisition of resistance-conferring mutations induced by initial treatment might be responsible

for the relative drug resistance noted at relapse[45]. For subsequent relapses and treatment attempts a significant decrease in CR rates is expected[19], which suggests the emergence of a new mechanism of resistance. According to this model, genomic studies carried out in samples from children at diagnosis and relapse demonstrated the acquisition of new genetic alterations at relapse, often involving cell proliferation and B-cell development pathways[45,46,48,49].

By contrast, late relapses may represent de novo development of a second leukemia from a common premalignant clone. Data regarding patients relapsing after being primarily treated within 10 Children Cancer Group (CCG) trials showed how the distribution of patients experiencing early, intermediate and late relapses were highly skewed toward National Cancer Institute (NCI) HR patients in the former group and NCI SR in the latter group. Although SR patients receive less intense therapy, these data suggest that intrinsic differences in the biology of the leukemic blasts are correlated with different mechanisms and the timing of relapse[16]. Distinct gene expression profiles were revealed for pediatric relapsed ALL patients at both early and late time points[49]. The analysis of microsatellite markers showed that some very late relapses of *TEL/AML1*+ positive leukemia most likely represent a new event that occurs in a quiescent precursor leukemia cell harboring an otherwise silent fusion gene that has escaped eradication during initial therapy[50]. Moreover, analysis of deletions at the non-translocated *TEL* allele study of relapsed *TEL-AML1*-positive ALL samples showed that the relapsed clone was related but distinct from the clone at initial diagnosis. This might explain the clinical responsiveness of many cases of late or off-treatment *TEL/AML1*+ ALL relapses[51]. Paired samples from patients experiencing early relapse are more similar in expression patterns than paired samples from those relapsing later[48]. Staal *et al*[52] using genome-wide expression array on purified leukemic cells, found that genes involved in a late or an early relapse identified clearly distinct pathways. Analyses of the TCR gene rearrangement status pattern of *NOTCH1* mutations and genome-wide copy number showed a common clonal origin between diagnosis and early relapses of T-cell ALL but not for the few cases of T-cell ALL late relapses, suggesting that these recurrences should be considered as a second T-ALL rather than a resurgence of the original clone[53]. These findings are suggestive of a model whereby late relapse is due to the acquisition of diverse secondary events that might occur in a distinct subpopulation such as a leukemic stem cell[48].

By comparing matched diagnosis and relapse samples, Bhojwani *et al*[48] found that certain genes involved in cell proliferation, protein biosynthesis, carbohydrate metabolism, and DNA replication/repair were among those highly expressed in relapsed *vs* newly diagnosed blasts. By contrast, some of the genes down-regulated at relapse compared with initial diagnosis included pro-apoptotic genes, antiproliferative genes and a putative tumor suppressor gene[48].

Treatment resistance related genes, such as *CDKN2A/B* and *MSH6*, *ETV6*, and cell cycle regulation and B-cell development (*PAX5*, *EBF1*, *IKZF1*) were shown to emerge at relapse, providing the opportunity to search for novel target therapies[37,45,48]. The discovery of these new genetic alterations associated with high rates of relapse (and shorter first remission), such as the rearrangement of *CRLF2*, *IKZF1* deletions/mutations and *JAK* family mutations, offers the potential for the identification of patients at diagnosis who should be treated more aggressively and with agents targeting those molecular lesions[45,54-56].

PROGNOSTIC FACTORS IN PATIENTS WITH RELAPSED ALL

During the last 2 decades several study groups such the Acute Lymphoblastic Leukemia-Relapse Study of the BFM Group (ALL-REZ BFM) have performed prospective controlled phase III trials to establish standardized treatment protocols with the primary goal of improving the prognosis of children with relapsed ALL and to evaluate risk factors, thereby allowing for risk-adapted treatment intensity[24].

Time to relapse (length of first remission), site of relapse and ALL-immunophenotype are well-established risk factors that can predict survival and constitute the most important prognostic determinants that can be used to stratify patients with a first relapse into different treatment groups[2,16,17,20,25-27,32,34,35,57].

Length of first complete remission

Before relapse, the median duration of the first complete remission (CR1) has been reported to be around 2.5 years[20,25,35]. Most ALL relapses occur during treatment or within the first 2 years after treatment completion, although relapses have been reported to occur even 10 years after diagnosis[2,18].

In a large series of 854 ALL relapses reported by the Nordic Society for Pediatric Hematology and Oncology (NOPHO), the median time from diagnosis to first relapse was 28 mo (range, 2-227 mo)[33]. According to Chessells *et al*[32] 74% of relapses occurred in the first 3 years after diagnosis, 4% after 6 years, and only 1% occurred more than 10 years after diagnosis. Reissmüller *et al*[22] reported a relative incidence of very early (within 18 mo from diagnosis), early (after 18 mo from diagnosis up to 6 mo after cessation of primary treatment) and late relapses (more than 6 mo after cessation of front-line therapy) of 41%, 22% and 37%, respectively. In a retrospective analysis of 1961 relapsed patients registered within 10 consecutive CCG studies, the duration of the CR1 for patients who relapsed varied according to NCI risk group at primary diagnosis, with shorter duration of remission coinciding largely with higher risk features at diagnosis[16]. The duration of the CR1 has been reported to vary with the site of relapse[34,35,41]. In the study re-

Table 4 Relative incidence of site of relapse

Isolated BM	Combined BM	Isolated EM	Isolated testis	Other isolated EM	Year	Ref.
42.90%	19.60%	37.50%			1996-2000	Malempati *et al*[34], 2007
47%	23%	30%			1995-2002	Roy *et al*[20], 2005
63%	16%	13%	7%		1981-1999	Reissmüller *et al*[22], 2009
57%	12%	15%	10%	2%	1972-1998	Chessells *et al*[32], 2003

BM: Bone marrow; EM: Extramedullary.

Table 5 Risk stratification after relapse

	Non-T			BCP		
	Isolated EM	Combined BM	Isolated BM	Isolated EM	Combined BM	Isolated BM
Risk stratification according to the BFM Group classification[42]						
Very early[1]	Intermediate	High	High	Intermediate	High	High
Early[1]	Intermediate	Intermediate	High	Intermediate	High	High
Late[1]	Standard	Intermediate	Intermediate	Standard	High	High
Risk stratification according to the United Kingdom ALLR3 Study classification (Ref [30])						
Very early[1]	High	High	High	High	High	High
Early[1]	Intermediate	Intermediate	High	Intermediate	High	High
Late[1]	Standard	Intermediate	Intermediate	Standard	High	High
Current approach to risk stratification according to I-BFM SG						
Very early[1]	High	High	High	High	High	High
Early[1]	Standard	Standard	High	Standard	High	High
Late[1]	Standard	Standard	Standard	Standard	High	High

[1]Very early, less than 18 mo from initial diagnosis; Early: More than 18 mo from initial diagnosis but < 6 mo from completion of primary treatment; Late: More than 6 mo after completion of primary treatment. BCP: B-cell precursor; BM: Bone marrow; EM: Extramedullary; I-BFM SG: International BFM Study Group.

ported by Malempati *et al*[34], the mean interval between day 28 of primary induction and relapse for all patients was 32.8 mo, CNS relapses tended to occur earlier (mean 23.1 mo), and testicular recurrences tended to occur later (40.5 mo) than BM relapses (mean 36.2 mo).

Timing of relapse has emerged as the most significant predictor of outcome and the most important factor for a second relapse is the duration of the first remission. Early relapse has worse prognoses than late relapse[16,17,20,22,25,32-35,38,57]. Some late relapses are thought to arise from a common precursor that retains the chemosensitivity of the original clone, which could explain the high cure rates achieved with chemotherapy alone in late relapses[30]. Ko *et al*[19] found CR rates of 83% for early first relapse and 93% for late first relapse. Breaking down early relapse into very early relapse (< 18 mo from diagnosis) and intermediate (18 to 36 mo from diagnosis), they found CR rates of 78% and 86%, respectively. EFS rates reported for early relapses ranged from 5% to 18% and 19% to 57% for late relapses[16,19,20,22,24,26,28]. Even when intensive salvage strategies including SCT are employed, longer-term EFS rates for early relapses are only 10% to 20%, compared with 40% to 50% for late relapses. These outcomes have been remarkably consistent over recent decades, irrespective of differences in the components of salvage regimens[21,24,28].

Site of relapse

The majority of relapses (60% to 80%) involve the bone marrow (BM) alone or together with extramedullary involvement, and more than 70% of relapses involving the BM are isolated BM relapses. Isolated CNS or testicular relapse or, much less frequently, relapse involving other extramedullary sites may also occur (Table 4)[20,22,32,34].

Bone marrow relapses are associated with a worse outcome than extramedullary relapses, with overall long-term survival rates of approximately 25%[16,17,19,22,33-34]. Survival at 3 to 6 years after relapse has been found to range from approximately 20% for isolated marrow relapse to 50%-80% for isolated extramedullary relapse, with combined-site (*i.e.*, marrow plus extramedullary) relapses having an intermediate outcome[16,20,22,25,32,34,40]. In extramedullary relapses, a clear distinction also has to be made for early relapses *vs* late relapses. Regarding early relapse, survival rates are higher for patients with isolated CNS relapse than for patients with either isolated or combined BM relapse, and this is also true for intermediate and late relapsing patients. Survival rates were also significantly higher for patients with concurrent marrow relapses compared to those with isolated marrow relapses[16,24].

Thus, involvement of an extramedullary site in patients with BM relapse has been identified as a favourable prognostic feature compared to patients without extramedullary involvement. To explain this fact, it has been hypothesized that combined BM relapses originate from the involved extramedullary compartment, in which the leukemic cells could survive the front-line chemotherapy

because they were protected by the blood-brain/testis barrier. Thus, relapses in extramedullary sites are often considered as relapses from malignant cells treated with suboptimal drug levels; due to their homing on these sanctuaries. Therefore, they may be more sensitive to chemotherapy than clones originating directly from the BM[24]. Five-year survival rates for isolated CNS range between 43.5% for early, and 78.2% for late relapses[16,40].

In the case of a testicular relapse, isolated relapse patients fare better, with an EFS of 58% *vs* 28% for combined relapses[20]. In the COG analysis reported by Nguyen *et al*[16], overall 5-year post-relapse survival rate after early isolated testicular relapse was lower (13%) than after intermediate (52%) or late (59.9%) relapses although this difference was not statistically significant.

Immunophenotype

The immunological lineage of the disease (B-cell precursor *vs* T-cell ALL) is another well recognized risk factor in childhood relapsed ALL. Late relapses of T-ALL are rare and make up approximately 10% of all recurrences[53]. The BFM group demonstrated that children with T-cell ALL BM relapses have a much worse prognosis than B-cell precursor ALL (BCP), irrespective of the time between diagnosis and recurrence[42]. In a report by investigators at St. Jude Children's Research Hospital, CR2 rates for this population were 60%, with a 5-year EFS of only 5% compared to 28.7% for B-cell lineage[25]. Other studies confirmed that the prognosis of patients with a first relapse of T-ALL is dismal, with only 15% to 25% of patients achieving durable remissions after second-line treatment[16,24]. Thus, apart from the fact that T-cell recurrences tend to occur early, T-cell immunophenotype itself is associated with a very poor outcome after relapse regardless of site and time to relapse[16,20-22,24-26,28,32].

Minimal residual disease

Minimal residual disease (MRD), measured either by flow cytometry or real-time polymerase chain reaction (PCR), may supplement morphologic response[29,58,59]. Rates of MRD positivity after reinduction for relapsed ALL are much higher than those observed in first-line ALL clinical trials[28]. The prognostic significance of MRD response at relapse has been assessed in several studies[28,31,60]. Persistence of MRD after re-induction/consolidation therapy (*i.e.*, after 5 and 12-13 wk from the beginning of treatment for relapse) influences prognosis in children with relapsed ALL. Children with MRD levels $< 1 \times 10^{-3}$ or 1×10^{-4} have been shown to carry a lower risk of recurrence than patients with higher levels of MRD[30,31,58,61,62].

Within the COG AALL01P2 study, five-year EFS probabilities differed in patients according to MRD response using flow cytometry-based assays at the end of the first block of chemotherapy (negative < 0.01%; positive ≥ 0.01%)[28]. The absence of MRD at the end of the first month of reinduction therapy portended better outcomes in all patients, and separately in early and late relapse patients. The combination of timing of relapse and MRD appeared to identify three groups of patients. Early relapse patients who were MRD positive had a dismal outcome, while late relapse patients who were MRD negative had an excellent outcome, approaching that seen in newly diagnosed patients. MRD-negative early relapse patients and MRD-positive late relapse patients appeared to form an intermediate group. MRD positivity was also correlated strongly with the duration of initial remission; those patients experiencing relapse at less than 18 mo from initial diagnosis had the highest proportion of MRD positivity[28,29]. In a prospective blinded study, Eckert *et al*[31] have recently reported that EFS and OS decreased and the cumulative incidence of relapse increased with increasing MRD level (quantified by PCR analysis of antigen receptors) after reinduction chemotherapy in intermediate-risk relapsed ALL patients treated by the ALL-REZ BFM P95/96 protocol. Patients of the lower MRD groups ($< 10^{-4}$ and $< 10^{-3}$ to $\geq 10^{-4}$) had an acceptable prognosis (EFS at 10 years 80% and 64%, respectively) compared to patients of the higher MRD groups ($< 10^{-3}$ to $\geq 10^{-2}$ and $\geq 10^{-2}$) who had EFS at 10 years of 36% and 4.8%, respectively. Multivariate analysis revealed that MRD after the second induction course was the only parameter independently predicting the occurrence of subsequent adverse events[31]. Conflicting results, however, were observed in the Medical Research Council (MRC) UKR3 trial, in which reinduction therapy with mitoxantrone was superior to that with idarubicin, yet no differences in the end of reinduction MRD were observed[30].

In a prospective and blinded study, the ALL-REZ BFM Study Group evaluated the impact of pre-transplantation MRD in children treated according to the ALL-REZ BFM 96 or 2002 protocol who received their transplantation in CR2 or third CR (CR3). MRD proved to be the most important determinant for subsequent relapse and survival after transplantation in univariate and multivariate analysis. The cut-off of less than 10^{-4} leukemic cells turned out to be a feasible discriminator between patients at high ($\geq 10^{-4}$ leukemic cells) or low risk ($< 10^{-4}$ leukemic cells) for subsequent relapse. According to these findings, patients classified as being intermediate risk with conventional clinical parameters could be further classified into a very HR subgroup if MRD proves to persist at a high level until transplantation[61]. In another study, classical risk factors such as immunophenotype, site of relapse, time to relapse, and others were only significant in patients who receive chemotherapy in CR2. These factors lost their relevance in patients undergoing SCT, and MRD remained the only independent prognostic variable in this setting[42]. Thus, MRD of leukemia both during second CR and before transplantation, has been reported to be a very strong prognostic factor for the ultimate outcome[61]. However, the Saint Jude group reported that, although MRD before transplantation was an independent predic-

tor of survival, patients with high levels of MRD (0.1% to < 5.0% leukemia cells) still had a reasonably good chance of survival (43%) after SCT, suggesting that the negative effect of MRD had been partially offset by recent improvements in the transplantation procedure[63].

Given its power as a prognostic factor, quantification of MRD at diagnosis of ALL relapse and regularly during therapy has become an essential tool to characterize the responsiveness of the disease and to allocate the patients to a risk adapted treatment. It is currently being incorporated for relapsed patients into a risk-classification algorithm for the management of relapsed ALL within the COG (Table 2)[28,29].

A similar stratification system was used in the UKALL R3 relapse trial[30], and is currently applied by the International BFM Study Group (I-BFM SG).

Although study designs are incorporating the use of MRD in order to quickly assess responses in patients with relapsed ALL who are treated with novel agents, at present MRD remains an unvalidated surrogate marker for this purpose[28,29]. To this regard, even when a clear superiority from one arm to the other was obtained regarding the primary outcome (*i.e.*, EFS), results from the UK ALLR3 trial failed to demonstrate a difference in MRD level at early assessment between both study arms[30].

Other prognostic factors

There is some debate in the literature on the prognostic factor of the white blood cell (WBC) count and the presence of blasts in the peripheral blood at the time of relapse[25,32,33]. There is some evidence that, among children with relapsed ALL, those who had WBC counts < 50000/μL at initial diagnosis are more likely to have favourable outcomes after relapse[16]. Age at primary diagnosis might influence outcome after relapse. Older age at diagnosis (≥ 10 years), as well as age younger than 1 year, has been associated with significantly inferior post-relapse outcome[16-17,33,35,57]. In a recent analysis of 1150 patients aged 0-18 years registered in four consecutive Austrian ALL-BFM trials, prognosis of relapsed leukaemia was significantly better for younger patients (patients aged 1-15 years at primary diagnosis) than for adolescent (*i.e.*, patients aged between 15 and 18 years at primary diagnosis) even when neither the time point or the site of relapse differed significantly between broth groups[64]. These results suggest that age at initial diagnosis is a prognostic factor in relapsed ALL, just as it is for newly diagnosed disease[29]. Certain unfavourable clonal cytogenetic abnormalities detected at primary diagnosis have been found to portend worse post-relapse survival[22,32]. Philadelphia chromosome-positive (Ph+) and 11q23 abnormalities were associated with early relapse and poorer prognosis[32]. The prognosis of children relapsing after first line treatment for Ph+ ALL, particularly for those relapsing after SCT, is poor[28,65]. The *ETV6/RUNX1* fusion gene has been associated with better outcome after relapse[22].

It has been debated whether the intensity of front-line treatment affects the outcome of patients after relapse[29]. Type of first treatment was reported to influence the outcome after relapse, with more recent regimens being associated with improved survival[32]. The Austrian BFM Study Group reported higher post-relapse EFS (but not survival) for 203 children with relapsed ALL who received treatment on the more recent of their front-line studies conducted during the 1980s and 1990s[22]. It might be expected that patients who relapse after receiving an inferior initial treatment regimen would have greater success in retrieval, and greater post-relapse survival than patients who relapse after receiving a superior initial treatment regimen, given that their leukemia clone at relapse should be "less resistant" after being exposed to less effective or intensive prior treatment[17]. However, data from 272 relapsed patients after primary therapy within the COG study CCG-1961 Study, demonstrated that there was no difference in 3-year post-relapse survival between two groups of patients having primarily received augmented *vs* standard intensity post-induction intensification. For subjects initially treated with augmented (n = 109) *vs* standard-intensity (n = 163) post-induction intensification, the 3-year post-relapse survival was 36.4% *vs* 39.2%, respectively (P = 0.72). There was no difference in the median time-to-death post-relapse according to initial regimen, (10.5 mo for augmented *vs* 16.2 mo for standard-intensity, P = 0.27), and no difference in post-relapse survival was seen after adjusting for timing of relapse, site of relapse, age at diagnosis, and lineage of the leukemia[17]. Similarly, in a report of post-relapse survival rates in 1961 children previously enrolled on 10 consecutive CCG clinical trials, according to treatment era at initial diagnosis (trials conducted from 1988-1995 *vs* 1996-2002), with treatment intensity increasing over time, the post-relapse outcomes were nearly identical[16]. Thus, differing intensity of initial treatment, as reflected in either the cross-regimen setting of single studies (CCG-1952 and CCG-1961) or the trans-era context of sequential CCG/COG studies involving both standard- and high-risk patients, does not alter the generally poor outcome associated with relapsed childhood ALL of any initial risk category[17]. Prognosis seems to be particularly poor for those patients relapsing after SCT[22].

Finally, male sex, African American or Hispanic ethnicity, and central nervous system (CNS) disease at diagnosis were reported to be significant predictors of inferior post-relapse survival in children with newly diagnosed ALL who had been enrolled on COG clinical trials from 1988 to 2002[16].

Risk-stratification

The BFM cooperative Group developed a relapse score incorporating duration of first complete remission, site of relapse, and immunophenotype to classify patients as standard-, intermediate-, and high-risk, with 6-year post-relapse survival rate reaching 78%, 41% and 19%, re-

Table 6 Children oncology group approach to relapsed acute lymphoblastic leukaemia[29]

Relapse	Site	Time		MRD	
B-lineage	Marrow	Early	Chemotherapy *vs* chemotherapy plus novel agents	Negative	SCT
				Positive	Bridging study before HSCT
		Late	Chemotherapy *vs* chemotherapy plus novel agents	Negative	Continuation therapy
				Positive	SCT
	IEM	Early	Chemotherapy *vs* chemotherapy plus novel agents	Any	SCT
		Late	Chemotherapy *vs* chemotherapy plus novel agents	Negative	Continuation therapy
				Positive	SCT
T-lineage	Marrow	Early	Chemotherapy *vs* chemotherapy plus novel agents	Negative	SCT
		Late		Positive	Bridging study before HSCT
	IEM	Early	Chemotherapy *vs* chemotherapy plus novel agents		
		Late		Any	SCT

IEM: Isolated extramedullary; SCT: Hematopoietic stem cell transplantation.

spectively for patients receiving more modern treatment. According to this classification, all children with T-cell relapse involving the bone marrow at any time, and children with very early combined and very early or early isolated marrow non-T cell are classified as HR; very early or early isolated extramedullary relapse, irrespective of immunophenotype, as well as early or late combined BM and late isolated marrow BCP ALL relapse, are classified as intermediate risk (IR); while SR category correspond to late isolated extramedullary (both T and non-T cell immunophenotype) (Table 5)[32,42]. Among 1556 patients up to 18 years of age with first relapse of ALL enrolled in trial of ALL-REZ BFM between June 1983 and April 2001, the SR group comprised 5% of patients while 55% and 40% of all patients were allocated to the IR and HR, respectively[42].

In a retrospective review of 150 relapsed patients form four large pediatric oncology units in the United Kingdom, Roy *et al*[20] found that children with a very early isolated extramedullary relapse had a significantly poor outcome when compared with the rest of the IR group, and suggested modifying this risk stratification system. Accordingly, within the United Kingdom ALLR3 Study these patients were classified as high risk patients[30]. However, only two risk groups are currently considered by the I-BFM SG. The standard risk group includes patients with: (1) a late or early isolated extramedullary relapse of BCP or T-cell ALL; (2) a late or early combined BM/extramedullary relapse; and (3) a late isolated BM relapse of BCP ALL. The high risk group comprises those with a very early isolated extramedullary relapse of BCP or T-cell ALL; early isolated or very early isolated or combined BCP ALL, and any BM relapse of T-ALL (Table 5).

TREATMENT FOR RELAPSED ALL

Risk-adjusted selection of treatment

Salvage treatment after ALL relapse involves inducing a CR2 with conventional intensive chemotherapy and apply consolidation, re-intensification and maintenance therapy, or allogeneic stem-cell transplantation (SCT) as further intensification of treatment. As occurs with primary diagnosed ALL, successful treatment of relapse largely relies upon the risk-based treatment allocation of patients in order to maximize response to therapy while minimizing toxicity and adverse effects. Using the prognostic criteria such as first remission duration; site and immunophenotype of relapse; genetic alterations; and initial response to relapse therapy, distinct subgroups of relapsed ALL can be identified that may either be treated with chemoradiotherapy only or by additional allogeneic SCT (Table 6 and Figure 1)[27,29].

Reinduction

Current treatment approaches for relapsed ALL begin with reinduction therapy in an attempt to induce a CR2. Reinduction of patients with relapsed ALL commonly includes conventional agents largely identical to those used at initial diagnosis except with increased dose intensity or alternative schedules with reported rates of toxic deaths around 4%-5%[26,42,47].

Few randomized trials comparing different reinduction regimens in risk-stratified children with relapsed ALL have been conducted[30,39,66], and it remains unclear whether any reinduction combination in use today is significantly superior to any other[18,47]. The Pediatric Oncology Group compared every 2 wk and weekly pegylated asparaginase with vincristine, doxorubicin, and prednisone in a population including both early and late marrow relapse and obtained CR2 rates of 82% and 97%, respectively[66].

The BFM group randomized dose and duration of infusional methotrexate in reinduction, demonstrating similar outcomes between intermediate-dose (1 g/m^2 over 36 h) and high-dose (5 g/m^2 over 24 h) infusions[39]. In a trial conducted by the United Kingdom Children's Cancer Group (UKCCG) patients were allocated to receive either idarubicin or mitoxantrone during induction; after 3 blocks of therapy, HR and IR patients with MRD $\geqslant 10^{-4}$ received allogeneic SCT, whereas SR and IR patients with MRD $< 10^{-4}$ continued chemotherapy. EFS and OS were significantly higher in the mitoxantrone group. The 3-year OS was 69% in the mitoxantrone group (45% in the idarubicin), which overall represented

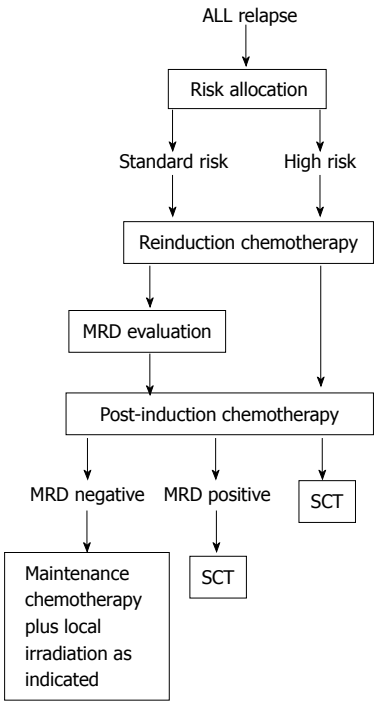

Figure 1 Algorithm for the management of relapsed acute lymphoblastic leukaemia in children. Risk allocation is based on immunophenotype, time and site of relapse (see Table 5). SCT: Allogeneic stem cell transplantation; MRD: Minimal residual disease.

a substantial improvement over preceding trials from the same investigators[30].

The conceptional backbone therapy of the ALL-REZ BFM Group is a series of short (*i.e.*, 5 to 7 d), intensive multiagent chemotherapy courses (block therapy), including most of the same traditional chemotherapy agents with known antileukemic efficacy, with an interval of 2 wk between the blocks to allow for regeneration of bone marrow aplasia, then followed by local irradiation therapy when indicated, and conventional maintenance therapy. This block therapy concept proved to be feasible, effective, and relatively well-tolerated and has been incorporated into many other treatment regimens for relapsed ALL and HR primary ALL worldwide[24,26]. A risk-adapted intensification of treatment by prolonging the intensive treatment phase in an ALL-REZ BFM Study could not prevent a high relapse rate in the high-risk group (early isolated or combined bone marrow relapse)[24]. Other treatment strategies with a more continuous therapy with repetitive application of comparably less intensive chemotherapy has been developed and used by the COG, and the UK ALL Relapse Study Group (MRC UKALLR), achieving comparable results[20,35]. It remains unclear whether short course intensive, or continuous less intensive chemotherapy constitute the most adequate and effective approach in treating childhood relapsed ALL or which subgroups of patients may benefit more from one approach or the other[24].

The COG conducted the AALL01P2 phase II pilot study with the primary objective of developing a safe and active reinduction regimen that could serve as a platform for evaluating the addition of promising new agents in future trials[28]. An objective of this study was to improve the depth of CR2 using three intensive non-overlapping blocks of chemotherapy derived from combinations that were previously shown to be effective in the management of recurrent ALL. With this regimen there was a 40% incidence of febrile neutropenia and a 19% incidence of documented infections[28]. Five toxic deaths occurred among 124 patients (4%) yielding a similar toxic death rate seen with other regimens[20,24,26,28,33,36,66].

The authors concluded that extending the duration of re-induction to three blocks appears to be beneficial for the group of patients with initial favourable morphologic responses and were MRD-negative at the end of the first month of treatment[28].

Reinduction remission rates for patients with a first relapse range from 71% to 95%, depending on the timing and site of relapse. CR2 rates for late bone marrow relapse typically approach 95%, whereas those for early relapse range from 70% to 85% and are frequently < 50% for very early relapses[16,19-22,24-26,28-30,32,34,35,42,66]. T-cell immunophenotype has been related with a lower remission rate[25]. Patients failing to achieve a CR2 after reinduction chemotherapy have a dismal prognosis[25,28,67]. Data from the BFM study group showed that only one third of children treated with curative intention after conventional reinduction failure obtained a CR2 (81% of them only after SCT) with a median survival of 255 d after the diagnosis of reinduction failure[67]. Given that further therapies with curative intent are associated with high treatment-related morbidity, mortality, and minimal survival, children with relapsed ALL and having no response to protocol-therapy should be eligible for innovative, ethically approved phase 1 or 2 trials[18,67].

For patients with HR relapsed ALL, the COG is currently exploring the role of adding novel agents to remission reinduction therapy (Table 6)[29]. The International Cooperative Group on Relapsed ALL conducts 2 randomized trials comparing the classic BFM reinduction therapy with that reported by the UKCCG in standard and intermediate risk patients, and with a novel regimen combining clofarabine, etoposide, and cyclophosphamide in high risk children, respectively[18,37].

Post-remission therapy: SCT vs continuation of chemotherapy

For patients with relapsed ALL who attain a second remission, no consensus on optimal therapy exists. All patients who reach a CR2 receive additional chemotherapy, even if SCT is planned. In general, a higher dose intensity is used than in first-line treatment but published data do not show one chemotherapy combination to be better than others[47].

For patients not allocated to SCT, a consolidation phase after induction chemotherapy followed by a prolonged continuation treatment is generally recommend-

ed[20,25,42]. According to the ALL-REZ BFM protocols, patients not allocated to SCT receive treatment consisting of alternating courses of polychemotherapy. At the end of intensive systemic chemotherapy, local radiotherapy is applied as indicated followed by conventional maintenance therapy up to 2 years[42].

Allogeneic SCT is a curative option for several hematologic malignancies and the current availability of several different stem cell sources has expanded this option for many children. High-dose myeloablative chemotherapy followed by SCT is an alternative to chemotherapy alone for relapsed ALL. Several published retrospective studies suggest some benefit for SCT, particularly for patients with early BM relapse[19,22,42,57]. With improved supportive care and better donor selection, the outcome after unrelated donor and matched sibling SCT for relapsed ALL has become more equal[68]. Therefore, the comparability of several different stem cell sources has expanded this option for many children and SCT has been widely used for patients with relapsed ALL. In the ALL-REZ BFM 87 study, SCT was associated with a superior EFS compared to chemotherapy/radiotherapy as postremission therapy alone, and the performance of SCT (included as time-dependent covariate) was an independent predictor of EFS. Results of autologous transplantation and chemotherapy were the same[24]. In the Austrian BFM Study Group report, patients who received SCT in second CR did significantly better than patients given chemotherapy only (10-year EFS 55% *vs* 33%) and this was even more obvious following an isolated BM relapse (10-year EFS 58% for SCT in second CR *vs* 22% for chemotherapy only)[22]. In a report by Eapen *et al*[57], children with an early BM first relapse of BCP ALL had lower rates of second relapse and higher rates of leukemia-free survival and OS if they received an HLA-matched sibling transplant with a TBI-containing regimen compared with a non-transplant approach. In contrast, for those with a late first relapse and second relapse, leukemia-free and OS rates were similar after chemotherapy alone and transplantation[57]. In a retrospective report from the NOPHO Study group, SCT led to increased long-term survival compared with chemotherapy irrespective of the length of first remission[33]. Matched-pair comparisons across BFM group ALL-REZ trials showed that unrelated donor transplantation achieved significantly better leukemia-free survival than chemotherapy in HR relapse but not in IR relapse. The EFS at 5 years was 17% for the chemotherapy group (0% for HR) and 42% for the SCT group (44% for HR) while rates of treatment related death were 4% and 30%, respectively[42].

Other studies suggest that the type of therapy after relapse does not affect outcome[21], and for a subgroup of patients with relapsed ALL, mainly represented by late BM and extramedullary relapses, combined chemotherapy and radiotherapy may yield durable second remissions[24]. From the BFM group, Borgmann *et al*[42] reported 39% EFS after transplantation for IR patients compared to 49% for non-transplanted patients. In line with such a statement are the data in a United Kingdom study including 256 patients, who were analyzed on the basis of HLA matched donor availability; no statistical benefit in outcome was seen[69]. Malempati *et al*[34] found no significant difference in EFS or OS between treatment with SCT or chemotherapy for any site of relapse or duration of the CR1, with a 2-year estimated EFS of 49.5% with SCT compared to 49.1% with chemotherapy for the entire group. For early BM relapse they also found no difference in treatment modality; the 2-year estimated EFS was 43.1% with SCT and 38.0% with chemotherapy; there was also no significant difference in EFS for late BM relapse according to treatment type: 2-year estimated EFS was 56.1% with SCT and 61.5% with chemotherapy. Similarly, 3-year estimated EFS after isolated CNS relapse was equivalent with either SCT or chemotherapy at 45% and 56.1%[34].

The analysis of the BFM ALL REZ BFM 90 Study showed that SCT did not improve EFS for IR patients (represented by late isolated or combined BM and isolated extramedullary regardless of time point of relapse) or for those who received allogeneic HLA-compatible grafts; however, EFS was significantly higher after SCT in HR patients (early BM, very early isolated or combined BM and any relapse of T-lineage) than after the administration of chemo-radiotherapy alone. This group of patients when treated with conventional chemo-radiotherapy had a low chance of cure[26]. In the study by van de Berg *et al*[38], the benefits of the conditioning and the possible graft-*vs*-leukemia effect on patients undergoing SCT did not outweigh the benefit of prolonged, rotational chemotherapy for late relapses (including BM relapses); patients treated with chemotherapy only achieved a 65% survival rate[38].

The impact of type of donor (matched related *vs* unrelated or mismatched related) on outcome has not been demonstrated. Some studies claimed a clear advantage for matched related donor SCT or for matched unrelated donor SCT[42]. Long-term EFS rates from of above 40% have been reported with HLA-matched sibling donor SCT in CR2 after early relapse[57]. By contrast, others found no significant difference in outcome according to type of donor[2,32]. Results with umbilical cord transplantation are comparable to that obtained with unrelated donor SCT[70]. Unrelated donor registries and cord blood banks have increased the donor availability for the majority of patients lacking an HLA matched familial donor but the process of searching for an unrelated donor usually takes several months during which patients in CR2 are at risk of new relapse or even death from treatment related complications[33]. In this regard, reported time to transplant after relapse is commonly around 3 mo[19,26,28,34].

Haploidentical hematopoietic SCT (haplo-SCT) from a mismatched family member donor offers an alternative option for patients who lack a human leucocyte antigen (HLA)-matched donor[71]. The main obstacles are graft rejection, delayed immune reconstitution, graft-*vs*-host

disease (GvHD) and vulnerability to infections[71]. T-cell depletion can prevent overwhelming GvHD allowing the graft to contain large numbers of stem cells. This approach can reduce the risk of graft failure retaining CD34-negative stem cells and most other immune cells, thus allowing expedite immune reconstitution during the early post-transplant period. However, the absence of the T cell-mediated graft-*vs*-leukemia effect would render the recipients of a T cell-depleted allograft more susceptible to leukemia relapse. In this scenario, donor-*vs*-recipient NK alloreactivity has emerged as a crucial factor for the outcome of haplo-SCT. Ruggeri *et al*[72] reported a low relapse risk for patients with acute myeloid leukaemia transplanted from NK-alloreactive donors. This NK-mediated graft-*vs*-leukemia effect has also been documented in children with ALL[73]. Data from the Pediatric Diseases and the Acute Leukemia Working Parties of the European Blood and Marrow Transplant showed 34% and 22% EFS for children undergoing haplo-SCT in CR2 and CR3, respectively[74]. Therefore, a T cell-depleted haplo-SCT should be included in the treatment algorithm as a valuable option for patients with ALL in need of transplantation and lacking a matched donor, especially if an NK alloreactive relative exists. An unmanipulated HLA-haploidentical SCT has been proposed for those few patients who are unable to locate an HLA-compatible donor, a suitable umbilical cord blood unit, or an NK-alloreactive relative[18]. As stated by Locatelli *et al*[18], for those patients considered candidates for allo-SCT, the preferred source of stem cells should be a matched sibling donor; for those lacking an HLA-compatible family donor, an unrelated donor, umbilical cord blood or haploidentical family donor are suitable options.

Intensive chemo-radiotherapy has been administered before transplantation to reduce the burden of disease and induce immunosuppression in the host. Total body irradiation (TBI)-containing regimens before SCT from a matched sibling donor proved to be superior to chemotherapy alone and a non-TBI regimen in children with early relapse and BCP ALL who achieve a CR2. Transplantation with a TBI-containing regimen resulted in significantly lower risks of relapse, treatment failure, and overall mortality compared to non-TBI regimens and this was independent of the duration of the first remission[57]. Such conditioning regimens reduce graft rejection but they can cause considerable mortality due to severe toxicity, delayed immune restoration and severe infection, especially in heavily pre-treated patients. Moreover, DFS estimates are not appreciably improved by aggressive chemo-radiotherapy, as recurrent or refractory malignancies have usually become resistant to chemotherapy. These observations have encouraged the reassessment of conditioning strategies for transplantation. Newer strategies aim to minimise toxicity while allowing rapid engraftment and expediting immune reconstitution during the early post-transplant period, thereby protecting the host from infection and perhaps generating a graft-*vs*-tumour effect against disease relapse[71,75]. In contrast to traditional myeloablative conditioning regimens that use high doses of radiation or chemotherapy or both to suppress host immune responses and eradicate diseases, this approach relies almost exclusively on graft-*vs*-host effects for eradication of the underlying diseases. Reduced intensity or non-myeloablative conditioning regimens for haplo-SCT reduced mortality and have an acceptable rate of engraftment. However, delayed immune reconstitution, severe GvHD and infection continue to be impediments[71,75]. Elimination of TBI may reduce damage to organs that generate immune cells, while avoidance of anti-thymocyte globulin may prevent complications that include delayed immune reconstitution and Epstein-Barr virus associated lymphoproliferative disease[75]. Using a reduced intensity conditioning regimen (fludarabine, thiotepa, melfalan and OKT3) without TBI and without anti-thymocyte globulin in children with refractory haematological malignancy, a more rapid and robust immune reconstitution when compared to patients transplanted with a myeloablative conditioning regimen was reported. Studies with melphalan-based reduced-intensity conditioning regimens and T/B cell-depleted grafts show high engraftment rates. The risk of acute and chronic GvHD was significantly reduced by graft manipulation procedures (T/B cell depletion) and is comparable to that after matched unrelated donor transplantations[71,75]. Furthermore, the overall incidence of cytomegalovirus, Epstein-Barr virus and adenovirus viremia in the reduced intensity conditioning regimen group was less than that in the myeloablative conditioning regimen group[75]. Transplant related mortality could be effectively reduced by improved T cell recovery and close monitoring of viral loads followed by preemptive therapy[71].

Many factors complicate the analysis of published results comparing outcomes after SCT *vs* chemotherapy only, including intrinsic selection biases, different lengths of the interval between diagnosis of relapse and transplantation as well as disparate conditioning regimens, supportive strategies and stem cell sources[21,34]. Very often, reported trials assigned HR with matched family donors to allogeneic transplantation and those without donors to chemotherapy or autologous transplantation[47].

Another impediment in comparing reports is the more favourable outcomes of non-sibling donors SCT over the years. A matched-pair analysis of unrelated donor SCT *vs* chemotherapy revealed that only high-risk patients benefited from unrelated donor transplantations[42]. In a prospective randomized trial, Gaynon *et al*[21] found poor protocol adherence with small numbers of patients recruited to the chemotherapy arm. The authors speculated how this might be related to the known poor outcome of relapsed ALL and "a desire to do everything possible for children for whom aggressive chemotherapy had already failed once"[21,47].

Thus, much debate has centered on optimal pos-

tremission therapy including stem cell transplantation[18,33,42]. A recent meta-analysis demonstrated the variability of outcomes and conclusions among studies comparing SCT with chemotherapy for the treatment of ALL in second remission[76]. However, excluding patients with late relapse[38], in no comparison is outcome after transplantation worse than after chemotherapy alone[47]. To this regard, after the induction of the CR2, options for ongoing continuation therapy are frequently risk based in order to allocate patients to treatment regimens with adequate intensity and justifiable toxicity[18,29,31]. There are some patients with an acceptable EFS rate with chemotherapy alone, while other patients need to undergo allogeneic SCT after the 2CR or are even eligible for phase Ⅰ/Ⅱ trials with the chance of benefiting from new agents. Both the COG and the BFM as well as the I-BFM groups developed formal criteria for risk stratification for relapsed ALL with the main intention of identifying children for whom SCT might be better than continuation chemotherapy once a second remission is attained (Tables 5 and 6)[2,42].

Children with a very early (< 18 mo after diagnosis) or early (between 18 mo after diagnosis and 6 mo after cessation of frontline chemotherapy) isolated BM relapse, a very early BM/extramedullary combined relapse and all T-cell ALL with BM involvement at relapse diagnosis should be categorized as HR patients and should be allocated to SCT given that nearly all will suffer a subsequent relapse when being treated solely with conventional intensive post-induction chemotherapy. Allogeneic SCT with a matched donor is currently the preferred therapeutic option for these children after the CR2[20,26,29,33,38,42,69].

The outcomes for very early extramedullary recurrences without SCT have been inferior to those of early or late extramedullary relapse, with an EFS of < 50%, and SCT in CR2 has also been considered for these patients a well[20,40,41,77].

It remains unclear as to what will be the most effective approach for HR patients who continue to have high levels of disease before or after transplantation, as this is associated with a high incidence of relapse post-allogenic SCT[20]. In this scenario, further cytoreductive chemotherapy (clofarabine), immunomodulation, the application of new agents, and/or innovative transplant procedures might be considered.

Children with early or late (> 18 mo from initial diagnosis) isolated extramedullary relapse represent the SR group and, for these patients, outcomes have been very good with chemotherapy and site-directed radiation and there is no indication for SCT. However, intensive systemic therapy is essential for preventing later BM recurrences[20,26,29,31].

The largest group of patients (more than 50%) belongs to the IR group, in which treatment choices are the most difficult[32,42]. This group includes patients with BCP ALL with either late (> 6 mo after cessation of frontline chemotherapy) isolated BM relapse, or with a late or early combined BM/extramedullary relapse as well as early (including T-cell) isolated extramedullary relapses (Table 5)[31]. The optimal post-remission therapy for children with late B-cell precursor BM relapse (either isolated or combined) is controversial[20]. Intensive systemic therapy is essential for preventing later BM recurrences. However, the benefit of SCT for these patients has not been firmly established. SCT is associated with a 10% to 20% risk of peri-transplantation mortality, depending on donor type, and still has a substantial relapse rate[32,57]. While some studies report comparable results with both SCT and chemotherapy[26,57] others argue that the outcome of patients undergoing a transplant is poorer, and that SCT in late relapses is not beneficial[38]. Within the NOPHO study, patients with late BM relapse but with initial HR features and combined BM relapses did not do well on conventional chemotherapy. The authors recommended considering allo-SCT for these subgroups of patients[33]. Data from the DCOG Relapse ALL 98 protocol showed that patients with a late BM relapse undergoing a transplant had poorer outcomes than those undergoing CT only. Although the majority of these patients died from a relapse of leukaemia, the benefits of the conditioning and the possible graft-*vs*-leukemia effect after SCT did not outweigh the benefit of the prolonged chemotherapy[38]. The COG is currently investigating if outcome for patients with late (≥ 36 mo) B-cell precursor marrow relapse, can be improved by using the same AALL01P2 triple re-induction regimen followed by 2 years of intensive chemotherapy[28,78].

In the IR patients (with EFS rates greater than 40%) additional risk factors, such as the dynamics of treatment response assessed by MRD, would help to identify those patients at a high risk of subsequent relapse who are thus eligible for SCT[26,30]. MRD response is being integrated into risk classification schemes[18,29]. A cut-off point MRD after reinduction of 10^{-3} (quantified by PCR) was recently proposed by Eckert *et al*[31] to discriminate between patients with a good or a poor prognosis. Patients belonging to the group with MRD between 10^{-3} and 10^{-4} can be categorised as molecular good responders and allogeneic SCT would not be appropriate for these patients. In the subsequent trial ALL-REZ BFM 2002, this level of MRD after induction was applied to decide whether chemotherapy or SCT should be used as consolidation post-induction therapy[31]. The same cut-off MRD of 0.01% (10^{-3}) (measured by flow cytometry) was applied by the COG for prognostic assessment after the first, second and third treatment block of the AALL01P2 study in IR and HR relapses[28]. Within the UK ALLR3 study, patients with MRD ≥ 0.001% (10^{-4}) (quantified by PCR) at the end of induction were eligible for SCT[30]. A different preceding treatment and quantification method within each protocol might explain these differences in MRD cut-off levels as being predictive of outcome.

A major task of ongoing and future trials is to predict subsequent relapses more precisely, thus clarifying which

patients benefit from post-remission intensification by allogeneic SCT. In this context, not only the acute mortality and toxicity, but also the long-term sequelae of allogeneic SCT have to be taken into account[24].

Local therapy for extramedullary ALL

Therapeutic irradiation of manifest extramedullary leukemia in addition to systemic chemotherapy for patients experiencing CNS recurrence can be regarded as standard of care, since the disease is protected from chemotherapy by biological blood barriers in extramedullary sanctuary sites such as the CNS and the testes[18]. In accordance with other investigators, for patients with CNS involvement at relapse, we would encourage the use of an Ommaya reservoir during intensification. Intraventricular therapy has several theoretical advantages: a more uniform distribution of chemotherapy throughout the cerebrospinal fluid (CSF), higher ventricular levels than those achieved by lumbar administration, and prolonged concentration over time exposure to cell cycle active chemotherapy[36].

For patients with late isolated CNS relapse (not allocated to SCT), cranio-spinal irradiation is generally postponed until the end of intensive chemotherapy or even after the end of maintenance treatment, in order to avoid intolerability for chemotherapy[38]. The administration of 24 Gy and 15 Gy to cranium and spine, respectively are commonly recommended[38], although the adequate dose (18 *vs* 24 Gy) and mode of CNS irradiation (cranial *vs* craniospinal) remains controversial[44]. A 4-year EFS of 78% can be achieved after reduction of the radiation dose to 18 Gy in patients with B-cell precursor ALL whose initial remission lasted > 18 mo while in patients relapsing before 18 mo the EFS was 52%[40]. Further dose reduction (15 Gy) is recommended for patients with prior irradiation[18]. The addition of cranial irradiation, even in patients without obvious CNS-involvement (prophylactic cranial irradiation), was reported to significantly improve the outcome of patients with isolated BM-relapse by the ALL REZ BFM Study Group and was introduced from 1989 onwards[26]. If the CNS was involved at the time of relapse, patients received more intense intrathecal triple chemotherapy with methotrexate, cytarabine and prednisone[26]. In addition, cranial or cranio-spinal radiation was delivered in an age-dependent manner to all patients[22,24,26]. This strategy was adopted by other study groups[21,36]. However, whether protective CNS irradiation is necessary in patients with isolated BM relapse, remains controversial and, given the well documented radiation associated late effects, it is omitted by several groups in favour of intensified intrathecal chemotherapy[25,33].

Most study groups recommend local irradiation of both testes at 24 Gy regardless whether only one or both testes are involved at relapse. Within the BFM studies, orchidectomy has been the treatment of choice for the involved testicle in the case of testicular relapse. In unilateral testicular disease the clinically affected testis is removed and the remaining testis irradiated (15-18 Gy according to the results of biopsy)[22]. In the case of clinical unilateral or bilateral testicular involvement and no resection 24 Gy local irradiation is generally recommended[22,25,26,33,36]. Radiotherapy (24 Gy) for bilateral testicular recurrence is expected to induce infertility and significantly impair hormone production[18]. Within the DCOG Relapse ALL 98 Protocol, patients with late testicular relapses were treated without irradiation and without surgery[38].

A variety of other extramedullary sites may be involved in ALL relapse. Little data are available regarding the prognostic impact of these manifestations and on the necessity of local therapy. Since a blood barrier is not present in these sites, systemic chemotherapy is supposed to be effective. Thus, for an extramedullary relapse other than CNS and testis, no local therapy is generally considered apart from cases where local persistence of the disease occurs after induction/consolidation chemotherapy. In this situation, it is recommended to take a biopsy and to apply local irradiation therapy if vital leukemic cells are still present.

Treatment for second and subsequent relapses

Most treatment failures after the CR2 are related to subsequent relapses[21]. For 74 patients experiencing a second relapse and enrolled into ALL-REZ BFM trials before 2006, the median duration of the second CR was 7.5 mo (range, 18 d to 4.4 years)[27]. In this situation, a significant decrease in CR rates is expected[21]. A variety of multidrug regimens provide a 40% CR rate in the second and subsequent relapses[28]. Ko *et al*[19] reported CR rates of 44%, 27%, and 12% for third, fourth, and further therapeutic attempts, respectively. The subsequent CR rate was lower when CR was not achieved or was of short duration after the prior treatment attempt[19]. In contrast, the NOPHO study group reported a third complete remission (CR3) as high as 72% in 274 patients after the second relapse. In this study, those who never achieved 3CR had a shorter first remission, more BM relapses and shorter time intervals between the relapses, indicating a more aggressive disease. However, long-term survival was only 12%[33]. Few other data appear for DFS rates in the CR3 and beyond[19,20,27,33]. According to Ko *et al*[19], DFS among patients who achieve CR decreased with an increasing number of prior treatment attempts. Two and 5-year DFS for patients achieving CR after third therapeutic attempts was 31% and 15%, respectively. DFS increased with increasing duration of the prior remission[19]. In a report from the Austrian BFM Study Group, the median duration of CR after second relapse was 13 mo, with 10-year EFS rates of only 9% and 6% after the second and third relapses, respectively[22].

Concerning prognostic factors, the length of the CR2 and relapse site are relevant[27,32]. Reismüller *et al*[27] found that the duration of the second CR seemed to have an influence on EFS: 6% *vs* 21% for patients with a CR2 duration of less or more than 1.5 years, respec-

tively. In this report, the only other prognostic factors that proved to be statistically significant were site of first and second relapse with isolated extramedullary relapses faring better than isolated and combined BM relapses, and duration of the first CR[27]. Other reported factors associated with survival are NCI risk criteria at initial diagnosis, immunophenotype, presenting leucocyte count and length of first remission[19,32]. Additional extramedullary sites of disease were not significantly associated with DFS[18]. The prognosis for children with BM relapse after SCT, and children with a second relapse of T-cell ALL is dismal. In the latter group, this is mainly due to the lack of ability to achieve a CR3[27]. Patients who relapse after allogeneic SCT often have refractory disease and are particularly susceptible to chemotherapy-related toxicity[79].

Survival after second relapse was reported to vary according to treatment. The role of SCT for patients with a second or third relapse has been debated. Overall survival ranging from 20% to 36% was reported for those undergoing SCT compared with 10% to 15% for those with chemotherapy only[32,33]. Ko and coworkers[19] found increased survival for patients undergoing SCT, regardless of time to relapse or the number of prior relapses.

Given that only a very small group of patients with second ALL relapse has a realistic chance of cure, these patients are ideal candidates for phase Ⅰ/Ⅱ trials exploring new innovative drugs, with the consideration of SCT in those achieving a durable remission[18,27,29].

OUTCOME OF RELAPSED ALL

Outcomes for children with relapsed ALL have changed little over time despite efforts by many investigators to intensify therapy with approaches that often include SCT. Although clinical remission can be achieved in most (85%) relapses, the chance to experience a second relapse is still high and long-term survival rates do not exceed 40% to 50%[20-22,24-26,33,35,43]. Results from the CCG 1941 marrow relapse study showed that 50% of patients failed to enter remission, died from toxicity, or relapsed again after achieving a brief second remission[21]. The overall outcomes are dismal for patients who do not achieve a CR2 after an initial attempt[18,28]. In a recent large retrospective review within the Therapeutic Advances in Childhood Leukemia Consortium (TACL), Ko *et al*[19] found 27% 5-year DFS for patients in CR2. These results are similar to those generally reported by other study groups with DFS rates ranging from 16% to 39% depending on the study, time to end point, and the patient population[16,20-22,24,25,32-34].

Second malignancies such as primary brain tumors and acute myeloid leukemia are another matter of concern in relapsed ALL patients with an estimated actuarial incidence at 15 years from diagnosis of around 11%[32,57].

NEW PERSPECTIVES

Unfortunately, retrieval therapy is inadequate in most cases of relapsed ALL and most of these children succumb to their disease. Further intensification of chemotherapy is unlikely to cure additional patients. The failure of intensive chemotherapy to cure most children, as well as its related toxicity, makes it essential to search for new treatment approaches. Approximately one third of relapsed patients can be assigned to a "poor prognosis group" (early BM-relapse or any BM relapse of T-cell ALL), for whom no promising therapy regimen exist[24]. Moreover, the extremely poor survival after relapse underscores the need to focus on improving the outcome of the primary therapy for those patients who are unlikely to be salvaged if they relapse. Promising new therapies should be integrated into trials for subsets of higher risk patients at initial diagnosis[16]. Using analyses of DNA copy number abnormalities, gene expression, DNA methylation and sequencing in matched diagnosis/relapsed ALL BM samples, investigations are under way regarding the evolution of genetic lesions from diagnosis to relapse that lead to drug resistance and disease progression with the aim of identifying new potential biomarkers and therapeutic targets[45,46,48,49,52,53,78]. Further development and the use of targeted therapies or immune modulators may decrease residual disease and may improve the outcome in children with relapsed ALL treated with either intensive chemotherapy or SCT[34]. Offering uniform clinical trials for patients with relapsed ALL while gathering biological data in order to identify new agents not generally used in the treatment of ALL at primary treatment, are the focus of several current collaborative study groups[16].

Because responses to single-agent therapy have been poor, integrating new agents in combination with established chemotherapy platforms in a randomized manner has been adopted as a therapeutic approach by de COG with the aim of exploring improvements in CR2 and MRD rates as a measure to define new agent activity and, potentially, to more efficiently select candidate agents for future study[29,78]. Novel approaches include new formulations of existing chemotherapeutic agents, new antimetabolites and nucleoside analogs, monoclonal antibodies directed against leukemia-associated antigens, adoptive therapy approaches such as chimeric antigen receptor (CAR)-modified T cells, and molecularly targeted drugs such as the proteasome inhibitor bortezomib and JAK kinase, aurora kinase, and mammalian target of rapamycin (mTOR) inhibitors[18,29,37,80].

Intrathecal lyposomal cytarabine may have a role in relapsed ALL with CNS involvement and resistance to conventional therapy[18].

Clofarabine is a second-generation purine analog capable of inhibiting DNA synthesis/repair and inducing cell death[81]. Clofarabine has been granted accelerated approval both in Europe and in the United States for the treatment of pediatric patients with relapsed or refractory ALL who received at least 2 prior regimens of chemotherapy[18]. O'Connor *et al*[82] reported an overall response rate of 67% in 23 pediatric patients diagnosed with relapsed ALL. Clofarabine was safe and effective

when used in combination with cyclophosphamide and etoposide although a high risk of severe infection was noted, including fungal and viral infection. The response rate to treatment with a clofarabine-based regimen was inversely proportional to the number of prior treatment attempts. Durable remissions were achieved, allowing patients the option of hematopoietic stem cell transplantation with the potential of long term cure. Treatment was effective in 3 out of 5 infants with relapsed *MLL* rearranged ALL[82]. Thus, the use of clofarabine-based regimens should be considered in children with either resistant or second or subsequent BM relapse[18]. Nelarabine is an inhibitor of purine nucleoside phosphorylase. The FDA approved nelarabine in October 2005 for third-line treatment of patients with T-cell ALL/lymphoma[18]. In the COG AALL00P2 trial, patients with T-ALL with a poor early treatment response that predicted poor outcomes in previous trials attained a 5-year EFS rate of 69% with intensive chemotherapy plus nelarabine without increased toxicities. Non HR patients (< 1000/μL peripheral blood blasts on prednisone prephase day 8 and MRD < 1% at induction therapy day 36) who received nelarabine had a 5-year EFS rate of 74%[83].

Monoclonal antibodies directed to cell surface antigens expressed by leukemic blasts (epratuzumab, blinatumomab, inotuzumab ozogamicin, and moxetumomab pasudotox, among others), are ideal candidates. Combinations of monoclonal antibody and cytotoxic therapies may hold particular promise in relapsed ALL[2,29]. Epratuzumab is a humanized monoclonal antibody that binds to the third extracellular domain of CD22. CD22, a B-cell surface antigen, is highly expressed in more than 90% of cases of childhood B-precursor ALL. After binding, the receptor/antigen complex is rapidly internalized and appears to modulate B-cell activation and signaling. Given the high CD22 expression levels in B-precursor ALL, its mechanism of action distinct from cytotoxic agents, and a toxicity profile that could allow for combining it with dose-intensive chemotherapy, epratuzumab became an attractive agent to explore in relapsed ALL. Epratuzumab was the first agent tested by the COG in combination with an established reinduction platform in children and young adults with first, early BM relapses of $CD22^+$ ALL in an effort to improve CR2 rates[84]. Patients received four intravenous doses of epratuzumab, 360 mg/m^2 per dose, twice weekly during the 14-d reduction phase, followed by four weekly doses, 360 mg/m^2 per dose, administered with block 1 of the AALL01P2 chemotherapy regimen[28,84]. Epratuzumab administration was tolerated with acceptable toxicity, both as a single agent and when combined with chemotherapy. MRD responses in those who achieved remission were significantly more favourable in those who received epratuzumab (42% MRD-negative compared with 25% among historical controls) suggesting that the antibody may enhance response to cytotoxic chemotherapy[29,78,84]. However, the rates of CR2 did not differ compared with a historical control population treated with chemotherapy alone[84]. Based on these results, the COG will not pursue epratuzumab further[78,85].

T-cell engaging antibodies are bispecific antibodies designed to transiently engage primed cytotoxic effector memory T lymphocytes for the lysis of target cells. The T-cell engaging CD19/CD3-bispecific antibody blinatumomab can redirect T lymphocytes against CD19+ ALL blasts, which represents a new approach to the treatment of BCP ALL. Handgretinger *et al*[79] reported the first clinical experience in three pediatric patients with BCP ALL showing that blinatumomab, administered as a continuous 24 h intravenous infusion at 15 mg/m^2 per day for several weeks, was well tolerated and able to rapidly induce MRD-negative complete responses in refractory BCP ALL after multiple relapses and allogeneic HSCT. Blinatumumab is an attractive drug to be explored in the near future for children with second or greater relapsed or refractory ALL[78].

Bortezomib is a proteasome inhibitor, which renders leukemic cells more sensitive to the apoptotic effects of chemotherapy. A phase 1 study conducted by the TACL (TACL study, T2005-003) demonstrated that a standard dose of bortezomib (1.3 mg/m^2 given on days 1, 4, 8, and 11) can be safely combined with an intensive 4-drug reinduction regimen in children with relapsed ALL and showed promising activity in relapsed childhood ALL[86]. Within the phase 2 expansion of this combination (TACL study T2005-003) patients were eligible only after they failed 2 or 3 previous treatment regimens. The CR rate was 64% with an additional 9% of CR without platelet recovery for an overall response rate of 73% which was significantly better than in previous trials. BCP ALL patients had an 80% overall response rate while no T-cell ALL patients showed a response. The study reached its predefined early stopping rule for efficacy when 14 complete responses were observed among the first 22 patients enrolled. OS at 24 mo was estimated to be 41%. Lethal bacterial sepsis was the principal toxicity[87]. A study combining bortezomib with a 4-drug reinduction platform (the AALL01P2 triple reinduction regimen) is also in progress within the COG for patients with early BM relapse occurring within 36 mo of diagnosis[29,78]. This approach will also be explored by the I-BFM SG.

In relapse Ph+ ALL, second complete remissions can be obtained with the combination of imatinib and intensive chemotherapy[28,88]. For patients relapsing after treatment with imatinib, the use of scalating doses of imatinib or alternative tyrosine kinase inhibitors may overcome imatinib resistance and help to induce a new remission and a second SCT should be considered in this situation[88,89]. High *FLT3* expression identifies *MLL-AF4*+ ALL patients at very high risk of treatment failure and poor survival, emphasizing the value of ongoing/future clinical trials for FLT3 inhibitors[90]. The COG is conducting a phase I trial (ADVL1011) of the JAK inhibitor ruxolitinib, and plans to develop a trial of ruxolitinib combined with chemotherapy in relapsed ALL patients with *JAK* mutations. These mutations are

present in a proportion of cases of so-called Ph-like ALL overexpressing *CRLF2*. Similarly, patients with other fusion genes activating *ABL1*, *JAK2*, and *PDGFRB* might be treated with *ABL/PDGFRB* class tyrosine kinase inhibitors[78].

The occurrence of defective immune recovery after haploidentical SCT was associated with a high risk of severe infections, which heavily affected morbidity and mortality. Post-transplant CD8-depleted donor lymphocyte infusions are feasible and promote immune reconstitution[91]. Further attempts might be directed at increasing the alloreactive potential of the transplantation. Donor lymphocyte infusions have been advocated to convert stable mixed chimerism into full chimerism and have been used successfully in patients with persistent, relapsed, or progressive disease both after conventional and nonmyeloablative SCT to exert graft-*vs*-tumor effects, most notably in patients with chronic myeloid leukemia[92,93].

Adoptive immuno therapy was investigated mostly in children who have a functional thymus and lower incidence of GvHD compared with adults. Donor-*vs*-recipient NK alloreactivity has emerged as a crucial factor for the outcome of haplo-SCT[72,73]. Genetic engineering to endow T cells with receptors that bind leukaemia cell surface antigens such as CD19 or CD22, is another promising adoptive therapy approach. Immune cells are genetically modified to express chimeric antigen receptors (CAR) that contain a target recognition domain linked to an intracellular component that activate a signalling cascade[80]. Impressive antileukemic effects have been reported using CD19-CAR constructs in pediatric patients with relapsed/refractory BCP ALL[80,94].

CONCLUSION

Relapsed ALL remains a significant challenge for pediatric oncologists. According to recent reports regarding genetic and epigenetic signatures, two different biological mechanisms seem to distinguish early *vs* late ALL relapse. This might partially explain their distinct behaviour, therapy response and outcome. While SCT is generally accepted as the best option as post-induction consolidation therapy for HR patients after CR2, this seems to apply to only a subgroup of patient categorized as IR. Early response evaluation in terms of MRD after reinduction therapy seems to offer the best chance to stratify IR to SCT or conventional chemotherapy and it is currently been applied by several study groups. However, leukemia-free survival remains dismally low for many patients after relapse and, despite efforts by many investigators to intensify therapy with approaches that often include SCT, outcomes for these children have changed little over time. Relapsed ALL represents the focus of considerable pediatric research and alternative treatment options exploring distinct mechanisms of action are being pursued. Given the rarity of the disease, prospective clinical trials need to be coordinated within international cooperative groups.

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REVIEW

Hemophagocytic lymphohistiocytosis: Recent progress in the pathogenesis, diagnosis and treatment

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Abstract

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome that develops as a primary (familial/hereditary) or secondary (non-familial/hereditary) disease characterized in the majority of the cases by hereditary or acquired impaired cytotoxic T-cell (CTL) and natural killer responses. The molecular mechanisms underlying impaired immune homeostasis have been clarified, particularly for primary diseases. Familial HLH (familial hemophagocytic lymphohistiocytosis type 2-5, Chediak-Higashi syndrome, Griscelli syndrome type 2, Hermansky-Pudlak syndrome type 2) develops due to a defect in lytic granule exocytosis, impairment of (signaling lymphocytic activation molecule)-associated protein, which plays a key role in CTL activity [*e.g.*, X-linked lymphoproliferative syndrome (XLP) 1], or impairment of X-linked inhibitor of apoptosis, a potent regulator of lymphocyte homeostasis (*e.g.*, XLP2). The development of primary HLH is often triggered by infections, but not in all. Secondary HLH develops in association with infection, autoimmune diseases/rheumatological conditions and malignancy. The molecular mechanisms involved in secondary HLH cases remain unknown and the pathophysiology is not the same as primary HLH. For either primary or secondary HLH cases, immunosuppressive therapy should be given to control the hypercytokinemia with steroids, cyclosporine A, or intravenous immune globulin, and if primary HLH is diagnosed, immunochemotherapy with a regimen containing etoposide or anti-thymocyte globulin should be started. Thereafter, allogeneic hematopoietic stem-cell transplantation is recommended for primary HLH or secondary refractory disease (especially EBV-HLH).

Key words: Alemtuzumab; Anti-thymocyte globulin; Cyclosporine A; Epstein-Barr virus; Etoposide; Hematopoietic stem-cell transplantation; Hemophagocytic lymphohistiocytosis; Hereditary diseases; Immunochemotherapy; Intravenous immunoglobulin; Molecular diagnosis; Rituximab; Steroids

Core tip: This review discusses the diagnostic criteria for hemophagocytic lymphohistiocytosis (HLH), the algorithms used to identify the underlying immune defects at the molecular level, and the optimal therapeutic approaches. For any HLH cases, a screening for primary HLH should be made following the diagnostic algorithm. During the process, immunosuppressive therapy should be started to control the hypercytokinemia with steroids, cyclosporine A, or intravenous immune globulin, and if primary HLH is confirmed, immunochemotherapy with a regimen containing etoposide or anti-thymocyte globulin should be given. Supportive measures to control hemorrhage/organ dysfunction are also required. In cases of primary HLH or secondary/refractory HLH, timely allogeneic hematopoietic stem cell transplantation is recommended.

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INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a primary (familial/hereditary) or secondary (non-familial/hereditary) hyperinflammatory and hypercytokinemic syndrome[1,2]. Immune homeostasis is maintained by regulating the proliferation and apoptosis of activated lymphocytes and their associated granule-dependent cytotoxic activity, which plays a critical role in defense against tumor cells and cells infected with viruses[3]. The hypercytokinemia conditions associated with HLH are caused by cytokine releases from activated T cells and macrophages as a result of impaired activation-induced cell death due to uncontrolled immune responses resulting from the impaired ability of cytotoxic T-cell (CTL) and natural killer (NK) cells to kill their target cells[4-6]. Thus, marked activation of macrophages occurs, which results in hypercytokinemia. Previous reviews of primary HLH identified a hereditary impairment in the molecules involved in the multistep processes of cytolysis (from cell activation to the release of perforin and granzymes)[7,8]. Indeed, primary HLH is caused by loss-of-function mutations in the genes encoding perforin and various molecules involved in the transport, fusion and exocytosis of secretory vesicles[4-8]. In other forms of HLH, particularly Epstein-Barr virus (EBV)-driven primary HLH, deficiency in SAP (which is important for CTL function) is responsible for the marked reduction in CTL and NK cell activity; however, in XIAP (which is important for apoptosis), CTL and NK cell activity are not altered[9-11]. Secondary HLH develops in apparently immunocompetent subjects; however, some of these subjects show acquired functional reductions in CTL and NK cell activity, which are associated with viral, bacterial or parasite infections, metabolic diseases, autoimmune diseases/rheumatological conditions (termed as macrophage activation syndrome; MAS), or malignancy[12-19]. Initial diagnostic work-up in the diagnosis of HLH includes the detection of the expansion of $CD8^+$ T cell subset in the peripheral blood[20,21] and the identification of the factors that trigger the development of HLH, particularly infectious agents[22,23]. In addition, for all HLH cases, molecular screening must be performed to determine whether the disease is primary or secondary[6,24,25]. The assay of NK or CTL activity[26,27] and flow cytometric analysis of molecules, such as perforin, Munc 13-4, SAP/XIAP, is essential for rapid diagnosis[6,24,25,28] (Figure 1). Age-related factors were emphasized in the past because primary HLH usually develops during the first 3 years of life; however, more recently, late-onset cases have been identified[29-33]. The diversity of clinical features associated with primary HLH has been examined using detailed genotype-phenotype analysis methods. Among infectious agents, EBV plays a major role in cases of infection-associated primary or secondary HLH. Thus, quantification of cell-free or peripheral mononuclear cell EBV-DNA levels is extremely useful for the diagnosis of EBV-HLH and chronic active EBV infection (CAEBV)-related HLH[34-36]. The HLH-94 or HLH-2004 regimens employed in many centers has led to a significant improvement in the therapeutic results due to the efficacy of a combination of immunochemotherapy and allogeneic HSCT for treating primary and secondary HLH, especially for refractory EBV-HLH[37-39]; however, the diversity of the clinical features associated with primary HLH raises questions regarding the appropriate timing of HSCT[40-48].

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Clinical features

The initial symptoms of HLH include persistent fever, hepatic and/or renal dysfunction, splenomegaly, hemorrhagic diathesis, neurological symptoms, and other features, caused by hyperinflammatory conditions[1,2,38,49-52]. The clinical features of primary and secondary cases are not significantly different; however, some types of primary HLH are associated with hypogammaglobulinemia-related symptoms (*e.g.*, FHL5 and XLP1)[45,53], enteropathy and renal tubular dysfunction due to the epithelial abnormalities in FHL5[54] and oculocutaneous albinism in patients with Griscelli syndrome type 2 (GS-2), Chediak-Higashi syndrome (CHS), and Hermansky-Pudlak syndrome type 2 (HPS-II)[55-59], although occurrence of HLH in HPS-II deficiency is limited to a single case[58]. The most ominous findings in cases of HLH are central nervous system (CNS) disease[60-62] or an association with primary or therapy-related hematological malignancies[63-65]. The HLH conditions do not show the same severity, which is determined by the type of NK deficiency[26,27] in association with the type of genetic mutations in the primary HLH[42-48], or the degree of lymphoproliferation as represented by serum soluble IL-2R levels in the secondary HLH[66]. HLH occurs in all age groups, from premature infants and neonates to the elderly, but the majority of primary HLH cases occur in early infancy. For cases occurring during the fetal and neonatal periods[67,68], pre- or post-natal molecular diagnosis is essential[69]. Primary HLH can also develop in adolescents and adults[29-33]. Thus, especially in this older age group, a molecular diagnosis is recommended to enable a definite diagnosis of primary or secondary HLH.

Triggers and underlying diseases

The most common "trigger" for HLH is infectious disease. Viral and other types of infection cause secondary HLH[13,14,22,23,49,50]. Among them, EBV-HLH and CAEBV-related HLH, which are defined by the specific diagnostic criteria[34-36], are the most common form of secondary HLH; however, infection-induced HLH also occurs in individuals with primary HLH, MAS, and malignancy. Post-organ transplant-HLH, or post-HSCT-HLH, is a distinct subtype of secondary HLH that was described recently[70-72]. Among the various malignancies, lymphoma-associated HLH (LAHS) is the most common[73-75]. Progress in molecular diagnostic techniques

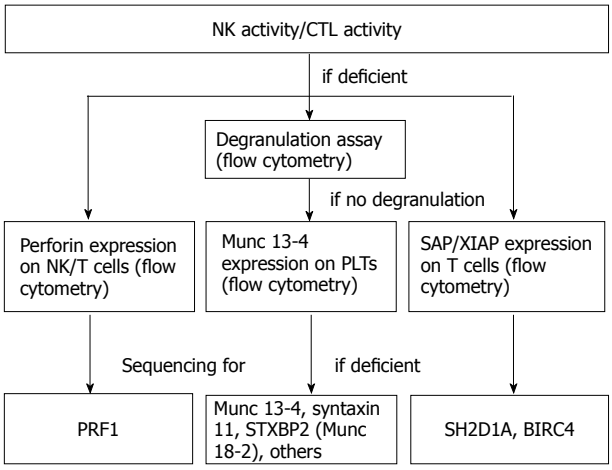

Figure 1 Diagnostic work up algorithm for a patient with primary hemophagocytic lymphohistiocytosis (familial hemophagocytic lymphohistiocytosis and X-linked lymphoproliferative syndrome)[6,22,23]. Degranulation assay is useful for familial hemophagocytic lymphohistiocytosis (FHL) type 3-5. It is advised to perform flow cytometry of peripheral blood mononuclear cells to detect the expansion of CD8+ T cells as first step of diagnosing hemophagocytic lymphohistiocytosis (HLH) prior to the work up algorism.

has led to the identification of molecular abnormality of primary HLH in cases of secondary HLH[76,77] as well as in hematological malignancies[63,64,78-80]. In addition, genotype-phenotype correlations have been identified in patients with primary HLH, particularly those associated with FHL2, FHL3, and FHL5[42-46] and with XLP[53,81-83]. It is these types of studies that identified the existence of atypical late-developing primary HLH cases in adolescents and adults[29-33], and the identification of which raises questions about how promptly HSCT should be introduced.

Laboratory findings and immunopathological features

The cardinal laboratory features associated with HLH include bicytopenia, high levels of serum ferritin, triglyceride, transaminases, lactate dehydrogenase and soluble IL-2R. Serum creatinine and BUN levels are often elevated, while plasma fibrinogen is decreased. Deficient NK activity has generally been noted[12,14-17,26,27]. Hemophagocytosis is observed on bone marrow smears or in lymph node or liver biopsies; however, the detection of hemophagocytes is not mandatory for the diagnosis. Although detection of abnormal karyotypes in bone marrow cells is rare in patients with HLH, they are occasionally detectable in cases of EBV-HLH, correlated with CAEBV[84]. Immunopathological features in HLH are characterized by uncontrolled activation of T cells, especially a significant increase in the subpopulation of CD8+ T cells with clonal expansion[20,21] and macrophages in association with overproduction of various cytokines[85,86].

Molecular genetics

The molecular defects associated with primary HLH are listed in Table 1. Molecular abnormalities have been identified in the perforin-granzyme cytotoxic molecule pathway (in FHL type 2-5, GS-2, CHS, HPS-Ⅱ), T-cell activation pathway (in XLP1), the apoptotic pathway (in XLP2), and the inducible T-cell kinase pathway (in ITK deficiency)[11,87-100]. More recently, CD27 deficiency[101,102] and magnesium transporter 1 (MAGT1) deficiency, also termed as XMEN (X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasm)[103], were identified. These EBV-driven ITK, or CD27 deficiency and XMEN give rise to EBV- associated lymphoproliferative disease (LPD), but does not primarily predispose to HLH; although hemophagocytosis was described in some of the cases[100,102]. These novel discoveries are expected to help elucidate the molecular mechanisms causing the inherited forms of EBV-LPD and HLH.

Table 1 List of primary hemophagocytic lymphohistiocytosis

Disease	Molecular abnormalities (chromosome location)
CTL molecule dysfunction	
Pore formation	
FHL2	Perforin (10q21-2)
Vesicle priming fusion	
FHL3	Munc13-4/Unc 13D (17q25)
FHL4	Syntaxin 11 (6q24)
FHL5	STXBP2/Munc18-2 (19p13)
Vesicle docking/trafficking	
Chediak-Higashi syndrome	LYST (1q42.1-42.2)
Griscelli syndrome, type 2	Rab27a (15q21)
Hermansky-Pudlak syndrome Ⅱ	AP-3 (3q24)
EBV-driven	
XLP1	SAP/SH2D1A (Xq25)
XLP2 (XIAP)	BIRC4 (Xq24-25)
ITK deficiency[1]	ITK (5q34)
CD27 deficiency[1]	CD27 (12p13)
XMEN[1]	MAGT1 (Xq21.1)

[1]Major clinical features in these diseases are not hemophagocytic lymphohistiocytosis (HLH) but Epstein-Barr virus (EBV)-associated lymphoproliferative disease. FHL: Familial hemophagocytic lymphohistiocytosis; XLP: X-linked lymphoproliferative disease; ITK: IL-2-inducible T cell kinase; XMEN: X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasia; MAGT1: Magnesium transporter 1, LYST is also called CHS1 gene. Association of hemophagocytosis was described in some cases.

Diagnosis and differential diagnosis

Achieving the definitive diagnosis of HLH is often challenging[104,105]. Currently, HLH is diagnosed according to globally accepted diagnostic criteria shown in Table 2[38]. Differential diagnoses include fulminant hepatitis or acute hepatic failure[106], severe sepsis, systemic inflammatory response syndrome, and other hyperinflammatory conditions[107]. In the differentiation of primary and secondary HLH, screening measures are employed, which include NK and CTL activity determination, degranulation assays as well as flow cytometric assay of the expression of perforin and other molecules (Figure 1)[6,24-27]. More recently, Western blot analysis was found to be useful to screen for primary HLH by detecting FHL-related proteins in platelets[28]. An accurate diagnosis is made by performing mutation analysis of the genes

Table 2 Diagnostic guidelines for hemophagocytic lymphohistiocytosis[38]

The diagnosis of HLH can be established if one of either (1) or (2) below is fulfilled	
(1) A molecular diagnosis consistent with HLH	
(2) Clinical diagnostic criteria fulfilled for 5 out of the 8 criteria below	
Clinical criteria	1 fever
	2 splenomegaly
Routine laboratory criteria	3 bicytopenia (Hb < 90 g/L, platelets < 100×10^9/L, neutrophils < 1.0×10^9/L)
	4 Hypertriglyceridemia (> 3.0 mmol/L) and/or hypofibrinogenemia (< 1.5 g/L)
Specific histopathological/marker criteria	5 hemophagocytosis
	6 low or absent NK cell activity
	7 hyperferritinemia (> 500 μg/L)
	8 hyper-sIL-2R-nemia (> 2400 U/mL)

HLH: Hemophagocytic lymphohistiocytosis; NK: Natural killer.

responsible for these hereditary diseases. EBV-HLH is diagnosed using a combination of HLH diagnostic criteria and EBV-specific data (*i.e.*, the number of EBV-DNA copies and antibody expression patterns in the serum)[34-36]. Although the majority of EBV-HLH cases in Asia are thought to be secondary HLH, molecular and genetic analyses need to be performed to determine whether they are in fact primary HLH, particularly in patients with refractory EBV-HLH[6,44,81-83,99-103]. In Europe, some patients with FHL3 or FHL5 presented with clinical features suggestive of CAEBV-related HLH[44]. Also, since the risk of malignancy is high in the condition of CTL dysfunction, patients presenting with hematological malignancies could be searched for primary HLH-related gene mutations[78-80].

Prognostic factors and clinical outcome

The ultimate treatment goal of HLH is to have disease-free survival without CNS sequelae and treatment-related acute myeloid leukemia (t-AML). The outcome of HLH depends on the severity of clinical features at the onset and types of HLH (primary or secondary). In particular, primary HLH, refractory EBV-HLH and LAHS without treatment have a poor outcome. In principle, primary HLH cases are fatal if HSCT is not performed[108]. In refractory secondary HLH, immunotherapy may not be curative, when the patients require both salvage chemotherapy and HSCT (Figure 2). Preferably, it is essential to perform HSCT before the development of CNS disease or of t-AML. In the prognostic analysis of HLH, it was found that after initial treatment, death during the acute phase occurs in 10%-15% of patients, usually due to life-threatening infections, hemorrhage, and/or irreversible organ dysfunction[37,109,110]. Death at the later stages of treatment is often due to reactivation of the disease and adverse effects associated with HSCT[109,110]. These data indicate the requirement of improved outcome of HSCT in the treatment of HLH. Although late onset cases of primary HLH are believed to carry a better prognosis, there is a report that adolescents and young adults with HLH who undergo allogeneic HSCT are at increased risk of mortality compared to younger patients[111]. The factors suggestive of a poor prognosis for those with EBV-HLH are summarized in Table 3.

Table 3 Poor prognostic factors in Epstein-Barr virus-hemophagocytic lymphohistiocytosis[34]

Persistent increase of cell-free EBV genome copies
Chromosome abnormality
Correlation with chronic active EBV infection (CAEBV)[1]
In association with primary HLH
Severe organ dysfunction, such as renal failure, CNS hemorrhage
Choice of treatment, such as timing of etoposide use, HSCT

[1]CAEBV is often associated with 1 and 2 of the above. CNS: Central nervous system; HSCT: Hematopoietic stem cell transplantation; EBV: Epstein-Barr virus; HLH: Hemophagocytic lymphohistiocytosis.

TREATMENT OF HLH

General considerations and supportive therapy

Any patients with HLH can be treated first with immunosuppressive regimens designed to control the hypercytokinemia and hyperinflammation. Such treatments include steroids (prednisolone or dexamethasone), cyclosporine A (CSA), or intravenous immune globulin (IVIG). During the initial period of therapy, finding out the triggering factors and underlying diseases as well as molecular diagnostic analyses are recommended to determine familial or non-familial diseases (Figure 1). If confirmed, primary HLH is similarly treatable with HLH-directed immunochemotherapy[6,37-41,112-116]. On the other hand, if apparent infection-triggered HLH is confirmed, rigorous treatment of any identified infectious agents is important. For any secondary HLH, application of treatment should aim to target the underlying diseases. Patients with very severe cases of HLH requiring hemodynamic and respiratory support are treated in the intensive care unit. Inotropic agents are life-saving for those that are hemodynamically unstable[117,118]. Antibacterial or antifungal agents are also required to treat opportunistic infections due to HLH-related neutropenia. Because severe thrombocytopenia and coagulopathy are both life-threatening conditions, the patient may require infusions of concentrated platelets, fresh frozen plasma, fibrinogen, and recombinant thrombomodulin[49,51,119]. Although there is no definite consensus on its benefit, plasma exchange or exchange transfusion may be used to treat the hypercytokinemia and reduce the hemorrhagic tendency during the initial treatment phase[120,121]. The addition of acyclovir to the therapeutic regimen for those with EBV-HLH is not thought to be beneficial because there is no objective evidence showing a clinical improvement using this drug[122]. However, acyclovir is useful for treating neonatal herpes simplex virus (HSV)-HLH in infancy[49,123,124]. Indeed, a combination of high-dose acyclovir, steroid pulse therapy, IVIG, and blood

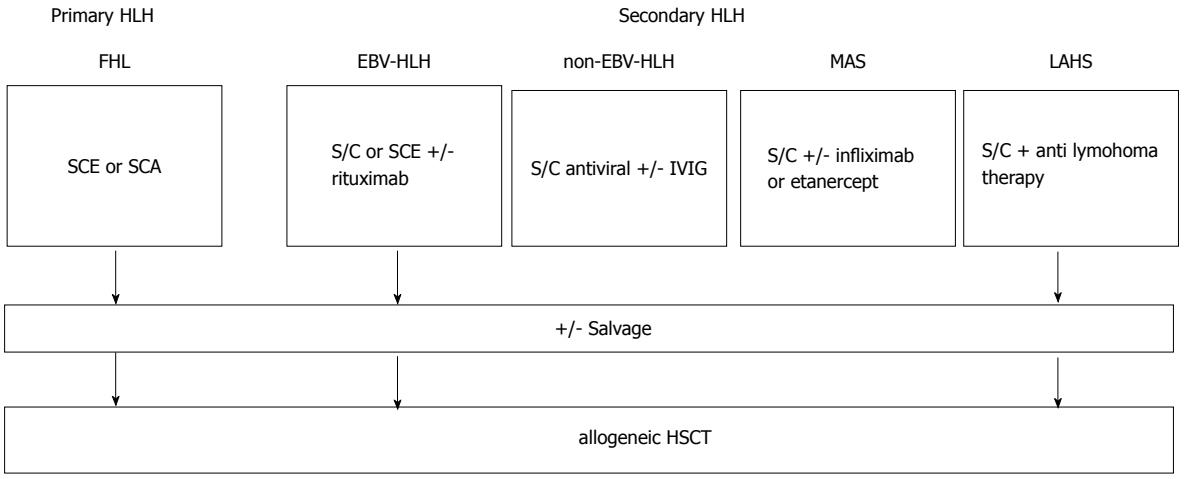

Figure 2 Flow chart illustrates the treatment pathways in hemophagocytic lymphohistiocytosis. Hematopoietic stem cell transplantation (HSCT) is required for the majority of primary hemophagocytic lymphohistiocytosis (HLH) and some of LAHS and EBV-HLH. FHL: Familial hemophagocytic lymphohistiocytosis; MAS: Macrophage activation syndrome; LAHS: Lymphoma-associated HLH; SCE: Steroids + cyclosporine A + etoposide; SCA: Steroids + cyclosporine A + ATG; IVIG: Intravenous immune globulin; S/C: Steroids alone or cyclosporine A alone or two drug combination.

transfusion has proved successful for treating neonatal HSV-HLH[125]. In patients with mycobacterium tuberculosis-associated HLH, early diagnostic confirmation and the timely administration of antituberculous medication is crucial for an improved outcome[126]. For patients with leishmania-related HLH, amphotericin B was shown to be effective[127]; however, for those with human immunodeficiency virus-related HLH, outcome remains poor even in the era of highly active antiretroviral therapy[128].

The efficacy of intrathecal chemotherapy for treating CNS disease in HLH patients has not been sufficiently evaluated. At present, the outcome for HLH patients with CNS disease is poor, even when treated with a combination of systemic immunochemotherapy and intrathecal chemotherapy[60-62]. The HLH-94 study tested the ability of a high systemic dose of dexamethasone to prevent the development of CNS disease. In addition, the study examined the use of intrathecal methotrexate in patients showing neurological symptoms at the time of disease onset; however, neither treatment appeared to prevent the exacerbation of CNS disease[37,62]. Data show that at the time of HLH diagnosis, neurological symptoms were already present in 37% of patients, and abnormal findings regarding the CSF were made in 52%; in all, 63% of patients had either neurological symptoms or abnormal CSF findings. CNS sequelae were more common in the latter group and, consistent with this, a substantial proportion of HLH survivors suffer neurological sequelae. Thus, early diagnosis of HLH and an evaluation of the CNS status including the CSF, coupled with early systemic HLH therapy, is crucial; in addition, the timely use of HSCT should be considered if reactivation of HLH with or without CNS disease is suspected to develop or to be exacerbated during the treatment[37,62,129]. However, since childhood survivors of HLH even after HSCT are shown to be at risk of long-term cognitive and psychosocial difficulties[130], prospective and systematic long-term follow-up of neurological function in these post-HSCT patients is essential.

Suppression of inflammation

In the past, IVIG was used to treat various types of HLH[49,131,132]; however, this form of treatment seems best suited to enterovirus-, hepatitis-, cytomegalovirus-, or bacteria-associated HLH[133-137]. Combined treatment with antibiotics and IVIG resulted in the full recovery of a patient with Group G streptococcal endocarditis-associated HLH[138]. Treatment with steroids alone can be effective for some HLH cases[139]. CSA quickly and efficiently suppresses the cytokines secreted by dysregulated T-cells and activated macrophages; indeed, CSA is able to control various cytokine-related pathological conditions[140,141]. Currently, the majority of HLH cases are treated first with a combination of steroids and CSA[49,140-142]. The prompt and continuous infusion of CSA (1-3 mg/kg per day over several days) is required to alleviate the cytokine "storm" as quickly as possible in patients with severe HLH, but without renal failure[49]. In addition, CSA treatment effectively supports neutrophil recovery especially in severely neutropenic Asian EBV-HLH patients during the acute phase[143].

Combined immunotherapy or immunochemotherapy

The etoposide/steroid/CSA triple combination was used in the HLH-94 or HLH-2004 regimen, consisting of 8 wk of initial therapy followed by continuation therapy and allogeneic HSCT if required. This regimen is now used in many centers to treat HLH, which comprises of dexamethasone (starting dose, 10 mg/d, Ⅳ or PO, followed by tapering), CSA (dose adjusted to obtain trough levels of 200 μg/L, PO, daily), and etoposide (150 mg/m^2, Ⅳ; a total of 10 doses during the initial 8 wk). This regimen has considerably improved the outcome for HLH patients: in the initial analysis comprising 113 patients, the 3-year survival rate was 45% (± 10%)[37]. The CNS outcomes in the patients treated with this regimen

were published in 2008[62]. Long-term follow-up results for 227 patients were published in 2011, where the estimated 5-year survival rate was 54% ± 6%, and the 5-year survival rate for 124 patients who received HSCT was 66% ± 8%[109]. The same group also suggested that this regimen should be revised as HLH-2004[58]; however, the therapeutic results of HLH-2004 have not yet been published. The HLH-94 regimen was also found to be effective when used to treat secondary EBV-HLH[144-146]. On the other hand, along the usage of HLH-94-type HLH treatment, several cases of t-AML were reported[65]. A French group used an ATG/steroid/CSA combination to treat patients with primary HLH[116,147,148], and similar to the etoposide/steroid/CSA regimen, this combination was also followed by HSCT. The effectiveness of this treatment was first described in 1993[147], where the regimen comprised steroids (2-5 mg/kg per day methylprednisolone, Ⅳ, followed by tapering), rabbit ATG (5-10 mg/kg per day for 5 d), and CSA (4-6 mg/kg per day, PO, daily). The study results were published in 2007[148]. In 38 consecutive patients with use of 45 courses of ATG, this regimen resulted in a rapid and complete response in 73%, a partial response in 24%, and no response in only one patient. Subsequent HSCT, when performed early after a complete or partial response, led to a high cure rate of 16 out of 19 cases. Overall, 21 of the 38 patients survived and there were four toxicity-related deaths. The same group also published the HSCT results for 48 patients in 2006[116]. Unfortunately, no direct comparison is possible between the therapeutic results performed after the ATG-regimen or after the HLH-94-type regimen. A regimen comprising HIT (hybrid immunotherapy)-HLH, which uses a combination of ATG/etoposide in the initial treatment phase, is currently being tested (unpublished; Jordan M, Histiocyte Society Clinical Studies 2013).

Other treatments

Rituximab is an effective treatment for some cases of EBV-HLH and has been used as a form of pre-emptive B-cell-directed therapy in patients with XLP1-related EBV-HLH or other severe forms of EBV-HLH in which EBV resides within B cells[149-152]. More recently, Chellapandian *et al*[153] examined 42 EBV-HLH cases and found that a combination of rituximab and conventional immunochemotherapy improved patient symptoms and reduced both the viral load and the level of inflammation. In the past, rituximab was thought to be unsuitable as a treatment for Asian patients with EBV-HLH in which EBV resides in T-cells or NK cells; however, the inclusion of rituximab in the initial treatment regimen may be useful in such cases[154]. R-CHOP (a combination of rituximab, doxorubicin, vincristine, cyclophosphamide and prednisolone) as well as R-etoposide are an effective combination for treating EBV-LPD-associated HLH[152]. The combination of rituximab and CSA induced remission in one patient with EBV-HLH occurring in association with CHS[57]. In addition, intrathecal rituximab is an effective treatment for post-transplant EBV-positive CNS lesions[155,156]. Alemtuzumab is effective as a bridge to allogeneic HSCT in primary HLH patients undergoing salvage treatment[157]. Marsh *et al*[158] reported that of 22 patients who received alemtuzumab (median dose, 1 mg/kg; range, 0.1-8.9 mg/kg) over a median of 4 d (range, 2-10), 64% experienced a partial response within 2 weeks. Indeed, 77% survived and underwent allogeneic HSCT, where the adverse events, including cytomegalovirus and adenovirus viremia, were reported to be "acceptable". Alemtuzumab has also been used to treat refractory MAS[159]. As other biological and experimental agents, the anti-CD25 antibody (daclizumab) was successfully used in a single adult patient with HLH[160] and the anti-TNF-α antibody (infliximab/etanercept) is an effective treatment for MAS[161-165]. Because IFN-γ plays a major role in the pathogenesis of HLH, a humanized anti-IFN-γ antibody, NI-0501(NovImmune), is currently being tested as a future treatment for the disease (unpublished; Arico M, Histiocyte Society Clinical Studies 2013), based on the murine model studies[166,167]. A study in XMEN patients showed that magnesium supplementation is an effective treatment because magnesium restores decreased intracellular free Mg^{2+} levels and corrects defective expression of NK activating receptor (NKG2D), while concurrently reducing the number of EBV-infected cells *in vivo*[103].

Allogeneic hematopoietic stem cell transplantation

Patients with primary HLH and those with refractory secondary HLH are candidates for allogeneic HSCT[37-41,112-116,168]. Primary HLH cases with nonsense (disruptive) gene mutations such as premature stop codon, or sequence frameshift generally develop symptoms in early infancy, thus require early introduction of HSCT. In these cases, delayed HSCT may have a risk of reactivation of HLH, development of CNS disease or hematological malignancies. Those with missense (hypomorphic) mutations may often wait for transplantation until adolescence or young adulthood. In secondary HLH, HSCT is planned whenever the disease becomes refractory to immunochemotherapy. For HSCT, reduced intensity conditioning (RIC) rather than myeloablative conditioning (MAC) is the preferred regimen because it results in better patient survival; however, the RIC regimen may result in mixed donor chimerism during the post-transplant period[41]. Landman-Parker *et al*[169] showed that partial engraftment of donor bone marrow cells after HSCT is sufficient to obtain long-term remission in patients with primary HLH. Experimental transplantation of perforin-deficient mice showed that 10%-20% perforin-expressing cells, with either mixed hematopoietic or CD8 (+) T-cell chimerism, are sufficient to re-establish immune regulation[170]. These data suggest that stable levels of donor chimerism (> 10%) could maintain remission in the HLH patients after HSCT. Of the 40 HLH patients who underwent allogeneic HSCT between 2003 and 2009 in Cincinnati, 14 received MAC comprising busulfan, cyclophosphamide,

and ATG plus or minus etoposide, while 26 patients received RIC comprising fludarabine, melphalan, and alemtuzumab. All patients engrafted successfully, and the overall estimated 3-year survival after HSCT was 43% for those receiving MAC and 92% for those receiving RIC ($P = 0.0001$)[41]. In Japan, 57 patients (43 with primary HLH and 14 with EBV-HLH) underwent HSCT between 1995 and 2005. Data show that EBV-HLH patients had a better prognosis after HSCT than primary HLH patients, also demonstrating that the RIC-conditioning regimen significantly improves the outcome of patients undergoing allogeneic HSCT[40].

CONCLUSION

Recent progress has been reviewed on how to understand the pathogenesis, how to diagnose and how to make treatment decisions in patients with HLH. Although the outcomes have significantly improved over the past decade, further refinement of treatment is required at the initial phase of the disease as well as pre- and post HSCT periods with special care for CNS disease in order to promise a cure with excellent quality of life in these patients with HLH.

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MINIREVIEWS

Myelofibrosis: Prognostication and cytoreductive treatment

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Abstract

Myeloproliferative neoplasms include three diseases: polycythemia vera, essential thrombocythemia and primary myelofibrosis (PMF), currently diagnosed according to the 2008 World Health Organization criteria. Patients with PMF may encounter many complications, and, among these, disease progression is the most severe. Concerning prognostication of Myelofibrosis (MF), the International Prognostic scoring system (IPSS) (International Prognostic Scoring System) model at diagnosis and the Dynamic IPSS (DIPSS) anytime during the course of the disease may be useful to define survival of MF patients. The IPSS and the DIPSS are based on age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational analysis seem to be the next step to implement MF prognostication. Concerning treatments, hydroxyurea has been considered until now the drug of choice when an anti-myeloproliferative effect is needed, but recent data on JAK inhibitors demonstrated a significant effect of these drugs on splenomegaly and symptoms.

Key words: Myelofibrosis; Prognosis; JAK2; Ruxolitinib; Fedratinib; Momelotinib

Core tip: Myelofibrosis (MF) is a mutational/clinical-complex disease. Prognostication of MF is based on the International Prognostic scoring system (IPSS) model at diagnosis and on the Dynamic IPSS thereafter. Factors included in both models are: age > 65 years, constitutional symptoms, hemoglobin < 10 g/dL, leukocytes > 25 × 10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational status help to better discriminate within each IPSS category. JAK inhibitors are new promising therapies with a molecular target, translating into a clinical benefit: spleen reduction MF-symptoms refief. Among JAK inhibitor, ruxolitinib has been approved for MF.

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PROGNOSTICATION IN PMF

Among myeloproliferative neoplasms (MPN), primary myelofibrosis (PMF) has the most heterogeneous clinical presentation, including anemia, splenomegaly, leukocytosis or leukopenia, thrombocytosis or thrombocytopenia, and constitutional symptoms (fever, weight loss, night sweats). Available estimate of survival in MF fixes the median value at 6 years ranging from few months to many years[1]. Causes of death may be summarized into bone marrow failure (severe anemia, bleeding due to thrombocytopenia, and infections due to leukopenia) in 25%-30% of patients, leukemic transformation, named blast phase (BP), in 10%-20% of patients, cardiovascular

complications in 15%-20%, and portal hypertension in 10%.

Many factors affect survival in PMF such as advanced age[1,2], anemia[1], red blood cell transfusion need[1], leukopenia[3], leukocytosis[1], thrombocytopenia[1], peripheral blast count[1], systemic symptoms[1], hepatic myeloid metaplasia[4-22], decreased marrow cellularity with higher degree of fibrosis[23], higher degree of microvessel density[1], high number of circulating CD34-positive cells[1], cytogenetic abnormalities[1], the *JAK2* (V617F) mutation[1], some new mutation[1] and high level of some cytokines[24-30].

PROGNOSTIC FACTORS IN PMF

Cytogenetic abnormalities

Cytogenetic analysis has a role to identify an abnormal profile that provides evidence of clonality. Although most PMF patients' bone marrow aspirate results in a "dry tap", karyotype analysis can be performed on peripheral blood[29]. Among MPN, PMF shows the highest aberration rate with approximately 30% of patients carrying an abnormal karyotype at diagnosis[9]. The most frequent isolated abnormalities in PMF involve chromosome 1, 8, 9, 13 and 20[1]. Sole abnormalities of chromosome 7 were reported in 7% of PMF in a dedicated analysis and 7q- was the most frequent[1]. Concerning prognostic relevance of cytogenetic changes in PMF, recent studies have been consistently claiming an impact on survival[1]. Three studies, each comprising 202, 200 and 131 patients showed a favorable prognostic value for sole 20q- or sole 13q-[1]. A further study of 433 PMF patients refined a two-tired cytogenetic-risk stratification: unfavorable and favorable karyotype[1]. In detail, this study identified a high risk profile for cytogenetics when patients carry sole abnormalities of i(17q), -5/5q-, 12p-, 11q23 rearrangement, inv(3), sole +8 or sole -7/7q-, complex karyotype (three or more abnormalities), and a low-risk profile when patients carry normal diploid, or sole abnormalities not included in the high-risk profile. The respective 5-year survival rates were 8% and 51%. The presence of monosomal karyotype, which is defined as two or more autosomal monosomies or a single autosomal monosomy associated with at least one structural abnormality, identified a subset of patients with unfavorable karyotype associated with extremely poor overall and leukemia-free survival, as demonstrated in a study of 793 PMF patients[31].

Mutational profile

In PMF the prognostic role of the oncogenic mutations involving *MPL* and *JAK2* has been assessed, overall, not showing a significant effect on survival[1]. Concerning *JAK2* (V617F) allele burden, there is evidence that having a lower allele load implies a worse survival. In one study[7], survival was significantly reduced in the lower quartile compared with upper quartiles and *JAK2*^{wt} patients, mostly because of infections. In the second paper[27], Kaplan-Meier plots revealed significantly shortened overall and leukemia-free survival for the lower quartile allele burden group, mostly related to BP transformation. Intriguing results were obtained in PMF patients receiving allogeneic hematopoietic stem cell transplantation (HSCT)[32]. In 139 out of 162 patients with known *JAK2* (V617F) mutation status who received HSCT after reduced-intensity conditioning, overall survival was significantly reduced in patients harboring *JAK2*^{wt} compared with *JAK2* mutated patients. In addition, patients who cleared *JAK2* in the peripheral blood six months post-HSCT had a significant lower risk of relapse: this highlights the importance of complete molecular response in MF.

Mutations in the *EZH2* gene, acting through modifying chromatin structure and rendering genes involved in apoptosis inaccessible for transcription, have been found in roughly 6% of PMF[1]. Recently, in 370 PMF and 148 post-PV/ET MF genotyped for mutations of *EZH2* a total of 25 different mutations were detected. *EZH2*-mutated PMF patients had significantly higher leukocyte counts, blast cell counts, and larger spleens at diagnosis, and most of them (53%) were in the high-risk International Prognostic scoring system (IPSS) category. Leukemia-free survival (LFS) and overall survival (OS) were significantly reduced in *EZH2*-mutated PMF patients[1].

Mutations in *IDH* were detected in 4% of patients: 7 patients with mutations of *IDH2* (5 R140Q, 1 R140W and 1 R172G) and 5 of *IDH1* (3 R132S and 2 R132C)[24]. The estimate of survival disclosed that *IDH* mutations are associated with inferior OS and LFS. In addition, a more pronounced effect for the mutant *IDH* on OS and LFS was demonstrated in the context of the *JAK2* mutation.

After the identification of recurrent somatic mutations that involved different components of the RNA splicing machinery and other spliceosome-related genes in myelodysplastic syndromes, these mutations have been investigated in MPNs. A study on 155 MF identified *SF3B1* mutations in 6.5% of the patients: 4 (40%) K700E, 4 (40%) K666T/N/M, 1 (10%) H662D and 1 (10%) N626S and failed to demonstrate any prognostic relevance[11]. In a subsequent study on 187 patients, 17% harbored *SRSF2* monoallelic mutations affecting residue P95[1]. Significant associations were demonstrated between *SRSF2* mutations and advanced age, *IDH* mutations, and higher DIPSS-plus risk category. Finally, *SRSF2* mutations were associated with shortened OS and LFS.

Very recently, a total of 879 MF patients were studied to determine the individual and combinatorial prognostic relevance of somatic mutations in *ASXL1, SRSF2, EZH2, TET2, DNMT3A, CBL, IDH1, IDH2, MPL* and *JAK2* in patients with MF[1]. Analysis was performed in 483 European patients and the seminal observations were validated in 396 patients from Mayo Clinic, Rochester, United States. Of these, *ASXL1, SRSF2*, and *EZH2* mutations inter-independently predicted shortened survival. However, only *ASXL1* mutations remained

significant in the context of the International Prognostic Scoring System (IPSS) with a hazard ratio (HR) of 2.02. These observations were validated in the Mayo Clinic cohort, where investigators found that *ASXL1, SRSF2* and *EZH2* mutations were independently associated with poor survival, but only *ASXL1* mutations held prognostic relevance independently from the Dynamic IPSS (DIPSS)-plus model, with a HR of 1.4. In the European cohort, LFS was negatively affected by *IDH1/2, SRSF2* and *ASXL1* mutations and in the Mayo cohort by *IDH1* and *SRSF2* mutations. In conclusion, the study identified *ASXL1* mutations as the most relevant to be included in the patient's evaluation besides the IPSS models. *ASXL1* mutations are however present at low frequency (less than 20%) in lower risk IPSS categories.

However, the most recent discovery in MF is the occurrence of *CALR* (calreticulin) mutation in patients with MPN without *JAK2* or *MPL* mutation[10,16] and in familial MPN[12]. In MF a clear association with survival has been documented[26].

Proinflammatory cytokines

The abnormal cytokine profile in PMF means that PMF is, at least in part, an inflammatory disease. Cytokines contribute to clinical phenotype, bone marrow fibrosis, angiogenesis, extramedullary hematopoiesis and constitutional symptoms. The interest on cytokines in PMF has recently arisen as JAK inhibitors may quickly reduce several proinflammatory cytokines[1]. Mayo Clinic investigators found that among 30 cytokines tested in a cohort of 90 treatment-naive patients with PMF, high levels of IL-8, IL-2R, IL-12, IL-15 correlate with inferior survival independently from conventional risk stratification[31]. In detail, the presence of 3-fold increased levels of one or both IL-8 and IL-2R may predict worse survival. C-reactive protein (CRP) is a simple marker of systemic inflammation mediated by cytokines, mainly IL-6, and it has been found in MF as well as in ET and PV at higher level than in healthy controls. In PV and ET, high-sensitivity CRP (hsCRP) correlated with a higher risk of vascular complications. In MF hsCRP seems to be associated with a higher incidence of BP and this seems independent from models used to predict survival in MF. To explain this association investigators even speculated on a mutagenic role of chronic oxidative stress on the stem cell, but this seems premature as, for example, new JAK inhibitors, which are able to reduce CRP expression, don't affect leukemic evolution.

Plasma immunoglobulin free light chain

Plasma immunoglobulin FLC might be considered as a surrogate marker of host immune response. free light chain (FLC) (κ or λ) values above the upper limit of normal have been documented in 33% of 240 patients with PMF[18]. Increases in FLC were significantly associated with increased creatinine and advanced age in PMF. In multivariable analysis, increased FLC predicted shortened survival independently from age, creatinine, and other conventional risk factors. No correlations were seen with LFS, karyotype, or *JAK2, MPL*, or *IDH* mutations. In patients with PMF who were studied by cytokine profiling, the prognostic value of an increased FLC level was independent of that from circulating IL-2R or IL-8 levels.

Red blood cell transfusion dependency

Criteria to define red blood cell (RBC) transfusion dependency in PMF have been published[5], and recently updated[6]. Experts considered a volume of 2 units of RBC/month over three months to be the most appropriate observational interval and RBC-transfusion frequency to define a person as RBC-transfusion-dependent. In general, the cutoff level of hemoglobin to define the need of RBC transfusion is 8.5 g/dL. The prognostic impact of RBC transfusion need was examined in 254 consecutive patients, of whom 24% required RBC transfusions at diagnosis and 9% became RBC transfusion dependent during the first year after diagnosis[1]. RBC transfusion need clearly separated two groups with different survivals: 35 mo (transfused from diagnosis), 25 mo (transfused within 1 year), and 117 months (not transfused). RBC transfusion need had an IPSS-independent prognostic power downgrading or upgrading prognosis within specific IPSS categories. This result was confirmed by a study on 288 consecutive patients with PMF[4].

Only 5% of transfusion-independent patients have iron overload as compared to 72% of transfusion-dependent patients[1]. Iron homeostasis is potentially an intriguing pathway in MF. Prognostic interdependence among serum hepcidin (key regulator of iron homeostasis), serum ferritin, hemoglobin of < 10 g/dL, and RBC transfusion requirement has been described although only increased hepcidin and ferritin levels had independent prognostic value for survival in MF. Homeostatic control of hepcidin by iron is preserved in MF, as demonstrated by the strong positive correlation between hepcidin and ferritin levels. In addition, the absence of correlation between hepcidin and circulating inflammatory cytokine levels indicates that hepcidin levels are mainly controlled by iron loading or advanced disease and not by inflammatory signal. The role of iron chelation in MF has not been yet investigated, but some reports showed improvement in term of iron deposits and hemoglobin level[15].

Prognostic models for survival at diagnosis of PMF

In the last years many prognostic models have been developed in PMF. The most used in the past was the Lille score[3], recently replaced by the IPSS[1].

IPSS score

The IPSS was defined through the collaboration of seven centers under the auspices of the IWG-MRT in 2009[1]. After a systematic individual case review, the database included 1054 patients with PMF defined accord-

Table 1 Score values for international prognostic scoring system and dynamic international prognostic scoring system

Parameter	Scores	
	IPSS	DIPSS
Age > 65 yr	1	1
Hemoglobin < 10 g/dL	1	2
Leukocyte count > 25 × 10^9/L	1	1
Blast cells ⩾ 1%	1	1
Constitutional symptoms	1	1

IPSS: Score 0 for low risk, score 1 for intermediate risk-1, score 2 for intermediate risk-2, score ⩾ 3 for high risk; DIPSS: Score 0 for low risk, score 1-2 for intermediate risk-1, score 3-4 for intermediate risk-2, score 5-6 for high risk.

Table 2 Score values dynamic international prognostic scoring system-plus

Parameter	Score value
DIPSS intermediate-1	1
DIPSS intermediate-2	2
DIPSS high risk	3
Unfavorable cytogenetics	1
Red blood cell need	1
Platelet < 100 ×10^9/L	1

DIPSS-plus: Score 0 for low risk, score 1 for intermediate risk-1, score 2-3 for intermediate risk-2, score 4-6 for high risk; DIPSS: Dynamic International Prognostic scoring system-plus.

ing to the WHO classification system, excluding post-PV and post-ET MF and prefibrotic PMF. This is the largest prognostic study ever performed in PMF. Median survival was 69 mo. Multivariate analysis of parameters obtained at disease diagnosis identified age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater as predictors of shortened survival. Based on the presence of 0 (low risk), 1 (intermediate risk-1), 2 (intermediate risk-2) or greater than or equal to 3 (high risk) of these variables, four risk groups with no overlapping in their survival curves were generated (Table 1). The four risk categories were well balanced: 22% of patients fell into the low risk category, 29% in the intermediate risk-1, 28% in the intermediate risk-2 and 21% in the high risk. Median survivals were 135 mo for low risk patients, 95 mo for intermediate-1 patients, 48 mo for intermediate-2 patients, and 27 mo for high risk patients.

Among these patients, 409 patients had available cytogenetic analysis at diagnosis: an abnormal karyotype implied a shorter survival primarily restricted to patients in the intermediate-1 and -2 risk categories. Concerning the *JAK2* (V617F) mutation, no association was observed between *JAK2* status and prognostic score or survival.

Dynamic models for survival in PMF

The progressive nature of PMF generated interest in defining new so-called dynamic models, such as the dynamic-IPSS (DIPSS) and the most recent DIPSS-Plus. In a non time-dependent analysis (models at diagnosis), patients are assigned to a risk group on the basis of the assessment of risk factors at diagnosis, and are followed in the same category irrespective of the acquisition of other risk factors during disease course. According to a dynamic model, patients contribute to the estimate of survival in a category only as long as they do not acquire further risk factors, then they shift to a higher category according to their new score.

DIPSS model

The DIPSS was developed in 525 PMF patients regularly followed[1]. DIPSS risk factors are age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater. The scoring system of DIPSS is different from IPSS (Table 1). The resulting DIPSS risk categories are low (score 0), intermediate-1 (score 1 or 2), intermediate-2 (score 3 or 4) and high (score 5 or 6). Median survival was not reached in low risk patients; it was 14.2 years in intermediate-1, 4 years in intermediate-2, and 1.5 years in high risk. From a practical point of view, anytime a decision has to be made on the basis of an updated prognostic status, the parameters of the DIPSS models will be checked and corresponding values will be assigned. The sum of the values will allow allocating the patient into a risk category (low, intermediate-1, intermediate-2, high) and cumulative survival can be estimated. It is obvious that the corresponding cumulative probability of survival at each time point of the follow-up should be read considering the time elapsed since diagnosis. This estimate remains applicable thereafter until the patient changes risk category. The DIPPS model was also able to predict also the evolution to BP[20].

Very recently Scott *et al*[23] found that DIPSS categories at the time of HSCT predict post-transplant outcome in 170 patients with PMF (related donor, 86; unrelated donor, 84). After a median follow-up of 5.9 years, the median survivals have not been reached for DIPSS low and intermediate-1 risk groups, and were 7 and 2.5 years for intermediate-2 and high-risk patients, respectively.

DIPSS-plus model

This model was produced in 793 patients with PMF of which 428 were referred within and 365 after their first year of diagnosis[1]. This composite model included as worse prognostic factors the unfavorable cytogenetics as previously grouped (complex, sole or two including +8, -7/7q-, i(17q), inv (3), -5/5q-, 12p-, 11q23 rearrangements), RBC transfusion need, platelet count lower than 100 × 10^9/L, and DIPSS categories. According to the model, 1 point each was assigned to DIPSS intermediate-1 risk, unfavorable karyotype, platelets lower than 100 × 10^9/L, and RBC transfusion need, while DIPSS intermediate-2 and high risk were assigned 2 and 3

points, respectively (Table 2). On the basis of this scoring system, four categories were generated: low risk (0 adverse points; median survival, 185 mo), intermediate-1 risk (1 adverse point; median survival, 78 mo), intermediate-2 risk (2-3 adverse points; median survival, 35 mo), and high risk (4-6 adverse points; median survival, 16 mo). It's interesting to note that DIPSS-plus investigators found a proportion of patients in each DIPSS risk category with RBC transfusion need, unfavorable karyotype, and thrombocytopenia, namely 0% (RBC transfusion need), 7% (unfavorable karyotype), and 7% (thrombocytopenia) in low risk patients, 13%, 12%, and 18% in intermediate-1 risk patients, 56%, 17%, and 32% in intermediate-2 risk patients; and 69%, 23%, and 47% in high risk patients, respectively. This sheds light into the possibility of better stratifying patients with lower risk categories.

ANTI-MYELOPROLIFERATIVE AGENTS

The ELN guidelines recommended to use hydroxyurea (HU) as drug of choice when an anti-myeloproliferative effect is needed in MPNs[1]. However, data available on HU are scant. The most complete study on HU in MF evaluated retrospectively 40 patients[1]. Reasons for treatment were constitutional symptoms (55%), symptomatic splenomegaly (45%), thrombocytosis (40%), leukocytosis (28%), pruritus (10%), and bone pain (8%). Responses on different symptoms/clinical findings were as follows: bone pain in 100%, constitutional symptoms in 82%, pruritus in 50%, splenomegaly in 40%, and anemia in 12.5%. According to the IWG-MRT criteria[1], clinical improvement was achieved in 16 patients (40%). Despite the high rate, the median duration of response was 13.2 mo. Worsening of anemia or appearance of pancytopenia were observed in half of the patients.

JAK inhibitors

In the last few years several medicines with anti JAK properties, named JAK inhibitors (JAKi) have been studied. Among these, ruxolitinib is the only approved in many States and available for clinical practice. Other compounds are nowadays under phase 3 investigation (fedratinib, momelotinib, pacritinib), while others are being tested in phase 1-2 studies (www.clinicaltrials.gov). For the practical purpose of this review only ruxolitinib, fedratinib and momelotinib will be discussed in detail as only data published as a full paper will be taken into account.

Ruxolitinib

A phase Ⅰ/Ⅱ trial with ruxolitinib (oral drug) was conducted in 152 patients with PMF or post-PV/post-ET MF. Eligible subjects were therapy-requiring patients, refractory, relapsed, intolerant to previous therapy, or patients with intermediate or high-risk Lille score, if at diagnosis. Main exclusion criteria were thrombocytopenia (platelets < 100×10^9/L) and neutropenia. Applying IWG-MRT criteria[1], 44% of patients obtained a clinical improvement of spleen size (≥ 50% reduction from baseline, measured by palpation) at 3 mo and responses were maintained at 12 mo in more than 70% of patients. The majority of patients had more than 50% improvement in constitutional symptoms mostly due to the activity against pro-inflammatory cytokines[1]. The reduction of the *JAK2*(V617F) allele burden was modest. This study was mainly conducted at MD Anderson Cancer Center (MDACC), Houston, and at Mayo Clinic, Rochester. Two comparisons of outcomes from this phase Ⅰ/Ⅱ trial with historical controls have been performed separately in the two centers to test the effect of ruxolitinib on survival. Mayo Clinic investigators compared 51 patients who received ruxolitinib at Mayo Clinic with 410 patients from the Institutional database not showing any difference in term of survival[28]. The second study compared 107 patients treated with ruxolitinib at MDACC with 310 patients (from three different centers) matched for the phase 1-2 study entry criteria, as controls[1]. A survival benefit for patients treated with ruxolitinib was demonstrated. In addition, the study demonstrated that patients treated with ruxolitinib who obtained a reduction of spleen size greater than 50% have a significantly better survival than those who did not[1].

Two prospective randomized trials with ruxolitinib have been published: COMFORT-1 (155 ruxolitinib *vs* 151 placebo)[1] and COMFORT-2 (146 ruxolitinib vs. 73 best available therapy, BAT)[1]. In COMFORT-1, the primary endpoint (reduction of spleen volume by MRI equal to or greater than 35%) at week 24 was reached in 42% of patients in the ruxolitinib arm and in 1% of those in the placebo arm. At week 24, 46% of patients receiving ruxolitinib and 5% of those receiving placebo experienced symptom alleviation by at least 50%, as measured by the modified Myelofibrosis Symptom Assessment Form (MF-SAF)[14]. Patients treated with ruxolitinib experienced relief of abdominal discomfort, early satiety, night sweats, itching, musculoskeletal pain[1]. In the COMFORT-2 trial the primary endpoint (the same as the COMFORT-1 study but evaluated at week 48) was reached in 28% of patients treated with ruxolitinib and in 0% of those receiving BAT; at week 24 the figures were 32% and 0%, respectively. Mean improvements from baseline in FACT-LymS (Functional Assessment of Cancer Therapy-Lymphoma System) were greater in the ruxolitinib arm.

Recently, the long-term (median time, 2 years) data from the COMFORT-1 trial has been published: 100 of 155 patients randomized to ruxolitinib were still receiving treatment[33]. Mean spleen volume reductions in the ruxolitinib group were 32% at week 24 and 35% at week 96; improvements in quality of life measures were also maintained. Improved survival was observed for ruxolitinib (n = 27 deaths) *vs* placebo (n = 41 deaths) with a hazard ratio of 0.58 (95%CI: 0.36-0.95). Dose-dependent anemia and thrombocytopenia were the most common adverse events in the ruxolitinib group, but these

events rarely led to discontinuation. The incidence of new-onset grade 3 or 4 anemia (29% and 11%, respectively) and thrombocytopenia (9% and 3%, respectively) reported in the first 6 mo of therapy decreased over time to less than 5% for anemia and less than 2% for thrombocytopenia. Mean hemoglobin values reached a nadir of 10%-12% below baseline between weeks 8 and 12 and stabilized over time to a new steady-state slightly below baseline by week 24, and then remained stable throughout the remaining follow-up. In the first 6 mo of treatment, the most common non-hematologic adverse events that occurred more frequently in the ruxolitinib group compared with the placebo group were ecchymosis, headache and dizziness. Under ruxolitinib the rate of non-hematologic adverse events reduced over time. Two patients originally randomized to receive ruxolitinib developed BP at the time of the primary analysis[1] and no further cases were reported in this group.

COMFORT-2 trialists updated the 3 year-follow with 45% (66 of 146) of those originally randomized to ruxolitinib remaining on treatment. The 3-year probability to maintain spleen response (greater than 35%, by MRI) was 50% among patients achieving such degree of response. Ruxolitinib continues to be well tolerated. Anemia and thrombocytopenia were the main toxicities, but they were generally manageable, improved over time, and rarely led to treatment discontinuation (1% and 3.6% of patients, respectively). Other adverse events of special interest included leukopenia, bleeding, infections, thromboembolic events, elevated transaminase levels, increased systolic blood pressure, weight gain. The rate of these events generally decreased with longer exposure to ruxolitinib treatment, with the highest rates occurring within the first 6 mo of treatment. Among these events, infections occurred in 50% of patients between weeks 0-24 and included bronchitis, gastroenteritis, nasopharyngitis, urinary tract infections. The rate of infections becomes 25% in weeks 144-168. Over the entire course of the study, 2 patients (1.4%) in the ruxolitinib arm had tuberculosis. No single non-hematologic adverse event led to definitive ruxolitinib discontinuation in more than one patient. Finally, patients randomized to ruxolitinib showed longer overall survival than those randomized to BAT (HR = 0.48, 95%CI: 0.28-0.85).

Both COMFORTs trial included patients with placebo or BAT who crossed to ruxolitinib: this makes impossible the evaluation of the net effect of ruxolitinib over comparators in the long term. Very recently, a comparison of survival from diagnosis of the DIPSS cohort (350 PMF, selection criteria, patients who become intermediate-2 and high risk IPSS, blast cell count lower than 10%) and the COMFORT-2 cohort (100 patients, intermediate-2 and high risk IPSS, blast cell count lower than 10%, selection criteria, PMF) has been published. This demonstrated an advantage in term of survival using ruxolitinib (COMFORT-2) *vs* standard therapy (DIPSS)[21].

Taken together the COMFORT trials showed that ruxolitinib, a drug with a good safety profile, improves two clinical needs of patients: splenomegaly and MF-related symptoms. However, reactivation of infections such as tuberculosis or viral hepatitis has been reported in very few case reports[1] and this underlines the need for a careful observation of patients during follow-up. *In vitro* data[8] demonstrated that ruxolitinib significantly affects dendritic cell differentiation and function leading to impaired T-cell activation[13], potentially resulting in increased infection rates in ruxolitinib-treated patients. Though requiring adequante monitoring for these potential side effects, data on survival advantage are really interesting and place this drug as a new potential first line therapy in MF patients at higher risk.

Fedratinib, SAR302503

In a phase Ⅰ-Ⅱ trial, fedratinib was administered orally once a day to 59 patients with intermediate and high-risk MF[1]. By six and 12 cycles of treatment, 39% and 47% of patients, respectively, had achieved a spleen response per IWG-MRT criteria. The majority of patients with leukocytosis or thrombocytosis at baseline achieved normalization of blood counts after six (57% and 90%, respectively) and 12 (56% and 88%, respectively) cycles. Beside the effect on splenomegaly, the majority of patients with constitutional symptoms, fatigue, pruritus had a durable resolution. Grade 3 to 4 hematologic adverse events included anemia (occurring in 35% of 37 patients who were not RBC transfusion dependent at baseline), thrombocytopenia (24%) and neutropenia (10%). At doses ranging between 240 mg and 520 mg, two of five RBC transfusion-independent patients became RBC transfusion-dependent and two of nine had grade 3/4 thrombocytopenia. The main non-hematologic adverse events included all grades nausea (69%), diarrhea (64%) vomiting (58%), all self-limited and controlled by symptomatic treatments. Asymptomatic increase of lipase, AST, ALT, and creatinine have been reported in roughly one quarter of patients. A randomized, blinded, placebo-controlled study of fedratinib (dose 400 mg or 500 mg daily), named JAKARTA, in patients with intermediate-2 or high risk MF is ongoing with the objective to evaluate the reduction of spleen volume by MRI equal to or greater than 35%. Unfortunately, despite this pivotal study met the primary endpoint in both dose groups, cases consistent with Wernicke's encephalopathy have been reported in patients participating in fedratinib trials. Following a thorough risk-benefit analysis, the risk to patient safety was considered to outweigh the benefit that fedratinib would bring to patients. All clinical trials involving fedratinib have been halted, and fedratinib treatment discontinued in patients enrolled in ongoing trials.

Momelotinib, CYT387

Momelotinib was studied in a phase 1/2 trial in patients with high or intermediate risk MF[17]. Pre-planned safety and efficacy analysis has been completed for the initial

60 patients. In the dose-escalation phase, the maximum-tolerated dose was 300 mg/d based on reversible grade 3 headache and asymptomatic hyperlipasemia. Twenty-one and 18 additional patients were accrued at two biologically effective doses, 300 mg/d and 150 mg/d, respectively. Anemia and spleen responses, per IWG-MRT criteria, were 59% and 48%, respectively. Among 33 patients who were RBC-transfused in the month prior to study entry, 70% achieved a minimum 12-wk period without transfusions. Most patients experienced constitutional symptoms improvement. Grade 3/4 adverse reactions included thrombocytopenia (32%), hyperlipasemia (5%), elevated liver transaminases (3%) and headache (3%). New-onset treatment-related peripheral neuropathy was observed in 22% of patients (sensory symptoms, grade 1). A phase 3 study to determine the efficacy of momelotinib *vs* ruxolitinib in MF patients naive of JAKi is ongoing.

CONCLUSION

Concerning prognostication of MF, the IPSS model at diagnosis and the DIPSS anytime during the course of the disease may be useful to define survival of MF patients. The IPSS and the DIPSS are based on age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25×10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational analysis seem to be the next step to implement MF prognostication. Taking together all available clinical data on MF, one may conclude that JAKi give a benefit to patients with MF, by reducing spleen size of about 50% in approximately 30%-40% of patients and by abolishing symptoms in the vast majority of patients. However, effect on these disease manifestations should be balanced with the safety profile[19]. Anemia and thrombocytopenia are on-target toxicities expected with all JAKi. Infections should be monitored with ruxolitinib, drug with the longest time of observation, but might be expected with all JAKi. Other toxicities may involve non-JAK2 targets, as in case of gastrointestinal events during therapy with fedratinib or in the case of neurological toxicity for momelotinib.

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ORIGINAL ARTICLE

Tbl3 encodes a WD40 nucleolar protein with regulatory roles in ribosome biogenesis

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Abstract

AIM: To investigate the subcellular localization and the function of mouse transducin β-like 3 (Tbl3).

METHODS: The coding sequence of mouse Tbl3 was cloned from the cDNAs of a promyelocyte cell line by reverse transcription-polymerase chain reaction. Fusion constructs of Tbl3 and enhanced green fluorescent protein (EGFP) were transfected into fibroblasts and examined by fluorescence microscopy to reveal the subcellular localization of tbl3. To search for nucleolar targeting sequences, scanning deletions of Tbl3-EGFP were constructed and transfected into fibroblasts. To explore the possible function of Tbl3, small hairpin RNAs (shRNAs) were used to knock down endogenous Tbl3 in mouse promyelocytes and fibroblasts. The effects of Tbl3 knockdown on ribosomal RNA (rRNAs) synthesis or processing were studied by labeling cells with 5,6-^{3}H-uridine followed by a chase with fresh medium for various periods. Total RNAs were purified from treated cells and subjected to gel electrophoresis and Northern analysis. Ribosome profiling by sucrose gradient centrifugation was used to compare the amounts of 40S and 60S ribosome subunits as well as the 80S monosome. The impact of Tbl3 knockdown on cell growth and proliferation was examined by growth curves and colony assays.

RESULTS: The largest open reading frame of mouse Tbl3 encodes a protein of 801 amino acids (AA) with an apparent molecular weight of 89-90 kilodalton. It contains thirteen WD40 repeats (an ancient protein-protein interaction motif) and a carboxyl terminus that is highly homologous to the corresponding region of the yeast nucleolar protein, utp13. Virtually nothing is known about the biological function of Tbl3. All cell lines surveyed expressed Tbl3 and the level of expression correlated roughly with cell proliferation and/or biosynthetic activity. Using Tbl3-EGFP fusion constructs we obtained the first direct evidence that Tbl3 is targeted to the nucleoli in mammalian cells. However, no previously described nucleolar targeting sequences were found in Tbl3, suggesting that the WD40 motif and/or other topological features are responsible for nucleolar targeting. Partial knockdown (by 50%-70%) of mouse Tbl3 by shRNA had no discernable effects on the processing of the 47S pre-ribosomal RNA (pre-rRNA) or the steady-state levels of the mature 28S, 18S and 5.8S rRNAs but consistently increased the expression level of the 47S pre-rRNA by two to four folds. The results of the current study corroborated the previous finding that there was no detectable rRNA processing defects in zebra fish embryos with homozygous deletions of zebra fish Tbl3. As ribosome production consumes the bulk of cellular energy and biosynthetic precursors, dysregulation of pre-rRNA synthesis can have negative effects on cell growth, proliferation and differentiation. Indeed, partial knockdown of Tbl3 in promyelocytes severely impaired their proliferation. The inhibitory effect of Tbl3 knockdown was also observed in fibroblasts, resulting in an 80% reduction in colony formation. Taken

together, these results indicate that Tbl3 is a newly recognized nucleolar protein with regulatory roles at very early stages of ribosome biogenesis, perhaps at the level of rRNA gene transcription.

CONCLUSION: Tbl3 is a newly recognized nucleolar protein with important regulatory roles in ribosome biogenesis.

Key words: Nucleolus; Nucleolar protein; Ribosome biogenesis; Ribosomal RNA; Pre-ribosomal RNA

Core tip: The mouse gene transducin β-like 3 (Tbl3) encodes a protein with thirteen WD40 protein-protein interaction motifs and is the mammalian homologue of yeast utp13. Virtually nothing is known about the function of tbl3. In this report, we provide the first direct evidence that Tbl3 is targeted to the nucleoli and plays an important role in regulating the synthesis of the 47S pre-ribosomal RNA, *i.e.*, at very early stages of ribosome biogenesis. This activity has never been described before and sets Tbl3 apart from all other known nucleolar proteins. TBL3 may provide an attractive target for anti-neoplastic therapy.

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INTRODUCTION

Ribosomes are essential for protein synthesis. Defects in ribosome biogenesis or "ribosomopathy" underlie several devastating hematological disorders such as the Diamond-Blackfan anemia, Shwachman-Bodian-Diamond syndrome, dyskeratosis congenita and the 5q- myelodysplastic syndrome[1,2].

The production of ribosomes is a highly coordinated, multi-step process that starts in the nucleoli, the organelles that form around the "nucleolar organizers" on the long arms of acrocentric chromosomes (human chromosomes 13, 14, 15, 21 and 22)[3]. The nucleolar organizers consist of 400 or so rRNA genes (rDNAs). The production of ribosomes begins with the transcription of the rDNAs by RNA polymerase I (RNA Pol I), which synthesizes a long primary transcript, the 47S pre-rRNA, that is then covalently modified (*e.g.*, 2'-O-ribose methylation and pseudouridylation) and processed into mature 28S, 18S and 5.8S rRNAs. The rRNAs are assembled into pre-ribosomes with about eighty ribosomal proteins plus the 5S rRNA, which is separately transcribed by RNA polymerase Ⅲ outside the nucleolus. A large number (> 150) of small nucleolar RNAs and nonribosomal proteins participate in the post-transcriptional modification, processing and assembly of rRNAs to produce pre-ribosomes, which undergo further maturation to become nascent 40S and 60S ribosomal subunits before being exported into the cytoplasm. Most steps of rRNA processing occur co-transcriptionally with pre-rRNA synthesis[4-7]. As the production of ribosomes consumes the bulk of cellular energy[8], defective or dysregulated ribosome production can affect many aspects of cellular physiology especially cell proliferation.

Due to the relative simplicity and the ease of genetic manipulations in the yeasts, considerably more has been learned about the functions of non-ribosomal nucleolar proteins in yeasts than in mammalian cells. Still, the functions of many yeast nonribosomal nucleolar proteins remain unknown. In mammals, the total number of non-ribosomal proteins involved in ribosome biogenesis is even larger, reflecting the greater complexities of eukaryotic rRNA modification, processing, ribosome assembly, transport and other nucleolar events. Recent proteomic analyses of purified human nucleoli listed over 200 to 700 nonribosomal nucleolar proteins[3,9-11]. The exact functions of many of these proteins are unknown.

In this report, we describe the initial functional characterization of murine transducin-β-like 3 (Tbl3). At least four proteins have been described as "transducin-β-like" based on DNA sequence analysis, including transducin-β-like 1 (Tbl1), transducin-β-like related 1 (Tblr1), transducin-β-like 2 (Tbl2) and tbl3. tbl1 and tblr1 have been shown to be transcription co-regulators (co-repressors) in the signal transduction pathway of retinoic acid receptor-α[12-15]. Tbl2 is a putative protein associated with the Williams-Beuren syndrome based on gene mapping but its function is entirely unknown[16]. Very little is known about tbl3[17]. A literature search yielded 8 publications that mentioned *Tbl3*. Of these, one is a gene mapping study and two are disease-gene association studies[18-20]. Three are proteomic studies[9-11]. The seventh describes the composition of the co-repressor complex associated with the retinal photoreceptor-specific nuclear receptor (PNR)[21]. The most recent report is from the authors and collaborators and focuses on the phenotypic characterization of a zebra fish mutant, *ceylon* (*cey*) with homozygous deletions of *Tbl3* (in addition to four other uncharacterized genes)[22]. The *cey* mutant embryos have normal tissue specification but the sizes of some organs or cell populations such eye, pancreas, T cells and erythrocytes are markedly diminished due to reduced cellular proliferation, which in turn is attributed to cell cycle slowing as a result of *Tbl3* deficiency. The proliferative defect of the *cey* mutant becomes apparent 3-4 d post fertilization (dpf). All mutant embryos die by 10-14 dpf due to depletion of maternally derived *Tbl3* mRNA in the embryos[22]. The embryonic lethality of *Tbl3* deletion highlights its importance in tissue/organ development. However neither the site of

Tbl3 expression nor the mechanism of cell cycle slowing was elucidated. Here, we provide direct experimental evidence that mouse Tbl3 is targeted to the nucleoli where it plays an important role(s) in regulating the production of the 47S pre-rRNA.

MATERIALS AND METHODS

Expression vectors

The full-length coding sequence of mouse Tbl3 was amplified from oligo d(T)-primed cDNAs of MPRO (Mouse Promyelocyte) cells[23], cloned in frame into the *Hin*dⅢ/*Sal* I sites of the pEGFP C3 (for N-terminal fusion with EGFP) and pEGFP N1 vector (for C-terminal fusion)(Clontech). The truncation mutants of Tbl3 (del 1-632, del 1-400, del 632-801, del 401-632) were generated by polymerase chain reaction (PCR) amplification of the desired coding sequences and cloned into the *Hind*Ⅲ/*Sal* I sites of pEGFP N1 or C3 vector. The N-terminal deletion mutants (del 1-10, del 1-12, del 1-16 del 1-20) and C-terminal deletion mutants (del 777-801, del 753-776, del 733-752, del 713-732, del 693-712, del 673-692, del 653-672, del 633-652) were also generated using PCR-based deletion methods. All plasmids were sequenced to ensure accuracy. DNAs were purified using a plasmid DNA purification kit (Qiagen) and further purified by phenol-chloroform extraction and ethanol precipitation.

Short hairpin RNA knockdown vectors

The short hairpin RNA or shRNA sequences for mouse *Tbl3* were designed using the shRNA Sequence Designer (Clontech). The sequences of the two shRNAs we used in the current study are: 5'ccggTGCCAAGGATCAGAGCATAttcaagaga-TATGCTCTGATCCTTGGCAttttttg and 5'ccggTGGCCATTACCTCTTCTGTttcaagagaACAGAAGAGGTAATGGCCAttttttg (uppercases denote *Tbl3*-derived sequences). Synthetic oligonucleotides were annealed and cloned into the *Age* I/*Eco*R I sites of the pMKO 1p vector (referred to as pMKO hereafter)[24]. pMKO contains a puromycin resistance gene expression cassette and confers puromycin resistance in transfected mammalian cells. Both constructs provided specific knockdown but the first shRNA exhibited a stronger knockdown effect and was used in most experiments. Either pMKO or pMKO-Luc shRNA (Luc stands for luciferase) was used as the negative control in all knockdown experiments with similar results.

Cell cultures

NIH 3T3 cells were transfected with the pEGFP N1 or C3 fusion constructs by calcium phosphate method. MPRO cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL) and the conditioned medium of BHK/HM-5 cell line as a source of murine granulocyte-macrophage colony stimulating factor (10% vol/vol)[23]. MPRO cells were transfected with 10 μg of pMKO (or pMKO-Luc shRNA) *vs* pMKO-Tbl3 shRNA DNAs per 2.5×10^6 cells by electroporation using Gene Pulser Xcell (Bio-Rad). Transfected cells were selected with puromycin (0.375 μg/mL)(Sigma) for 8-10 d to establish stable transfectants.

Fibroblast colony assay

LAP-3 fibroblasts[25] were transfected in triplicates with 5 μg of pMKO (or pMKO-Luc shRNA) *vs* pMKO-Tbl3 shRNA by the Lipofectamine (Life Technologies) method in parallel to minimize variation in toxicity and transfection efficiency. After 24-48 h, transfected cells were detached by trypsin/EDTA and subcultured at 1:10-20 ratios in new 60-mm tissue culture dishes and selected with puromycin (1.5 μg/mL) for 5-10 d. Colonies were fixed and stained *in situ* with Coomassie Stain (BioRad).

Northern analyses

Total cellular RNAs were extracted from the same starting numbers of MPRO cells stably transfected with pMKO (or pMKO-Luc shRNA) or pMKO-Tbl3 shRNA in parallel using an RNeasy kit (Qiagen). Total RNAs extracted from equal numbers of starting cells were electrophoresed in 1% formaldehyde-agarose gels, blotted onto Hybond-N (Amersham) and hybridized with a randomly primed, ^{32}P-labled *Tbl3* probe in RapidHyb buffer (Amersham) at 70 ℃ for 3 h, followed by two washings with 2 × standard sodium citrate buffer (SSC) plus 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min. The final wash was done with 0.1 × SSC plus 0.1% SDS at 56 ℃ for 15 min.

^{3}H-uridine labeling and fluorography

To study the synthesis and processing of pre-rRNA, equal numbers of stably transfected MPRO/pMKO (or pMKO-Luc shRNA) and MPRO/pMKO-Tbl3 shRNA were labeled with 2.5 μCi/mL of 5,6-^{3}H-uridine (Perkin Elmer) for 30 min at 37 ℃, washed with phosphate buffered saline (PBS) and chased for 0-180 min at 37 ℃ in nonradioactive media. Total RNAs were purified using an RNeasy kit. In some experiments, Trizol agent was used to recover the 5.8S rRNA quantitatively. Total RNAs from equal numbers of starting cells were electropheresed in 1% formaldehyde-agarose gels and blotted onto Hybond-N. For fluorography, Northern blots were spray-coated with En^{3}Hance Spray (Perkin Elmer) and exposed to X-ray films directly without covering at -80 ℃ for 1-14 d. The fluor graphs were analyzed using a BioRad Gel Doc XR+.

Ribosome profiling by sucrose gradient centrifugation

For ribosome profiling, equal numbers of MPRO/pMKO (or MPRO/pMKO-Luc shRNA) and MPRO/pMKO-Tbl3 shRNA were washed with PBS and pelleted at 4 ℃ and re-suspended in polysome lysis buffer containing 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 20 mmol/L HEPES (pH 7.4), 1% NP-40, 1 mmol/L dithiothreitol, heparin sodium (200 μg/mL), phenylmethylsulfonyl

fluoride (1.0 mmol/L) (Sigma) and RNasin (100 unit/mL)(Promega). Cells were homogenized using a 1-ml syringe with a 25-gauge needle for eight times and centrifuged at 8000 *g* for 10 min at 4 ℃. The supernatant was layered on a 10%-45% (wt/vol) sucrose density gradient made in the polysome gradient buffer (100 mmol/L KCl, 5 mmol/L $MgCl_2$, 20 mmol/L HEPES, pH 7.4) at 38000 *g* for 3 h at 4 ℃ in a Beckman SW55Ti rotor and analyzed using a UA-6 Absorbance Detector (Isco). Of note, no cycloheximide was added to the lysates or sucrose gradients to allow the polysomes to dissociate into a single peak of 80S monosomes (*i.e.,* 40S plus 60S subunits) to facilitate the comparison of the total amounts of ribosomes.

Western blots

NIH3T3 cells expressing Tbl3-EGFP fusion protein were lysed in RIPA buffer. Lysates were denatured and electrophoresed in a 4%-12% denaturing SDS PAGE (NuPage; InVitrogen) along with Magic Mark XP Western Protein Standard (Life Technologies), blotted onto Immobilon-P (Millipore), probed with a rat monoclonal anti-GFP antibody (Pierce), followed by biotinylated goat anti-rat Igs (Pierce) and streptavidin-conjugated horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence.

RESULTS

Tbl3 contains thirteen WD40 repeats

The cDNA of the largest open reading frame of mouse *Tbl3* was cloned from the MPRO[23] cell line by RT-PCR. It contains 2,406 nucleotides (nt) and encodes a protein of 801 aa. Sequence alignments show that it is highly homologous to human TBL3, *Schizosaccharomyces pombe* utp13, and *Saccharomyces cerevisiae* utp13 (Figure 1). Thirteen WD40 repeats (also known as beta-transducin repeats) are present in mouse tbl3. The WD40 repeat consists of the consensus aa sequence [$X_{6\text{-}94}$-(GH-$X_{23\text{-}41}$-WD)] and is found in many proteins involved in signal transduction, rRNA processing, gene regulation, vesicular trafficking and cytoskeletal assembly[26,27]. Crystallography studies reveal that the WD40 repeats cluster together to form a β-propeller structure, which serves as a rigid platform for multiple protein-protein interactions[27]. A second region of homology is found in the C-terminus of mouse Tbl3 and yeast utp13. The conserved sequence in the C-terminus has not been found in any other protein. It is likely that this region contains a functional domain(s) unique to mouse Tbl3 and yeast utp13.

Tbl3 expression correlates with cell proliferation and/or biosynthetic activity

Tbl3 is expressed in all cell lines and tissues that we have examined. In all cases, there is only one band of *Tbl3* mRNA measuring approximately 3 kb in length in each cell type (Figure 2). The level of *Tbl3* mRNA varies significantly from one cell type to another and roughly correlates with the proliferative or biosynthetic activity of the cells. Among the cell lines surveyed, erythroleukemia (HCD57)[28], macrophage (J774), B-cell lymphoma (BaF3), T-cell lymphoma (EL4) and NIH3T3 fibroblasts express the highest levels of *Tbl3*. In normal tissues, hepatocytes, myocardium, testes, hematopoietic progenitors, $CD8^+$ T cells and $CD14^+$ monocytes express 3-5 times more *Tbl3* than the average tissue (not shown).

Tbl3 is targeted to the nucleoli

To study the subcellular localization of mouse tbl3, we constructed an expression vector expressing mouse Tbl3 as a fusion protein with EGFP in its C-terminus. The vector was transfected into NIH3T3 cells (or CV-1 or 293 cells). Fluorescence microscopy revealed that the overwhelming majority of the fusion protein was targeted to the nucleoli with a very small fraction appearing in the nucleoplasm (Figure 3A-E). The same result was obtained when EGFP was fused to the N-terminus of Tbl3 (not shown). The small nucleoplasmic pool (in a very fine punctate pattern at high magnifications) may represent genuine extra-nucleolar distribution of Tbl3 or an overexpression artefact. Western blot analysis using a monoclonal anti-GFP antibody yielded a single band of tbl3-EGFP fusion protein of approximately 116 kD (Figure 3F). Thus, the deduced apparent molecular weight of mouse Tbl3 is 89-90 kDa.

Sequence requirement for nucleolar targeting by tbl3

No previously reported nucleolar targeting motifs have been identified in Tbl3. To determine the aa sequence required for nucleolar localization of tbl3, we constructed a series of scanning deletion mutants of Tbl3 as EGFP fusion proteins (Figure 4). These constructs were transfected into NIH3T3 fibroblasts and examined by fluorescence microscopy. Among the constructs examined, only the full-length Tbl3 and those with very short (10-16 aa) N-terminal deletions could localize to the nucleoli. The remaining constructs yielded a diffuse pattern throughout the cytoplasm and nucleoplasm (but largely excluded from the nucleoli), a pattern that is indistinguishable from that of EGFP *per se*. This result suggests that the overall topology and/or the WD40 repeats rather than a unique localization signal are responsible for the nucleolar localization of tbl3.

shRNA-mediated knockdown of *Tbl3* increases the level of newly synthesized 47S pre-rRNA

To study the function of *Tbl3*, we used the MPRO murine promyelocyte cell line as a model system. MPRO was established from a normal mouse marrow[23]. The differentiation of MPRO is reversibly blocked at the promyelocyte stage. DNA array and proteomic studies have shown that MPRO, unlike most transformed leukemia cell lines, closely reflects the physiology of normal promyelocytes[29]. To investigate the function of *Tbl3* in MPRO, we trasnsfected the MRPO cells with either

```
S.pombe utp13     -----MAPIGEKKRFELEKSIEPIYTG--GPVAFDSNEKILVTALTDRIIGTRSETGER- 52
S.cerevisiae      ---------MDLKTSYKGISLNPIYAGSSAVATVSENGKILATPVLDEININIDLTPGSRK 51
Mouse tbl3        MAETAAGLCRFKANYAVERKIEPFYKG--GKAQLDQTGHYLFCVCGTKVNILDVASGAL- 57
Human TBL3        MAETAAGVGRFKTNYAVERKIEPFYKG--GKAQLDQTGQHLFCVCGTRVNILEVASGAV- 57
                                .:::*:* *  . . .... : *      .:      .*

S.pombe           -LFSIKKDEDDYVTALAITSDSKKLIAAFRSRLLTIYEIPSGRRIKSMK-AHETPVITMT 110
S.cerevisiae      ILHKISNEDEQEITALKLTPDGQYLTYVSQAQLLKIFHLKTGKVVRSMK-ISS-PSYILD 109
Mouse             -LRSLEQEDQEDITSFDLSPDDEVLVTASRALLLAQWAWREGTVTRLWKAIHTAPVASMA 116
Human             -LRSLEQEDQEDITAFDLSPDNEVLVTASRALLLAQWAWQEGSVTRLWKAIHTAPVATMA 116
                   * .:.::::: :*:: ::.*.: *  . :: **  :    *   :  *     *   :

S.pombe           IDPTNTLLATGGAEGLVKVWDIAGAYVTHSFRGHGGVISALCFGKHQN--TWVLASGADD 168
S.cerevisiae      ADSTSTLLAVGGTDGSIIVVDIENGYITHSFKGHGGTISSLKFYGQLNSKIWLLASGDTN 169
Mouse             FDATSTLLATGGCDGAVRVWDIVQHYGTHHFRGSPGVVHLVAFHPDPTR--LLLFSSAVD 174
Human             FDPTSTLLATGGCDGAVRVWDIVRHYGTHHFRGSPGVVHLVAFHPDPTR--LLLFSSATD 174
                   *.*.****.** :* : * **   * ** *:*  *.:  : *  . .    :* *.  :

S.pombe           SRVRLWDLNSSRSMAVFEGHSSVIRGLTFEPTGS------FLLSGSRDKTVQVWNI---K 219
S.cerevisiae      GMVKVWDLVKRKCLHTLQEHTSAVRGLDIIEVPDNDEPSLNLLSGGRDDIINLWDFNMKK 229
Mouse             TSIRVWSLQDRSCLAVLTAHYSAVTSLSFSEGGH------TMLSSGRDKICIVWDL---Q 225
Human             AAIRVWSLQDRSCLAVLTAHYSAVTSLAFSADGH------TMLSSGRDKICIIWDL---Q 225
                    ::*.* .  .: .:  * *.: .* :            :**..**.   :*::   :

S.pombe           KRSAVRTIPVFHSVEAIGWVNGQPE--------EKILYTAGEGNLILAWDWKSGSRLDPG 271
S.cerevisiae      KCKLLKTLPVNQQVESCGFLKDGDG--------KRIIYTAGGDAIFQLIDSESGSVLKR- 280
Mouse             SYQTTRTVPVFESVEASVLLPEQPAPALGVKSSGLHFLTAGDQGILRVWEAASGQCVYTQ 285
Human             SCQATRTVPVFESVEAAVLLPEEPVSQLGVKSPGLYFLTAGDQGTLRVWEAASGQCVYTQ 285
                  . .  :*:** ..**:   :                : ***    :   :  **. :

S.pombe           VDTTHSETNAIIQVVPFSENTLLSVHSDLSLLLR-----KRVPGEGFITIKKLNGSFDEV 326
S.cerevisiae      TNKPIEELFIIGVLPILSNSQMFLVLSDQTLQLINVEEDLKNDEDTIQVTSSIAGNHGII 340
Mouse             PQMPGLRQELTHCTLARAADLLLTVTADHNLLLY--------EAHSLQLQKQFAGYSEEV 337
Human             AQPPGPGQELTHCTLAHTAGVVLTATADHNLLLY--------EARSLRLQKQFAGYSEEV 337
                   : .             : . :: . :* .* *          :   ..: *    :

S.pombe           IDCAWIG--DDHLAVCSNTEFIDVISTDGT--------QVFGVLEGHTDIVLTLDSSEDG 376
S.cerevisiae      ADMRYVGPELNKLALATNSPSLRIIPVPDLSGPEASLPLDVEIYEGHEDLLNSLDATEDG 400
Mouse             LDVRFLGPSDSHIVVASNSPCLKVFELQTL---------ACQILHGHTDIVLALDVFRKG 388
Human             LDVRFLGPEDSHVVVASNSPCLKVFELQTS---------ACQILHGHTDIVLALDVFRKG 388
                   *  ::*   .::.:.:*:  : ::                : .** *:: :**  ..*

S.pombe           VWLATGAKDNTVRLWNLNIEDNVYKCIHVFTGHTASVTAVALGPLDVNGYPTFLASSSQD 436
S.cerevisiae      LWIATASKDNTAIVWRYNENSCKFDIYAKYIGHSAAVTAVGLPNIVSKGYPEFLLTASND 460
Mouse             WLFASCAKDQSIRIWKMN-KAGQVACVAQGSGHTHSVGTICCSRLKES----FLVTGSQD 443
Human             WLFASCAKDQSVRIWRMN-KAGQVMCVAQGSGHTHSVGTVCCSRLKES----FLVTGSQD 443
                    :*: :**::  :*. * :           **: :* ::    :  .    ** :.*:*

S.pombe           RTLKRFNLGSQLN-------KSDFSNRAVWTIKAHDRDVNAIQVSKDGRIIASASQDKTI 489
S.cerevisiae      LTIKKWIIPKPTASM-----DVQIIKVSEYTRHAHEKDINALSVSPNDSIFATASYDKTC 515
Mouse             CTVKLWPLPEALLAKSTAADSGPVLLQAQTTRRCHDKDINSLAVSPNDKLLATGSQDRTA 503
Human             CTVKLWPLPKALLSKNTAPDNGPILLQAQTTQRCHDKDINSVAIAPNDKLLATGSQDRTA 503
                   *:* : : .          .  .   :  * :.*::*:*:: :: :. ::*:.* *:*

S.pombe           KLWDSSTGEVVGVLRGHRRGVWACSFNPFSRQLASGSGDRTIRIWNVDTQQCVQTLEGHT 549
S.cerevisiae      KIWNLENGELEATLANHKRGLWDVSFCQYDKLLATSSGDKTVKIWSLDTFSVMKTLEGHT 575
Mouse             KLWALPQCQLLGVFTGHRRGLWNVQFSPTDQVLATASADGTIKLWALQDFSCLKTFEGHD 563
Human             KLWALPQCQLLGVFSGHRRGLWCVQFSPMDQVLATASADGTIKLWALQDFSCLKTFEGHD 563
                  *:*     :: ..: .*:**:*  .*   .: **:.*.* *:::* ::  . ::*:***

S.pombe           GAILKLIYISQGTQVVSAAADGLVKVWSLSSGECVATLDNHEDRVWALASRFDGSLLVSG 609
S.cerevisiae      NAVQRCSFINKQKQLISCGADGLIKIWDCSSGECLKTLDGHNNRLWALSTMNDGDMIVSA 635
Mouse             ASVLKVAFVSRGSQLLSSGSDGLLKLWTIKSNECVRTLDAHEDKVWGLHCSQLDDHAITG 623
Human             ASVLKVAFVSRGTQLLSSGSDGLVKLWTIKNNECVRTLDAHEDKVWGLHCSRLDDHALTG 623
                   :: :  ::.: .*::*..:***:*:*  ...**: *** *::::*.*     ..  ::.

S.pombe           GADAVVSVWKDVTEEYIAKQAEELERRVEAEQLLSNFEQTEDWQQAIALALSLDRPHGLL 669
S.cerevisiae      DADGVFQFWKDCTEQEIEEEQEKAKLQVEQEQSLQNYMSKGDWTNAFLLAMTLDHPMRLF 695
Mouse             GSDSRIILWKDVTEAEQAEEQAKREEQVIKQQELDNLLHEKRYLRALGLAISLDRPHTVL 683
Human             ASDSRVILWKDVTEAEQAEEQARQEEQVVRQQELDNLLHEKRYLRALGLAISLDRPHTVL 683
                   :*. . .*** **    ::  . : :*  :* *.*      : .*: **::**:*  ::

S.pombe           RLFERVMTAPHQPNSITG-------NKDVDNVLVQLPDHQLIILFQRIRDWNTNSKTSMV 722
S.cerevisiae      NVLKRALGESRSRQDTEEGKIEVIFNEELDQAISILNDEQLILLMKRCRDWNTNAKTHTI 755
Mouse             TVIQAIRRDPE-------------ACEKLEATVLRLRRDQKEALLRFCVTWNTNSRHCHE 730
Human             TVIQAIRRDPE-------------ACEKLEATMLRLRRDQKEALLRFCVTWNTNSRHCHE 730
                   :::     ..               :.:: .:  *  .*   *::    ****::

S.pombe           AQRLLRLLLHSYSPEHLLKLSGIKDILDSMIPYTDRHLARVNDLIEDSYIVDYVI----- 777
S.cerevisiae      AQRTIRCILMHHNIAKLSEIPGMVKIVDAIIPYTQRHFTRVDNLVEQSYILDYALVEMDK 815
Mouse             AQAVLGVLLRHEAPEELLAYDGVRGSLEALLPYTERHFQRLSRTLQAATFLDFLWHNMKL 790
Human             AQAVLGVLLRREAPEELLAYEGVRAALEALLPYTERHFQRLSRTLQAAAFLDFLWHNMKL 790
                  **  :  :*      .*    *:   :::::***:**: *:.  :: : ::*:

S.pombe           -------------------
S.cerevisiae      LF----------------- 817
Mouse             SPCPAAAPPAL-------- 801
Human             -PVPAAAPTPWETHKGALP 808
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Figure 1 Sequence analyses of mouse transducin β-like 3, human transducin β-like 3 and yeast upt13. Amino acid sequence alignment of mouse transducin β-like 3 (Tbl3), human TBL3, *Schizosaccharomyces pombe* (*S. pombe*) utp13 and *Saccharomyces cerevisiae* (*S. cerevisiae*) utp13 using the Clustal program. "*" indicates identical aa; ":" indicates conserved substitution; "." indicates semiconserved substitution.

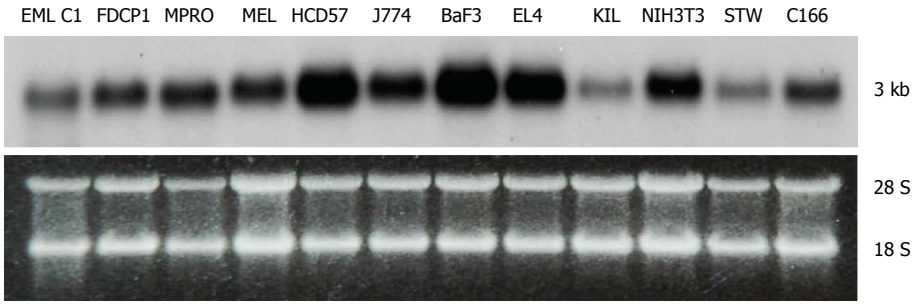

Figure 2 Tissue expression of transducin β-like 3. Northern blot analysis of the transducin β-like 3 (Tbl3) message (approximately 3 kb) in select mouse cell lines. The bottom panel is the corresponding ethidium bromide-stained gel showing similar loading. Each lane contained 10 μg of total RNA. The 28S and 18S rRNAs are indicated. EML C1: A multipotent hematopoietic progenitor line[38]; FDCP1: A bipotent granulocyte-monocyte progenitor line; MPRO: A promyelocytic line[23]; MEL: A murine erythroleukemia line; HCD57: An erythroblast line[28]; J774: A macrophage line; BaF3: A pre-B lymphoma line; EL4: A T cell-like lymphoma line; KIL: A natural killer line[39]; NIH3T3: An embryonic fibroblast-like line; STW: A bone marrow stromal cell line; C166: A yolk sac-derived endothelial line.

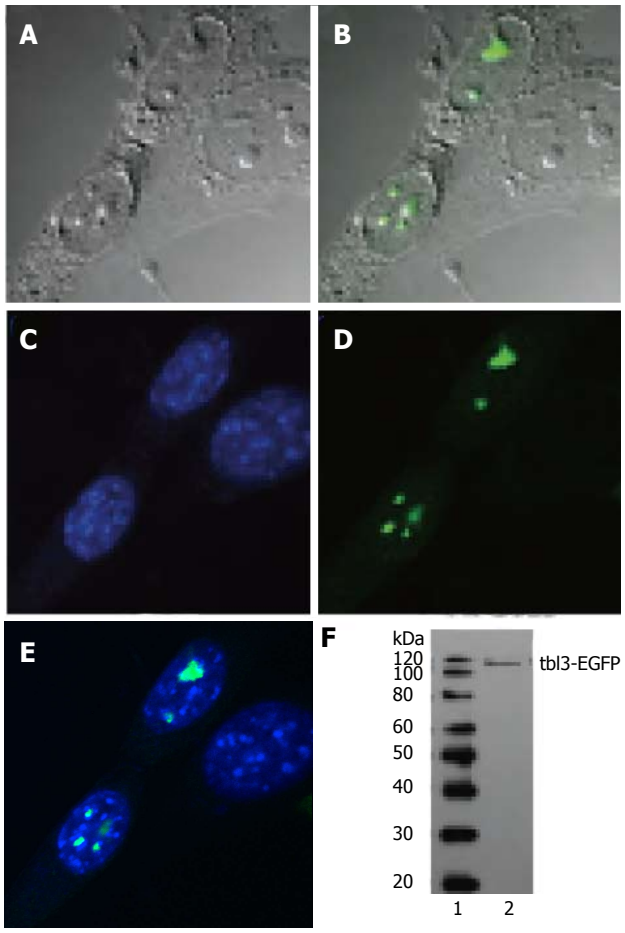

Figure 3 Nucleolar localization of Tbl3-enhanced green fluorescent protein. NIH 3T3 cells were transfected with the p-enhanced green fluorescent protein (EGFP) N1-transducin β-like 3 (Tbl3) construct. Cells were examined by fluorescence microscopy 20 h. after transfection. A: Differential interference contrast (DIC) microscopy of three fibroblasts revealing the nucleoli; B: Merged tbl3-EGFP green fluorescence and DIC of the cells shown in panel A. The nucleolar localization of tbl3-EGFP was verified by confocal microscopy; C: DAPI staining of DNA in the nuclei of the same field; D: Green fluorescence alone of the same field; E: DAPI plus tbl3-EGFP; F: Western detection of the tbl3-EGFP fusion protein. NIH 3T3 cells expressing tbl3-EGFP were lysed in RIPA buffer. The lysate (5 μg protein) was run on a 4%-12% NuPAGE gel (lane 2) along with Magic Mark size markers (lane 1), blotted and probed with an anti-GFP monoclonal antibody and visualized by enhanced chemiluminescence.

pMKO (or pMKO-Luc shRNA; negative controls) or pMKO-Tbl3 shRNA (1 or 2) vector and selected with puromycin to obtain stable transfectants. To avoid natural selection of variants with growth advantages, only low-passage (< 6) stable transfectants were used. Northern blot analyses demonstrated that the levels of *Tbl3* mRNA were knocked down by approximately 50%-70% in the pMKO-Tbl3 shRNA transfectants while the levels of *Tbl3* mRNA remained unchanged in the pMKO transfectants (Figure 5A, upper panel). The levels of housekeeping genes such as β-actin were unaffected by pMKO-Tbl3 shRNA (Figure 5A, middle panel).

To investigate the effects of *Tbl3* knockdown on newly transcribed pre-rRNA and its subsequent processing, we performed ^{3}H-uridine pulse-chase experiments in the MPRO transfectants. ^{3}H-uridine is phosphorylated after entering the cells and incorporated into newly transcribed RNAs, most of which are rRNAs. There was no detectable difference in the amounts of steady-state 28S and 18S rRNAs as revealed by ethidium bromide staining (Figure 5B and C, lower panels) and spectrophotometry. There was also no difference in the steady-state levels of the 5.8S rRNA on prolonged exposure (not shown). Intriguingly the levels of ^{3}H-uridine-labeled, newly transcribed 47S pre-rRNA were consistently increased by 2 to 4 fold in pMKO-Tbl3 shRNA transfectants (Figure 5B and C, upper panels). Furthermore, there was no obvious delay or defect in the processing of the 47S pre-rRNA as evidenced by similar rates of disappearance in the negative control and shRNA groups (Figure 5D) and by the proportional and contemporaneous appearance of the ^{3}H-uridine-labeled 28S and 18S rRNAs (and 5.8S rRNA; not shown) and the 41S, 36S and 32S processing intermediates (Figure 5B and C, upper panels). The key bands in lane 10 (Neg-1) and lane 12 (shRNA) of Figure 5B were further analyzed and the signal volume of the 28S rRNA was assigned a value of 1.00 to facilitate comparison. The calculated ratios of ^{3}H-labeled 32S:28S:18S rRNAs are 0.47:1.00:0.66 for the negative control group and 0.28:1.00:0.65 for the shRNA group. This result is consistent with the interpretation that there is no obvious delay in processing. Ribosome profiling by sucrose gradient centrifugation revealed that there was also no significant difference in the amount or ratio of the 40S and 60S ribosomal subunits and the 80S monosomes in

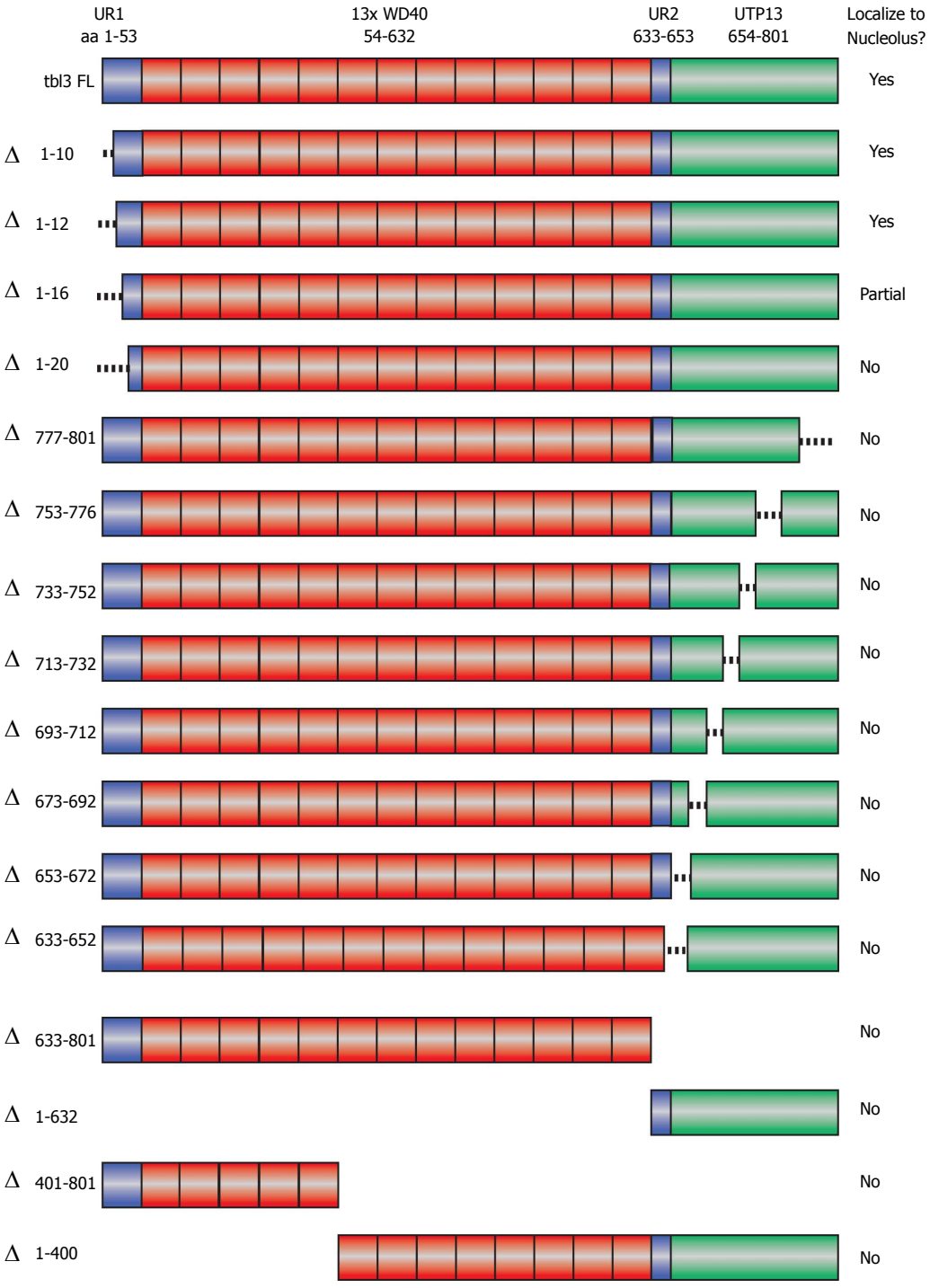

Figure 4 Scanning deletions of tbl3 and their effects on nucleolar targeting. A schematic summary of the deletions of transducin β-like 3 (tbl3) tested in the localization study. Shown at the very top is the modular structure of tbl3. "Δ" indicates deleted aa. Deletion mutants were expressed as C-terminal enhanced green fluorescent protein (EGFP) fusion proteins in NIH3T3 and examined by fluorescence microscopy. The results of localization are summarized in the right column. UR1: Unique region 1 (aa 1-53); WD40: Region containing thirteen WD40 repeats (aa 54-632); UR2: Unique region 2 (aa 633-653); UTP13: Conserved C-terminal domain (aa 654-801).

MPRO/pMKO (or MPRO/pMKO-Luc shRNA; negative controls) *vs* MPRO/pMKO-Tbl3 shRNA cells (Figure 6). This is consistent with the previous finding that the steady-state levels of 28S, 18S and 5.8S rRNA were not affected by Tbl3 knockdown (Figure 5B and C, lower panels). Together, the results of ^{3}H-uridine pulse-chase and ribosome sucrose gradient centrifugation indicate that *Tbl3* knockdown leads to increased levels of the 47S pre-rRNA but has no detectable effects on the processing of pre-rRNAs or the amount of steady-state 28S, 18S, 5.8S rRNAs or the amounts of the 40S and 60S subunits or the 80S monosomes.

Tbl3 knockdown impairs the proliferation of promyelocytes

During the course of the study we noticed that the

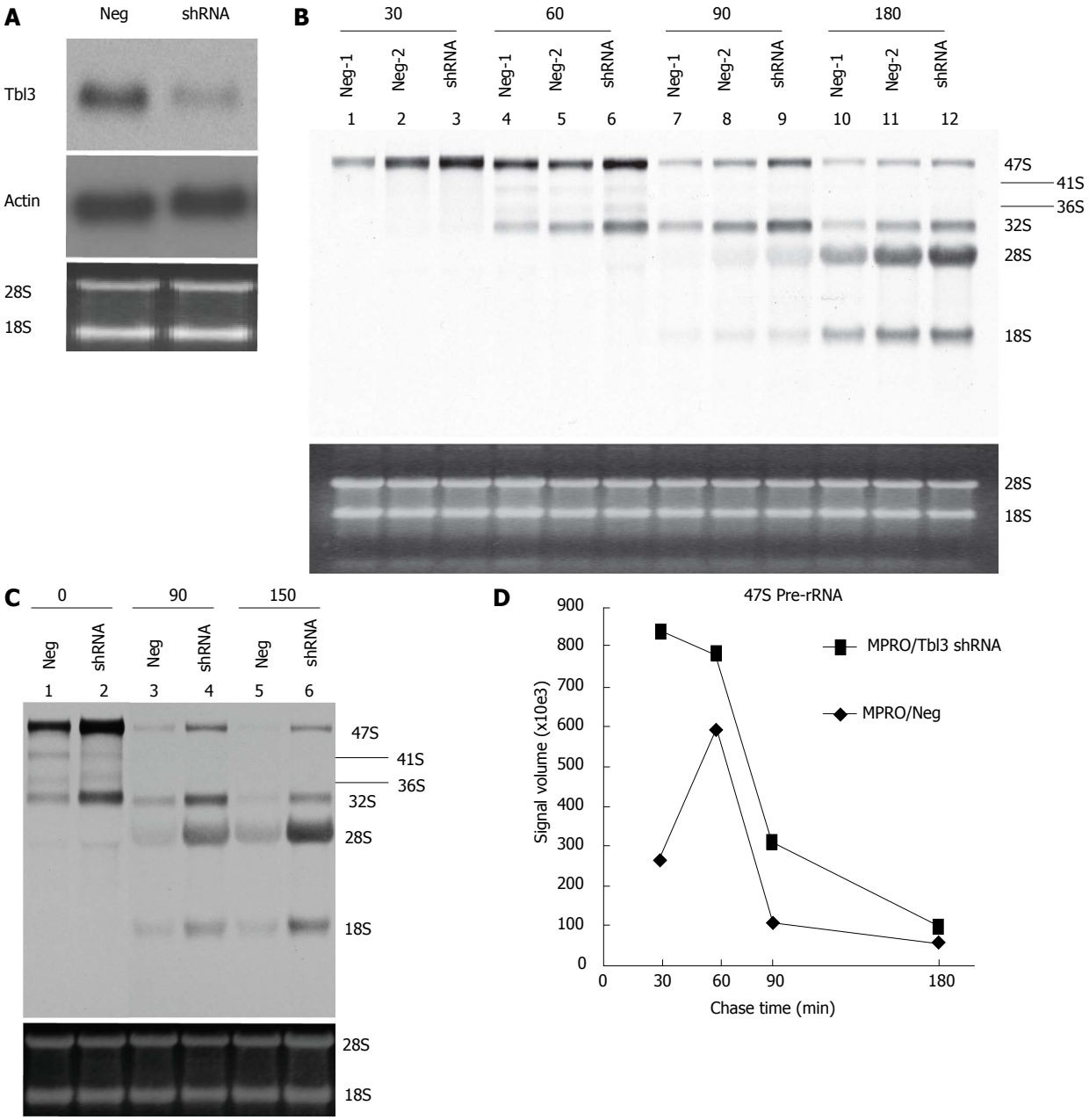

Figure 5 Effects of transducin β-like 3 knockdown on rRNA levels. A: Specific knockdown of transducin β-like 3 (Tbl3) by small hairpin RNAs (shRNAs). MRPO cells were transfected with pMKO (negative control) *vs* pMKO-Tbl3 shRNA and selected with puromycin to establish stable transfectants. Ten μg of total RNAs of stable transfectants were subjected to Northern analysis using a ^{32}P-labeled Tbl3 probe. The knockdown effect is 50%-70%. Middle panel: The same blot probed with a β-actin probe. Bottom panel: ethidium bromide-stained gel showing equal loading. The positions of the 28S and 18S rRNA are indicated; B: Tbl3 knockdown increases the level of newly synthesized 47S pre-rRNA but has no discernible effect on rRNA processing. MPRO stably transfected with pMKO (negative control) or pMKO-Tbl3 shRNA were pulse labeled with ^{3}H-uridine for 30 min, washed and chased for 0, 30, 60, 90 and 180 min with fresh medium without ^{3}H-uridine. Total RNAs were purified, electrophoresed and Northern blotted. ^{3}H-uridine-labeled rRNAs were visualized by fluorography. Tbl3 knockdown consistently increases the level of the 47S pre-rRNA by about 2-4 fold. Two negative controls (Neg-1 and Neg-2) are included to show the range of variation in signal strength in the negative control group. Bottom panel: Ethidium bromide-stained gel showing similar steady-state levels of 28S and 18S rRNAs (and the 5.8S rRNA; not shown). Each lane contained total RNAs purified from equal numbers of starting cells. No adjustment was made on the basis of RNA concentration or yield; C: An independent Tbl3 knockdown experiments similar to that described in B but the analysis was performed after 0, 90 and 150 minutes of chase. The 41S and 36S are better visualized in this blot. Bottom panel: Ethidium bromide-stained gel showing similar steady-state levels of 28S and 18S rRNAs (and the 5.8S rRNA; not shown). Each lane contained total RNAs purified from equal numbers of starting cells. No adjustment was made on the basis of RNA concentration or yield; D: Time course of disappearance of ^{3}H-uridine-labeled 47S pre-rRNAs. The fluorograph in B was analyzed and the measured signal volume was plotted against time. Note that ^{3}H-uridine incorporation peaked earlier (30 min *vs* 60 min) in the shRNA group, consistent with the notion that tbl3 knockdown increased the rate of pre-rRNA synthesis. There is no evidence of delay of processing of the 47S pre-rRNA in the shRNA group during the most relevant (60-90 min) or later (90-180 min) period judging from the slopes of decline.

growth of newly established MPRO/pMKO-Tbl3 shRNA transfectants was very slow compared with MPRO/pMKO (or MPRO/pMKO-Luc shRNA) or the parental MPRO. Calculation based on the time it took for the same number of newly established MPRO/pMKO-Tbl3 shRNA stable transfectants to reach the same population

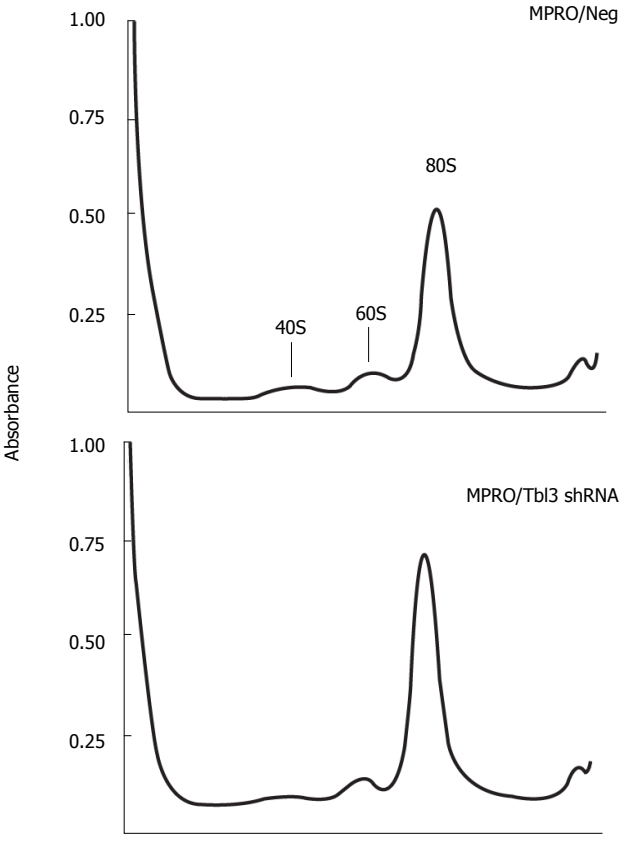

Figure 6 Knockdown of transducin β-like 3 has no discernable effect on ribosome profiles. Cell lysates prepared from equal numbers of MPRO/pMKO *vs* pMKO-transducin β-like 3 (Tbl3) small hairpin RNAs (shRNAs) stable transfectants were analyzed by sucrose gradient centrifugation in the absence of cycloheximide, followed by absorbance measurement at 254 nmol/L. (The omission of cycloheximide allowed polysomes to dissociate into 80S monosomes to facilitate the comparison of the total amount of 80S monosomes.) The peaks corresponding to the 40S and 60S ribosomal subunits and the 80S monosomes are indicated. The slight difference in the 80S monosome peaks is due to unequal loading and well within experimental variations.

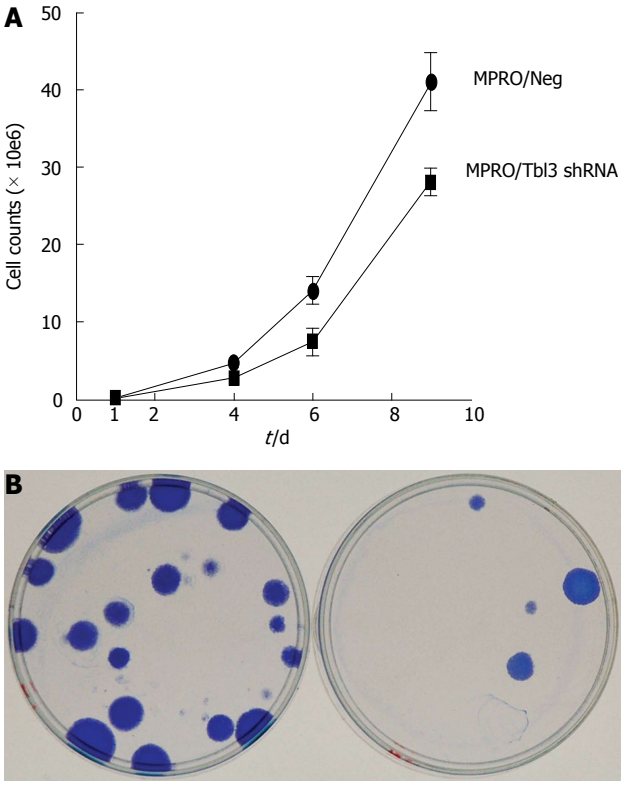

Figure 7 Knockdown of transducin β-like 3 inhibits cellular proliferation. A: Growth curves of stable MPRO/pMKO (negative control) vs MPRO/pMKO-transducin β-like 3 (Tbl3) small hairpin RNAs (shRNAs) transfectants. Each culture was started with 105 low-passage stable transfectants. Each data symbol represents the mean of triplicates and the standard deviation; B: Tbl3 knockdown markedly inhibits the proliferation of fibroblasts, left: LAP3/Neg; right: LAP3/Tbl3 shRNA. LAP-3 fibroblasts were transfected with pMKO or pMKO-Tbl3 shRNA and selected with puromycin (1.5 μg/mL) for 5-10 d to allow colony formation of stable transfectants. Colonies were fixed and stained with Coomassie blue *in situ* and photographed. Plates shown are representative of three independent experiments.

size as MPRO/pMKO indicates that the doubling time of MPRO/pMKO-Tbl3 shRNA was 3-4 times longer than that of MPRO/pMKO in newly established transfectants. As the population expanded from a few transfectants to approximately 10^6 cells, the doubling time of MPRO/pMKO-Tbl3 shRNA became progressively shorter, apparently due to the selection or outgrowth of variants that were less affected by the knockdown effects and hence enjoyed a higher proliferative rate. Nevertheless, the growth rate of MPRO/pMKO-Tbl3 shRNA was still slower compared with MPRO/pMKO in the 2-3 wk after the initial expansion following transfection and puromycin election (Figure 7A).

Tbl3 knockdown markedly impairs the proliferation of fibroblasts

To further examine the effects of *Tbl3* knockdown on cellular proliferation, we used the LAP3 fibroblast cell line as the model system. The LAP3 fibroblast cell line is derived from NIH3T3 (transformed embryonic cells) but is substantially "weakened" compared with the parental line[25]. Theoretical considerations and empiric experience indicate that it is particularly suitable for uncovering defects in ribosome biogenesis[25]. LAP3 cells were transfected with pMKO (or pMKO-Luc shRNA; negative controls) *vs* pMKO-Tbl3 shRNA (1 or 2) in triplicates in parallel and allowed to form macroscopic colonies in the presence of puromycin to select for stable integrants. The colonies were stained with Coomassie blue *in situ* to allow direct comparison. As shown in Figure 7B, the pMKO-Tbl3 shRNA-transfected group showed a great reduction (by about 80%) in colony numbers, indicating that *Tbl3* knockdown also has deleterious effects on fibroblast cell growth and proliferation.

DISCUSSION

In this report we provide direct evidence that mouse Tbl3 is targeted to the nucleoli (Figure 3A-D). This finding implicates Tbl3 in the ribosome biogenesis pathway and/or other nucleolar events. The results of targeting studies using a series of scanning deletion mutants of Tbl3 suggest that no particular nucleolar targeting

sequence is involved (Figure 4). Thus, Tbl3 likely associates with other proteins in the nucleoli *via* its WD40 protein-protein interaction motifs and/or other topological features and this forms the basis for its nucleolar localization.

To explore the function of mammalian *Tbl3*, we used the shRNA approach to achieve a partial knockdown. In retrospect, a partial rather than complete knockdown is desirable since a complete knockdown is likely incompatible with cellular survival based on what we have learned from our cell line studies and from the *cey* zebra fish embryos[22]. First, we looked at the processing of the 28S, 18S and 5.8S rRNAs. Then, we focused on the newly synthesized 47S pre-rRNA by pulse-labeling cells with ^{3}H-uridine. The latter approach allowed us to focus on newly synthesized pre-rRNA and minimized the contribution of post-transcriptional processing. As shown in Figure 5B and C (bottom panels), *Tbl3* knockdown had no discernable effect on the steady-state levels of the mature 28S, 18S and 5.8S rRNAs. Instead, it consistently increased the level of newly synthesized 47S pre-rRNA by two to four folds (Figure 5B and C, upper panels and Figure 5D). The higher levels of newly synthesized 47S pre-rRNA could result, *a priori*, from increased synthesis or decreased processing of the 47S pre-rRNA or both. If the elevated level of 47S pre-rRNA resulted from decreased processing, then we expect to see a slower rate of disappearance of the ^{3}H-labeled 47S pre-rRNA or a higher 47S to 28S (or 47S to 18S or 47S to 58S) ratio or the ratio of 28S to any processing intermediate in cells with *Tbl3* knockdown. However, our results showed that rates of disappearance of 47S pre-rRNA were very similar (Figure 5D) and the various ratios remained very similar in MPRO/pMKO-Tbl3 shRNA and in MPRO/pMKO (negative control) at all time points examined (Figure 5B and C, upper panels). This finding argues against a processing defect that would alter the size or quantity of the 28S, 18S and 5.8S rRNAs and raises the possibility that *Tbl3* knockdown primarily increased the synthesis of the 47S pre-rRNA. This interpretation is consistent with the previous conclusion that higher levels of steady-state 47S pre-rRNA reflect higher rates of rDNA transcription in a given cell type[30].

What could be the potential mechanisms by which Tbl3 regulates the synthesis of the 47S pre-rRNA? As Tbl3 is the mammalian homologue of yeast utp13, a review of the data on yeast utp13 may shed some light. Both mouse Tbl3 and yeast utp13 contain thirteen WD40 protein-protein interaction repeats and a conserved region in the C-terminus that is unique to both Tbl3 and utp13. The presence of many WD40 repeats in Tbl3 and utp13 indicates that they very likely form a complex or complexes with other proteins. Indeed, proteomic studies indicate that yeast utp13 forms a primary subcomplex, the so-called "utp-B" complex, with five other nucleolar proteins, namely utp 6, utp 18, utp 21, dip2 and pwp2[31]. This primary subcomplex in turn associates with other subcomplexes such as "UTP-A" and "UTP-C" complexes as well as additional nucleolar proteins to form a 90S megacomplex, which is known as the "small subunit (SSU) processome". The SSU is assembled co-transcriptionally at the 5' end of the newly transcribed pre-rRNA[31]. The SSU megacomplex is visible on electron microscopy as a terminal knob on the leading end of the elongating pre-rRNA still attached to the rDNA chromatin in yeast nucleoli[32]. By analogy, mammalian Tbl3 may similarly form a megacomplex *via* its multiple WD40 motifs with other proteins involved in the synthesis and/or processing of pre-rRNA and associates co-transcriptionally with the 5' end of the pre-rRNA. Given the observation that Tbl3 knockdown increases the production of 47S pre-rRNA, we hypothesize that Tbl3 is an important component of a putative pathway that normally provides feedback inhibition to the rDNA transcription machinery to coordinate the synthesis of the 47S pre-rRNA with the subsequent processing of rRNAs and ribosome assembly. Without coordination or feedback regulation, the rate of pre-RNA synthesis may become out of sync with that of processing and assembly and this mismatch may cause errors or waste in ribosome biogenesis[33,34].

Since the production of rRNAs and ribosomes consumes the lion share of cellular energy and biosynthetic precursors, over production of rRNAs inevitably will deprive cells of the energy and biosynthetic precursors needed for other physiologic processes and result in decreased cellular proliferation. Thus the consumption or siphoning away of cellular energy and biosynthetic precursors in overproduction of pre-rRNA may explain at least in part the inhibitory effect of *Tbl3* knockdown on cellular proliferation in mammalian cells (Figure 7A and B). As Tbl3 contains thirteen WD40 repeats it is possible that Tbl3 interacts with other nuclear or nucleolar proteins directly or indirectly involved in cell cycle regulation or DNA synthesis in addition to its interaction with components of SSU. The previously described detection of Tbl3 in the co-repressor complex of PNR in retina may well represent such an extra-nucleolar role[21]. However, we believe that the main role of Tbl3 is in the nucleoli as most (approximately 99%) if not all Tbl3 protein is found in that organelle (Figure 3A-D).

As we mentioned to earlier, complete depletion of yeast utp13 results in severe 18S rRNA processing defects and lethality in yeasts[32]. Considering the fact that Tbl3 is the mammalian homologue of yeast utp13, it is surprising that no rRNA processing defect was detected in the current study of mouse cells with partial *Tbl3* knockdown (Figure 5). Similarly, there were no detectable defects in rRNA processing in the *Tbl3*-/*Tbl3*- (*cey*) zebra fish embryos (supplemental Figure 6 of ref. 22). How can one reconcile these differences? The simplest explanation is that yeast utp13 and mammalian Tbl3 (or zebra fish tbl3) have evolved divergently in functionality. It is also quite possible that additional or redundant factors or mechanisms have evolved in mammals (or zebra fish) for rRNA processing such that a deficiency of Tbl3

alone has no discernible impact on rRNA processing. Still another possibility is that Tbl3 has more than one function and while a total deficiency is required to completely block the rRNA processing function, a partial deficiency is sufficient to interfere with the regulation of pre-rRNA synthesis.

The clinical importance of ribosomes is underscored by the fact that defects in ribosomes or ribosome biogenesis are the causes of several bone marrow failure syndromes such as the Diamond-Blackfan anemia (defective erythropoiesis due to rRNA processing defects caused by mutations in ribosomal protein genes *RPS17* or *RPS19* or *RPS24*)[35] and Shwachman-Bodian-Diamond syndrome (bone marrow failure, exocrine pancreas insufficiency, skeletal abnormalities and disposition to myelodysplastic syndrome and acute myeloid leukemia; caused by uncoupling of GTP hydrolysis from eIF6 release on the 60S ribosome subunit)[36]. More recently, a subtype of myelodysplastic syndrome, the so-called "5q- syndrome", has been experimentally phenocopied by RNAi-mediated haploinsufficiency of *RPS14*[37]. The propensity of ribosomopathy disorders to afflict hematopoiesis probably reflects the sensitivity of the highly proliferative hematopoietic progenitors to any disturbances in DNA or protein synthesis. Given the pronounced inhibitory effect of partial *Tbl3* knockdown on cellular proliferation in the current study, it is tempting to speculate that a loss of function mutation or deletion of human *TBL3* may also lead to a bone marrow failure-like syndrome. Furthermore, TBL3 may provide an attractive target for anti-tumor therapy.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Lester Lau and Dmitri Pestov for providing the LAP-3 fibroblasts.

COMMENTS

Background

Transducin β-like 3 (tbl3) encodes a protein with thirteen WD40 protein-protein interaction motifs. Virtually nothing is known about the function of Tbl3 including its subcellular localization and the general nature of its function. This report describes the first direct evidence that Tbl3 is targeted to the nucleoli and plays an important role in regulating the synthesis of the 47S pre-ribosomal RNA (pre-rRNA), *i.e.*, the first step in the production of ribosomes, which are indispensable for protein synthesis and hence the survival and normal functions of all cells.

Research frontiers

This article addresses a sizable gap in the understanding of the very early stage of the production of ribosomes. Understanding the function(s) of Tbl3 may lead to the development of new antibiotics and anti-tumor drugs.

Innovations and breakthroughs

Tbl3 is a newly identified nucleolar protein with a previously unrecognized regulatory function in the very early stage of ribosome production.

Applications

Given the pronounced inhibitory effect of *Tbl3* knockdown on cellular proliferation as demonstrated in the current study, the authors suspect that a loss of function mutation or deletion of human *TBL3* may also lead to a bone marrow failure-like syndrome or other developmental defects. Furthermore, TBL3 may provide a good target for anti-cancer therapy by interfering with the production of ribosomes at a very early stage.

Terminology

Ribosomopathy: defects in ribosome production and/or function including the synthesis, modification and processing of ribosomal RNA and assembly of ribosomes. Bone marrow failure syndromes: diseases caused by proliferative and/or differentiation defects in the production of blood cells by bone marrow stem cells.

Peer review

Authors provided the direct evidence that murine Tbl3 protein is targeted to compartment of nucleoli and plays an important role in synthesis of 47S pre-rRNA. Thus, this protein is important for regulation of ribosome biosynthesis. Manuscript is well written and new information about localization of Tbl3 protein in nucleolus is very interesting from the complex view on proteome of nucleolus.

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OBSERVATIONAL STUDY

Age is an independent adverse prognostic factor for overall survival in acute myeloid leukemia in Japan

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Abstract

AIM: To elucidate risk factors for survival of elderly acute myeloid leukemia (AML) patients in a real-world practice by observational study.

METHODS: We conducted a population-based study in 213 adult and elderly AML patients (127 males and 86 females) in Kagawa Prefecture, Japan. To construct this cohort, we gathered all data for patients diagnosed with AML at 7 hospitals in Kagawa between 2006 and 2010. The primary end point was overall survival (OS) after AML diagnosis. Unadjusted Kaplan-Meier survival plots were used to determine OS in the overall cohort. Multivariate analysis was used to determine the independent adverse prognostic factors for OS, with the covariates of interest including age, gender, race/ethnicity, CCI, education, median income, metropolitan statistical area size and history of myelodysplastic syndrome.

RESULTS: The average population of Kagawa during the study period was 992489, and the incidence of AML was 4.26 per 100000 person-years. A total of 197 patients with non-acute promyelocytic leukemia (non-APL) (119 males and 78 females) were also included. The median age of non-APL patients was 70 years (average 67, range 24-95). The 5-year OS rate was 21.1%. Subsequent analysis by age group showed that the survival rate declined with age; the 5-year OS rates of non-APL patients younger than 64 years, 65-74 years, and older than 75 years were 41.5%, 14.1%, and 8.9%, respectively. Multivariate analysis revealed that unfavorable risk karyotype, older age, poor performance status (PS) (3-4), lack of induction chemotherapy, and antecedent haematological disease were independent prognostic predictors. In the subgroup analysis, we also found that older patients with non-APL had lower complete remission rates and higher early death rates than younger patients, irrespective of PS. However, intensive chemotherapy was a significant predictor for longer survival

not only in the patients < 75 years of age, but also in those over 75 with PS 0-2.

CONCLUSION: Age would contribute considerable life expectancy to indicate induction chemotherapy with eligible dose of cytotoxic drugs for a favorable case even in advanced elderly.

Key words: Acute myeloid leukemia; Elderly; Adverse prognostic factor; Overall survival; Population-based study

Core tip: The prevalence of acute myeloid leukemia (AML) is increasing among elderly patients in Japan. Our population-based observational study revealed that age was an independent prognostic factor in a real-world practice for the treatment of AML patients. Although we found that AML patients older than 75 years had lower complete remission rates and higher early death rates than patients younger than 75 years, an appropriately intensified induction chemotherapy would be helpful to prolong the survival of elderly AML patients with better performance status (PS) (1-2). The intensity of chemotherapy should thus be adjusted according to age and PS.

Ohnishi H, Imataki O, Kawachi Y, Ide M, Kawakami K, Waki M, Takimoto H, Hoshijima Y, Fukumoto T, Matsumoto K, Waki F, Matsuoka A, Shintani T, Uemura M, Yokokura S, Taoka T, Matsunaga T. Age is an independent adverse prognostic factor for overall survival in acute myeloid leukemia in Japan. *World J Hematol* 2014; 3(3): 105-114 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/105.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.105

INTRODUCTION

Elderly acute myeloid leukemia (AML) patients often have several comorbidities and poor performance status (PS) at the time of diagnosis, and may be intolerant to intensive chemotherapy, making them poor candidates for intensive induction chemotherapy[1-4]. Compared to young adult AML patients, elderly AML patients also have higher frequencies of adverse prognostic factors such as unfavorable risk karyotype and secondary AML (therapy-related AML)[5], leading to a poorer prognosis[6-8].

Juliusson *et al*[9] analyzed a population-based cohort of patients aged ≥ 16 years in the Swedish National Acute Leukemia Registry. They found that onset of AML may occur at any age, but is most common in the elderly population, with the highest incidence in individuals aged 80-85 years. The median age of onset was 72 years (range 16-97): 71 years for males and 72 years for females. A number of prospective clinical trials have studied treatments for young adult patients with non-acute promyelocytic leukemia (non-APL)[10-13]. These trials showed that the 5-year overall survival (OS) rate increased to 35%-48% in patients treated with induction chemotherapy using idarubicin and cytosine arabinoside. However, these trials excluded patients with poor PS (3-4) and elderly patients. Considering the distribution of the age onset of AML, these clinical trials included only a small proportion of the total AML population, and the results therefore do not accurately reflect treatment options for the overall AML population.

To accurately evaluate the overall AML population, several retrospective population-based studies have been conducted in Sweden[9,14], the United Kingdom[15,16], and the United States[17]. These studies found relatively low 5-year OS rates of 10%-20%[16,18,19]. Older age and poor PS were reported to be adverse prognostic factors for OS in these population-based studies, but not in prospective clinical trials. However, the retrospective population-based studies did not include multivariate analyses[9,18]. It is therefore still unclear whether older age and poor PS are independent adverse prognostic factors for OS in patients with AML.

In this study, multivariate analysis identified older age, lack of induction chemotherapy, poor PS (3-4), antecedent hematological disease, and unfavorable risk karyotype as independent prognostic factors for poor OS. We also found that older patients had lower complete remission (CR) rates and higher early death rates than younger patients, irrespective of PS. This analysis provides data describing the overall AML population.

MATERIALS AMD METHODS

Patients and survey methods

We performed a multicenter observational study of adult AML patients aged ≥ 17 years from seven institutions within Kagawa Prefecture (Kagawa Rosai Hospital, Takamatsu Municipal Hospital, Sakaide City Hospital, Kagawa Prefectural Central Hospital, Takamatsu Red Cross Hospital, Mitoyo General Hospital, and Kagawa University Hospital) between January 1, 2006 and December 31, 2010. Data were collected from the medical records. Diagnosis of AML was made according to the criteria of the French-American-British classification. We excluded patients with a previous diagnosis of myelodysplastic syndrome (MDS). Antecedent haematological disease was defined as benign hematological disease other than MDS. The potential effects of consolidation therapy and hematopoietic stem cell transplantation are beyond the scope of this analysis, and patients were not censored at the time of transplantation or any other treatment in the survival analysis. This study was approved by the Institutional Review Boards of Kagawa Rosai Hospital, Takamatsu Municipal Hospital, Sakaide City Hospital, Kagawa Prefectural Central Hospital, Takamatsu Red Cross Hospital, Mitoyo General Hospital, and Kagawa University Hospital.

Non-APL karyotype classifications

The karyotypes of non-APL patients were grouped according to the criteria of the National Comprehensive Cancer Network (NCCN) clinical practice guidelines[20], the Southwest Oncology Group (SWOG) classification[21], and the Cancer and Leukemia Group B (CALGB) classification[22].

Initial therapy regimens

Induction therapy regimens were chosen by each treating physician based on available clinical data and local standards of care, but not on karyotype. None of the patients were enrolled in clinical trials.

APL patients with a white blood cell (WBC) count of $< 3.0 \times 10^9$ per liter and a blast plus promyelocyte count of $< 1.0 \times 10^9$ per liter were started on oral all-*trans*-retinoic acid (ATRA) (45 mg/m^2 per day) alone until the start of consolidation therapy. Patients with a WBC count between 3.0×10^9 per liter and 10.0×10^9 per liter or a blast plus promyelocyte count of $\geqslant 1.0 \times 10^9$ per liter were started on oral ATRA until the start of consolidation therapy plus idarubicin (IDR) (12 mg/m^2 per day by 30-min infusion, days 1-2) plus cytarabine (Ara-C) (80 mg/m^2 per day by continuous infusion, days 1-5). Patients with a WBC count of $\geqslant 10.0 \times 10^9$ per liter were started on oral ATRA until the start of consolidation therapy plus IDR (days 1-3) plus Ara-C (100 mg/m^2 per day by continuous infusion, days 1-5).

Non-APL patients were treated with one of 15 regimens, which we categorized as intensive chemotherapy, less intensive chemotherapy, or best supportive care. The intensive chemotherapy regimens were: (1) full dose IDR (12 mg/m^2 per day by 30-min infusion, days 1-3) plus Ara-C (100 mg/m^2 per day by continuous infusion, days 1-7); (2) 80% dose IDR (days 1-3) plus Ara-C (days 1-7); (3) full dose daunorubicin (DNR) (50 mg/m^2 per day by 30-min infusion, days 1-5) plus Ara-C (days 1-7); (4) 80% dose DNR (days 1-5) plus Ara-C (days 1-7); (5) full dose IDR (days 1-3) plus enocitabine (BHAC) (200 mg/m^2 per day by 180-min infusion, days 1-8); (6) 80% dose IDR (days 1-3) plus BHAC (days 1-8); (7) full dose DNR (days 1-5) plus BHAC (days 1-8); (8) 80% dose DNR (days 1-5) plus BHAC (200 mg/m^2 per day by 180-min infusion, days 1-8). The less intensive chemotherapy regimens were: (1) less than 80% dose IDR (days 1-3) plus Ara-C (days 1-7); (2) less than 80% dose DNR (days 1-5) plus Ara-C (days 1-7); (3) less than 80% dose IDR (days 1-3) plus BHAC (days 1-8); (4) less than 80% dose DNR (days 1-5) plus BHAC (days 1-8); (5) CAG [Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14) plus aclarubicin (ACR) (14 mg/m^2 per day by 30-min infusion, days 1-14) plus granulocyte colony-stimulating factor (G-CSF) (200 g/m^2 per day by subcutaneous injection, days 1-14)]; (6) CA [Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14) plus ACR (14 mg/m^2 per day by 30-min infusion, days 1-14)]; (7) Low dose Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14).

Statistical analysis

The χ^2 test was used to analyze the significance of differences between two or three groups (Microsoft Excel 2010, version 14.0; Microsoft Corporation Japan, Tokyo). A *P* value less than 0.05 was considered statistically significant. All other data were analysed using JMP 7.0.1 (SAS Institute Japan, Tokyo, Japan). The Kaplan-Meier method was used to estimate probabilities of OS, and the log-rank test was used to analyse the significance of differences in OS between two or three groups. For the survival analysis, patients were censored at the time of the last follow-up. Multivariate analysis of prognostic factors for OS was performed using the Cox proportional hazards method. All prognostic factors were first analyzed using univariate analysis. Early death was defined as 8-wk mortality after the diagnosis or initiation of chemotherapy. Factors with a *P* value of less than 0.05 on univariate analysis were included in the multivariate analysis using a stepwise method.

RESULTS

A total of 219 patients were diagnosed with AML between January 1, 2006 and December 31, 2010 at the 7 participating institutions. Considering the average population of Kagawa Prefecture during the study period, the incidence of AML was 4.26 per 100000 person-years.

We focused on analyzing adult patients with AML. Six patients were excluded due to a lack of available clinical data, and the remaining 213 patients were included. These patients were 127 men and 86 women (male to female ratio 1.48) with a median age of 70 years (range 24-95). Thirty-five patients underwent allogeneic hematopoietic stem cell transplantation. The cohort included 16 APL patients and 197 non-APL patients. The estimated 5-year OS rate of the APL patients was 69.2% (95%CI: 55.5-82.9). The median follow-up period for APL survivors was 23.5 mo (range 0-56).

The non-APL patients had a median age of 70 years (range 24-95), including 74 patients (37.6%) aged $\leqslant 64$ years, 50 patients (25.6%) aged 65-74 years, and 73 patients (36.8%) aged $\geqslant 75$ years. Table 1 shows the characteristics of the non-APL patients. In our study some data were missing for each parameter. Therefore, the total numbers of patients in each parameter group-that is, the sum of each column-are sometimes different. Some clinical features varied among the different age groups. Overall, 93 patients (47.2%) had one or more features of myelodysplasia (not satisfy the diagnosis criteria for MDS). The frequency of myelodysplastic features was higher in patients aged $\geqslant 75$ years than in younger patients. Approximately half of the patients (48.3%) had Eastern Cooperative Oncology Group PS of 2-4. As age increased, the proportion of patients with good PS (0-1) decreased and that with poor PS (3-4) increased. The chromosomal karyotype is known to be the strongest predictor of prognosis, but the systems used to classify the karyotypes vary among studies. We divided our co-

hort into three karyotype groups: 5.1%-6.6% of patients were classified as having a favourable risk karyotype, 22.8%-29.9% of patients were classified as having an unfavourable risk karyotype, and category not recognized. A favorable risk karyotype was more frequent in patients aged ≤ 64 years than patients aged ≥ 65 years. The serum lactase dehydrogenase (LDH) level was lower in patients aged 65-74 years than in the other age groups (≤ 64 years and ≥ 75 years); there were significantly more patients with a normal LDH level (< 250 U/L) and fewer patients with an increased LDH level (> 500 U/L) in the group aged 65-74 years than in the other age groups. Twenty-nine patients (14.7%) had renal dysfunction with a serum creatinine level of > 1.3 mg/dL, and 48 patients (24.3%) had an infection at the time of diagnosis. Intensive induction chemotherapy was administered to 102 patients (51.7%) in total, including 71.6% of patients aged ≤ 64 years, 46.0% of patients aged 65-74 years, and 35.6% of patients aged ≥ 75 years.

In non-APL patients, the estimated 5-year OS rate was 21.1% (95%CI: 1.7-40.5) (Figure 1A). The median follow-up period among non-APL survivors was 32 mo (range 1.0-59.5). Analysis by age group showed that the 5-year OS rate decreased with increasing age. In non-APL patients aged ≤ 64 years, 65-74 years, and ≥ 75 years, the 5-year OS rates were 41.5% (95%CI: 34.5-48.5), 14.1% (95%CI: 8.8-19.4), and 8.9% (95%CI: 4.1-13.7), respectively; and the median survival times were 19, 10 and 7 mo, respectively (Figure 1B). In addition, poor PS (3-4), lack of induction chemotherapy or less intensive induction chemotherapy, presence of antecedent hematological disease (except for MDS), and unfavourable risk karyotype according to the NCCN, SWOG, or CALGB classifications adversely affected the OS rate (Figure 1C-H). Multivariate analysis revealed that older age, poor PS (3-4), lack of induction chemotherapy, presence of antecedent hematological disease, and unfavourable risk karyotype according to any karyotype classification were adverse prognostic factors (Table 2). Detailed information regarding karyotype categories according to the NCCN, SWOG, and CALGB classifications is shown in Table 3.

The rates of CR and early death (within 8 wk of diagnosis) according to PS in different age groups are shown in Figure 2. The proportion of patients with poor PS (3-4) increased with age (Figure 2A). Early death rates were related to both age and PS (Figure 2B). Older patients had higher early deaths rates than younger patients, and patients with poor PS had higher early death rates than patients with good PS. CR rates were also related to both age and PS (Figure 2C). Older patients had lower CR rates than younger patients, and patients with poor PS had lower CR rates than patients with good PS.

DISCUSSION

This study was limited to AML patients in a specific area of Japan, unlike the large scale study conducted by Juliusson *et al*[9], which included data from the whole of Sweden. However, Kagawa Prefecture is surrounded by ocean and mountain ranges, and residents almost never seek treatment for malignancies elsewhere due to the inconvenience of travelling. This study has almost complete capture of the patient population. We therefore consider that the present results were highly representative of the patient representation. Furthermore, the median and interquartile range of age, and incidence of AML, were similar between this study and the Swedish study by Juliusson *et al*[9]. This similarity in the distributions of patients between the two reports indicates that the present study was a reliable population-based study. In Japan, all population-based studies of this sort are conducted under a strict registration system which is facilitated by a nation-wide organization. In all of the cases in the present cohort, a primary physician had reached consensus in referring the patient to a general community hospital due to a haematological malignancy.

Dores *et al*[17] reported that there were no differences in the age of onset or the incidence of AML among non-Hispanic whites, Hispanic whites, Blacks, and Asians/Pacific Islanders in the United States. They also reported that the frequencies of APL, a subtype of AML, differed among these subpopulations, accounting for 6.1%, 14.2%, 9.3%, and 7.0% of the four groups, respectively. Nakase *et al*[23] reported that although the frequency of t(8;21) AML was higher in the Japanese population than the Australian population (33.1% *vs* 15.3%, $P < 0.05$), there was no difference in the frequency of APL between these populations (14.8% *vs* 11.1%). However, these data may not be generalizable as the patients selected for the study were all diagnosed at a single hospital. The frequency of APL in our cohort was 7.5%, which is similar to the frequencies reported among Australians[23], Asians/Pacific Islanders and non-Hispanic white[17], higher than the frequency reported in the Swedish study[9], and lower than the frequencies reported among both Hispanic whites and Blacks in the United States[17], suggesting that the frequency of APL differs among races.

Several studies[7,8,24,25] have reported on differences between elderly and young adult AML patients in terms of host factors such as physiological functions and biological factors such as characteristics of AML cells. Pollyea *et al*[8] reported that outcomes in elderly AML patients were affected by host factors such as decreased drug metabolism, compromised immune defence systems, increased frequency of poor PS, increased frequency of hemorrhagic complications, and increased frequency of psychiatric medications. They also demonstrated that AML cells in elderly patients had more immature morphology and expressed higher levels of the multidrug resistance gene MDR1 than AML cells in young adult patients. Dombert *et al*[7] showed that the frequencies of myelodysplasia and unfavourable risk karyotype were higher in elderly AML patients than young adult AML patients. Our study showed similar results, with elderly patients having higher frequencies of poor PS, myelodysplasia, and unfavourable risk karyotype compared with young adult patients. These data suggest that our study results are a reliable reflection of the overall AML population.

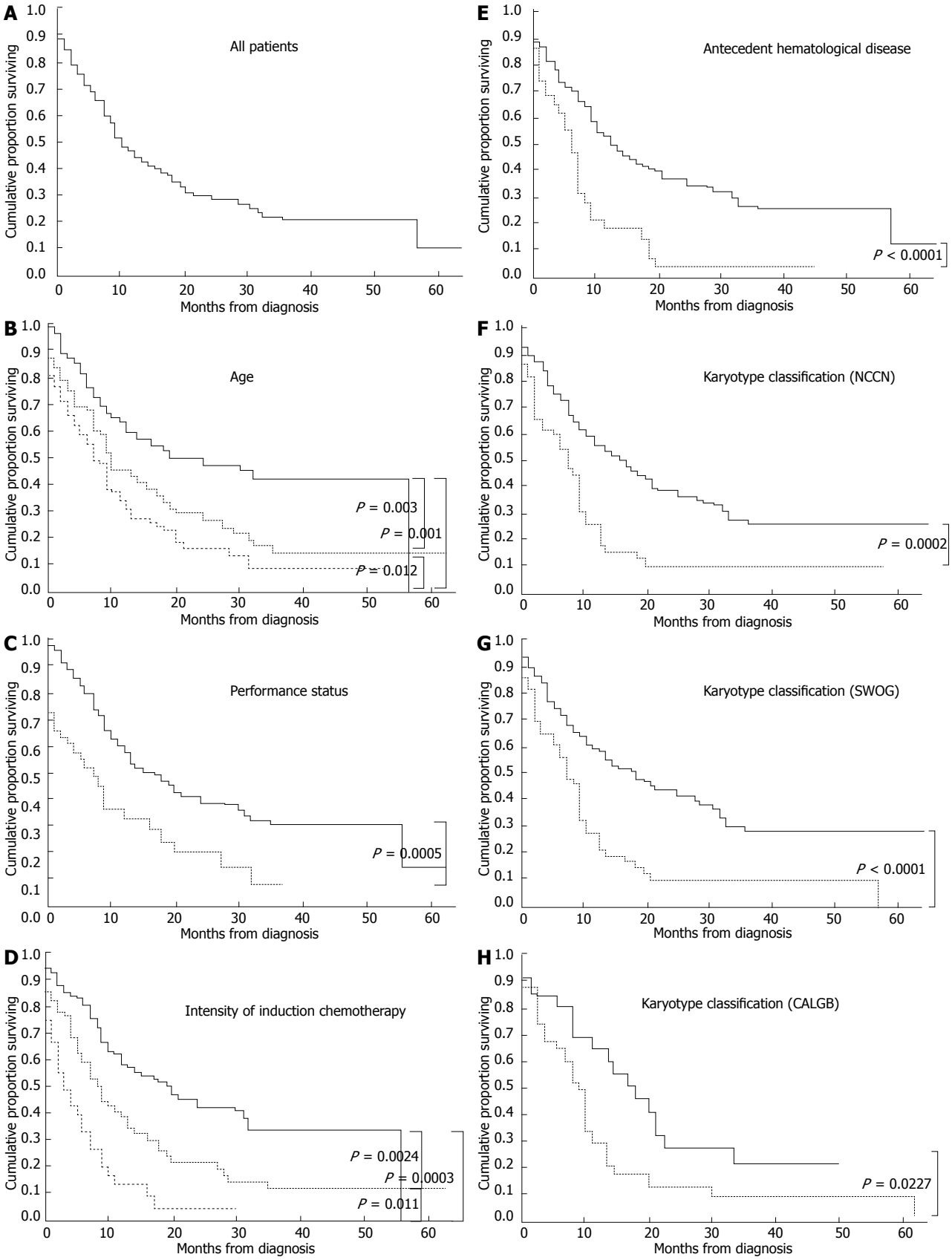

Figure 1 Kaplan-Meier estimate of overall survival in patients with non-acute promyelocytic leukemia. A: All patients; B: Patients categorised by age group. Black line, ≤ 64 years; dotted line, 65-74 years; grey line, ≥75 years; C: Patients categorised by performance status (PS). Black line, PS 0-2; dotted line, PS 3-4; D: Patients categorised by intensity of induction chemotherapy. Black line, intensive chemotherapy; dotted line, less intensive chemotherapy; grey line, best supportive care; E: Patients categorised by antecedent hematological disease. Black line, without antecedent hematological disease; dotted line, with antecedent haematological disease; F: Patients categorised according to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes; G: Patients categorised according to the Southwest Oncology Group (SWOG) karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes; H: Patients categorised according to the Cancer and Leukemia Group B (CALGB) karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes.

Table 1 Characteristics of non-acute promyelocytic leukemia patients

Parameter	Category	Number of patients								
		All patients (n = 197)		Age in year						
				⩽ 64 (n = 74)		65-74 (n = 50)		⩾ 75 (n = 73)		
Gender	Female	78	39.60%	30	40.50%	19	38.00%	29	39.70%	
	Male	119	60.40%	44	59.50%	31	62.00%	44	60.30%	
FAB classification	M0	9	4.60%	3	4.10%	0	0.00%	6	8.20%	
	M1	24	12.20%	10	13.50%	4	8.00%	10	13.70%	
	M2	100	50.80%	34	45.90%	27	54.00%	39	53.40%	
	M4	24	12.20%	11	14.90%	7	14.00%	6	8.20%	
	M5	10	5.10%	5	6.80%	3	6.00%	2	2.70%	
	M6	13	6.60%	3	4.10%	4	8.00%	6	8.20%	
	M7	4	2.00%	2	2.70%	1	2.00%	1	1.40%	
Myelodysplasia	Yes	93	47.20%	32	43.20%	20	40.00%	41	56.20%	[a]
	No	100	50.80%	40	54.10%	29	58.00%	31	42.50%	
Performance status	0-1	98	49.70%	45	60.80%	28	56.00%	25	34.20%	
	2	46	23.40%	13	17.60%	8	16.00%	25	34.20%	
	3-4	49	24.90%	14	18.90%	13	26.00%	22	30.10%	
Karyotype risk category										
NCCN	F	13	6.60%	9	12.20%	2	4.00%	2	2.70%	[a]
	I	122	61.90%	43	58.10%	27	54.00%	52	71.20%	
	U	46	23.40%	16	21.60%	19	38.00%	11	15.10%	
SWOG	F	10	5.10%	7	9.50%	2	4.00%	1	1.40%	[a]
	I	97	49.20%	35	47.30%	23	46.00%	39	53.40%	
	U	59	29.90%	20	27.00%	21	42.00%	18	24.70%	
	others	15	7.60%	6	8.10%	2	4.00%	7	9.60%	
CALGB	F	13	6.60%	9	12.20%	3	6.00%	1	1.40%	[a]
	I	100	50.80%	33	44.60%	26	52.00%	41	56.20%	
	U	45	22.80%	15	20.30%	16	32.00%	14	19.20%	
	others	39	19.80%	17	23.10%	5	10.00%	17	23.20%	
Antecedent	No	158	80.20%	60	81.10%	37	74.00%	61	83.60%	
hematologic disease	Yes	36	18.30%	13	17.60%	11	22.00%	12	16.40%	
Prior chemotherapy	No	174	88.30%	65	87.80%	41	82.00%	68	93.20%	
	Yes	19	9.60%	7	9.50%	7	14.00%	5	6.80%	
Laboratory findings										
WBC (× 10^3/mL)	< 100	178	90.40%	65	87.80%	48	96.00%	65	89.00%	
	⩾ 100	16	8.10%	7	9.50%	1	2.00%	8	11.00%	
Hemoglobin (g/dL)	< 8.0	93	47.20%	36	48.60%	23	46.00%	34	46.60%	
	⩾ 8.0	101	51.30%	36	48.60%	26	52.00%	39	53.40%	
Platelet (× 10^4/mL)	< 5.0	85	43.10%	29	39.20%	23	46.00%	33	45.20%	
	5.0-10.0	64	32.50%	25	33.80%	20	40.00%	19	26.00%	
	⩾ 10.0	45	22.80%	18	24.30%	6	12.00%	21	28.80%	
% Blast in blood	< 20	89	45.20%	28	37.80%	29	58.00%	32	43.80%	
	20-50	43	21.80%	17	23.00%	11	22.00%	15	20.50%	
	> 50	59	29.90%	27	36.50%	8	16.00%	24	32.90%	
% Blast in marrow	< 50	98	49.70%	33	44.60%	30	60.00%	35	47.90%	
	⩾ 50	84	42.60%	35	47.30%	18	36.00%	31	42.50%	
LDH (IU/L)	< 250	58	29.40%	15	20.30%	24	48.00%	19	26.00%	[a]
	250-500	75	38.10%	27	36.50%	14	28.00%	34	46.60%	
	> 500	59	29.90%	29	39.20%	11	22.00%	19	26.00%	
Creatinine (mg/dL)	⩽ 1.3	162	82.20%	66	89.20%	41	82.00%	55	75.30%	
	> 1.3	29	14.70%	5	6.80%	8	16.00%	16	21.90%	
Infection at	No	146	74.10%	52	70.20%	39	78.00%	55	75.30%	[a]
induction therapy	Yes	48	24.30%	20	27.00%	10	20.00%	18	24.60%	
Intensity of	Intensive	102	51.70%	53	71.60%	23	46.00%	26	35.60%	[a]
induction therapy	Less-intensive	56	28.40%	13	17.50%	19	38.00%	24	32.80%	
	BSC	39	19.70%	8	10.80%	8	16.00%	23	31.50%	

[a]P < 0.05 *vs* total. F: Favorable; I: Intermediate; U: Unfavorable; WBC: White blood cell; NCCN: National Comprehensive Cancer Network; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B; LDH: Lactase dehydrogenase.

The estimated 5-year OS rate of our non-APL patients was 21.1%, which is similar to the 5-year OS rates reported by retrospective population-based studies conducted in Sweden[9,26,27], the United Kingdom[16], and the United States[17,28,29], but lower than the 5-year OS rates reported by prospective clinical trials for young adult non-APL patients[10-13]. When the data of all AML patients are analysed in a population-based study, patients with poor PS, organ dysfunction, documented infection, and severe comorbidities are included; this lowers the

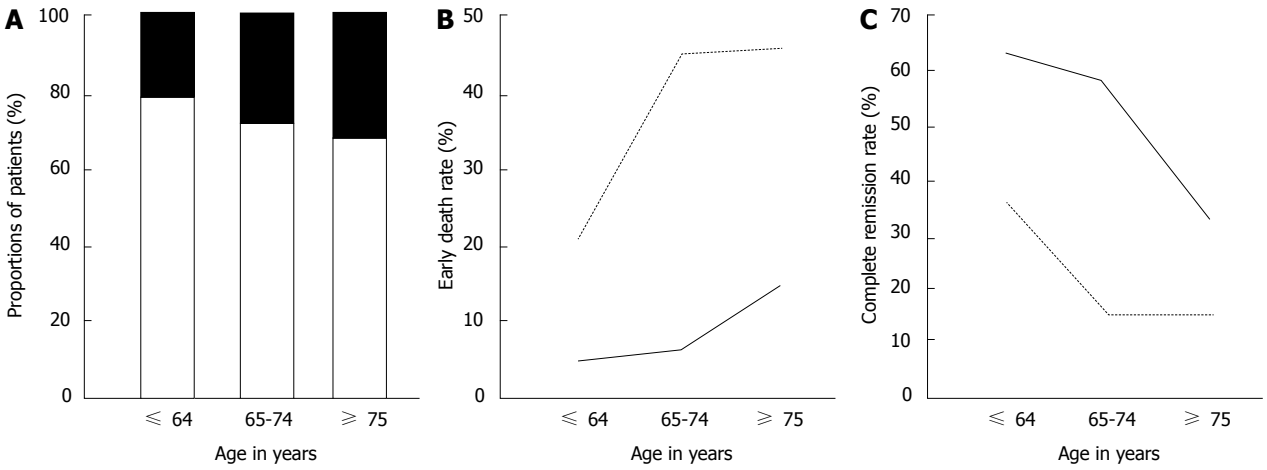

Figure 2 Early death rate and complete remission rate according to performance status in different age groups. A: Proportions of patients with good and poor performance status (PS) in different age groups. Grey, PS 0-2; black, PS 3-4; B: Early death rate according to PS in different age groups. Black line, PS 0-2; dotted line, PS 3-4; C: Complete remission rate according to PS in different age groups. Black line, PS 0-2; dotted line, PS 3-4.

Table 2 Multivariate analysis of prognostic factors affecting survival in non-acute promyelocytic leukemia patient

Risk factors			
Chromosomal abnormality	**NCCN**	**SWOG**	**CALGB**
	HR (95%CI) *P*	**HR (95%CI) *P***	**HR (95%CI) *P***
Age in years	0.031	0.043	0.026
≤ 64	1	1	1
65-74	1.33 (1.06-1.58)	1.39 (1.13-1.64)	1.40 (1.15-1.66)
≥ 75	1.88 (1.59-2.04)	1.82 (1.60-2.05)	1.91 (1.67-2.13)
Performance status	0.012	0.028	0.019
0-2	1	1	1
3-4	1.94 (1.15-3.26)	1.80 (1.06-3.05)	1.88 (1.11-3.20)
Intensity of induction therapy	0.041	0.029	0.027
Intensive, less-intensive	1	1	1
Best supportive care	1.74 (2.02-2.96)	1.80 (1.06-3.07)	1.83 (1.07-3.14)
Antecedent hematological disease	0.007	0.007	0.004
No	1	1	1
Yes	1.92 (1.20-3.08)	1.90 (1.19-3.05)	2.03 (1.25-3.27)
Chromosomal abnormality	0.001	< 0.001	< 0.001
Favorable, intermediate	1	1	1
Unfavorable	1.98 (1.31-2.99)	1.44 (0.55-3.78)	1.56 (0.63-3.87)

NCCN: National Comprehensive Cancer Network practice guidelines in oncology- v.2.2010; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B.

overall long-term OS rates compared with the survival rates in prospective clinical trials of young adult AML patients without these conditions.

Juliusson *et al*[9] analysed and compared the CR rates of patient cohorts grouped by PS and found that the CR rate decreases as age increases, indicating that PS and age are independent adverse prognostic factors. Our data are consistent with these findings. However, they did not conduct multivariate analysis to determine adverse prognostic factors associated with OS in non-APL patients. Our multivariate analysis identified older age, poor PS (3-4), lack of induction chemotherapy, presence of antecedent haematological disease, and unfavourable risk karyotype as independent adverse prognostic factors for OS. In prospective clinical trials for young adult patients with non-APL, good PS and no organ dysfunction, the presence of antecedent hematological disease and unfavourable risk karyotype were found to be independent adverse prognostic factors for OS[10-12]. Our retrospective population-based study yielded similar results, indicating that the presence of antecedent hematological disease and unfavourable risk karyotype are adverse prognostic factors for OS in all non-APL patients. It has been postulated that the frequencies of myelodysplasia, poor PS, and unfavourable risk karyotype are higher in elderly non-APL patients than young adult non-APL patients, leading to poorer long-term survival in elderly patients[6-8]. As elderly patients and those with poor PS are excluded from prospective clinical trials, population-based studies are necessary to determine whether older age and poor PS are adverse prognostic factors for OS. As described above, a study in Sweden found that older

Table 3 Detailed information regarding karyotype categories according to the National Comprehensive Cancer Network, Southwest Oncology Group, and Cancer and Leukemia Group B classifications

Category	Favorable	Intermediate	Unfavorable	Category not recognized
NCCN	t(8;21)	+8, t(9;11), -X, -Y, -6, +1, +4, +7, +11, +13, +21, del(9), del(20), add(12), add(16), add(17), inv(3), t(1;16), t(3;21), t(8;18), t(8;20), t(11;16), t(11;17)	–5, del(5q), -7, non-t(9;11) abn11q23, inv(3), t(9;22), complex karyotype ⩾ 3	-
SWOG	t(8;21)	-Y, +8	abn(3q), -5, -7, t(9;22), abn(9q), abn(11q), abn(17p), abn(20q), abn(21q), complex karyotype ≥ 3	t(9;11), -X, -6, +1, +4, +7, +11, +13, +21, add(12), add(16), inv(3), t(1;16), t(3;21), t(8;18), t(8;20)
CALGB	t(8;21), del(9q)	-Y, del(5q), t(9;11), +11, del(11q), abn(12p), +13, del(20q), +21	inv(3), -7, +8, complex karyotype ≥ 3	-X, -6, +1, +4, +7, add(12), add(16), add(17), t(1;16), t(3;21), t(8;18), t(8;20), t(11;16), t(11;17)

NCCN: National Comprehensive Cancer Network; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B.

age and poor PS independently affected prognosis in terms of the CR rate and early death rate, but these results were not obtained through statistical analyses[9]. It therefore could not be ruled out that factors such as myelodysplasia and unfavourable risk karyotype were related to older age and influenced the results. Our multivariate analysis shows for the first time that older age is an independent adverse prognostic factor for OS.

This study has several limitations: (1) it is a retrospective study; (2) the number of patients is small; (3) the study includes Japanese patients only; (4) analyses of the data regarding comorbidities and expression of MDR1 on AML cells at the start of treatment could not be performed; and (5) analyses of haemorrhagic and infectious complications and their severity could not be performed.

We propose the following three reasons why older age is an independent adverse prognostic factor for OS in non-APL patients, leading to poor prognosis: (1) epigenetic changes to genes affect the pharmacokinetics of anticancer drugs; (2) preclinical organ dysfunction may not be reflected in the findings of blood tests and functional investigations; and (3) other unknown factors.

It is necessary to perform further large-scale, prospective, population-based observational studies, which take the various parameters that change with age into consideration, in order to definitively determine the adverse prognostic factors associated with older age. Age would contribute a considerable life expectance to indicate induction chemotherapy with eligible dose of cytotoxic drugs for a favorable case even if advanced elderly.

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COMMENTS

Background

Elderly acute myeloid leukemia (AML) patients have higher frequencies of myelodysplasia, unfavorable risk karyotype, and poor performance status (PS), which are the leading causes of poor long-term prognosis, compared with young adult AML patients. Elderly patients and those with poor PS are often excluded from prospective clinical trials for AML therapies. In such cases, the results of clinical practice may differ from the results reported in a clinical trial. Therefore, it is necessary to include a full cohort of consecutive patients diagnosed with AML in order to elucidate the real-world outcome for patients suffering from AML.

Research frontiers

In the field of medical oncology, it remains controversial whether age itself is an independent prognostic factor for prognosis. Generally it is axiomatic that older patients are more likely to have a poorer PS and have more underlying diseases, both of which result in morbid prognosis. Thus, the age and coexistence of related prognostic factors can be confounding. However, biological aging is not always associated with cognitive dysfunctions, and recent studies in geriatric oncology have aggregated sufficient evidence that geriatric assessment for chemotherapy is independent from age.

Innovations and breakthroughs

These multivariate analysis shows for the first time that older age is an independent adverse prognostic factor for overall survival (OS). This was validated in several chromosomal risk categories, *i.e.*, NCCN, SWOG, and CALGB. Though this cohort was limited to a local community in Japan, our results are expected to change the realistic planning of practical treatments for very elderly AML patients, who are not usually assessed in clinical trials.

Applications

The clinical outcome of patients over age 65 with AML is poor. However, intensive chemotherapy is a significant predictor for longer survival not only in patients younger than 75, but also in patients over age 75 with PS 0-2. Based on these results, patients with AML over 75 years of age could be candidates for intensive or less-intensive induction chemotherapy to obtain a better remission rate and further survival.

Terminology

The population-based study is an observational study for longitudinally registered patients without any medical interventions. The population-based cohort is set up to investigate whole populations in order to avoid intentional bias. It is crucial that the cohort be representative of a defined population. The population-based study offers three advantages: (1) it can illustrate the distributions, prevalence, and treatment outcome of the disease; (2) it can assess the risk factors for disease in a realistic manner; and (3) it can carry out unbiased evaluations of relations including confounders. Therefore, the authors believe that the present population-based study has reached a robust conclusion about whether advanced age and poor PS are adverse prognostic factors for OS.

Peer review

Ohnishi H *et al* reported for the first time that older age is an independent adverse prognostic factor for overall survival in AML patients through a population-based study cohorting 213 adult AML patients, by using multivariate analysis. Overall, This is a well-written and cafefully discussed paper.

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CASE REPORT

Unusual cytogenetic abnormalities associated with Philadelphia chromosome

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Author contributions: Sharma SK designed and wrote the paper; Handoo A provided the laboratory data; Choudhary D and Gupta N provided the clinical data.

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Abstract

Cytogenetic abnormalities are the hallmark of leukemias. We report here two cases of unusual cytogenetic abnormalities associated with Philadelphia chromosome, one with mixed phenotypic acute leukemia showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.

Key words: Philadelphia chromosome; Cytogenetic abnormalities

Core tip: Cytogenetic abnormalities are the hallmark of leukemias. We report here two cases of unusual cytogenetic abnormalities associated with Philadelphia chromosome, one with mixed phenotypic acute leukemia showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.These two cases highlight the novel additional cytogenetic abnormalities associated with Ph chromosome.

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INTRODUCTION

Cytogenetic abnormalities are the hallmark of leukemias. Translocation (9;22) is the characteristic feature of chronic myeloid leukemia and is also seen in variable number of patients with acute lymphoblastic leukemia. Monosomy 7 is seen in patients with myelodysplastic syndrome, acute myeloid leukemia and few cases of acute lymphoblastic leukemia. Mixed phenotypic acute leukemia (MPAL) is commonly associated with t(9;22) and sometimes with monosomy or deletion 7, but combined cytogenetic abnormality involving t(9;22) and monosomy 7 has rarely been reported in MPAL. We report here two cases, one of MPAL showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.

CASE REPORT

Case 1

A 27 years old man was admitted with weakness and low grade fever for 20 d. On examination he was found to have pallor without lymphadenopathy or hepato-splenomegaly. Hemogram showed hemoglobin 5.8 g/dL, total leukocyte count 62 × 10^9/L with 60% blasts and platelet count 170 × 10^9/L. Flowcytometry revealed two distinct

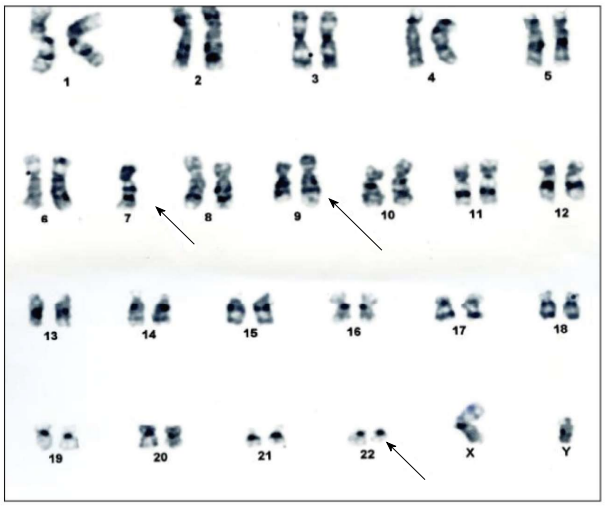

Figure 1 Karyotyping of the patient with mixed phenotype acute leukemia showing (arrows) 45, XY, monosomy 7, t(9;22) (q34;q11.2)[18]/46,XY[2].

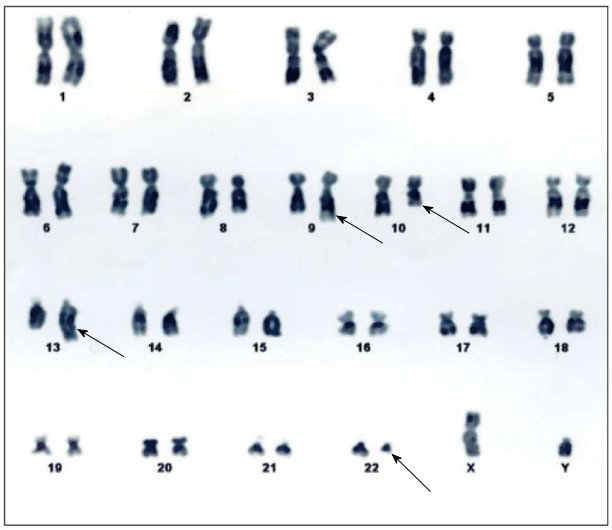

Figure 2 Karyotyping of the patient with chronic myeloid leukemia showing (arrows) 46 XY, t(9;22)(q34;11.2), t(10;13)(q23;q34)[20].

clusters of cells, one expressing CD34, HLD-DR, MPO, CD117, CD13 and CD33. The other cluster of blasts expressed cytoplasmic CD79a, CD19 and CD10. The blasts were negative for surface and cytoplasmic CD3, CD5, CD20 and CD16. The patient was diagnosed as a case of MPAL (Blymphoid/myeloid). Karyotyping revealed 45,XY, monosomy7, t(9;22)(q34;q11.2)[18]/46,XY[2] (Figure 1). RT-PCR for BCR-ABL detected genomic breakpoint at e13a2 corresponding to p210. Patient was treated with hyper-CVAD chemotherapy along with imatinib, which resulted in complete remission.

Case 2

A 50 years old man presented with complaints of mild weakness for 3 wk. His hemoglobin was 10.5 g/dL, total leukocyte count 46.7 × 10^9/L and platelet count 3.6 × 10^9/L, differential count showed neutrophils 62%, myelocytes 10%, metamyelocytes 8%, lymphocytes 12%, monocytes 3%, eosinophils 2% and basophils 3%. Leukocyte alkaline phosphatase was low. Karyotyping revealed 46 XY, t(9;22)(q34;11.2), t(10;13)(q23;q34)[20] (Figure 2). Real-time polymerase chain reaction for BCR-ABL was positive for p210. He was diagnosed as chronic myeloid leukemia-chronic phase and treated with imatinib. He achieved complete hematological remission in 2 mo.

DISCUSSION

Philadelphia (Ph) chromosome results from reciprocal translocation of chromosome 9 and 22. This translocation leads to the generation of a chimeric gene that results from the fusion of the *ABL* gene on chromosome 9 with the BCR gene on chromosome 22. MPAL is a rare leukemia arising from a hematopoietic pluripotent stem cell with a frequency of 0.5%-1%[1,2]. In a study by Matutes *et al*[2], comprising 100 patients of MPAL, cytogenetics revealed t(9;22) in 20%, 11q23/MLL-rearrangements in 8%, complex in 32%, aberrant in 27% and normal in 13% karyotypes. Three cases of CML[3,4] and one case of T-cell/myeloid MPAL[5] associated with del 7, t(9;22)(q34;q11) have been reported. Deletion/monosomy 7 is associated with poor prognosis in AML and t(9;22) confers a bad prognosis in ALL. Additional chromosomal abnormalities in CML may appear in about 5% of cases[6-8]. In a study by Luatti *et al*[9], 21 patients (5.6%) had additional chromosomal abnormalities; the overall cytogenetic and molecular response rates in these patients were significantly lower. None of these patients had translocations involving chromosome 10 and 13. Our patient with MPAL demonstrated characteristic bilineage leukemia and showed complete remission following hyper-CVAD plus imatinib therapy. The patient with CML also responded to imatinib. These two cases highlight the novel additional cytogenetic abnormalities associated with Ph chromosome. Whether this association of Philadelphia chromosome with these additional cytogenetic abnormalities adversely affect the prognosis needs to be evaluated. Though some studies have shown poor outcome with additional chromosomal abnormalities[9], our patients showed good initial response to imatinib based therapy.

COMMENTS

Case characteristics

A 27 years old man was admitted with weakness and low grade fever for 20 d. A 50 years old man presented with complaints of mild weakness for 3 wk.

Clinical diagnosis

Case 1: Hemogram showed hemoglobin 5.8 g/dL, total leukocyte count 62 × 10^9/L with 60% blasts and platelet count 170 × 10^9/L. Case 2: His hemoglobin was 10.5 g/dL, total leukocyte count 46.7 × 10^9/L and platelet count 3.6 × 10^9/L, differential count showed neutrophils 62%, myelocytes 10%, metamyelocytes 8%, lymphocytes 12%, monocytes 3%, eosinophils 2% and basophils 3%.

Laboratory diagnosis

Real-time polymerase chain reaction for BCR-ABL was positive for p210.

Treatment

Patient was treated with hyper-CVAD chemotherapy along with imatinib, which

resulted in complete remission.

Peer review

The manuscript demonstrates good initial response to imatinib based therapy of two rare cases of leukemia patients one with mixed phenotypic acute leukemia bilineage (B-lymphoid/myeloid) and the second with CML bearing an additional to t(9;22) chromosomal abnormality.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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