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Intravascular large B-cell lymphoma: the heterogeneous clinical manifestations of its classical and hemophagocytosis-related forms

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In this issue, Ferreri and the International Extranodal Lymphoma Study Group document that the clinical features of patients with intravascular lymphoma (IVL) vary based on the presence or absence of hemophagocytosis (HPC), rather than geographical region, differentiating IVL into classical and HPC-related forms. IVL is defined morphologically by the distribution of tumor cells exclusively in the lumina of blood vessels. IVL has unique clinical characteristics due to massive involvement of extranodal sites, without lymphadenopathy or leukemic manifestations, and is currently regarded as a rare entity listed in the category of diffuse large B-cell lymphomas of the World Health Organization (WHO) classification.[1] However, since

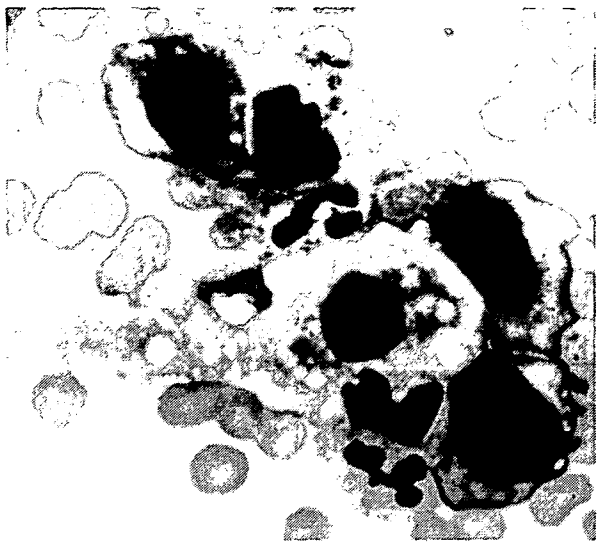

Figure 1. Asian variant of intravascular lymphoma. Bone marrow smear showing lymphoma cells and seemingly benign histiocytes with hemophagocytosis (May-Grünwald-Giemsa stain, ×1000)

most of the literature on IVL is formed of single case reports or small series of patients, the broad clinical spectrum of the disease is underappreciated. In the last decade, an increasing number of reports have shed light on this disease and have led to the recognition of Asian and cutaneous variants of IVL.[2-7]

IVL and HPC

HPC is accompanied by fever, cytopenia, and hepatosplenomegaly, frequently occurs in patients with infectious or autoimmune disorders, as well as malignant lymphoma, and appears to be prevalent in Asians. Therefore, the hemophagocytic syndrome often presents diagnostic and therapeutic challenges for pathologists and clinicians, in Asia. Lymphoma-associated HPC develops rapidly and, in most cases, is fatal within several months of onset, despite intensive chemotherapy.[8,9] In children and young adults, HPC tends to occur with Epstein-Barr virus (EBV)-associated lymphoproliferative disorders, including nasal type NK/T-cell lymphoma. In elderly patients, HPC is more commonly seen in B-cell lymphomas. Interestingly, the majority of B-cell lymphomas with HPC as a main clinical symptom are reported in Asian patients, and it remains to be determined whether those patients constitute a clinicopathologically distinct population. In 1997, Murase *et al.* suggested that malignant histiocytosis-like B-cell lymphoma, a symptomatically descriptive diagnostic term, may fall within the framework of IVL, and they proposed the term Asian variant of IVL for this peculiar disease.[3] Since then, despite considerable skepticism, Japanese clinicians have regarded HPC as a key sign of the Asian variant of IVL, providing a nation-wide group of 96 IVL patients, which to our knowledge represents the largest series of patients, for our recent analysis in Japan.[7] In that series, bone marrow was the most frequently involved organ and this involvement was usually accompanied by HPC (Figure 1), causing symptomatic anemia (66% of the cases), thrombocytopenia (58%), and leukocytopenia (27%). However, it was rarely accompanied by neurological abnormalities or cutaneous lesions, providing additional support for our assertion that clinicopathological features in many Japanese IVL patients are consistent with the Asian variant of the disease. Of note, the Asian variant of IVL has been observed almost exclusively in Asian countries, especially Japan, with a few observations in Western countries.[10] It should also be emphasized that hemo-erythrophagocytosis is not a mandatory morphological marker of Asian variant of IVL, indicating that clinical findings of bicytopenia or pancytopenia are extremely important.[4]

Heterogeneous symptoms of IVL

Dermatological signs specific to IVL patients were first described by Pfleger and Tappeiner in 1959.[2] While most of those cases had a fatal course, some exceptional cases of untreated long-term survival were documented as indolent lymphomas for IVL, although the diagnosis was often incidental.[11] Based on these traditional ideas about IVL, Ferreri and the International Extranodal Lymphoma Study Group (IELSG) highlighted a distinct IVL subgroup, the cutaneous variant, primarily diagnosed in Europeans.[5] The cutaneous variant is characterized by skin tumors. It is predominant in females and associated with a normal platelet count; it is regarded as a favorable prognostic factor. Subsequently, the same group investigated the frequency of HPC among IVL patients in different geographical regions, including Western countries, Japan, and other Asian countries.[12] They concluded that clinical features of IVL patients vary according to the presence or absence of HPC rather than to geographic region. There is a paucity of HPC in Western countries, where IVL is characterized as classical or HPC-related. Japanese patients with IVL and HPC frequently have advanced disease (i.e., stage IV) and related symptoms, although IVL with HPC is usually considered to be a disseminated disease, and there are few technologies and methods available to delineate the clinical stages precisely. On the other hand, the analysis by Ferreri *et al.* as well as our own analysis, demonstrated that, beyond clinical forms or variants, the prognosis of IVL is poor even when the age of onset, gender, and lactate dehydrogenase levels are considered. Of note, nearly all of the tumor cells are characterized by a B-cell immunophenotype with B-cell lymphoma-2 (Bcl-2) and multiple myeloma oncogene-1/interferon regulatory factor 4 (mum1/IRF4) proteins, indicating that they should be classified as non-germinal center B-cell type lymphomas.[7,13]

Future issues regarding aggressive extranodal lymphomas

Anatomic localization is now appreciated as a parameter for classifying diffuse large B-cell lymphomas, as exemplified by primary mediastinal large B-cell lymphoma. Of course, distinct groups of extranodal lymphomas exist, which are defined by heterogeneous presentation syndromes related to the preferentially involved organs, such as primary central nervous system lymphoma and primary testicular lymphoma, in addition to IVL. These lymphomas are consistently characterized by an aggressive clinical course and predominant or exclusive extranodal distribution, with or without mass formation and sometimes with CD5 expression, despite histological differences, such as angiotropic and intravascular patterns.[14,15] Concerning IVL, Ferreri *et al.* state, "*Extensive phenotypic and molecular characterization is needed to test whether these different clinical forms may also have different biological backgrounds, and, therefore, international co-operative studies are warranted*". We agree with their proposal and believe that such trials should be extended to aggressive extranodal lymphomas, since there are few perspectives concerning the distinct clinicopathological profiles of such diseases or the basis of their mechanisms of lymphomagenesis, adhesion, and dissemination.[16,17]

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Prognostic analysis of aberrant somatic hypermutation of *RhoH* gene in diffuse large B cell lymphoma

Leukemia (2007) **21**, 1846–1847; doi:10.1038/sj.leu.2404717; published online 19 April 2007

Previous reports demonstrated the aberrant somatic hypermutation (SHM) of proto-oncogenes *PIM-1, MYC, RhoH* and *PAX5* as a novel mechanism of genetic lesion in diffuse large B-cell lymphoma (DLBCL).[1] The Rho family of small GTPases including Rho, Rac, and Cdc42 has been well characterized as molecular switches to transduce signals from plasma membrane to the downstream effectors. RhoH, a member of the Rho family, is specifically expressed in hematopoietic cells, especially in T and B lymphocytes, and has been reported to downregulate Rac[2] and LFA-1.[3] As RhoH is GTPase-deficient and constitutively active, GTP-bound form,[4] the activity of RhoH is directly related to the level of expression. Aberrant SHM of *RhoH* gene are distributed in intron 1 (+300∼1700) and have been reported in 46% of DLBCL,[1] 11% of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL),[5] 11% of primary cutaneous follicular center lymphoma (PCFCL),[6] 31% of primary cutaneous large B cell lymphoma, leg type (PCLBCL),[6] and 13% of hepatitis C virus (HCV) associated non Hodgkin lymphoma.[7] Although several studies reported the SHM of DLBCL and other lymphoid malignancy, the prognostic values of SHM remain elusive. Here, we have screened for SHM of *RhoH* gene in the lymph node samples from 100 previously untreated DLBCL cases.

These patients were diagnosed and treated at Nagoya University Hospital since 1987 to 2002, and median duration of follow-up was 1026 days. Informed consent was obtained from all patients. Samples from 12 cases of reactive lymphadenopathy were also screened for negative control. Intron 1 of *RhoH* gene was amplified and screened by the denaturing HPLC (DHPLC) analysis using the WAVE Maker System (Transgenomic Inc., San Jose, CA, USA) as previously described.[8] DHPLC gradients and temperatures were determined using WAVE Maker System software. When heterozygous profiles were identified by visual inspection of the chromatograms, amplified products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and were sequenced on the DNA sequencer (310; Applied Biosystems, Foster City, CA, USA) using the BigDye terminator cycle-sequencing kit (Applied Biosystems). Statistical analyses were performed using the Fisher exact test for 2 × 2 tables with StatView-J 5.5 software (Abacus Concepts Inc., Berkeley, CA, USA).

We found 78 mutations (76 point mutations, two insertions) unique to individual tumor DNAs in 35 of 100 DLBCL cases (35%) in *RhoH* gene (Table 1). Overall, 73 out of 78 were novel SHM, and five were same mutations as reported previously.[1] They shared most of the features of the immunoglobulin variable gene-associated SHM mechanism such as single nucleotide substitution ($n=76$) with rare deletion/insertions ($n=2$) and a predominance of transitions ($n=48$) than transversions ($n=28$).[5] The number of mutations in one patient ranged from 1 to 6, and 20 patients had more than one mutation. These mutations were distributed in a region throughout the first 1.6 kb of the *RhoH* gene. No mutation was detected in DNA from reactive lymphadenopathy patients except for the known polymorphism T486G in 2/12 samples.[1] The absence of aberrant SHM tends to correlate with advanced stage (stages III and IV, $P=0.06$), although there was no statistical significance (Table 2). The presence of aberrant SHM in *RhoH* gene impacted neither on overall survival (Figure 1a) nor disease-free survival (Figure 1b) ($P=0.57$ and 0.75, respectively). Taken together, our study demonstrated that although the

Table 1 Mutational analysis of *RhoH* gene in DLBCL patients

UPN	*Mutation*	*UPN*	*Mutation*
1	C999T	47	G538A
	G1070A		
	T1117A		
	A1192G		
10	A775G	49	A548C
	G798C		G859A
	T1043C		T1036A
	C1119T		C1057A
			G1098T
			G1102A
11	T676C	50	A563C
			A591T
18	C746T	52	C999T
22	C505G	53	C1081T
	G1165A		
23	T523C	58	C1057T
	G1061A		C1062T
28	G555C	60	A591G
	G694A		A593G
	G743A		
	C788G		
29	G743T	63	G530A
	G750A		
	G753A		
	G1012A		
31	T412C	67	A721G
	C598T		
	G1053A		
	G1061A		
32	A993T	68	C715T
	G1029C		G817T
	G1084A		ins1077T
			ins1110C
			T1127C
33	G983A	73	G743T
	T1085A		
34	T851A	80	C544T
			T590G
39	C885T	86	C443T
			A512G
			A587C
40	T939G	105	C521T
			C695T
			C850G
			G1053T
			G1107T
			A1198T
41	C443T	106	T420C
	C890T		C544T
42	G1029A	107	A1039C
45	G859A	113	T613C
			A748C
46	T814A		

Table 2 *P*-values of each parameter

	Mutation (+) 35 cases (%)	*Mutation (−) 65 cases (%)*	*P-value*
Age > 60 years	42.9	44.6	0.87
Stage ≥ 3, 4	34.3	53.8	0.06
LDH > Normal	62.9	49.2	0.19
PS ≥ 2	11.4	24.6	0.12
Extranodal sites ≥ 2	22.9	23.1	0.98
Male	54.3	66.2	0.25

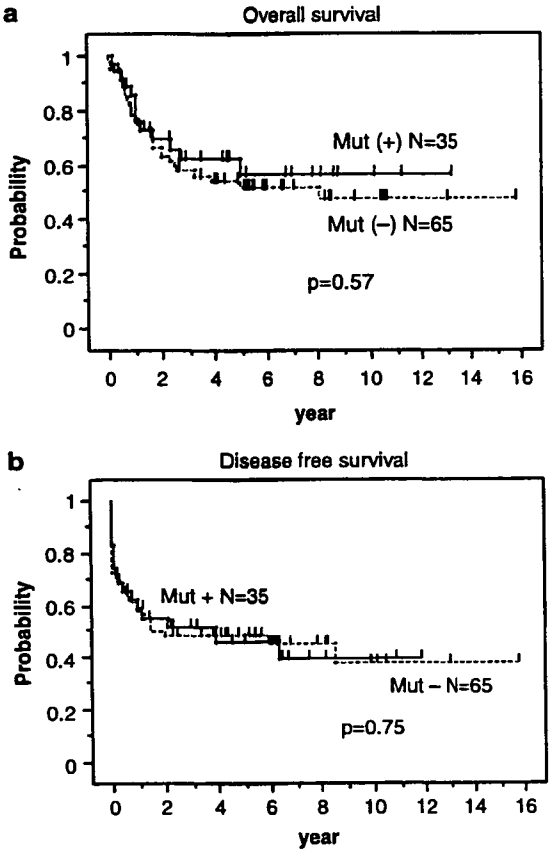

Figure 1 Kaplan–Meier curves according to the SHM of *RhoH*. (a) Overall survival in all patients. (b) Disease-free survival in all patients.

SHM might contribute to the lymphomagenesis through regulation of *RhoH* gene expression, it has less impact on survival of DLBCL patients.

Acknowledgements

This work was supported by Grants-in-Aid from the Sankyo Foundation of Life Science, National Institute of Biomedical Innovation, and the Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Ms Satomi Yamaji and Chika Wakamatsu for their excellent technical help.

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Downregulation of microRNAs-143 and -145 in B-cell malignancies

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(Received June 15, 2007/Revised August 6, 2007/Accept August 15, 2007/Online publication September 24, 2007)

Recently, it has been found that inappropriate expression of microRNAs (miRNAs) is strongly associated with carcinogenesis. In this study, we demonstrated that the expression of miRNAs (miRs) -143 and -145, the levels of which were previously shown to be reduced in colon cancers and various kinds of established cancer cell lines, was also decreased in most of the B-cell malignancies examined, including chronic lymphocytic leukemias (CLL), B-cell lymphomas, Epstein-Barr virus (EBV)-transformed B-cell lines, and Burkitt lymphoma cell lines. All samples from 13 CLL patients and eight of nine B-cell lymphoma ones tested exhibited an extremely low expression of miRs-143 and -145. The expression levels of miRs-143 and -145 were consistently low in human Burkitt lymphoma cell lines and were inversely associated with the cell proliferation observed in the EBV-transformed B-cell lines. Moreover, the introduction of either precursor or mature miR-143 and -145 into Raji cells resulted in a significant growth inhibition that occurred in a dose-dependent manner and the target gene of miRNA-143 was determined to be ERK5, as previously reported in human colon cancer DLD-1 cells. Taken together, these findings suggest that miRs-143 and -145 may be useful as biomarkers that differentiate B-cell malignant cells from normal cells and contribute to carcinogenesis in B-cell malignancies by a newly defined mechanism. (*Cancer Sci* 2007; 98: 1914–1920)

MicroRNAs are endogenous ~22-nt non-coding RNAs that regulate gene expression by inhibiting the translation of mRNAs in a sequence-specific manner.[1–8] With more than 400 already identified, the human genome may contain up to 1000 miRNAs[8] (http://microrna.sanger.ac.uk/). Up to one-third of human mRNAs are predicted to be miRNA targets.[9] Each miRNA can target more than 200 transcripts directly or indirectly,[10,11] whereas more than one miRNA can converge on a single mRNA target.[9,12–15] Therefore, the potential regulatory circuitry afforded by miRNA is enormous. These findings support the notion that alterations of miRNAs copy number and their regulatory genes highly prevalent in cancer because of genomic aberrations is closely associated with carcinogenesis.

Recent increasing evidence shows that the expression of miRNA genes is deregulated in human cancer.[16–19] Specific over- or underexpression has been shown to correlate with particular tumor types.[20–24] miRNA overexpression can result in downregulation of tumor suppressor genes, whereas their underexpression can lead to oncogene upregulation.[16–19] For example, let-7, downregulated in lung cancer,[25–27] suppresses *Ras*,[26] miR-15 and miR-16, deleted or downregulated in CLL,[28] suppress *BCL2*,[29] miR-17-5p and miR-20a control the balance of cell death and proliferation driven by the proto-oncogene *c-Myc*.[30] Clear evidence indicates that miRNA polycistron miR-17-92 serves as an oncogene in lymphoma,[23] and lung cancer,[31] and that miR-372 and miR-373 are novel oncogenes in testicular germ cell tumors that act by numbing the p53 pathway.[32] Thus, miRNA expression profiles may predict the outcome of disease.[25,27,33]

First, by differential hybridization using a DNA microarray for miRNAs and then by semi-quantitative RT-PCR analysis between tumor and non-tumor tissues, we previously found that miRs-143 and -145, whose genes are located within 1.8 kb of each other in the chromosome 5q32 region, were downregulated in colon cancer,[34] as also reported by Michael *et al.*[35] Furthermore, all of the various kinds of human cancer cell lines tested exhibited an extremely low-expression of miRs-143 and -145, whereas the normal tissues in which they originate showed a good expression of both.[34]

In the present study, we investigated the expression of miRs-143 and -145 in hematopoietic malignancies, and found that the expression levels of both miRs were significantly decreased in B-cell malignancies, thus suggesting that they are good markers for B-cell malignancies, especially for CLL in combination with miRs-15 and -16.[28] Furthermore, the expression levels of miRs-143 and -145 were consistently low in Burkitt lymphoma cell lines and were inversely related to the growth of EBV-transformed cell lines. The transfection experiment of Raji cells with either precursor miR-143 or -145 demonstrated that both miRs negatively contributed to the cell growth.

Materials and Methods

Patients and tissue preparation. All human blood and lymph node samples were obtained from patients who had undergone collection for diagnosis at Nagoya University Hospital and its collaborating hospitals in Nagoya, Aichi Prefecture. Human tonsils were also obtained by tonsilectomy. Informed consent in writing was obtained from each patient. Collection and distribution of the samples were approved by the appropriate Institution Review Board. The patients comprised 13 cases of chronic lymphocytic leukemia (CLL) and nine cases of B-cell lymphoma (8DLBCL, 1MALT). Pathological review and FACS analysis showed B-cell non-Hodgkin lymphoma. PBL from three healthy donors were used as normal controls. Fresh lymphoma biopsy specimens and tonsils obtained by operation were gently minced over a wire mesh screen to obtain a cell suspension, which was then centrifuged over Ficoll-Hypaque (Amersham Biosciences AB, Uppsala, Sweden). $CD19^+$ B-cells from the tonsils were used as control cells. Purity was assessed,

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Abbreviations: 5-Aza, 5-Aza-2'-deoxycytidine; C/EBP, CCAAT/enhancer binding protein; CLL, chronic lymphocytic leukemia; Ct, threshold cycle; DLBCL, diffuse large B cell lymphoma; DNMT-1, DNA methyltransferase; EBV, Epstein-Barr virus; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal regulated kinase; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; *GAPDH*, glyceraldehyde phosphate dehydrogenase; HRP, horseradish peroxidase; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; MAPK, mitogen-activated protein kinase; miRNAs, microRNAs; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLSD, protected least significant difference; PVDF, polyvinylidene fluoride; RISC, RNA-induced silencing complex; RT, reverse transcription; SDS, sodium dodecyl sulfate; TSA, Tricostatin A; UTR, untranslated region.

doi: 10.1111/j.1349-7006.2007.00618.x

and isolation carried out by FACS. All samples were prepared by gravity centrifugation through Ficoll-Hypaque and then frozen in liquid nitrogen until the experiments could be carried out. Such thawed specimens were used for the extraction of total RNA.

Cell culture, viability and treatment with 5-Aza-2'-deoxycytidine or tricostatin A. Human Burkitt cell lines Raji, Daudi, P3, and KHM-10B; and human EBV-transformed cells IC, L11, L22, and L25 were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO_2 at 37°C.

The number of viable cells was determined by use of the trypan-blue dye exclusion test. 5-Aza (Sigma) was used for demethylation of DNA and histone. TSA (Sigma), a histone deacetylase inhibitor, was also used to examine the effect of acetylation of histone on miR-143 and -145 expression. The cells were treated with these agents for 18 h at various concentrations.

Quantitative RT-PCR and genomic PCR. Total RNA was isolated from the cells by the phenol/guanidium thiocyanate method with DNase I treatment. To determine the expression of miRNAs by semi-quantitative RT-PCR, we measured their levels by using a mirVana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA) and mirVana qRT-PCR Primer set (Ambion). Briefly, after reverse transcription of 50 ng of total RNA, cDNA was generated. The PCR reaction consisted of 22 cycles (95°C for 15 s, 60°C for 30 s) after an initial denaturation step (95°C for 3 min). The cycle number was initially determined by quantitative PCR. The PCR primer pairs for miRs-143, -145, and -15a were obtained commercially from Ambion. The PCR products obtained by using such primer pairs were confirmed to be from loci of miRs-143 and -145 by DNA sequencing. U6 was used as a control and was determined in each case. In addition, in order to examine the expression level in detail we also carried out TaqMan® MicroRNA Assays using real-time PCR.[16] The Ct is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. miR-143 and miR145 concentration in each type of cell were measured and were normalized to U6, which was used as an internal control. To determine the level of *ERK5* mRNA we prepared cDNA from the total RNA samples by using a PCR purification kit (Qiagen, Hilden, Germany) and used them for PCR (Takara, Ohtsu, Japan). The primers for *ERK5* were as follows: ERK5-sense-211, 5'-CCTTCGATGTGA-CCTTTGAC-3'; and ERK5-antisense-1418, 5'-TGACACCATTG-ATCTGACCC-3'. To examine the presence of the genomic loci of miRs-143 and -145, we extracted DNA from the cell lines tested and used it for PCR (Takara). The primers for genomic loci of miRs-143 and -145 were as follow: 5q32-sense, 5'-TTGGTCCTGGGTGCTCAAAT-3'; and 5q32-antisense, 5'-AGGAACTCCCAAGCTCAAGT-3'. The primers amplified the DNA fragment including both loci at 5q32. The genomic locus of *GAPDH* was used as an internal control. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). The PCR products were analyzed by electrophoresis on 2% agarose gels.

Transfection of Raji cells with precursor or mature miR-143 and -145 miRNAs. Raji cells were seeded in six-well plates at a concentration of $1–2 \times 10^5$/well on the day before the transfection. The miRs-143 and -145 precursors (20–100 nM/mL; Ambion) and mature miRNAs (20–60 nM/mL) were used for the transfection of the cells, which was achieved by using cationic liposomes (i.e. TransIT-TKO) (Mirus Bio Company, Madison, WI, USA) according to the manufacturer's lipofection protocol. The transfection efficiency was evaluated by the transfection of the cells with a duplex siRNA-FITC (Dharmacon, Lafayette, CO, USA). Non-specific control miRNA (NS, 57% GC content; Ambion) was used as a control for non-specific effects. The sequences of mature miRNA-143 (miRNA-143 m) and -145 (miRNA-145 m) were as follows: UGAGAUGAAGCACU-GUAGCUCA and GUCCAGUUUUCCCAGGAAUCCCUU, respectively. The effects manifested by the introduction of the precursor or mature miRNAs into the cells were assayed at 36 h after the transfection. At the same time, semi-quantitative RT-PCR was carried out on the cells transfected with the precursors.

Western blotting. The cells were homogenized in chilled lysis buffer comprising 10 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% protease inhibitor cocktail (Sigma) and stood for 30 min on ice. After centrifugation at 16 000*g* for 20 min at 4°C, the supernatants were collected as protein samples. Protein contents were measured with a DC protein assay kit (Biorad, Hercules, CA, USA). 10 μg of lysate protein for western blotting of ERK5 and c-myc was separated by SDS-PAGE using a 10% polyacrylamide gel and electroblotted onto a PVDF membrane (DuPont, Boston, MA, USA). After blockage of non-specific binding sites for 1 h with 5% non-fat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight at 4°C with antihuman ERK5 antibody (Cell Signaling Tec. Inc., Beverly, MA, USA), antihuman c-myc antibody (Santa Cruz, Santa Cruz, CA, USA) or with DNMT-1 (Santa Cruz). The membranes were then washed three times with PBS containing 0.1% Tween 20, incubated further with HRP-conjugated sheep antimouse or donkey antirabbit Ig antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature, and then washed three times with PBS containing 0.1% Tween 20. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Statistics. Differences were statistically evaluated by one-way ANOVA followed by Fisher's PLSD. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Expression levels of miRs-143 and -145 were significantly decreased in B-cell malignancies. We examined the expression of miRs-143 and -145 in the samples from patients with B-cell malignancies by conducting semi-quantitative RT-PCR and TaqMan assays using real-time PCR. Representative bands of expression obtained by the former and the mean values of TaqMan assays from two independent experiments using the latter are presented in Fig. 1. There was no patient with any abnormality of chromosome 5q32, where miRs-143 and -145 are colocalized within a 1.8-kb distance from each other. In TaqMan assays, Ct values of the samples detected by TaqMan probes corresponding to U6, miR-143 and miR-145 are shown in Table 1. Notably, all of the cases of CLL showed an extremely low-expression (Fig. 1a). Among the B-cell lymphomas, all of the samples except Bl-18 exhibited a low level (Fig. 1b). Recent reports by Calin *et al.*[28] demonstrated that the expression levels of mir-15a and -16 from 13q13.4 were decreased in CLLs with an incidence of approximately 68%. However, the incidence of reduced miR-15a expression in our Japanese CLLs was not so high (approximately 54%, Fig. 1a).

Relationship between the expression levels of miRs-143 and -145 and cell growth. In order to clarify the relationship between the

Table 1. The Ct values of U6, miR-143, and miR-145 in real-time PCR using the TaqMan probes.

Standard Curve	Ct	
	Mean ± SD	CV (%)
U6	27.1 ± 0.12	0.4
miR-143	31.0 ± 0.12	0.4
miR-145	28.1 ± 0.08	0.3

Ct, cycle threshold; CV, coefficient variation; miR, miRNA; SD, standard deviation.

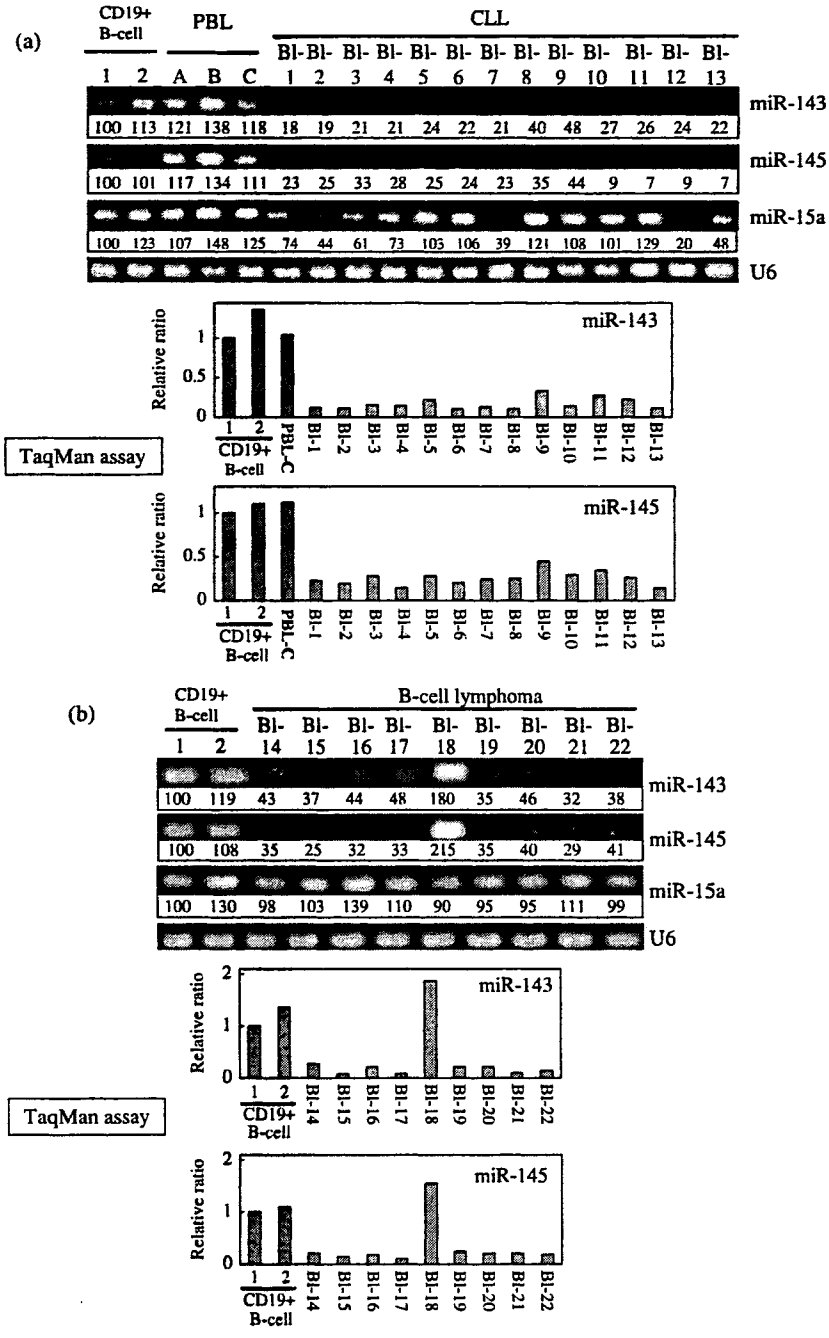

Fig. 1. Expression of microRNAs (miRNAs)-143 and -145 in human B-cell malignancies examined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Blood samples or lymph nodes (Bl) from patients were obtained on admission or after relapse of the disease. Thirteen cases of chronic lymphocytic leukemia (CLL) (a) and nine cases of B-cell lymphoma (b) were examined. Peripheral blood lymphocytes (PBL) from individuals a, b, and c and CD19+ B-cells from the tonsils (1 and 2) were used as controls. In CLLs, the expression of miR-15a[28] was also examined by semi-quantitative RT-PCR. U6 was used as an internal standard. The intensity of the bands for miR-143, -145 and -15a expression was determined by densitometry and the value is given under each band. The levels of CD19+ B-cells for CLL and B-cell lymphoma were designated as 100 in semi-quantitative RT-PCR and as 1 in quantitative RT-PCR by TaqMan assays using a real-time PCR. The results of real-time PCR are expressed as the mean values of two independent experiments.

expression levels of miRs-143 and 145 and cell growth in B-cell lines, we examined the expression levels of EBV-transformed B-cell lines from healthy donors (IC, L11, L22, and L25) and established Burkitt cell lines (Raji, Daudi, P32, and KHM-10B; Fig. 2a). It should be noted that the expression levels were inversely related to the cell growth of EBV-transformed cells from the data of semi-quantitative RT-PCR (Ambion) (Fig. 2a,b; expression level, L25 < L22 = L11 < IC; growth, IC < L22 < L11 < L25). Furthermore, all of the Burkitt cell lines, in which cells have genetic aberrations including *c-Myc*, showed a fairly low level of the expression of both miRNAs like the L25 cells (Fig. 2a), which showed the highest cell proliferation among the EBV-transformed B-cell lines (Fig. 2b). The data in Fig. 2a,b clearly indicate an inverse relationship between the expression levels of miR-143 and -145 and cell growth.

Transfection of RAJI cells with precursor or mature miR-143 or -145, respectively, causes growth inhibition. In order to examine the suppressive function of miR-143 and -145 with respect to cell growth and to examine which enzymatic modification during miRNA biogenesis is perturbed, we transfected low-expressant Raji cells with precursor miR-143 (miR-143p) or -145 (miR-145p), in which transfection resulted in a significant growth inhibition that occurred in a dose-dependent manner (Fig. 3a). Semi-quantitative RT-PCR using the primers for miR-143 or -145 demonstrated a significant increase in the levels of miR-143 and 145 in the Raji cells transfected with the respective precursors compared with their levels in the control cells

doi: 10.1111/j.1349-7006.2007.00618.x

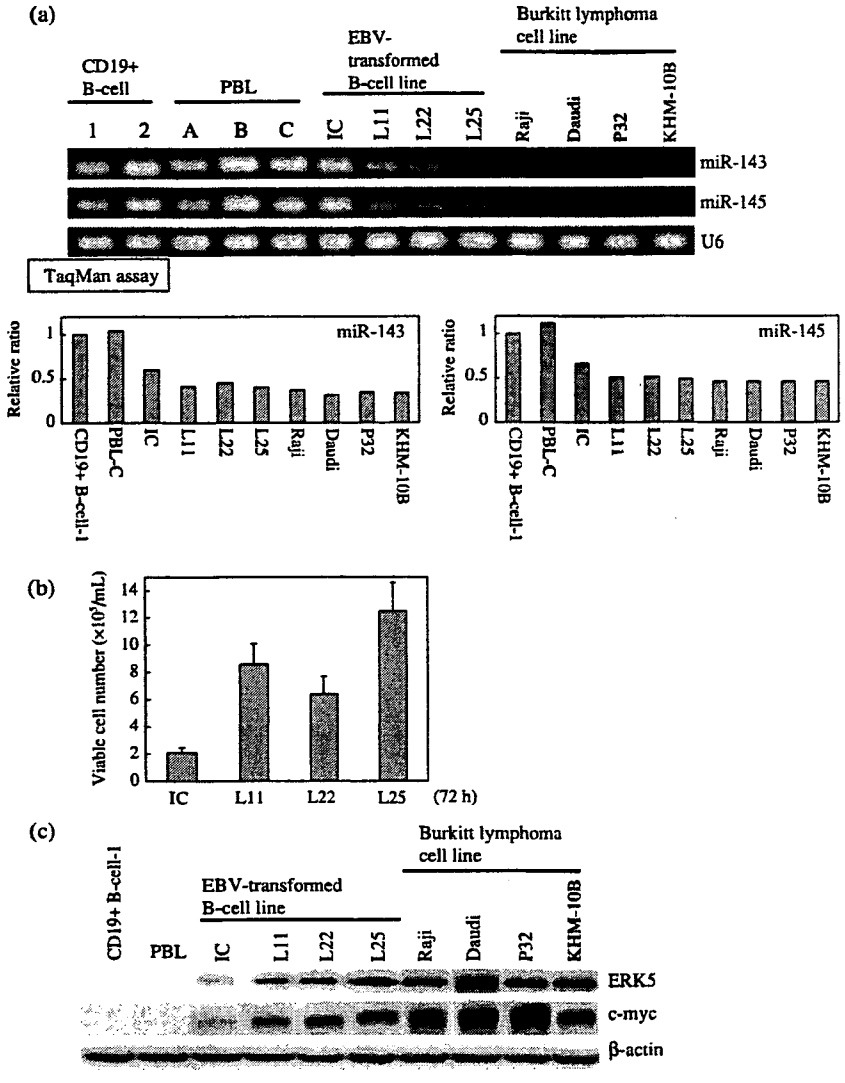

Fig. 2. Expression of microRNAs (miRNAs)-143 and -145 and cell growth in B-cell cultured cell lines. (a) Evaluation of expression of miRNAs-143 and -145 in human Epstein-Barr virus (EBV)-transformed B-cell lines and Burkitt lymphoma cell lines by use of semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and TaqMan MicroRNA assays using real-time PCR. U6 was used as an internal standard. $CD19^+$ B-cell-1 was designated as 1 in quantitative RT-PCR by TaqMan miRNA assays using real-time PCR. The results of real-time PCR were expressed as the mean values of two independent experiments. (b) Steady-state cell growth of EBV-transformed B-cell lines at 72 h after seeding at the concentration of 1×10^5/mL (c) Western blot analysis of ERK5 and c-myc in control and B-cell lines in the same samples as in (b), and in human Burkitt lymphoma cell lines. β-actin was used as an internal control.

(Fig. 3a). In order to further confirm that miR-143 or -145 has a growth inhibition, we carried out a transfection experiment using mature miR-143 (miR-143 m) or -145 (miR-145 m) (Fig. 3b). In both experiments, a dose-dependent growth inhibition by the introduction of mature miR-143 or -145, which results were very similar to those obtained when the transfection was carried out with precursor miR-143 or -145. These findings indicate that miR-143 and -145 negatively contribute to cell growth, because the compensation of miR-143 or -145 by the transfection induced growth inhibition and that the perturbation of processing of miR-143 or -145 in the nucleus including transcription and microprocessor of Drosha and DG8 could cause the cells to reduce their expression of both.

Genomic status and epigenetic change in miR-143 and -145 loci on 5q32. In order to examine the chromosomal aberrations of miRs-143 and -145 loci on 5q32, we carried out genomic PCR on the B-cell lines by using a primer pair covering both loci.[34] As shown in Fig. 4a, more than one allele was confirmed to be present in all EBV-transformed and Burkitt lymphoma cell lines, as in the placenta, used as the positive control. Furthermore, the treatment with 2–10 μM 5-Aza, which completely reduced the level of DNMT-1, did not upregulate the expression of miRNAs-143 and -145 at all in L25 cells (Fig. 4b); nor did that with TSA. Thus, it appears that some genomic aberration or epigenetic change did not cause the low expression of miR-143 and -145 in the cells.

MicroRNA-143 targets the *ERK-5* mRNAs. Previously, we determined that one of the target genes of miR-143 was *ERK5* in the human colon cancer cell line DLD-1.[34] ERK5 is a recently characterized MAPK, which is most similar to the well-studied ERK1/2 subfamily, but uses distinct mechanisms to elicit responses. The physiological importance of this signaling cascade is underscored by the early embryonic death caused by the targeted deletion of the *erk5* or the *mek5* genes in mice.[35] In Fig. 2c, the level of ERK5 expression in Burkitt lymphoma cell lines was approximately several times higher than that in the other B-cells including $CD19^+$ B-cells and EBV transformed B-cell lines except L25, where the pattern was very similar to that of c-myc. We clearly demonstrated that the expression levels between miR-143 and ERK5 were inversely correlated in regard to cell growth in EBV-transformed cell lines (Fig. 2). Based on these results, ERK-5 was shown to be a growth-related MAPK even in B-cells. In order to further confirm that the target mRNA of miR-143 is *ERK5*, we examined the expression of ERK5 and the mRNA in the RAJI cells transfected with precursor miR-143 (Fig. 3). Expectedly the protein expression levels were significantly decreased in a dose-dependent

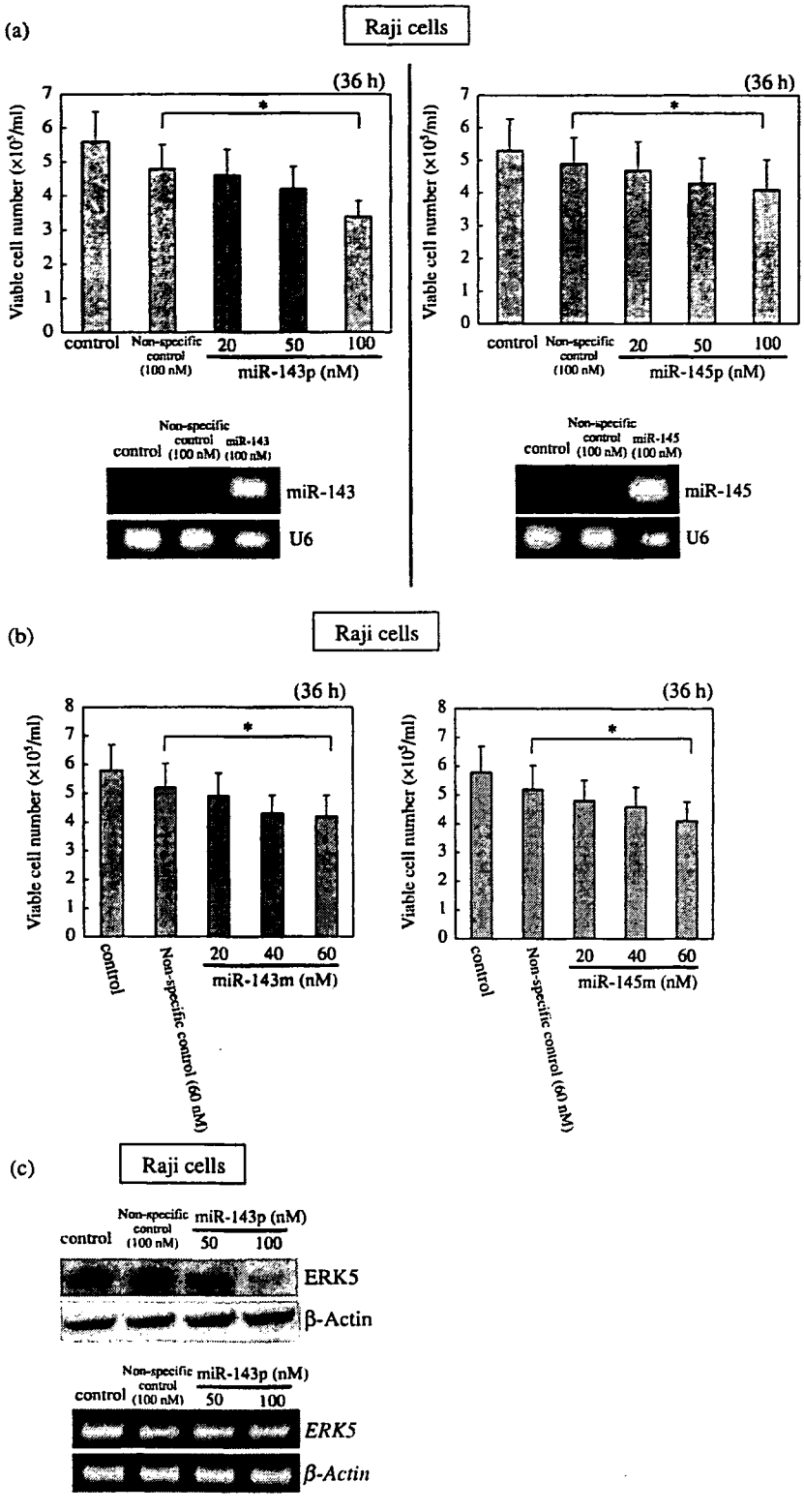

Fig. 3. Effect of transfection of human Burkitt lymphoma Raji cells with either precursor (a) or mature type (b) of microRNAs (miRNAs)-143 and -145, and ERK5 expression in the cells transfected with the precursor miR-143 (c). (a, b) Number of viable transfected or control cells at 36 h after transfection is shown. Data are presented as the mean ± standard deviation (SD) of three different experiments, each carried out in duplicate. Levels of miRNAs-143 and -145 in Raji cells at 36 h after the transfection of the cells with miR-143 or -145 precursor miRNAs at 100 nM are shown in (a). U6 was used as an internal standard. The difference between the non-specific control and 100 (a) or 60 (b) nM treatment was significant ($^*P < 0.01$). (c) Expression levels of ERK5 protein and the mRNA at 36 h after the transfection of Raji cells with miR-143 precursor miRNAs, as evaluated by western blot analysis (upper panel) and by quantitative RT-PCR (lower panel), respectively. β-actin was used as an internal standard. Control cells were incubated in medium containing transfection reagent alone.

manner, whereas the mRNA levels were almost unchanged (Fig. 3c). Thus, these findings and the previous results in colon cancer[34] altogether indicate that miR-143 could target the *ERK5* gene even in B-cells.

Discussion

In the current study, we demonstrated that the expression levels of miRs-143 and -145 were significantly reduced in the B-cell

doi: 10.1111/j.1349-7006.2007.00618.x

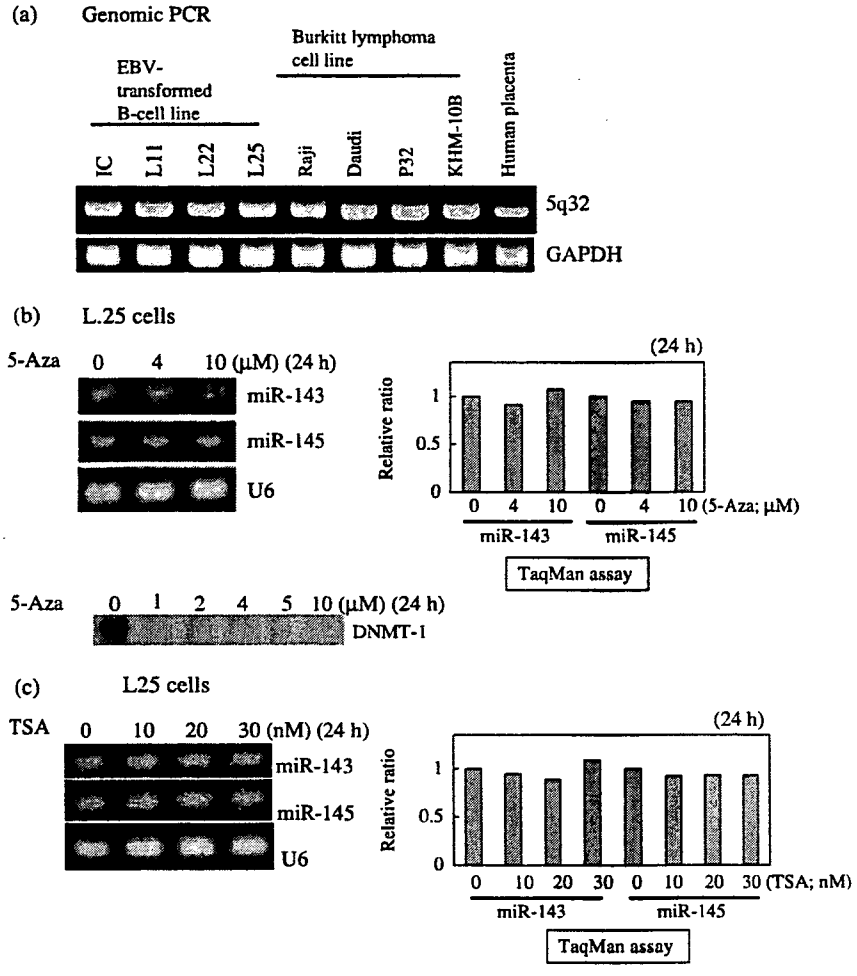

Fig. 4. Confirmation of the presence of the genomic loci of microRNAs (miRNAs)-143 and -145 at chromosome 5q32 by genomic polymerase chain reaction (PCR) (a) and expression of the miRNAs in Epstein-Barr virus (EBV)-transformed L25 cells after 24-h treatment with 5-Aza-2'-deoxycytidine (5-Aza, b) or tricostatin A (TSA, c). (a) The primers amplified the DNA fragment including the loci of miRs-143 and -145. Placental DNA was used as a positive control. The genomic locus of glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as an internal control. (b, c) The expression of the miRNAs in L25 cells after the treatment with either agent at the indicated concentrations is shown. The value for the control cells is designated as 1 in quantitative reverse transcription (RT)-PCR by TaqMan miRNA assays using a real-time PCR. The results of real-time PCR are expressed as the mean values of two independent experiments. U6 was used as an internal standard. Results of Western blot analysis of DNA methyltransferase (DNMT)-1 are also given in (b).

malignancies tested and further that the expression levels were inversely related to the cell growth in the EBV-transformed B-cell lines, which have no genomic aberrations. The introduction of miR-143 or -145 precursors or mature types into the Raji cells led to a significant growth inhibition. Such data suggest that miRs-143 and -145 could negatively contribute to cell growth by targeting growth-related genes.

The increased expression of miR-143 by the transfection with precursor miR-143 into Raji cells lowered the protein expression levels of ERK5, whereas the mRNA level of *ERK5* remained unchanged, which suggests that *ERK5* is one of the target genes of miR-143, as shown in previous reports by us,[34] and Esau *et al.*[37]

Recent studies indicate that proteins involved in miRNA biogenesis, including Drosha and double-stranded-RNA-binding protein DGCR8, Dicer 1, Argonaute 2, and RISC, may also participate in the complex interactions that regulate miRNA expression, together with additional mechanisms that regulate miRNA at the epigenetic, transcriptional level. Since the transfection of Raji cells with the precursor miR-143 or -145 exhibited growth inhibition, transcription and/or processing by Drosha and DGCR8 microprocessor in the nucleus was possibly perturbed, which is true in the case of human colon cancer DLD-1 cells.[34] Furthermore, we did not obtain any data that indicated an inappropriate expression caused by genomic aberration or epigenetic change such as methylated DNA and histone. Recently, miR-223 was shown to be upregulated by the retinoic acid-induced replacement of NFI-A with C/EBP α, resulting in promotion of human granulocyte differentiation.[38] As miR-223 repressed NFI-A translation, the upregulation of miR-223 by C/EBP α and granulopoiesis was further accelerated through positive feedback. Therefore, the machinery involved in the transcription step of miRs-143 and -145, whose primary miRNAs are most likely identical, because their genomic loci are located within a 1.8-kb span, should be clarified for achieving a fully comprehensive view of the processes operating in carcinogenesis.

Rescued expression of downregulated or functionally-deficient miRNAs and/or inhibitors of overexpressed miRNAs may contribute to rebalanced the expression of large gene clusters implicated in oncogenesis, tumor progression, and cell death. One of the target genes for miR-143 was presently shown to be *ERK5* MAPK, which was also shown to be targeted in DLD-1 cells by us,[34] and in adipocytes by others.[37] Also, we suggest that miR-145 may target *MAP3K3* and *MAPK4K4* and that other potential targets for miR-145, which have oncogenic functions, are *MYCN*, *FOS*, *YES*, and *FLI* (http://microrna.sanger.ac.uk/) and cell-cycle promoters such as *cyclin D2* and *L1*. Particularly, the MAPK signal via ERK5 contains c-myc in the downstream of ERK5.[39] Thus, the reduced expression of both miRNAs directly or indirectly affects cell fate such as growth, survival, and death signals; that is, low-expression of miR-143 and -145 possibly by perturbed transcription and/or microprocessing, could result in an unbalanced signaling cascade including MAPK, which would lead to a sustained cell proliferation.

To our knowledge, upregulation of mir-155 from BIC RNA in B-cell lymphomas,[40] and downregulation of miR-14 and -15, which originated from the deletion of a region of chromosome

13q13.4,[28] have been reported in B-cell malignancies. Furthermore, the target gene of miR-15 and -16 was shown to be the antiapoptotic gene *BCL-2*.[29] Therefore, the genomic deletion of 13q13.4 causes an inappropriate expression of miR-15 and -16 that targets antiapoptotic *BCL-2* mRNAs, which negatively works against cell death. We found that the level of miR-15a was reduced in seven of 13 CLLs; however, the level of decrease was not as great as in the case of miRNAs-143 or -145. Our results suggested that the incidence of the 13q13.4 deletion in Japanese CLLs may be lower than that in Caucasian CLLs.[28] Recently, the same group also evaluated the miRNA expression profiles of 41 samples of CLL, and found 25 genes (of 161 analyzed) to have a unique miRNA expression signature.[41] However, miR-143 or -145 was not found to be significant with respect to the miRNA expression profile, although it is not clear whether miR-143 and -145 were examined. Previously, we reported that the expression of miRs-143 and 145 was strongly related to tumorigenesis in colon cancer,[34] because more than 80% of the cases were shown to have low-expression compared with non-cancerous tissues. In addition, all of the malignant cell lines tested were shown to be downregulated for these miRs.[34]

Thus, miRs-143 and -145 play pivotal roles in the pathogenesis of B-cell malignancies and could be plausible biomarkers to differentiate B-cell malignant cells from normal B-cells, being even better ones than miRs-15a and -16 in Japanese CLLs. Also, we propose miR-143 or -145 as a candidate for the development of RNA medicine against cancer. Further detailed study to decipher the transcription machinery of miR-143 and -145 including their promoter region on 5q32 and their binding sites in the 3′ UTR of *ERK5* will be needed for a better understanding of the carcinogenesis involving miRNAs.

Acknowledgments

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan. We thank Dr Ogio for giving tonsil as control B-cells.

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doi: 10.1111/j.1349-7006.2007.00618.x

● 悪性リンパ腫

濾胞性リンパ腫に対する治療の進歩

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木 下 朝 博

要 旨

濾胞性リンパ腫はこれまで化学療法などの効果が不十分で難治性リンパ腫とされてきた．近年抗 CD20 モノクローナル抗体や放射性同位元素標識モノクローナル抗体，プリンアナログなどといった新規薬剤の開発が進み，治療成績が改善しつつある．また，リツキシマブによる *in vivo* purging を応用した自己造血幹細胞移植や骨髄非破壊的同種造血幹細胞移植などの新しい移植法が開発されている．

はじめに

濾胞性リンパ腫（FL）は代表的な低悪性度リンパ腫病型であり，経過が緩慢で進行期でも生存期間中央値は 7 ～ 10 年と長い．しかし，化学療法の効果が不良で多くは組織学的進展（histologic transformation）などによって最終的に死亡する難治性疾患であり，長期予後は中・高悪性度リンパ腫よりもむしろ悪い．ただし，多くの新規治療法が開発導入されていることや支持療法が進歩しているため予後が改善しつつある[1]．本稿では近年著しく進歩した FL に対する治療について，進展期に対する治療を中心に解説する．

キーワード：濾胞性リンパ腫，化学療法，モノクローナル抗体，
リツキシマブ，放射線免疫療法

薬物療法

1．無治療での経過観察—watchful waiting

これまでFLは化学療法による治癒が困難なことや病状の進行が緩徐であるため，無症状，高齢者，腫瘍量が少ない，予後不良因子を有しない，合併症を有する場合には，病状が進展したり症状が出現したりするまで無治療で経過観察を行う（watchful waiting）ことも診療方針の1つとされてきた[2]．現在でもwatchful waitingは診療方針の1つとなりうるが，近年，後述するようなさまざまな新規治療法が開発されているため，より積極的に治療を開始することが多くなっていると推定される．

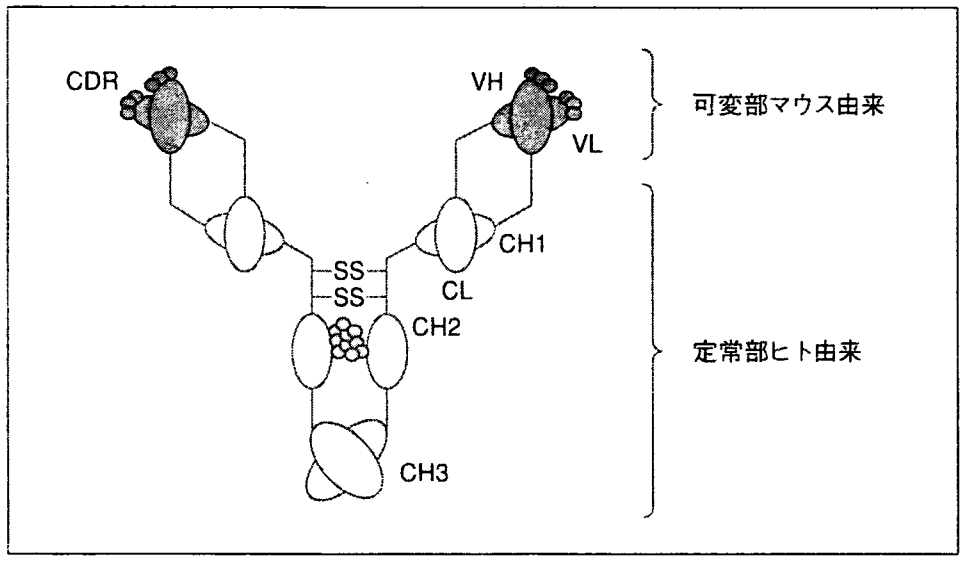
2．化学療法

FLに対する化学療法としてはシクロホスファミドなどのアルキル化剤，多剤併用療法［COPないしCVP（シクロホスファミド，ビンクリスチン，プレドニゾロン）療法やCHOP（シクロホスファミド，ドキソルビシン，ビンクリスチン，プレドニゾロン）療法など］，プリンアナログ（フルダラビン，クラドリビンなど）などがある．しかし，進展期FLに治癒をもたらしたり生存期間を延長したりすることが証明された化学療法はない．

プリンアナログのうちフルダラビンは慢性リンパ性白血病に対して広く使用されており，クラドリビンは有毛細胞白血病（hairy cell leukemia）に対する標準的治療薬と位置づけられている．これらの薬剤はFLを始めとする低悪性度リンパ腫にも広く用いられている．FLに対するフルダラビン単剤療法では完全寛解（CR）37％，奏効率65％が得られた[3]．また，フルダラビンはシクロホスファミドやデキサメタゾン，ミトキサントロンなどとの併用療法も行われており，良好な奏効率が報告されている[4]．クラドリビンも低悪性度リンパ腫に対して有効な薬剤であり，高い奏効率を示す[5]．

現在ではこれらの化学療法は後述する抗CD20モノクローナル抗体リツキシマブと併用されることが多く，優れた治療成績が報告されている．

図1　抗 CD20 モノクローナル抗体，リツキシマブの構造



略語：巻末の「今号の略語」参照

3．抗 CD20 モノクローナル抗体，リツキシマブ

マウス・ヒトキメラ型抗 CD20 モノクローナル抗体であるリツキシマブはB細胞表面の分化抗原である CD20 を標的とする薬剤であり，B細胞リンパ腫に対して高い治療効果を示す（図1）．再発・再燃低悪性度B細胞リンパ腫を対象として行われた単剤投与試験では，CR 6％，部分寛解（PR）42％ で奏効率は 48％，効果持続期間の中央値は 13ヵ月だった[6]．

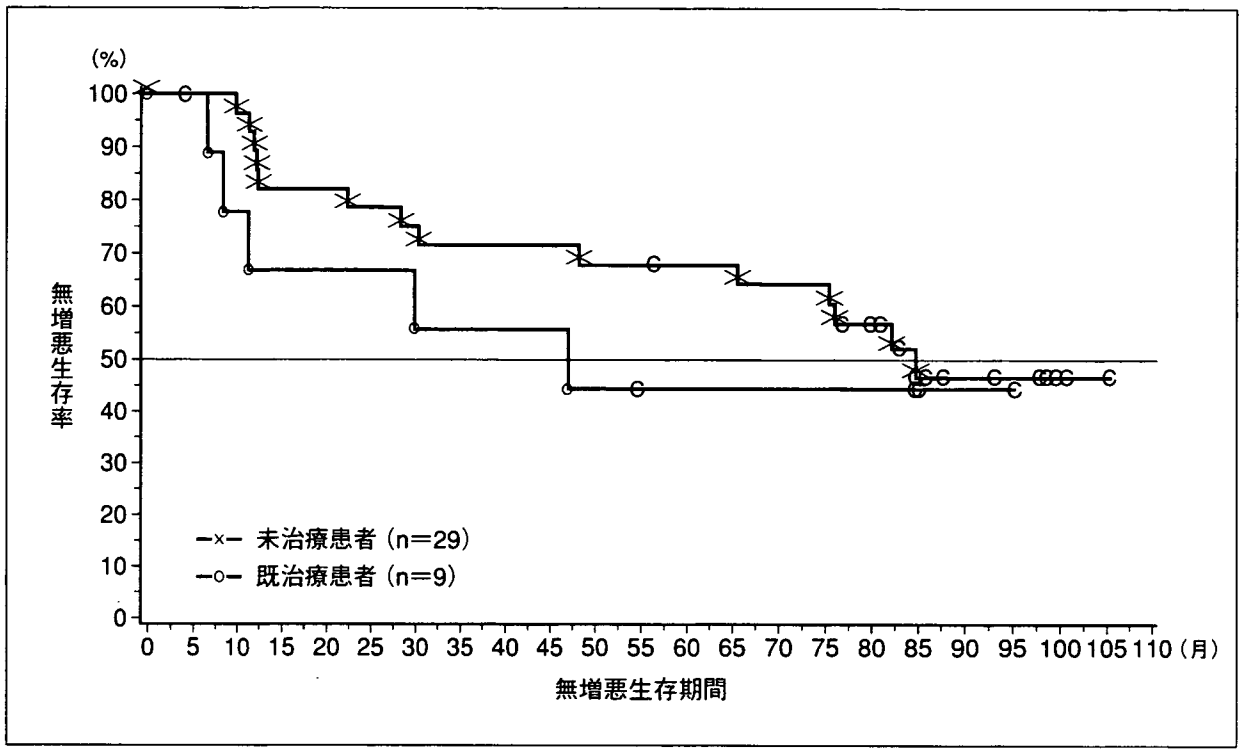
一般的に FL ではポリメラーゼ連鎖反応（PCR）レベルで検出される微少残存病変（MRD）を通常化学療法で消失させることは困難とされる．これに対してリツキシマブは MRD レベルでみた場合にも高い治療効果を示す．単剤での治療の場合，FL で 22～62％ に MRD 消失が得られる[7]．

4．リツキシマブと化学療法の併用

リツキシマブは通常の化学療法剤と有害反応が重複しないことから化学療法への併用が可能である．FL を中心とする低悪性度Bリンパ腫に対して，CHOP 療法との併用により CR 55％，奏効率 95％ という極めて良好な治療効果が報告された（図2）[8]．

リツキシマブ併用化学療法は併用しない化学療法よりも良好な治療成績を示す．未治療進展期 FL を対象としてリツキシマブ併用 CVP

図2 R-CHOP療法による低悪性度B細胞リンパ腫の無増悪生存期間(未治療患者と既治療患者のデータ)
(文献[8]より引用改変)



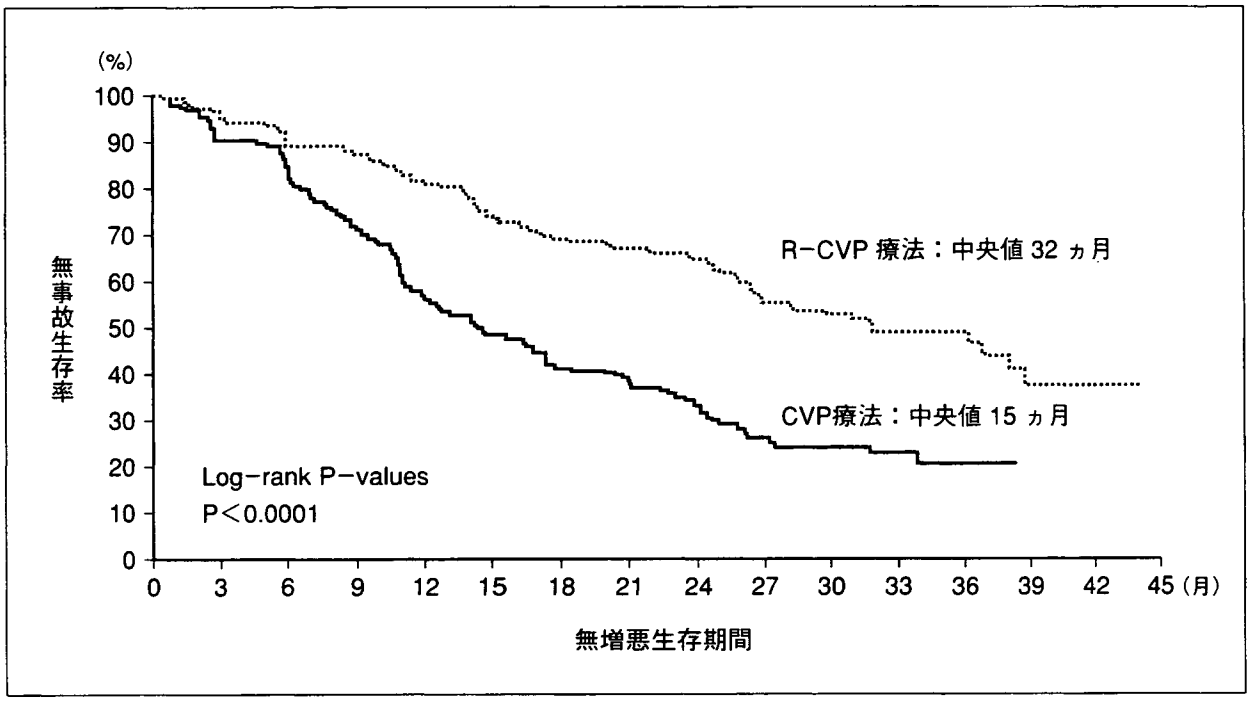
(シクロホスファミド，ビンクリスチン，プレドニゾロン)(R-CVP)療法をCVP療法と比較する試験が行われ，全奏効率およびCR率，無増悪生存期間すべてでR-CVPがCVPに対して勝った（図3）[9]．シクロホスファミド，フルダラビン，ミトキサントロン（FCM）療法とリツキシマブの併用療法についてもFCM療法との無作為化比較試験が行われ，R-FCMがFCMに対してCR率や全奏効率で有意に勝っていた[10]．このようにリツキシマブ併用化学療法はFLに対して高い治療効果を示すことから，リツキシマブ併用化学療法がFLに対する標準的治療と位置づけられつつある．

その他のリツキシマブ併用化学療法としてはプリンアナログとの併用の試みがあり，例えばフルダラビンとの併用ではCR 80％を含む奏効率90％，およびMRD消失効果が88％に得られた[11]．

5．リツキシマブ維持療法

リツキシマブによる維持療法もFLに対する重要な治療法の1つである．再発・再燃FLに対するCHOPまたはR-CHOP治療後のリツキシマブ維持療法に関する検討では，CHOPまたはR-CHOPい

図3 未治療進展期濾胞性リンパ腫（FL）に対するR-CVP療法とCVP療法の比較試験における無増悪生存期間（文献[9]より引用改変）

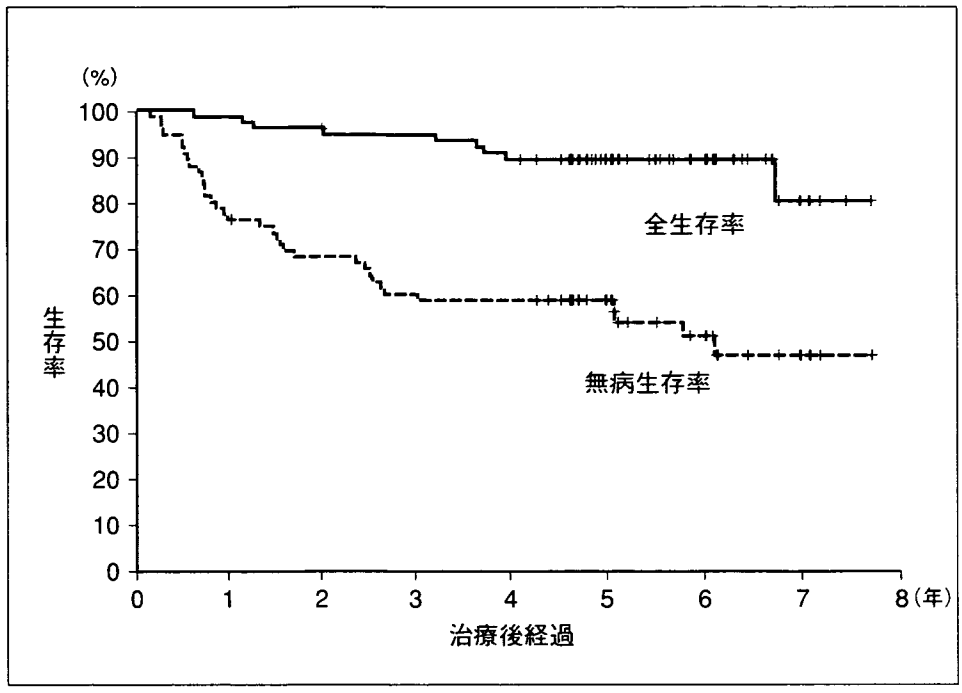


ずれで治療されたFLにおいてもリツキシマブ維持療法によって無増悪生存期間，全生存期間の改善が認められた[12]．今後我が国においてもリツキシマブ維持療法の承認が期待される．

6．放射性同位元素標識モノクローナル抗体

リンパ腫細胞は放射線治療に対する感受性が高いため，アイソトープ抱合モノクローナル抗体（radioimmunoconjugates；RIC）の開発が進められた．RICは抗体が直接結合できなかった近接細胞に対してもクロスファイアー効果によって放射線治療効果を発現する．CD20を標的とするものとしては，yttrium-90（^{90}Y）ibritumomab tiuxetan[13]，iodine-131（^{131}I）tositumomab[14]がある．未治療進展期FLに対する^{131}I tositumomab治療では，CR 75％，全奏効率は95％で，CR例の80％はPCRレベルのMRDも陰性化した[15]．5年無増悪生存率と全生存率はそれぞれ59％と89％，無増悪生存期間の中央値は6.1年と極めて良好だった（図4）．また，未治療FLを対象として，CHOP療法に引き続いて^{131}I tositumomabを投与する第Ⅱ相試験が行われ，CR率は69％，5年全生存率と無増悪生存率はそれぞれ87％と67％と極めて良好だった[16]．

図4 未治療進展期濾胞性リンパ腫（FL）に対する ^{131}I tositumomab 治療の無病生存曲線および全生存曲線（文献[15]より引用改変）



このようにRICはFLに対して高い有効性を示す新規薬剤であり，移植前処置への応用も含め，今後FL治療において大きな役割を果たすことが期待される．

7．造血幹細胞移植

1）自己造血幹細胞移植併用大量化学療法

FLは通常化学療法による治癒が困難な難治性疾患であることから，自己造血幹細胞移植（AHSCT）を併用した大量放射線化学療法が盛んに研究されてきた．FLでは高率に骨髄浸潤を認めるため移植片中のリンパ腫細胞を除去することが重要である．現在では *ex vivo* purgingに代わりリツキシマブを用いた *in vivo* purgingが広く応用されている[17]．

初発進行期FLに対するupfront AHSCTの有用性に関する比較試験は少ない．未治療FLを対象としたAHSCTとインターフェロンによる維持療法の無作為化比較試験では，5年無増悪生存率では移植群がインターフェロン群に勝った．しかし，生存期間についてはデータが未公表である[18]．また，再発・治療抵抗性FLを対象とした通常化学療法とAHSCTの比較試験では，化学療法群に比べてAHSCT

群で無増悪生存期間が有意に良好だったが生存期間には有意差を認めなかった[19].

近年リツキシマブに代表される新規薬剤の導入によって FL の治療成績が向上しつつあることや，AHSCT に伴う二次発がん，特に骨髄異形成症候群が問題となることなどから，FL に対して実地医療として早期に AHSCT を行うべきではないと考えられている．今後 AHSCT の適応や移植時期についての検討が必要である．

2）同種造血幹細胞移植

一般的には同種造血細胞移植は AHSCT 後の再発症例を対象に行われる場合が多く，移植片対白血病（GVL）効果によって FL に対して治癒が期待できる治療と言えるが，一方，治療関連死亡率が 30～40％と高いことが問題である．近年，移植前処置の毒性を軽減した骨髄非破壊的同種造血幹細胞移植（RIST）が盛んに試みられており，少数例ながら有望な治療成績が報告されている[20].

おわりに

このように FL に対してはリツキシマブに代表される有効な新規薬剤の開発が進んでおり，高い治療効果が得られている．今後これら薬剤や造血細胞移植療法を効果的に組み合わせた治療戦略を確立する必要がある．

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