Table 5
Anti-proliferative activity of 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives against several cell lines

Compound	MOLM-13 GI_{50} (nmol/L)	ML-1 GI_{50} (nmol/L)	FLT3-Δ599GI_{50} (nmol/L)	FLT-D835Y GI_{50} (nmol/L)
2	36 ± 3.4	4318 ± 905	36 ± 9.2	52 ± 13
21a	56 ± 8.6	5958 ± 1552	76 ± 22	116 ± 38
23c	19 ± 3.6	>10,000	37 ± 11	31 ± 5.9
23f	29 ± 1.9	9108 ± 1045	74 ± 14	65 ± 21
23k	102 ± 5.1	>10,000	114 ± 13	63 ± 10
23r	94 ± 10	>10,000	74 ± 14	48 ± 19

GI_{50} values are ±SEM means of three experiments. See Ref. 16 for assay details.

Table 6
Antitumor activity of 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives

Compound	Dose[a] (mg/kg)	T/C_{min}[b] (on day)	Regression V/V_0 min[c] (on day)	Mortality
Control		1.00	–	0/5
2	50	0.47 (7)	–	0/5
	100	0.34 (7)	–	1/5
23a	50	0.45 (7)	–	0/5
23c	50	0.27 (7)	–	0/5
23f	50	0.25 (10)	–	0/5
	100	0.10 (7)	0.571 (4)	0/5
	200	0.006 (10)	0.098 (10)	0/5
23k	50	0.24 (7)	–	0/5
23r	50	0.20 (7)	1.39 (7)	0/5
	100	0.014 (7)	0.12 (7)	0/5

[a] Compounds were orally administrated at b.i.d. intervals for 5 days.
[b] Averages of tumor volume ratio in the treated to control mice.
[c] Averages of the relative tumor volume to the initial tumor volume on day 0. See Ref. 10 for assay details.

metabolism (**23f** vs **23q**) at the 4-position was also effective; however, cycloalkyl moieties (**23l** and **23o**) exhibited relatively low metabolic stability. Introduction of an alkoxy unit (**23m**) resulted in a substantial reduction in activity likewise for **7i**.

Among them, several compounds were subjected to evaluation for anti-proliferative activity against human leukemia cell lines (Table 5).[20,21] These selected compounds showed significant inhibition for mutant FLT3-expressing cells in addition to MOLM-13 cells, while weak or no inhibition for ML1 cells expressing wild-type FLT3 was observed. Compound **23f** also exhibited selective kinase inhibitory profile against FLT3 kinase as previously described.[10]

Finally, these antitumor effects were evaluated by oral administration to sc MOLM-13 tumor xenograft model in SCID mice (Table 6). All compounds except for **23a** revealed more potent activity than that of **2** without mortality nor body weight loss. Above all, **23f** and **23r** showed significant antitumor activity including tumor regression in a dose dependent manner.

In summary, we have demonstrated a novel class of 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives showed anti-proliferative activity against MOLM-13 cells by SAR investigations to aim for metabolic stability. Decreased lipophilicity by incorporating polar group into the 1,3,4-oxadiazolyl group at the 5-position of the pyrimidine ring was effective to improve metabolic stability. Interestingly, our compounds showed selective anti-proliferative activity against multiple leukemia cell lines having FLT3/ITD mutation without inhibition of wild-type FLT3 expressing cells. As a result, some of them showed potent in vivo antitumor activity with tumor regression against sc tumor xenograft model in SCID mice. Further SAR studies of this class of FLT3 inhibitors will be reported elsewhere together with detailed biological data.

Acknowledgments

We are grateful to Ms. Miyako Mizuguchi, Ms. Hiroko akamura, and Ms. Naoko Kondo for their helpful technical assistance. We are indebted to Dr. Tatsuhiro Ogawa for his helpful discussion. We also thank Ms. Nana Oiwa, Mr. Satoshi Tashiro, Ms. Shinobu Shioya, Ms. Yoko Tahara, and Ms. Masayo Suzuki for conducting the human liver microsomal stability studies and in vitro cytotoxic assays.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.031.

References and notes

1. Rosnet, O.; Schiff, C.; Pebusque, M. J.; Marchetto, S.; Tonnelle, C.; Toiron, Y.; Birg, F.; Birnbaum, D. *Blood* **1993**, *82*, 1110.
2. Lyman, S. D.; James, L.; Zappone, J.; Sleath, P. R.; Beckmann, M. P.; Bird, T. *Oncogene* **1993**, *8*, 815.
3. Kiyoi, H.; Towatari, M.; Yokota, S.; Hamaguchi, M.; Ohno, R.; Saito, H.; Naoe, T. *Leukemia* **1998**, *12*, 1333.
4. Kiyoi, H.; Yanada, M.; Ozeki, K. *Int. J. Hematol.* **2005**, *82*, 85.
5. Levis, M.; Small, D. *Leukemia* **2003**, *17*, 1738.
6. Stone, R. M.; DeAngelo, D. J.; Klimek, V.; Galinsky, I.; Estey, E.; Nimer, S. D.; Grandin, W.; Lebwohl, D.; Wang, Y.; Cohen, P.; Fox, E. A.; Neuberg, D.; Clark, J.; Gilliland, D. G.; Griffin, J. D. *Blood* **2005**, *105*, 54.
7. Knapper, S.; Burnett, A. K.; Littlewood, T.; Kell, W. J.; Agrawal, S.; Chopra, R.; Clark, R.; Levis, M. J.; Small, D. *Blood* **2006**, *108*, 3262.
8. Fiedler, W.; Serve, H.; Döhner, H.; Schwittay, M.; Ottmann, O. G.; O'Farrell, A.-M.; Bello, C. L.; Allred, R.; Manning, W. C.; Cherrington, J. M.; Louie, S. G.; Hong, W.; Brega, N. M.; Massimini, G.; Scigalla, P.; Berdel, W. E.; Hossfeld, D. K. *Blood* **2005**, *105*, 986.
9. DeAngelo, D. J.; Stone, R. M.; Heaney, M. L.; Nimer, S. D.; Paquette, R. L.; Klisovic, R. B.; Caligiuri, M. A.; Cooper, M. R.; Lecerf, J.-M.; Karol, M. D.; Sheng, S.; Holford, N.; Curtin, P. T.; Druker, B. J.; Heinrich, M. C. *Blood* **2006**, *108*, 3674.
10. Kiyoi, H.; Shiotsu, Y.; Ozeki, K.; Yamaji, S.; Kosugi, H.; Umehara, H.; Shimizu, M.; Arai, H.; Ishii, K.; Akinaga, S.; Naoe, T. *Clin. Cancer Res.* **2007**, *13*, 4575.
11. (a) Brown, P.; Best, D. J.; Broom, N. J. P.; Cassels, R.; O'Hanlon, P. J.; Mitchell, T. J.; Osborne, N. F.; Wilson, J. M. *J. Med. Chem.* **1997**, *40*, 2563; (b) Rajapakse, H. A.; Zhu, H.; Young, M. B.; Mott, B. T. *Tetrahedron Lett.* **2006**, *47*, 4827.
12. Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*; John Wiley and Sons: New York, 1991. chapter 2, p 41 and references cited therein.
13. Landreau, C.; Deniaud, D.; Reliquet, A.; Reliquet, F.; Meslin, J. C. *J. Heterocycl. Chem.* **2001**, *38*, 93.
14. Gerstenberger, B. S.; Konoperski, J. P. *J. Org. Chem.* **2005**, *70*, 1467.
15. *FLT3 kinase assays.* To evaluate the kinase inhibitory activities against FLT3 by ELISA, GST-tagged FLT3 cytoplasmic domain and biotinylated poly-(Glu/Tyr 4:1) substrate (CIS bio International) bound to the surface of 96-well assay plates were used. Phosphorylated substrate was bound to anti-phosphotyrosine antibody conjugated to europium. The bound antibody was measured using ARVO (Perkin-Elmer) at excitation/emission of 340/615 nm.
16. *Growth inhibition assays.* MOLM-13, ML-1, FLT3/Δ599, and FLT3/D835Y were maintained in RPMI1640 with 10% heat-inactivated FBS, 1% penicillin, and 1% streptomycin. HMC-1 cells were maintained in IMEM with 10% heat-inactivated FBS, 0.01% α-thioglycerol, 1% penicillin, and 1% streptomycin. These cell lines were seeded in 96-well culture plates in appropriate media and incubated for 72 h in the presence of test compounds. Cell viability was measured using the cell proliferation reagent WST-1 (Roche) according to the instructions of the manufacture; see also Ref. 19 and 20.
17. *Human liver microsomes (HLM) stability procedure.* Human pooled liver microsomes were purchased from Human and Animal Bridging Research Organization. Reaction mixtures ($n = 1$ or 2) containing test compound (0.1 or 1 μmol/L), liver microsomes (0.2 mg protein/mL), nicotinamide adenine dinucleotide phosphate, reduced form (5 mmol/L), phosphate buffer (100 mmol/L, pH 7.4), $MgCl_2$ (6 mmol/L), EDTA (0.1 mmol/L), and DMSO (0.01 or 0.001 vol%) or methanol (1 vol%) were incubated at 37 °C for constant time. Just before and after incubation, aliquots of the reaction mixtures were added to twofold volume of CH_3CN containing internal standard to stop the reaction (samples for quantitative analysis). After centrifugation, the supernatant was

analyzed by LC–MS or LC–MS/MS. The values of intrinsic clearance were calculated from percent remaining of the test compound.
18. Cain, G. A.; Beck, J. P. *Heterocycles* **2001**, *55*, 439.
19. Daylight version 4.83; Daylight Chemical Information Systems, Inc.: Mission Viejo, CA, 2003.
20. Yamamoto, Y.; Kiyoi, H.; Nakano, Y.; Suzuki, R.; Kodera, Y.; Miyawaki, S.; Asou, N.; Kuriyama, K.; Yagasaki, F.; Shimazaki, C.; Akiyama, H.; Saito, K.; Nishimura, M.; Motoji, T.; Shinagawa, K.; Takeshita, A.; Saito, H.; Ueda, R.; Ohno, R.; Naoe, T. *Blood* **2001**, *97*, 2434.
21. Kiyoi, H.; Ohno, R.; Ueda, R.; Saito, H.; Naoe, T. *Oncogene* **2002**, *21*, 2555.

European Journal of Haematology ISSN 0902-4441

ORIGINAL ARTICLE

Prognostic implication and biological roles of RhoH in acute myeloid leukaemia

Toshihiro Iwasaki[1], Akira Katsumi[1], Hitoshi Kiyoi[2], Ryohei Tanizaki[1], Yuichi Ishikawa[1], Kazutaka Ozeki[2], Miki Kobayashi[1], Akihiro Abe[1], Tadashi Matsushita[1], Takashi Watanabe[3,4], Mutsuki Amano[4], Tetsuhito Kojima[5], Kozo Kaibuchi[4], Tomoki Naoe[1]

[1]Department of Hematology and Oncology; [2]Department of Infectious Diseases; [3]Institute for Advanced Research; [4]Department of Cell Pharmacology; [5]Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract

The Rho family of small GTPases, including Rho, Rac and Cdc42, has been well characterised as a molecular switch that transduces signals from plasma membrane to the downstream effectors. *RhoH* gene, a member of the Rho family, is specifically expressed in haematopoietic cells. The known function of RhoH is antagonising Rac and mediating activation of ZAP-70 in T lymphocytes; however, biological roles of RhoH in myeloid cells remain unknown. Here, we analysed the prognostic implication of the expression level of the *RhoH* gene transcript in bone marrow samples from 90 newly diagnosed acute myeloid leukaemia (AML) patients using a real-time fluorescence detection method. Kaplan–Meier analysis demonstrated that low expression of the *RhoH* transcript was a predictor of worse prognosis in both overall and disease-free survival. Multivariate analysis demonstrated that low expression of *RhoH* was an independent unfavourable prognostic factor for both overall and disease-free survival of AML patients. Overexpression of *RhoH* leads to dephosphorylation of Bad at Serine 75 residue possibly through deactivation of Rac. It is possible that low expression of *RhoH* (i.e. high GTP-Rac) contributes to chemotherapy resistance in leukaemia cells. Our result suggests that inhibition of Rac and its signalling components might provide a useful anti-leukaemic strategy.

Key words RhoH; Rac; gene expression; acute myeloid leukaemia; prognosis

Correspondence Akira Katsumi, MD, PhD, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: 81 52 744 2145; Fax: 81 52 744 2161; e-mail: katsumi@med.nagoya-u.ac.jp

Accepted for publication 26 July 2008

doi:10.1111/j.1600-0609.2008.01132.x

The Rho family of small GTPases, including Rho, Rac and Cdc42, has been well characterised as a molecular switch that regulates actin organisation, cell adhesion and migration. *RhoH* gene, also known as Translocation Three Four (*TTF*) gene, was first isolated as a fusion partner with the *BCL6* gene, which is frequently disrupted in diffuse large B-cell lymphoma (DLBCL) (1). Previous reports demonstrated that the aberrant somatic hypermutation (SHM) of *RhoH* is a novel mechanism of lymphomagenesis, possibly through its deregulated expression, suggesting that *RhoH* is the proto-oncogene (2). Although SHM might contribute to the onset of malignant lymphoma through regulation of *RhoH* gene expression, it has less impact on survival of DLBCL patients (3).

RhoH expression is widespread in haematopoietic cells, including bone marrow progenitor and differentiated myeloid and lymphoid cells (4). RhoH has been reported to downregulate Rac (4) and LFA-1 (5), although the effects on LFA-1 are still controversial (6). Recent reports demonstrated that RhoH is the critical regulator of thymocyte development and T-cell receptor (TCR) signalling by mediating recruitment and activation of ZAP-70 (7).

As RhoH is GTPase-deficient and constitutively active (8), the activity of RhoH is directly related to its level of expression in a GTP-bound form. Although the function of RhoH in lymphoid cells has been clarified, the biological roles of RhoH in the myeloid cells remain unknown. The question that whether *RhoH* expression affects the prognosis of acute myeloid leukaemia (AML) patients still remains unanswered. To address this question, we screened bone marrow samples obtained from 90 previously untreated AML cases for the expression level of the *RhoH* gene.

Materials and methods

Patients and samples

The diagnosis of AML was based on the French–American–British (FAB) classification. Genomic DNA was obtained from the bone marrow of 90 adult patients with *de novo* AML. Patients comprised the subtypes M0 (n = 1), M1 (n = 22), M2 (n = 39), M4 (n = 19), M5 (n = 8) and M6 (n = 1). Bone marrow samples from the AML patients were subjected to Ficoll–Hypaque (Pharmacia LKB, Uppsala, Sweden) density-gradient centrifugation. All samples were confirmed to contain more than 90% leukaemia cells and were then cryopreserved in liquid nitrogen before analysis. The cytogenic G-banding analysis was performed with standard methods. The cytogenetic risk groups were stratified according to the Medical Research Council (9). Among the 90 AML patients analysed, patients younger than 65 yr old were treated with the AML87 (10), AML89 (11) or AML92 (12) protocol of the Japan Adult Leukemia Study Group (JALSG) or their modification. The patients over 65 yr old were treated with the dose-reduced regimens of the JALSG protocols. The patients receive 3–4 consolidation therapies after confirmation of complete remission (CR). Informed consent was obtained from all the patients and healthy controls to use their samples for banking and molecular analysis, and approval for these studies was obtained from the Ethics Committee of Nagoya University.

Total RNA was extracted from the samples using a QIAamp RNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). cDNA was synthesised from each RNA sample using a random primer and Moloney murine leukaemia virus reverse transcriptase (Super-Script II; Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The expression level of the *RhoH* transcripts was quantified using a real-time fluorescence detection method on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described previously (13). The relative gene expression levels were calculated from standard curves and adjusted on the basis of the expression level of the *GAPDH* gene. Each gene expression level was analysed in triplicate, and the mean was subjected to analysis. cDNA extracted from the Raji cells was used as the standard. *FLT3* gene mutation of the internal tandem duplication (ITD) (14) and kinase domain mutation (15), *NRAS* gene mutation of codons 12, 13 and 61 (16), *NPM1* gene mutation of exon 12 (17) were examined as described previously.

Plasmids and cells

Total RNA was isolated from cells of a human leukaemia cell line, Jurkat, as described above. The RT-PCR product encoding human *RhoH* cDNA was subcloned into the pCR-BluntII-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequence analysis, followed by introduction of the N-terminal FLAG sequence by PCR mutagenesis. The FLAG-human *RhoH* cDNA was subcloned into the pcDNA3.1 vector (Invitrogen). pcDNA3-Tiam1-C1199-HA was kindly provided by Dr John G. Collard, the Netherlands Cancer Institute. pEGFP-C1 Rac wild type was kindly provided by Dr Miguel A. del Pozo, Centro Nacional de Investigaciones Cardiovasculares, Spain.

Statistical analyses

Differences in median variables in age and peripheral white blood cell (WBC) counts were analysed with the Mann–Whitney *U*-test for distribution among the two groups, or the Kruskal–Wallis test and Bonferroni test for distribution among more than three groups. Survival probabilities were estimated by Kaplan–Meier methods, and differences in survival distributions were evaluated by the log-rank test. The patients undergoing haematopoietic stem cell transplantation were censored at the time of transplantation. The prognostic significance of the clinical variable was assessed using the Cox proportional hazards model. These statistical analyses were performed with the STATVIEW-J 5.0 (Abacus Concepts, Berkeley, CA, USA). For all analysis, the *P*-values were twotailed, and a *P*-value of less than 0.05 was considered statistically significant.

Rac activation assay

Rac activation assay was performed as described previously (18). Briefly, the treated cells were washed with ice-cold phosphate-buffered saline and lysed in 400 μL of buffer containing 0.5% NP-40, 50 mM Tris–HCl (pH 7.0), 500 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 20 μg GST-PBD. The lysates were centrifuged to remove

particulates and incubated with glutathione-agarose beads (Sigma, St Louis, MO, USA) for 30 min at 4°C, washed thrice and eluted with SDS sample buffer. Rac protein was detected by Western blotting using a monoclonal antibody against Rac1 (Millipore, Billerica, MA, USA). Densitometry analysis was performed using the SCION IMAGE software (Scion Corporation, Frederick, MD, USA).

Bad phosphorylation assay

The cells were lysed with lysis buffer containing 0.5% NP-40, 50 mM Tris-HCl (pH 7.0), 50 mM NaCl, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and phosphatase inhibitor cocktail 2 (Sigma). To this, SDS-PAGE sample buffer was added. Phosphorylated and total Bad were detected by Western blotting using antibodies specific to phospho-Bad S112 (Cell Signaling Technology Inc, Danvers, MA, USA) and phospho-Bad S136 (Cell Signaling Technology) and total Bad (BD Transduction Laboratories, Franklin Lakes, NJ, USA), respectively.

Results

Clinical characteristics and prognosis of AML patients according to the RhoH transcript level

We screened for the expression level of *RhoH* gene in the bone marrow samples from AML patients using a real-time fluorescence detection method. We also quantified the expression level of *RhoH* in CD34 positive fraction of normal peripheral blood mononuclear cells and granulocytes. *RhoH* mRNA level of leukaemic blasts is within the same range in normal mononuclear cells (Fig. 1). There were no significant differences in *RhoH* transcript levels among the FAB subtypes. The *RhoH/GAPDH* ratio was calculated and the median of 90 samples was 1.57. The 90 patients were then divided into *RhoH* high (i.e. *RhoH/GAPDH* above 1.58) and *RhoH* low groups. The expression level of *RhoH* was not related to the FAB classification, age, WBC counts, cytogenetics or CR rate (Table 1). In addition, *RhoH* expression was not associated with the known gene mutations such as *NRAS*, *FLT3* and *TP53* (Table 2). Kaplan–Meier analysis demonstrated that low expression of the *RhoH* transcript is a predictor of worse prognosis for both overall and disease-free survival (P = 0.028 and 0.003, respectively) (Fig. 2A,B). Patients were then categorised into favourable, intermediate, unfavourable and unknown cytogenetic groups based on the Southwest Oncology Group and Medical Research Council cytogenetic risk category definitions (19). Kaplan–Meier analysis for both overall and disease-free survival was performed for 65 (72.2%) patients in the intermediate risk group. The results indicated that low expression of the *RhoH* transcript is a worse prognostic factor for both overall and disease-free survival in the intermediate risk group (P = 0.03 and 0.001, respectively) (Fig. 3A,B).

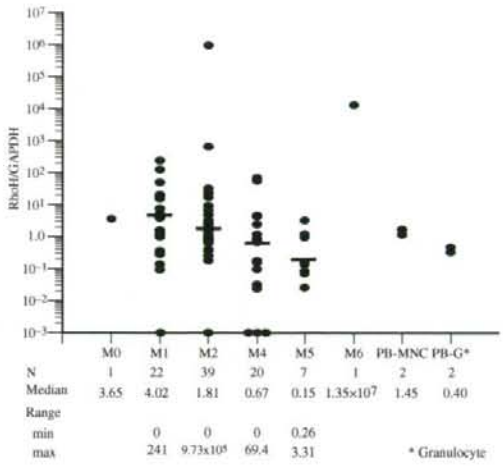

Figure 1 Expression level of the *RhoH* transcript according to the FAB classification. Distribution of the expression level of *RhoH* transcript is indicated according to the FAB classification. The relative gene expression levels were calculated using standard curves and adjusted on the basis of the expression level of the *GAPDH* gene. Normal haematopoietic cells included each of two peripheral blood mononuclear cells and granulocytes. Horizontal bars indicate each mean value.

Table 1 Relationship between expression of the *RhoH* transcript and clinical characteristics in 90 AML cases

	High group (n=45)	Low group (n=45)	P-value
FAB			0.05
M0	1	0	
M1	14	8	
M2	22	17	
M4	6	14	
M5	1	6	
M6	1	0	
Age, yr (range)	48 (16–76)	51 (15–85)	0.224
WBC count, $\times 10^9$/L (range)	23.8 (1.3–300)	33.2 (2.4–180)	0.175
Cytogenetics*			0.19
Favourable	8	12	
Intermediate	34	31	
Normal karyotype	18	19	
Unfavourable	3	2	
Outcome (CR or failure)			0.657
CR	37	35	
Failure	8	10	

Table 2 Associations of *RhoH* expression with previously reported gene mutations

Gene mutations	*n*	Relative *RhoH* transcript level (median)	*P*-value
FLT3 mutations			0.934
mutation (+)	23	1.58	
mutation (−)	67	1.56	
NRAS mutation			0.403
mutation (+)	8	0.935	
mutation (−)	82	1.583	
TP53 mutation			0.081
mutation (+)	3	4.941	
mutation (−)	87	1.53	
NPM1 mutation			0.8532
mutation (+)	23	1.21	
mutation (−)	67	1.602	

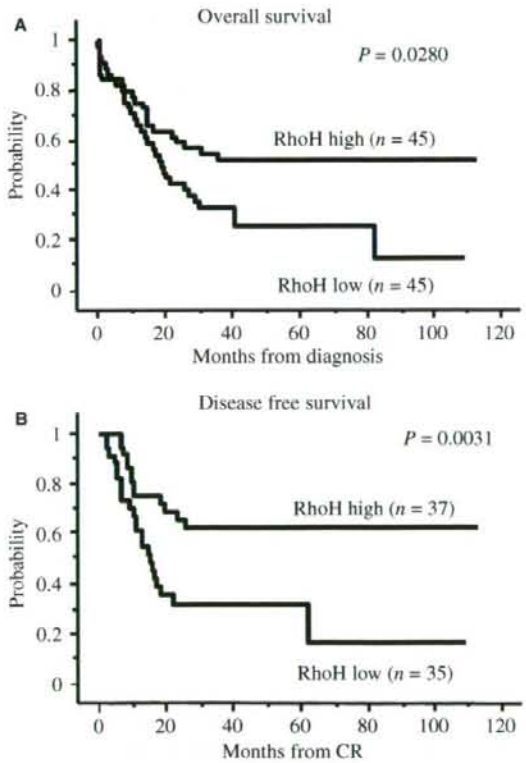

Figure 2 Overall and disease-free survival of AML patients according to the expression level of *RhoH*. Kaplan–Meier analysis of both overall (A) and disease-free (B) survival of all patients demonstrated that low expression of the *RhoH* transcript is a worse prognostic factor ($P = 0.028$ and 0.003, respectively). Statistical difference was evaluated using the log-rank test.

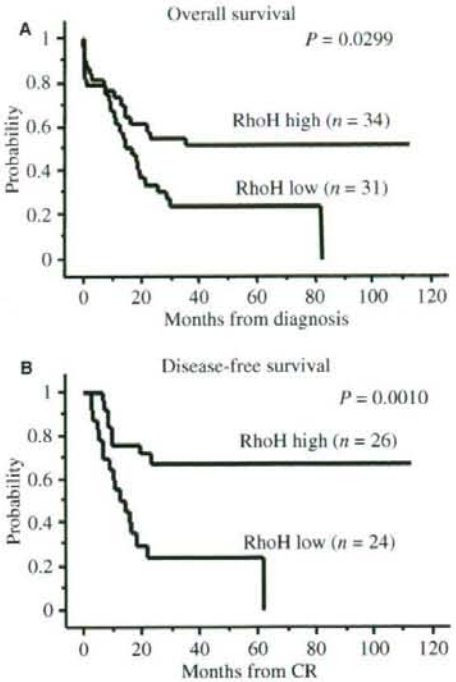

Figure 3 Overall and disease-free survival according to the expression level of *RhoH* in the intermediate risk group AML patients. Kaplan–Meier analysis for both overall (A) and disease-free (B) survival was performed for patients in the intermediate risk group [i.e. normal karyotype, +8, +6, −Y, del (12p)]. The results indicated that low expression of the *RhoH* transcript is a worse prognostic factor in the intermediate risk group ($P = 0.03$ and 0.001, respectively). Statistical difference was evaluated using the log-rank test.

Multivariate analyses showed that presence of the*TP53* mutation [relative risk (RR) 10.175; $P = 0.0036$), high WBC count (more than 100×10^9/L; RR 3.457; $P = 0.0003$) and low *RhoH* expression (RR 2.272; $P = 0.0091$) were independent poor prognostic factors for overall survival (Table 3). The same analysis revealed that the presence of the *TP53* mutation (RR 14.292; $P = 0.0168$), low *RhoH* expression (RR 4.854; $P = 0.0002$), high WBC count (RR 2.901; $P = 0.0187$) and presence of *FLT3*/ITD (RR 2.515; $P = 0.0419$) were independent poor prognostic factors for disease-free survival (Table 3).

RhoH downregulates Bad phosphorylation at serine-75

Next, we investigated the biological roles of RhoH in AML. Previous studies demonstrated that RhoH antagonises the Rac-mediated signalling (4, 8). COS7 cells were

Table 3 Multivariate analysis according to expression *of RhoH in* 90 AML cases

Prognostic factors	*P*-value	Relative risk (95% CI)
Overall survival		
WBC count more than 100×10^9/L	0.0003	3.457 (1.778–6.720)
TP53 mutation positive	0.0036	10.175 (2.136–48.470)
Low *RhoH* expression	0.0091	2.272 (1.225–4.201)
Disease-free survival		
FLT3 ITD positive	0.0419	2.515 (1.034–6.117)
WHO count more than 100×10^9/L	0.0187	2.901 (1.194–7.049)
TP53 mutation positive	0.0168	14.292 (1.616–126.384)
Low *RhoH* expression	0.0002	4.854 (2.083–11.235)

co-transfected with a vector coding for GFP-wild type Rac and NH_2-terminally truncated active form Tiam-1, a GEF-specific for Rac (20). Co-expression of RhoH in these cells induces Rac deactivation (Fig. 4A). Rac is known to stimulate the phosphorylation of serine(S)-75 (corresponding to murine S112) of Bad via PAK1, thereby suppressing drug-induced caspase activation and apoptosis in human lymphoma cells (21). Overexpression of RhoH dephosphorylates S75 of Bad (Fig. 4B). Contrastingly, RhoH expression does not affect phosphorylation of S99 (corresponding to murine S136) of Bad and S473 of Akt (data not shown).

Discussion

The human *RhoH* gene encodes a 191-amino acid protein belonging to the Rho family of GTPases. As RhoH is naturally GTPase-deficient and remains constitutively active, its activity is directly related to the level of expression. *RhoH* gene is expressed widely in the haematopoietic cells, including bone marrow, spleen and predominantly in T and B cells. RhoH is known to antagonise Rac by suppressing its ability to target membrane (22). Recent studies have demonstrated that RhoH acts as a critical regulator in thymocyte development and TCR signalling, by mediating recruitment and activation of non-receptor tyrosine kinase ZAP-70 (6, 7).

Although the roles of RhoH in T lymphocytes have been extensively studied, its function in myeloid cells is unknown. In this report, we have identified RhoH as a prognostic factor for AML. No significant differences in the FAB classification, age, WBC count, cytogenetics and CR ratio were noted between *RhoH* high and low groups (Table 1). In addition, *RhoH* transcript level is not associated with any known gene mutations such as *FLT3*, *NRAS TP53* and *NPM1* (Table 2). These findings, as well as multivariate analysis, demonstrated that low expression of *RhoH* is the independent unfavourable prognostic factor for both overall and disease-free survival in AML.

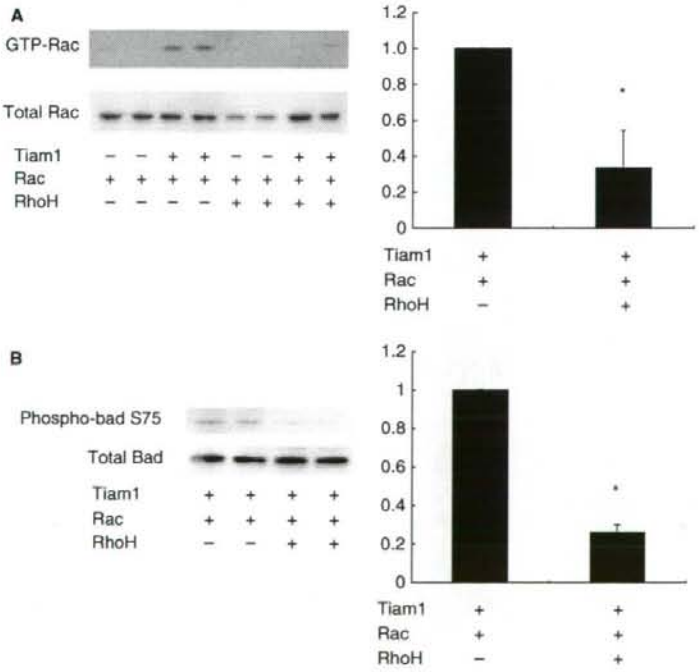

Figure 4 Biological effects of *RhoH* expression. (A) COS7 cells were transiently transfected with the indicated vectors. Cells were lysed and Rac pull-down assays were performed. Rac was detected by Western blotting. One representative blot of three independent experiments is shown (left). For densitometric quantification, relative Rac activity was calculated from the amount of PBD-bound Rac normalised to the amount of total Rac. Results were mean ± standard deviation from three experiments. *A *P*-value of <0.02 (right). (B) COS7 cells were transiently transfected with the indicated vectors. Cells were lysed and both total Bad and phospho-S75 of Bad were detected by Western blotting. One representative blot of four independent experiments is shown (left). For densitometric quantification, relative Bad phosphorylation was calculated from the phospho-Bad normalised to the amount of total Bad. Results were mean ± standard deviation from four independent experiments. *A *P*-value of <0.01 (right).

Cytogenetic characteristics revealed during diagnosis are associated with both induction and postremission therapy responses in adult *de novo* AML (19). The intermediate risk group is heterogeneous and includes a large proportion of patients with normal karyotypes, and a more effective prognostic marker had been warranted to enable stratification of this group of patients (19). Low expression of *RhoH* is the independent unfavourable prognostic factor for both overall and disease-free survival of AML in the intermediate risk group (Fig. 3). This result highly suggests that *RhoH* transcript level is an effective molecular marker to further stratify patients in the intermediate risk group of AML.

No significant difference was observed in the CR ratio between *RhoH* high and low groups, suggesting that low expression of *RhoH* might affect the response to the postremission therapy. A previous study claimed that RhoH is required for maintenance of lymphocyte LFA-1 ($\alpha L\beta 2$ integrin) in a non-adhesive state (5). On the other hand, *RhoH*-null thymocytes bound to intercellular adhesion molecule-1 with an efficiency equal to that of control cells (6). The same study also demonstrated that adhesion to the $\alpha 4\beta 1$ integrin ligand vascular cell adhesion molecule-1 (VCAM-1) was indistinguishable between wild-type and *RhoH*-null thymocyte (6). In our study, the adhesion of 32D or HL60 cells to VCAM-1 did not depend on the presence of RhoH (data not shown). These data highly suggest that RhoH does not affect cell adhesion-dependent drug resistance through LFA-1 and $\alpha 4\beta 1$ integrin.

A previous study demonstrated that overexpression of RhoH in cytokine-stimulated haematopoietic stem cells is associated with reduced proliferation and increased apoptosis (4), suggesting that RhoH is a proapoptotic molecule, although the mechanism is unknown. Bad belongs to the BH3-only subfamily of proapoptotic proteins. The serine residues 112, 136 and 155 are the main phosphorylation sites in the mouse protein. These correspond to S-75, -99 and -118 in the human protein, respectively (23). In another study, increased Rac1 signalling was shown to stimulate Bad phosphorylation at human S-75 in human lymphoma cells. When this phosphorylation is selectively inhibited, increased apoptosis occurs in response to chemotherapy (21).

Our results demonstrated that overexpression of RhoH led to Bad dephosphorylation at S-75. Together with the previous findings that Rac1 and its family members are frequently activated in human cancers (24–26), our data suggest that it is highly possible that low expression of *RhoH* (i.e. high GTP-Rac) contributes to chemotherapy resistance in leukaemia cells. Our work adds to this hypothesis by suggesting that inhibition of Rac and its signalling components might provide a useful anti-leukaemic strategy.

Acknowledgements

This work was supported by Grants-in-Aid from the National Institute of Biomedical Innovation and the Sankyo Memorial Foundation, and the Grants-in-Aid for the Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors thank Dr John G. Collard and Dr Miguel A. del Pozo for providing resources for our research. The authors also thank Ms Satomi Yamaji and Chika Wakamatsu for their excellent technical help.

References

1. Dallery E, Galiegue-Zouitina S, Collyn-d'Hooghe M, *et al.* TTF, a gene encoding a novel small G protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene* 1995;**10**:2171–8.
2. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, Dalla-Favera R. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 2001;**412**:341–6.
3. Hiraga J, Katsumi A, Iwasaki T, Abe A, Kiyoi H, Matsushita T, Kinoshita T, Naoe T. Prognostic analysis of aberrant somatic hypermutation of RhoH gene in diffuse large B cell lymphoma. *Leukemia* 2007;**21**:2146–7.
4. Gu Y, Jasti AC, Jansen M, Siefring JE. RhoH, a hematopoietic-specific Rho GTPase, regulates proliferation, survival, migration, and engraftment of hematopoietic progenitor cells. *Blood* 2005;**105**:1467–75.
5. Cherry LK, Li X, Schwab P, Lim B, Klickstein LB. RhoH is required to maintain the integrin LFA-1 in a nonadhesive state on lymphocytes. *Nat Immunol* 2004;**5**:961–7.
6. Dorn T, Kuhn U, Bungartz G, *et al.* RhoH is important for positive thymocyte selection and T-cell receptor signaling. *Blood* 2007;**109**:2346–55.
7. Gu Y, Chae HD, Siefring JE, Jasti AC, Hildeman DA, Williams DA. RhoH GTPase recruits and activates Zap70 required for T cell receptor signaling and thymocyte development. *Nat Immunol* 2006;**7**:1182–90.
8. Li X, Bu X, Lu B, Avraham H, Flavell RA, Lim B. The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function. *Mol Cell Biol* 2002;**22**:1158–71.
9. Grimwade D, Walker H, Oliver F, *et al.* The importance of diagnostic cytogenetics on outcome in AML: analysis of 1612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* 1998;**92**:2322–33.
10. Ohno R, Kobayashi T, Tanimoto M, *et al.* Randomized study of individualized induction therapy with or without vincristine, and of maintenance-intensification therapy between 4 or 12 courses in adult acute myeloid leukemia. AML-87 Study of the Japan Adult Leukemia Study Group. *Cancer* 1993;**71**:3888–95.

11. Kobayashi T, Miyawaki S, Tanimoto M, *et al.* Randomized trials between behenoyl cytarabine and cytarabine in combination induction and consolidation therapy, and with or without ubenimex after maintenance/intensification therapy in adult acute myeloid leukemia. The Japan Leukemia Study Group. *J Clin Oncol* 1996;**14**:204–13.
12. Miyawaki S, Tanimoto M, Kobayashi T, *et al.* o beneficial effect from addition of etoposide to daunorubicin, cytarabine, and 6-mercaptopurine in individualized induction therapy of adult acute myeloid leukemia: the JALSG-AML92 study. Japan Adult Leukemia Study Group. *Int J Hematol* 1999;**70**:97–104.
13. Ozeki K, Kiyoi H, Hirose Y, *et al.* Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. *Blood* 2004; **103**: 1901–8.
14. Kiyoi H, Naoe T, Yokota S, *et al.* Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia* 1997;**11**:1447–52.
15. Yamamoto Y, Kiyoi H, Nakano Y, *et al.* Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001;**97**:2434–9.
16. Kiyoi H, Naoe T, Nakano Y, *et al.* Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 1999;**93**:3074–80.
17. Suzuki T, Kiyoi H, Ozeki K, *et al.* Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. *Blood* 2005;**106**:2854–61.
18. Katsumi A, Milanini J, Kiosses WB, del Pozo MA, Kaunas R, Chien S, Hahn KM, Schwartz MA. Effects of cell tension on the small GTPase Rac. *J Cell Biol* 2002;**158**:153–64.
19. Slovak ML, Kopecky KJ, Cassileth PA, *et al.* Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood* 2000;**96**:4075–83.
20. van Leeuwen FN, van der Kammen RA, Habets GG, Collard JG. Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. *Oncogene* 1995;**11**:2215–21.
21. Zhang B, Zhang Y, Shacter E. Rac1 inhibits apoptosis in human lymphoma cells by stimulating Bad phosphorylation on Ser-75. *Mol Cell Biol* 2004;**24**:6205–14.
22. Chae HD, Lee KE, Williams DA, Gu Y. Cross-talk between RhoH and Rac1 in regulation of actin cytoskeleton and chemotaxis of hematopoietic progenitor cells. *Blood* 2008;**111**:2597–605.
23. Rapp UR, Fischer A, Rennefahrt UE, Hekman M, Albert S. BAD association with membranes is regulated by Raf kinases and association with 14-3-3 proteins. *Adv Enzyme Regul* 2007;**47**:281–5.
24. Schnelzer A, Prechtel D, Knaus U, Dehne K, Gerhard M, Graeff H, Harbeck N, Schmitt M, Lengyel E. Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. *Oncogene* 2000;**19**:3013–20.
25. Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat Rev Cancer* 2002;**2**:133–42.
26. Sun D, Xu D, Zhang B. Rac signaling in tumorigenesis and as target for anticancer drug development. *Drug Resist Updat* 2006;**9**:274–87.

Diffuse large B-cell lymphoma after transformation from low-grade follicular lymphoma: morphological, immunohistochemical, and FISH analyses

Akiko Miyagi Maeshima,[1,4] Mutsuko Omatsu,[1] Junko Nomoto,[2] Dai Maruyama,[2] Sung-Won Kim,[2] Takashi Watanabe,[2] Yukio Kobayashi,[2] Kensei Tobinai[2] and Yoshihiro Matsuno[3]

[1]Clinical Laboratory and [2]Hematology and Stem Cell Transplantation Divisions, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, and [3]Department of Surgical Pathology, Hokkaido University Hospital, Kita 14 Nishi 5, Kita-ku, Sapporo 060-8648, Japan

(Received March 12, 2008/Revised April 27, 2008/Accepted May 6, 2008/Online publication June 28, 2008)

Follicular lymphoma (FL) is one of the most common subtypes of non-Hodgkin lymphoma and frequently transforms to diffuse large B-cell lymphoma (DLBCL). To clarify some aspects of the natural history of FL, we retrospectively examined 43 consecutive patients who had DLBCL with pre- or coexisting FL grade 1 or 2. The patients comprised 22 men and 21 women with a median age of 53 years. Most of the patients (34/43) showed advanced-stage (III or IV) disease initially. We examined both FL and DLBCL components morphologically, immunohistochemically, and by interface fluorescence *in situ* hybridization (FISH: IGH/BCL2 fusion, *BCL6 translocation*) analysis. Most of the DLBCLs were classified as the centroblastic subtype, with two exceptions of the anaplastic subtype. Immunohistochemical analysis of both the FL and DLBCL components revealed the following respective positivity rates: CD20 100%/100%, CD10 86%/66%, Bcl-2 96%/91%, Bcl-6 84%/88%, MUM1 16%/34%, CD30 0%/20%, CD138 0%/0%, and CD5 0%/3%. Loss of CD10 (*6/36, 17%*) and gain of MUM1 (*7/28, 25%*) and CD30 (*5/21, 24%*) through transformation were not infrequent. High positivity rates for Bcl-2 and Bcl-6 were maintained throughout transformation. Among the DLBCLs, 84% were classified as the germinal center B-cell phenotype (GCB) and 16% as non-GCB in accordance with the criteria of Hans *et al.* IGH/BCL2 fusion was detected by FISH in 89% of FLs and 82% of DLBCLs. BCL6 translocation was detected in 1/6 (17%) DLBCLs without IGH/BCL2 fusion. Thus, although the morphological features and FISH results for DLBCL were consistent with transformed FL, the immunophenotype showed wide heterogeneity. (*Cancer Sci* 2008; 99: 1760–1768)

Follicular lymphoma (FL) is one of the most common subtypes of non-Hodgkin lymphoma in the Western world, accounting for 22% of all cases worldwide.[1] FL occurs over a broad range of ages, and most cases are manifested initially in lymph nodes.

The risk of FL transformation has been reported as being approximately 20% at 8 years.[2,3] Transformation to DLBCL is observed frequently, with cells most commonly resembling centroblasts,[4] but occasionally resembling anaplastic large cells with CD30 expression.[5] Rare cases have transformed to Burkitt or Burkitt-like lymphoma,[6] or precursor B-lymphoblastic lymphoma/acute lymphoid leukemia.[7] Moreover, composite FL and Hodgkin lymphoma have been suggested to represent two morphologic manifestations of the same tumor clones.[8,9]

In recent years, several analyses of genetic alterations that appear to affect the risk for FL transformation have been reported, including c-MYC translocation,[6] p53 mutation,[10,11] deletions of the tumor suppressor genes p15 and p16,[12,13] and chromosomal 6q23-26 and 17q aberrations.[14] However, there have been few immunohistochemical analyses of transformed FL. FL is positive for the pan-B-cell marker CD20, and frequently positive for CD10, Bcl-2 and Bcl-6, but is usually negative for the postgerminal center B-cell or plasma cell markers CD30, MUM1 and CD138, and CD5. Because only one study has demonstrated gain of CD30 expression through FL transformation,[5] we considered that more analyses were needed to clarify the immunophenotypic changes occurring during the transformation of FL to DLBCL.

Since 2000 DLBCL has been subdivided into GCB and non-GCB (including the activated B-cell phenotype [ABC] and type 3 phenotype) using the cDNA microarray technique.[15,16] The GCB group shows better outcomes and includes cases with translocation (14;18)(q32;q21). For clinical practice, Hans *et al.* showed that a panel of immunohistochemical markers comprising CD10, Bcl-6, and MUM1 could be used on paraffin-embedded tissues to separate DLBCL into tumors with a GCB or non-GCB phenotype.[17] Davies *et al.* examined 35 cases of transformed FL, and found that 89% of them had a GCB phenotype and 9% had a non-GCB phenotype.[18]

The translocation (14;18)(q32;q21) is present in 80–100% of FLs in Western countries,[19,20] whereas in South-East Asia, including Japan, the incidence of translocation is considerably lower: about 60%.[21] It is unclear whether FL with t(14;18) frequently transforms to DLBCL.

The aim of this study was to clarify the natural history of FL, mainly in view of immunophenotypic changes through transformations. We evaluated low-grade FLs and their transformant DLBCLs using morphological, immunhistochemical, and FISH analyses to delineate the heterogeneity of DLBCL after transformation from low-grade FL.

Materials and Methods

Patients. The criteria used for identification of transformed FL were those reported for aggressive B-cell lymphomas in a review based on the workshop of the XIth Meeting of the European Association for Haematopathology.[22] Briefly, it was considered that the term 'transformation' should be used only when there was morphological evidence of simultaneous or prior low-grade FL. Therefore, in the present study, we chose DLBCL with simultaneous or prior low-grade (grade 1 or 2) FL. We retrospectively studied 43 consecutive patients with DLBCL with pre- (20 cases) or coexisting (23 cases) FL grade 1 or 2 treated at the National Cancer Center Hospital, Tokyo, Japan, between 1997 and 2005. The total number of DLBCL specimens was 47 (1–3 per case), and the total number of specimens of low-grade FL was 53 (1–5 per case). A total of 400 FLs and 653 DLBCLs were registered during the same

[4]To whom correspondence should be addressed. E-mail: akmaeshi@ncc.go.jp

doi: 10.1111/j.1349-7006.2008.00873.x

period. Clinical information was extracted from the medical records, and is summarized in Table 1.

Morphological review. The materials were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-μm thick sections, and stained with hematoxylin–eosin (HE) for histologic evaluation. All specimens were reviewed by two pathologists (AMM and YM) to confirm that the morphologic characteristics fulfillled the criteria for FL and DLBCL in the 2001 World Health Organization classification of lymphoid neoplasms.[23] Tumors were judged to be FL grade 1 when neoplastic follicles contained 0–5 centroblasts/10 HPF, and FL grade 2 when they contained 6–15 centroblasts/10 HPF. We diagnosed DLBCL when the tumor cells were spread diffusely without a follicular pattern and large lymphoid cells accounted for more than 30% of the tumor cells. DLBCL was subclassified as the centroblastic, anaplastic, immunoblastic, or T-cell/histiocyte rich variant. The centroblastic variant was subclassified as monomorphous (comprising only large lymphoid cells) or polymorphous (comprising a mixture of large- and medium-sized lymphoid cells).

Immunohistochemistry and *in situ* hybridization. We performed immunohistochemical staining for both FL and DLBCL components on formalin-fixed paraffin-embedded tissues using a panel of monoclonal and polyclonal antibodies. Sections 4-μm thick were cut from each paraffin block, deparaffinized, and incubated at 121°C in pH 6.0 citrate buffer for 10 min for antigen retrieval. Antibodies included those against the following antigens: a pan-B-cell marker, CD20 (L26, ×100; Dako, Glostrup, Denmark); a pan-T-cell marker, CD3 (PS1, ×25; Novocastra, Newcastle-upon-Tyne, UK); FL markers, CD10 (56C6, ×50; Novocastra), Bcl-2 (124, ×100; Dako); and Bcl-6 (poly, ×50; Dako, Kyoto, Japan); postgerminal center B-cell or plasma cell markers, CD30 (Ber-H2, ×100; Dako, Denmark); MUM1 (MUM1p, ×50; Dako, Japan); and CD138 (5F7, ×25; Novocastra); and CD5 (4C7, ×50; Novocastra), employing an autostainer with the standard polymer (Dako autostainer plus: CD3, CD5, CD10, and CD30) or labeled streptavidin-biotin method (Biogenex autostainer: CD20 and Bcl-2), or manually by the standard avidin-biotin complex method (Bcl-6, MUM1, and CD138). Immunoreactivity was judged positive if more than 30% of the tumor cells were stained. All immunohistochemical specimens were judged by AM Maeshima, and Y Matsuno confirmed them.

To classify each case as having either a 'GCB phenotype' or a 'non-GCB phenotype', a panel of three antigens (CD10, Bcl-6, MUM1) was used according to the protocol reported by Hans *et al.*[17] Briefly, cases were assigned to the 'GCB phenotype' if the specimens were positive for CD10. If the specimens were negative for both Bcl-6 and CD10, the corresponding cases were assigned to the 'non-GCB phenotype'. If the specimens were positive for Bcl-6 and negative for CD10, the expression of MUM1 was used to determine the group: if MUM1 was negative, the case was assigned to the 'GCB phenotype', and if positive, to the 'non-GCB phenotype'.

Interphase fluorescence *in situ* hybridization (FISH) analysis. Sections 4-μm thick were cut from each paraffin block and used for FISH analysis. The specimens were treated with a 2× saline sodium citrate buffer (SSC, pH 7.3), digested with 0.005% and 0.3% pepsin/0.01 N HCl for 14 min at 37°C, rinsed in 1× phosphate buffer saline (PBS, pH 7.4) for 5 min, formalin $MgCl_2$/PBS for 10 min, rinsed in 1× PBS for 5 min twice, and dehydrated in an ethanol series. Next, the samples were denatured in 70% formamide/20× SSC for 2 min at 37°C and dehydrated with 70% ethanol for 5 min, followed by 100% ethanol. Denatured probes (10 μL) were dropped onto the slides, covered with a coverslip, and sealed with rubber cement. The slides were then treated using a microwave procedure to intensify the signals. The microwave (MI-77; Azumaya Company, Tokyo, Japan) was set to irradiate the samples for 3-second periods at intervals of 2 s, for a total of 60 min at a frequency of 2.45 GHz and an output power of 250 W with the temperature sensor set to 37°C. After incubation overnight at 37°C, the slides were washed with 50% formamide/2× SSC for 10 min at 45°C, and then washed twice more for 10 min each at room temperature; the slides were then washed with 2× SSC for 10 min. The specimens were rinsed in 4× SSC/0.05% Triton for 5 min, 2× SSC for 5 min at 45°C, and 0.2× SSC at room temperature. The slides were covered with antifade solution and viewed under a BX60 fluorescence microscope (Olympus, Tokyo, Japan) using a 100× oil immersion lens and appropriate filters.

LSI IGH Spectrum Green/LSI BCL2 Spectrum Orange Dual Fusion Translocation Probe (Vysis, Downers Grove, IL, USA) was used to detect t(14;18): IGH/BCL2 fusion. LSI BCL6 Dual Color, Break Apart Rearrangement Probe (Vysis) was used to detect 3q27: BCL6 translocation. Judgment of the fusion gene was performed as described previously.[21] Briefly, a total of 50–200 nuclei per case were scored, and if more than 2% of the tumor cells had two fusion signals in the IGH/BCL2 examination, they were judged positive for fusion. If more than 1.5% of tumor cells had split signals in the BCL6 examination, they were judged positive for translocation.

Statistical analysis. Five-year and 10-year overall survival rates were calculated by the Kaplan–Meier method. Univariate analysis was performed using the log-rank test for clinicopathologic parameters, as shown in Tables 1–3. Clinical information of patients with pre-existing FL and those with coexisting FL was compared by the Fisher's exact test, Mann–Whitney *U*-test or log-rank test in Table 2. Differences were considered significant when *P*-value was less than 0.05.

Results

Patients. Clinical information is summarized in Table 1. The patients comprised 22 men and 21 women, ranging in age from 25 to 80 years with a median age of 53 years. Most of them (34/43) had advanced-stage (III or IV) initially. All of the patients received treatments after initial diagnoses: cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) ± radiation (12 cases), rituximab (R)-CHOP ± radiation (22 cases), or other types of treatment (cyclophosphamide, vincristine, prednisone and procarbazine (C-MOPP), vincristine, cyclophosphamide, prednisone and doxorubicin (VEPA), methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin (MACOP-B), vincristine/vindesine, doxorubicin and prednisone (VCR/VDS + DOX + PSL), R+ radiation and radiation) (8 cases). Overall survival after initial diagnosis was 76.5% at 5 years and 57.3% at 10 years. Overall survival after transformation was 60.0% at 5 years. Clinical information was compared between 19 patients with pre-existing FL and 24 patients with coexisting FL, and is summarized in Table 2.

Morphology. The results of morphological analysis, immunohistochemical staining for each antibody, and FISH analysis are summarized in Table 3 and Table 4. A total of 47 DLBCL specimens from 43 patients were reviewed. The biopsy sites were lymph node (27), tonsil (6), spleen (1), and extranodal sites (13: gingiva 1, stomach 1, small intestine 1, terminal ileum 1, rectum 3, bone marrow 2, skin 3, lung 1). The subtypes of DLBCL in the final specimens were centroblastic monomorphous (30), centroblastic polymorphous (11), and anaplastic (2). Notably, one case (no. 38) finally transformed to classical Hodgkin lymphoma, mixed cellularity, after transformation of FL to DLBCL. Massive necrosis was detected in four cases (9%).

A total of 53 FL specimens from 43 patients were reviewed. The biopsy sites were lymph node (21), tonsil (2), spleen (1), and extranodal sites (29: nasopharynx 1, esophagus 1, stomach 6, duodenum 6, small intestine 2, colon 1, bone marrow 11, skin 1). The grades of FL were grade 1 (20), grade 2 (27), judged as limited to low-grade FL due to the very small amount

Table 1. Patient characteristics

Case	Age/ gender	Stage	IPI	Initial diagnosis (site)	Initial therapy /response	Interval to transformation in months (time of relapse)	Second diagnosis (site)	Subtype of DLBCL	Follow-up months	Outcome from initial diagnosis
1	50/F	2	L	DLBCL + FL, gr.3a + FL, gr.1 (spleen)	Rx3 + CHOPx7/CR	0		anaplastic	40	AWD
2	47/M	2	L	FL, gr.2 (LN)	CHOPx8/CR	91 (2nd)	DLBCL (tonsil)	centroblastic	96	AWD
3	77/M	3	HI	FL, gr.1 (LN)	VCR/VDS, DOX, PSL/unknown	24 (1st)	DLBCL (LN)	centroblastic	31	DOD
4	45/M	3	L	FL, gr.1 (LN)	C-MOPPx11/CR	71 (4th)	DLBCL + FL, gr.3a (LN)	centroblastic	71	AWD
5	44/M	3	L	FL, gr.2 (LN, BM)	R-CHOPx6/PR	69 (1st)	DLBCL + FL, gr.2 (BM)	centroblastic	69	AWD
6	40/F	4	LI	FL, gr.1 (LN)	R-CHOPx6/CR	38 (2nd)	DLBCL + FL, gr.3a (tonsil), DLBCL (rectum), DLBCL (small intestine)	centroblastic	69	AWD
7	66/F	4	LI	FL, gr.1 (LN)	CHOPx3/PR	11 (1st)	DLBCL + FL, gr.3a (LN), DLBCL (skin)	centroblastic	66	AWD
8	80/F	3	HI	DLBCL + FL, gr.3a + FL, gr.2 (LN)	CHOPx5/PR	0		centroblastic	46	DOD
9	51/F	1	L	FL, gr.2 (duodenum)	radiation 40Gy/CR	10 (1st)	DLBCL (LN)	centroblastic	71	AWOD
10	67/M	3	LI	FL, gr.2 (LN)	VEPAx14/CR	93 (1st)	DLBCL (tonsil)	centroblastic	101	DOD
11	60/M	4	LI	DLBCL + FL, gr.2 (small intestine)	CHOPx8 + radiation 39Gy/CR	0		centroblastic	59	AWOD
12	25/F	4	LI	FL, gr.1 (duodenum), DLBCL + FL, gr.3a (LN)	Rx4 + CHOPx8/CR	0		centroblastic	69	AWOD
13	53/M	4	HI	DLBCL + FL, gr.2 (skin)	CHOPx8/PR	0		centroblastic	23	AWD
14	49/M	4	LI	FL, gr.2 (BM)	MACOP–Bx4/PR	49 (2nd)	DLBCL (skin)	centroblastic	55	DOD
15	61/F	3	HI	DLBCL + FL, gr.2 (LN)	Rx4 + CHOPx8/CR	0		centroblastic	44	AWOD
16	46/M	2	L	DLBCL + FL, gr.3a + FL, gr.2 (LN)	R-CHOPx8 + radiation 40Gy/PD	0		centroblastic	19	DOD
17	66/F	3	HI	DLBCL + FL, gr.3a + FL, gr.2 (LN)	CHOPx2 + radiation 40Gy/CR	0		centroblastic	43	AWOD
18	43/M	2	L	DLBCL + FL, gr.1 (LN)	R-CHOPx6 + radiation 40Gy/CR	0		centroblastic	72	AWOD
19	60/F	3	LI	FL, gr.1 (LN, duodenum)	CHOPx8/CR	40 (1st)	DLBCL (tonsil)	centroblastic	80	AWOD
20	41/F	4	LI	FL, gr.2 (tonsil), DLBCL (LN)	R-CHOPx8 + radiation 40Gy/CR	0		centroblastic	38	AWOD
21	72/F	2	LI	FL, gr.2 (LN)	R-CHOPx8 + radiation 40Gy/PR	84 (1st)	DLBCL (LN)	centroblastic	116	AWD
22	46/F	4	H	FL, gr.2 (duodenum, stomach), DLBCL (LN)	R-CHOPx8 + radiation 40Gy/CR	0		centroblastic	32	AWD
23	44/M	3	L	DLBCL + FL, gr.2 (LN)	R-CHOPx8/CR	0		centroblastic	30	AWOD
24	53/F	4	LI	FL, gr.2 (LN)	R + radiation 39Gy/CR	29 (1st)	DLBCL (lung)	centroblastic	29	AWD
25	53/F	4	HI	FL, gr.1 (duodenum, stomach, BM, colon, tonsil), DLBCL + FL, gr.3a (tonsil)	R-CHOPx5/CR	0		centroblastic	33	AWD
26	42/F	4	L	DLBCL + FL, gr.3a (LN), FL, gr.1 (BM)	R-CHOPx6/CR	0		centroblastic	24	AWOD
27	54/M	4	L	FL, gr.2 (LN, BM)	CHOPx8/PR	49 (1st)	DLBCL + FL, gr.3a (BM)	centroblastic	49	AWD

doi: 10.1111/j.1349-7006.2008.00873.x

Table 1. *Continued*

Case	Age/ gender	Stage	IPI	Initial diagnosis (site)	Initial therapy /response	Interval to transformation in months (time of relapse)	Second diagnosis (site)	Subtype of DLBCL	Follow-up months	Outcome from initial diagnosis
28	54/M	4	H	DLBCL + FL, gr.3b + FL, gr.2 (LN)	R-CHOPx8/PR	0		centroblastic	16	AWOD
29	47/M	3	LI	FL, gr.1 (BM)	CHOPx6/CR	46 (2nd)	DLBCL + FL, gr.3b (LN)	centroblastic	47	AWOD
30	59/M	3	L	FL, gr.1 (duodenum), DLBCL + FL, gr.3a (LN)	R-CHOPx6/CR	0		centroblastic	20	AWOD
31	52/M	3	L	DLBCL + FL, gr.3a + FL, gr.2 (LN)	R-CHOPx5/CR	0		centroblastic	18	AWOD
32	57/M	4	L	FL, gr.2 (BM)	R-CHOPx6/CR	26 (1st)	DLBCL + FL, gr.3a (LN)	anaplastic	27	AWD
33	47/F	3	L	DLBCL + FL, gr.1 (LN)	CHOPx6/CR	0		centroblastic	38	AWD
34	47/M	4	LI	FL, low grade (BM), DLBCL (LN)	R-CHOPx8/PR	0		centroblastic	13	AWD
35	47/F	4	L	FL, gr.2 (stomach, BM), DLBCL + FL, gr.3a (LN)	R-CHOPx8/CR	0		centroblastic	7	AWOD
36	59/F	1	L	FL, gr.1 (LN)	CHOPx3 + radiation/CR	24 (1st)	DLBCL (LN)	centroblastic	24	AWD
37	54/M	4	L	FL, low grade (BM), DLBCL + FL, gr.3b (LN)	R-CHOPx6/CR	0		centroblastic	12	AWOD
38	61/F	4	H	FL, low grade (esophagus, stomach), DLBCL + FL, gr.3a (LN), DLBCL (terminal ileum, rectum)	R-CHOPx8/NC	0	HL, MC (LN)	centroblastic	28	DOD
39	68/M	1	L	FL, low grade (BM), DLBCL + FL, gr.3a (LN)	CHOPx3 + radiation/CR	0		centroblastic	8	AWOD
40	79/M	1	L	FL, gr.2 (nasopharynx)	radiation 40Gy/CR	54 (2nd)	DLBCL (stomach)	centroblastic	152	DOD
41	70/M	4	HI	FL, gr.1 (stomach)	Rx4 + CHOPx6/NC	37 (1st)	DLBCL (gingival)	centroblastic	133	DOD
42	45/F	4	L	FL, gr.2 (LN)	C–MOPPx7/CR	21 (1st)	DLBCL (rectum)	centroblastic	37	DOD
43	56/F	4	LI	FL, low grade (stomach), DLBCL (tonsil)	unknown	0		centroblastic	0	unknown

AWD, alive with disease; AWOD, alive without disease; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; C-MOPP, cyclophosphamide, vincristine, prednisone and procarbazine; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; DOD, dead of disease; FL, follicular lymphoma; gr. grade; H, high; HI, high intermediate; HL, MC, Hodgkin lymphoma, mixed cellularity; IPI, international prognostic index; L, low; LI, low intermediate; MACOP-B, methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin; NC, no change; PD, progressive disease; PR, partial remission; R, rituximab; VCR/VDS + DOX + PSL, vincristine/vindesine, doxorubicin and prednisone; VEPA, vincristine, cyclophosphamide, prednisone and doxorubicin.

Table 2. Patients' clinical data at initial diagnosis

	19 cases with pre-existing FL	24 cases with coexisting FL	*P*-value[†]
Gender (male/female)	11/8	11/13	0.54
Age (median, range)	57 (40–79)	52 (25–80)	0.30
Stage (I, II/III, IV)	5/14	4/20	0.47
LDH (normal/higher than normal)	13/6	14/10	0.54
PS (0/≥1)[‡]	13/2	13/7	0.24
Extranodal involvement (0/≥1)	13/6	11/13	0.21
B symptom (−/+)[‡]	14/1	18/2	0.72
Bulky mass (−/+)	18/1	21/3	0.62
IPI (L, LI/HI, H)[‡]	14/1	14/6	0.10
5-year OS from initial diagnosis	80.2%	65.2%	0.43
5-year OS from transformation	47.7%	65.2%	0.11

[†]Fisher's exact test, Mann–Whitney *U*-test or log-rank test.
[‡]data for PS, B symptom, and IPI were not obtained in eight cases.
FL, follicular lymphoma; H, high; HI, high intermediate; IPI, international prognostic index; L, low; LDH, serum lactate dehydrogenase; LI, low intermediate; OS, overall survival; PS, performance status.

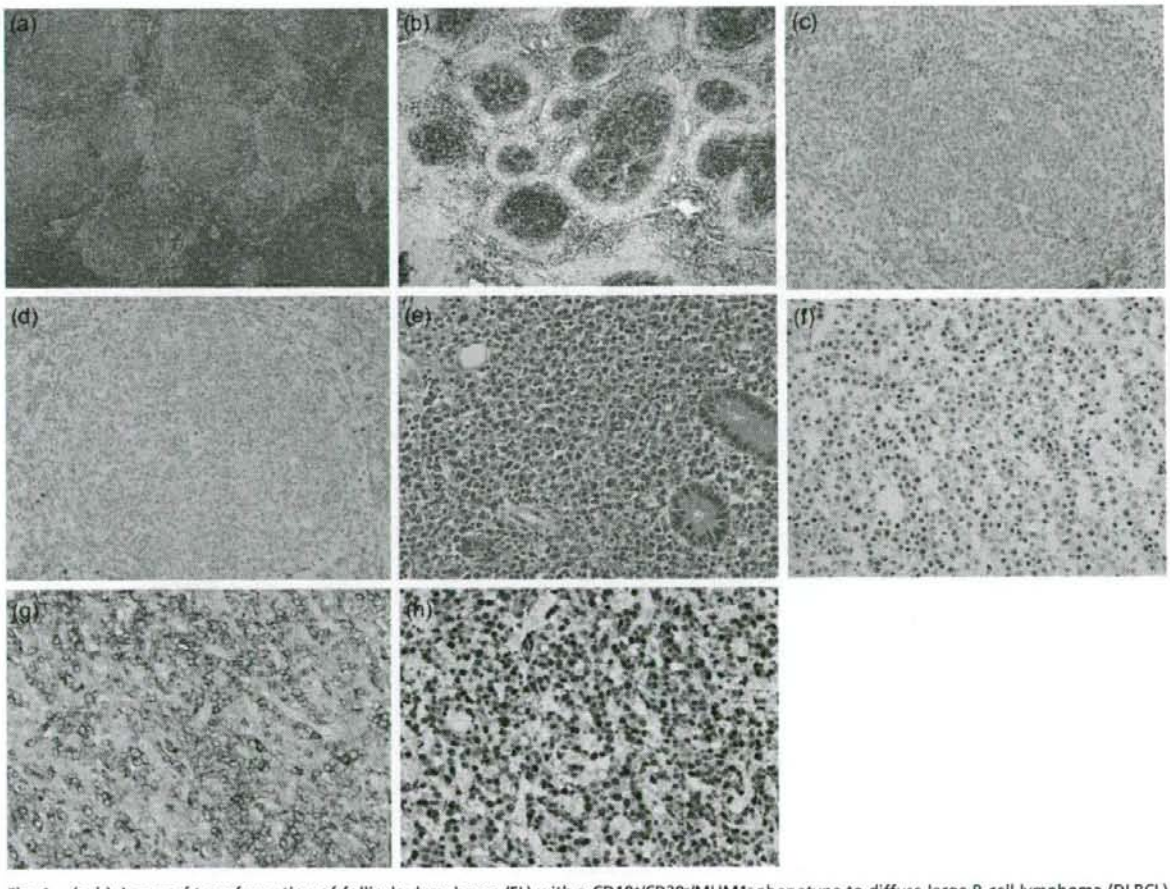

Fig. 1. (a–h) A case of transformation of follicular lymphoma (FL) with a $CD10^+/CD30^-/MUM1^-$ phenotype to diffuse large B-cell lymphoma (DLBCL) with a $CD10^-/CD30^+/MUM1^+$ phenotype. FL, lymph node. (a) Hematoxylin–eosin (HE) (×40), (b) $CD10^+$ (×40), (c) $CD30^-$ (×200), (d) MUM1– (×200). DLBCL, small intestine, (e) HE (×400), (f) $CD10^-$ (×200), (g) $CD30^+$ (×200), (h) $MUM1^+$ (×200).

of material available (6). There were 17 cases with a grade 3a component and three with a grade 3b component.

Immunohistochemistry. The results of immunohistochemistry are summarized in Table 3, Table 4, and Fig. 1. Paraffin sections were available for 41 FL specimens and 43 DLBCL specimens, but in relatively few of the FL and/or DLBCL cases were not available for some of the markers. All tumors were positive for CD20 and negative for CD3. CD10 was positive in 86% (31/36)

doi: 10.1111/j.1349-7006.2008.00873.x

Table 3. Results of immunohistochemistry and FISH analysis

Case no.	Immunophenotype of a low-grade (grade 1 or 2) FL component								FISH[†]		Immunophenotype of a DLBCL component[‡]								FISH		
	CD20	CD10	Bcl-2	Bcl-6	MUM1	CD30	CD5	CD138	IGH/BCL2	BCL6 translocation	CD20	CD10	Bcl-2	Bcl-6	MUM1	CD30	CD5	CD138	GC/non-GC	IGH/BCL2	BCL6 translocation
1	+	+	+	+	−	−	−	−	+		+	+	+	+	−	−	nt	−	GC	+	
2	+	+	+	−	−	nt	−	−	+		+	+	+	+	+	+	+	−	GC	+	
3	+	+	+	+	−	−	nt	−	nt		+	+	+	+	+	−	−	−	GC	+	
4	nt	nt	nt	nt	nt	nt	nt	nt	nt		+	+	+	+	−	−	−	−	GC	+	
5	+	+	+	+	−	−	nt	−	nt		+	nt	+	+	−	−	−	−	GC	nt	
6	+	+	+	+	−	−	−	−	+		+	−	+	−	+	+	−	−	nonGC	+	
7	+	+	+	+	−	−	nt	−	nt		+	−	+	+	+	−	−	−	nonGC	+	
8	+	+	+	+	−	nt	nt	−	+		+	+	+	+	−	−	−	−	GC	+	
9	+	+	+	+	−	−	−	−	+		+	−	nt	nt	nt	nt	nt	nt		+	
10	+	+	+	nt	nt	nt	nt	nt	nt		+	+	−	+	−	−	−	−	GC	+	
11	+	+	+	−	−	−	−	−	nt		+	+	+	−	−	−	−	−	GC	nt	
12	+	+	+	+	+	−	−	−	nt		+	+	+	+	+	−	−	−	GC	−	−
13	+	+	+	+	−	−	nt	−	+		+	+	+	+	−	−	−	−	GC	+	
14	+	nt	+	−	−	−	nt	−	nt		+	+	+	+	−	−	−	−	GC	+	
15	+	+	+	+	−	−	nt	−	+		+	+	+	+	−	nt	−	−	GC	+	
16	+	+	+	+	+	nt	−	nt	+		+	+	+	+	−	nt	−	nt	GC	+	
17	nt	nt	nt	nt	nt	nt	nt	nt	nt		+	+	+	nt	nt	nt	−	nt	GC	nt	
18	+	−	+	+	−	nt	−	nt	nt		+	−	+	+	+	nt	−	nt	nonGC	nt	
19	+	+	+	+	−	−	−	−	nt		+	−	+	nt	nt	nt	nt	nt		nt	
20	+	+	+	+	−	−	nt	−	nt		+	+	+	+	+	−	−	−	GC	+	
21	+	−	−	nt	nt	nt	nt	nt	−	nt	+	−	+	+	−	−	−	−	GC	−	
22	+	+	+	+	−	−	−	−	nt		+	+	+	+	−	−	−	−	GC	+	
23	+	−	+	+	−	−	nt	−	+		+	−	+	nt	nt	nt	−	nt		+	
24	+	+	+	+	−	−	−	−	nt		+	−	+	+	+	+	−	−	nonGC	+	
25	+	+	+	−	+	−	−	−	+		+	+	+	+	+	−	−	−	GC	+	
26	+	+	+	−	−	−	nt	−	+		+	+	+	+	−	nt	nt	nt	GC	+	
27	+	+	+	+	−	nt	nt	nt	+		+	nt	−	+	−	−	−	−	GC	+	
28	+	+	+	nt	nt	nt	nt	nt	+		+	+	+	nt	nt	nt	−	nt	GC	+	
29	+	nt	+	+	+	−	−	−	nt		+	−	+	+	+	+	−	−	nonGC	−	−
30	+	+	+	nt	nt	nt	−	nt	+		+	+	+	+	−	−	−	−	GC	+	
31	+	−	+	nt	nt	nt	nt	nt	nt		+	−	+	nt	−	nt	nt	nt		nt	
32	nt	nt	+	+	+	−	nt	−	nt		+	−	+	nt	+	+	−	nt	nonGC	+	
33	+	+	+	nt	nt	nt	−	nt	nt		+	+	+	nt	nt	−	−	nt	GC	nt	
34	+	+	−,+	+	−	−	−	−	nt		+	+	+	nt	−	nt	−	nt	GC	+	
35	+	+	+	+	−	−	−	−	nt		+	+	+	+	−	−	−	−	GC	+	
36	+	+	+	+	−	−	−	−	nt		+	+	−	+	−	−	−	−	GC	−	−
37	+	nt	+	nt	nt	−	nt	nt	−	nt	+	−	−	nt	nt	nt	−	nt		nt	
38	+	+	+	nt	nt	nt	−	nt	+		+	+	+	−	+	−	−	−	GC	+	
39	+	nt	+	nt	nt	−	nt	nt	nt		+	−	+	+	−	+	−	−	GC	−	+
40	+	+	+	+	−	−	nt	−	nt		+	+	+	−	−	−	−	−	GC	−	−
41	+	+	+	+	−	−	nt	−	nt		+	+	+	+	−	−	−	−	GC	+	
42	+	+	+	+	−	−	−	−	nt		+	−	+	+	−	−	−	nt	GC	nt	
43	+	−	+	nt	nt	nt	−	nt	+		+	+	+	nt	nt	nt	−	nt	GC	+	

[†]judged from the final biopsy specimen, [‡]fluorescence *in situ* hybridization for IGH/BCL2 fusion.
DLBCL, diffuse large B-cell lymphoma; FISH, fluorescence *in situ* hybridization; FL, follicular lymphoma; GC, germinal center B-cell phenotype; nt, not tested.

Table 4. Summary of results of immunohistochemistry and FISH analysis

Antibody	FL component	DLBCL component	Gain/loss/no change
CD20	100% (40/40)	100% (43/43)	
CD10	86% (31/36)*	66% (27/41)†	1/6/29
Bcl-2	96% (42/44)	91% (38/42)	1/4/37
Bcl-6	84% (26/31)	88% (28/32)	4/2/20
MUM1	16% (5/31)	34% (12/35)	7/1/20
CD30	0% (0/28)	20% (6/30)	5/0/16
CD138	0% (0/28)	0% (0/28)	
CD5	0% (0/21)	3% (1/38)	1/0/19
GCB, non-GCB		84% (31/37), 16% (6/37)	
FISH: IGH/BCL2	89% (16/18)	82% (28/34)	

†excluding bone marrow specimens.
DLBCL, diffuse large B-cell lymphoma; FISH, fluorescence *in situ* hybridization; FL, follicular lymphoma; GCB, germinal center B-cell phenotype.

of FLs and 66% (27/41) of DLBCLs, representing a gain in one case, loss in six cases, and no change in 29 cases (including 21 positive cases and eight negative cases). Bone marrow specimens were excluded because only these materials showed extremely low CD10 expression. Among six cases of DLBCL showing loss of CD10, two were diagnosed as DLBCL several times (nos. 6 and 7), and both cases showed loss of CD10 expression between the first and second occasions. Bcl-2 and Bcl-6 were frequently expressed in both FL and DLBCL. Bcl-2 was positive in 96% (42/44) of FLs and 91% (38/42) of DLBCLs, representing a gain in one case, loss in four cases, and no change in 37 cases through transformation. Bcl-6 was positive in 84% (26/31) of FLs and 88% (28/32) of DLBCLs, representing a gain in four cases, loss in two cases, and no change in 20 cases through transformation. Among 32 DLBCL cases for which both Bcl-2 and Bcl-6 immunohistochemistry could be performed, 25 were Bcl-2+/Bcl-6+, four were Bcl-2+/Bcl-6–, 3 were Bcl-2–/Bcl-6+, and no case was Bcl-2–/Bcl-6–.

The postgerminal center B-cell and plasma cell marker MUM 1 was positive in 16% (5/31) of FLs and in 34% (12/35) of DLBCLs, representing a gain in seven cases, loss in one case, and no change in 20 cases (including four positive cases and 16 negative cases). CD30 was negative in all FLs and positive in 20% (6/30) of DLBCLs. Two cases of DLBCL with anaplastic morphology were positive for CD30. CD30 showed scattered expression in marginally and sparsely distributed large lymphoid cells of low-grade FL and FL grade 3, but no case showed positivity in over 30% of the cells. CD5 was negative in all FLs and positive in only one case (no. 2) of DLBCL. This positive case was FL grade 2 in an abdominal lymph node with a $CD10^+$/Bcl-2+/Bcl-6–/$CD5^-$/cyclin D1-immunophenotype and IGH/BCL2 fusion by FISH analysis initially, and showed transformation to centroblastic monomorphous DLBCL in the tonsil, revealing a $CD10^-$/Bcl-2+/Bcl-6+/$CD5^+$/cyclin D1-immunophenotype and IGH/BCL2 fusion by FISH analysis. In the one case of classical Hodgkin lymphoma after transformation from FL via DLBCL (no. 38), FL in the stomach and esophagus and DLBCL in a cervical lymph node had a $CD20^+$/$CD30^-$/$CD10^+$ phenotype and IGH/BCL2 fusion by FISH analysis, but Hodgkin/Reed-Sternberg cells in an inguinal lymph node had a $CD20^-$/$CD30^+$/$CD15^+$/$CD10^-$ phenotype, and were negative for EBER-1 *in situ* hybridization and positive for IGH/BCL2 fusion by FISH (Fig. 2).

Thirty-one (84%) DLBCLs were classified as GCB, and six (16%) DLBCLs were classified as non-GCB. Two cases of DLBCL (nos. 6 and 7) for which several sequential biopsies were taken were judged from the final biopsy specimen: these were non-GCB in the final DLBCL specimens, but had been GCB in the initial specimens.

FISH analysis. Paraffin-embedded sections were available for 18 FL cases and 34 DLBCL cases. IGH/BCL2 fusion was detected in 89% (16/18) of FL cases and in 82% (28/34) of DLBCL cases. In all six DLBCL cases without IGH/BCL2 fusion, BCL6 translocation was detected in one case (17%). Two FL cases without IGH/BCL2 fusion were not available paraffin-embedded sections.

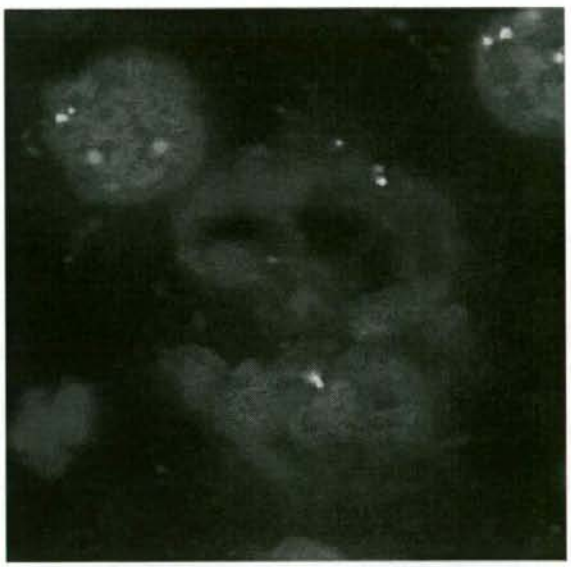

Fig. 2. The result of fluorescence *in situ* hybridization of classical Hodgkin lymphoma transformed from follicular lymphoma (FL). IGH and BCL2 fusion pattern with the LSI IGH Spectrum Green/LSI BCL2 Spectrum Orange Dual Fusion Translocation Probe (Vysis, Downers Grove, IL, USA). Two fusion IGH/BCL2 signals are present.

Statistical analysis. Only the initial treatment regimen was a significant prognostic factor: patients who received CHOP or R-CHOP showed a better outcome than patients who received other treatments ($P < 0.001$). No other significant prognostic factors were detected, including GCB *versus* non-GCB. However, patients with DLBCL showing CD30-positivity (six cases) did not die as a result of disease progression.

Discussion

As transformation of FL to DLBCL is currently the focus of widespread clinical and pathological interests, we studied the

doi: 10.1111/j.1349-7006.2008.00873.x

heterogeneity of the DLBCL component using morphological, immunohistochemical, and FISH analyses.

FL transformed most commonly to the DLBCL centroblastic subtype,[4] but occasionally to the DLBCL anaplastic subtype with CD30 expression.[5] We confirmed that most of the DLBCLs were of the centroblastic subtype, with two exceptional cases of the anaplastic subtype.

CD10 shows restricted expression in germinal center B-cells of reactive lymphoid tissue. Although the reported frequency of CD10 expression in FL varies, about 60% of FLs express CD10.[4] However, no previous report has documented changes in CD10 expression through transformation from low-grade FL to DLBCL. In this study, 14 DLBCLs were negative for CD10, and among them, six showed loss of CD10 expression through transformation. Previous reports have indicated that CD10 expression is often stronger in follicles than in interfollicular neoplastic cells, and that the frequency of CD10 expression is lower in FL grade 3 than in low-grade FL.[24] It is suggested that loss of CD10 expression through FL transformation is possible in the process of escape from the follicular dendritic cell meshwork and diffusion, and tumor cell enlargement.

Bcl-2 is expressed on resting and B and T cells, but not on normal germinal center cells. Bcl-2 protein has an antiapoptotic function,[25] and is expressed in the majority of FLs ranging from nearly 100% in grade 1–75% in grade 3,[26] and in about 30–50% of de novo DLBCLs.[23] Bcl-6 is expressed in germinal center B-cells and a subset of $CD4^+$ T cells.[27] Bcl-6 is expressed in 88% of FLs,[28] and 55–97% of de novo DLBCLs.[29] Most of the DLBCLs that had transformed from FL retained a high frequency of Bcl-2 and Bcl-6 expression, which tended to be higher than that in de novo DLBCL. Because Bcl-2 and Bcl-6 expression was retained during transformation in most cases, Bcl-2 and Bcl-6 positivity might be a precondition for transformation of DLBCL from FL.

MUM1 is a lymphoid-specific member of the interferon regulatory factor family of transcription factors.[30] MUM1 is normally expressed in plasma cells and a minor subset of germinal center B cells, and has been reported to be expressed in 50–77% of DLBCLs.[31–33] In this study, MUM1 was positive in 16% of low-grade FLs and 34% of DLBCLs. This rate was lower than that in de novo DLBCL, but it was surprising that 34% of transformed FLs expressed MUM1. Twenty cases that were MUM1-positive in both the FL and DLBCL components were suggested to be derived from germinal center MUM1-positive B cells, and seven cases showing MUM1-gain indicated that this event was not infrequent during FL transformation.

CD30 was positive in Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma, anaplastic large cell lymphoma, anaplastic variant of DLBCL, and a subset of non-neoplastic activated B and T cells.[1,5] Most FLs contain a small number of CD30-positive cells, located mainly at the edge of the neoplastic follicles,[34] and we confirmed this feature. Transformation of FL into CD30-positive large B-cell lymphoma with anaplastic features has been reported.[5] In this study, 20% of the cases gained CD30 expression in DLBCL, which included two cases of the DLBCL anaplastic variant, indicating that CD30-positive large lymphoid cells tended to increase gradually during transformation.

CD5 is reported to be an unfavorable prognostic marker in de novo DLBCL.[35] Richiter's syndrome, a transformant of chronic lymphocytic leukemia/small lymphocytic lymphoma, is a well-known secondary $CD5^+$ DLBCL. Manazza *et al.* reported that CD5 and CD10-double-positive FL transformed to $CD5^+$ DLBCL.[36] Our present study is the first to indicate that $CD5^-$/$CD10^+$ FL with IGH/BCL2 fusion can transform to secondary $CD5^+$/$CD10^-$ DLBCL with IGH/BCL2 fusion.

Notably, our series included one case of classical Hodgkin lymphoma that had transformed from FL via DLBCL. Previous reports have suggested that composite follicular lymphoma and Hodgkin lymphoma represent two morphologic manifestations of the same tumor clones.[8,9] In the present case, IGH/BCL2 fusion was detected in both the FL and the Hodgkin/Reed-Sternberg cells by FISH analysis, strongly suggesting transformation from FL.

Although transformed FL is generally considered to have a GCB phenotype, we demonstrated that a proportion of FLs can show a dramatic change in immunophenotype through transformation. Davies *et al.*[18] examined 35 cases of transformed FL, and found that 89% of them had a GCB phenotype and 9% had a non-GCB phenotype. In our study, six (16%) of the DLBCLs had a non-GCB phenotype. Some previous studies examining the difference in prognosis between patients with a GCB phenotype *versus* those with non-GCB-phenotype DLBCL revealed that the former group had a more favorable prognosis.[15,16] However, Colomo *et al.* found no prognostic difference between them,[37] and recently therefore this issue has been controversial. In the present study, GCB *versus* non-GCB was not a significant prognostic factor. However, as the number of cases was small, further studies are necessary to clarify the prognostic difference between GCB and non-GCB in transformed FL.

We detected a high relative frequency (82%) of IGH/BCL2 fusion in transformed FL. Although the rate is higher than that in Japanese FL, it is almost equal to that in FL grade 1 (10/12, 83%).[21] Because the present cases of DLBCL had transformed from low-grade FL, we were unable to conclude whether cases showing IGH/BCL2 fusion transformed more frequently than cases without it.

In conclusion, our study has clearly demonstrated heterogeneity of the immunophenotype in DLBCL transformed from low-grade FL, suggesting that various mechanisms may affect FL transformation. As many genetic changes including c-MYC amplification and p53 mutation have been detected in transformed FL, it will be necessary to analyze the relationship between these genetic abnormalities and morphological, immunohistochemical, and IGH/BCL2 fusion status in transformed FL.

Acknowledgments

The authors would like to thank C. Kina and S. Miura for technical assistance with immunohistochemistry. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan.

References

1 A clinical evaluation of the International Lymphoma Study Group Classification of Non-Hodgkin's Lymphoma. The non-Hodgkin's Lymphoma Classification Project. *Blood* 1997; **89**: 3909–18.
2 Horning SJ, Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphoma. *N Eng J Med* 1984; **311**: 1471–5.
3 Gallagher CJ, Gregory WM, Jones AE *et al.* Follicular lymphoma: prognostic factors for response and survival. *J Clin Oncol* 1986; **4**: 1470–80.
4 Harris NL, Ferry JA. Follicular lymphoma. In: Knowles DM, ed. *Neoplastic Hematopathology*, 2nd edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2001; 823–53.
5 Alsabeh R, Medeiros LJ, Glackin C *et al.* Transformation of follicular lymphoma into CD30-large cell lymphoma with anaplastic cytologic features. *Am J Surg Pathol* 1997; **21**: 528–36.
6 Yano T, Jaffe ES, Longo DJ *et al.* MYC rearrangements in histologically progressed follicular lymphomas. *Blood* 1992; **80**: 758–67.
7 de Jong D, Voetdujk B, Bavestock G *et al.* Activation of the c-myc oncogene in a precursor B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. *N Eng J Med* 1998; **318**: 1373.
8 Brauninger A, Hansmann ML, Strickler JG *et al.* Identification of common germinal-center B-cell precursors in two patients with both Hodgkin's disease and non-Hodgkin's lymphoma. *N Eng J Med* 1999; **340**: 1239–47.
9 Marafioti T, Hummel M, Anagnostopoulos I *et al.* Classical Hodgkin's disease and follicular lymphoma originating from the same germinal center B cell. *J Clin Oncol* 1999; **17**: 3804–9.

10 Sander CA, Yano T, Clark HM *et al.* p53 mutation is associated with progression in follicular lymphomas. *Blood* 1993; **82**: 1994–2004.
11 Lo Coco F, Gaidano G, Louie DC *et al.* p53 mutations are associated with histologic transformation of follicular lymphoma. *Blood* 1993; **82**: 2289–95.
12 Pinyol M, Cobo F, Bea S *et al.* p16 (INF4a) gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. *Blood* 1998; **91**: 2977–84.
13 Elenitoba-Johnson KS, Gascoyne RD, Lim MS *et al.* Homozygous deletions at chromosome 9p21 involving p16 and p15 are associated with histologic progression in follicle center lymphoma. *Blood* 1998; **91**: 4677–85.
14 Tilly H, Rossi A, Stamatoullas A *et al.* Prognostic value of chromosomal abnormalities in follicular lymphoma. *Blood* 1994; **84**: 1043–9.
15 Alizadeh AA, Eisen MB, Davis RE *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; **403**: 503–11.
16 Rosenwald A, Wright G, Chan WC *et al.* The use of molecular profiling to predict survival after chemotherapy for diffuse large B-cell lymphoma. *N Eng J Med* 2002; **346**: 1937–47.
17 Hans SP, Weisenburger DD, Greiner TC *et al.* Conformation of molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; **103**: 275–82.
18 Davies AJ, Rosenwald A, Wright G *et al.* Transformation of follicular lymphoma to diffuse large B-cell lymphoma proceeds by distinct oncogenic mechanisms. *Br J Haematol* 2007; **136**: 286–93.
19 Godon A, Moreau A, Talmant P *et al.* Is t(14;18) (q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. *Leukemia* 2003; **17**: 255–9.
20 Biagi JJ, Suymour JF. Insights into the molecular pathogenesis of follicular lymphoma arising from analysis of geographic variation. *Blood* 2002; **99**: 4265–75.
21 Sekiguchi N, Kobayashi Y, Yokota Y *et al.* Follicular lymphoma subgrouping by fluorescence in situ hybridization analysis. *Cancer Sci* 2005; **96**: 77–82.
22 Leoncini L, Delsol G, Gascoyne RD *et al.* Aggressive B-cell lymphomas: a review based on the workshop of the XI meeting of the European association for haematopathology. *Histopathology* 2005; **46**: 241–55.
23 Jaffe ES, Harris NL, Stein H *et al. World Health Organization Classification of Tumors, Pathology and Genetics, Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press, 2001; **162–167**: 171–4.
24 Dogan AMQ, Aiello A *et al.* Follicular lymphomas contain a clonally linked but phenotypically distinct neoplastic B-cell population in the interfollicular zone. *Blood* 1998; **91**: 4708–14.
25 Nunez G, London L, Hockenbery D *et al.* Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemoopietic cell lines. *J Immunol* 1990; **144**: 3602–10.
26 Lai R, Arber DA, Chang KL *et al.* Frequency of bcl-2 expression in non-Hodtkin lymphoma: a study of 778 cases with comparison of marginal zone lymphoma and monocytoid B-cell hyperplasia. *Mod Pathol* 1998; **11**: 864–9.
27 Falini B, Fizzotti M, Pileri S *et al.* Bcl-6 protein expression in normal and neoplastic lymphoid tissues. *Ann Oncol* 1997; **8**: 101–4.
28 Peh SC, Shaminie J, Tai YC *et al.* The pattern and frequency of t(14;18) translocation and immunophenotype in Asian follicular lymphoma. *Histopathology* 2004; **45**: 501–10.
29 Anagnostopoulos I, Dallenbach F, Stein H. Diffuse large cell lymphomas. In: Knowles DM, ed. *Neoplastic Hematopathology*, 2nd edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2001; 860.
30 Mamane Y, Heylbroeck C, Genin P *et al.* Interferon regulatory factors: the next generation. *Gene* 1999; **237**: 1–14.
31 Natkunam Y, Warnke RA, Montgomery K *et al.* Analysis of MUM1/IRF4 protein expression using tissue microarrays and immunohistochemistry. *Mod Pathol* 2001; **14**: 686–94.
32 Falini B, Fizzotti M, Pucciarini A *et al.* A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. *Blood* 2000; **95**: 2084–92.
33 Tsuboi K, Iida S, Inagaki H *et al.* MUM1/IRF4 expression as a frequent event in mature lymphoid malignancies. *Leukemia* 2000; **14**: 449–56.
34 Piris M, Gatter KC, Mason DY. CD30 expression in follicular lymphoma. *Histopathology* 1991; **18**: 25–9.
35 Yamaguchi M, Seto M, Okamoto M *et al.* De novo CD5$^+$ diffuse large B-cell lymphoma: a clinicopathologic study of 109 patients. *Blood* 2002; **99**: 815–21.
36 Manazza AD, Bonello L, Pagano M *et al.* Follicular origin of a subset of CD5$^+$ diffuse large B-cell lymphomas. *Am J Clin Pathol* 2005; **124**: 182–90.
37 Colomo L, Lopez-Guillermo A, Perales M *et al.* Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. *Blood* 2003; **101**: 78–84.

doi: 10.1111/j.1349-7006.2008.00873.x

Jpn J Clin Oncol 2008;38(8)547–552
doi:10.1093/jjco/hyn069

Follicular Lymphoma of the Duodenum: A Clinicopathologic Analysis of 26 Cases

Kazuhiro Sentani[1], Akiko Miyagi Maeshima[1], Junko Nomoto[2], Dai Maruyama[2], Sung-Won Kim[2], Takashi Watanabe[2], Yukio Kobayashi[2], Kensei Tobinai[2] and Yoshihiro Matsuno[1,3]

[1]Clinical Laboratory, [2]Hematology and Stem Cell Transplantation Divisions, National Cancer Center Hospital, Tokyo and [3]Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan

Received March 31, 2008; accepted July 7, 2008; published online August 7, 2008

Objective: Follicular lymphomas (FLs) occur commonly in the lymph nodes, and duodenal FL (DFL) is reported to be rare.
Methods: We analysed the clinical, morphological, immunohistochemical and genetic features of 26 cases of DFL. Primary DFLs and systemic FLs that involved the duodenum at any point during the clinical course were included in the analysis.
Results: Typically, primary DFLs (14 cases) were found incidentally at routine medical check-ups, whereas involvement of the duodenum by systemic FLs (12 cases) was found through staging procedures. All cases involved the second portion of the duodenum. *Helicobacter pylori* infection was common (71%). In all cases, the histologic grade was low (either grade 1 or 2), and CD20, CD10 and Bcl-2 were positive by immunohistochemistry. Immunoglobulin heavy chain gene (IGH) and bcl-2 gene (BCL2) fusion was frequently shown by fluorescence *in situ* hybridization (FISH) analysis: nine of 12 cases (75%) of primary DFL and 10 of 12 cases (83%) of systemic DFL were positive. Treatment regimens employed were rituximab (R) plus chemotherapy (10), R (6), chemotherapy (3), irradiation (3) and the other three patients were subjected to observation. After a median follow-up duration of 40 months (ranging 11–96 months), 17 patients were alive without disease, seven were alive with disease and one had died of lymphoma.
Conclusions: Primary DFLs resemble systemic and nodal FLs, except that the former has high incidence of early stage and low-grade histology. The duodenum appears to be a frequently involved extranodal site of FL with IGH/BCL2.

Key words: duodenum – follicular lymphoma – immunohistochemistry – FISH

INTRODUCTION

Follicular lymphoma (FL) is a neoplasm of follicular centre B cells and is one of the most common subtypes of non-Hodgkin lymphoma (NHL) in Europe and the USA (1). The most common subtype was reported to be diffuse large B-cell lymphoma (33%), followed by FL (18%) in Japan (2). Most patients with FL present with nodal involvement, and extranodal presentation occurs at the advanced stage. Primary extranodal FL has been reported to occur in the skin (3), salivary gland (4), ocular adnexa (5) and female genital tract (6). The gastrointestinal (GI) tract is the most commonly involved extranodal site of NHL, accounting for ~40% of all extranodal primary NHLs (7). However, FL of the GI tract represents only ~1–3.6% of all GI tract NHLs (8).

Duodenal FL (DFL) is a rare entity, with only a few reported cases (9–11). DFL is reported to be a characteristic clinicopathologic entity due to its localized nature and good prognosis (9,10). Yoshino et al. (9) reported that DFL was present around the Vater's papilla and showed multiple small-size polyps. Sato et al. (11) reported that DFL had intermediate characteristics of FL and mucosa-associated lymphoid tissue (MALT) lymphoma.

Most cases of nodal FL have a Bcl-2-positive immunophenotype and show immunoglobulin heavy chain gene (IGH) and bcl-2 gene (BCL2) fusion; up to 85% of tumours shows IGH/BCL2 fusion in Europe and the USA (12), whereas 60% does so in Japan (13). However, the situation might differ for cases originating from other sites. For example, FL

For reprints and all correspondence: Yoshihiro Matsuno, Department of Surgical Pathology, Hokkaido University Hospital, Kita 14 Nishi 5, Kita-ku, Sapporo 060-8648, Japan. E-mail: ymatsuno@med.hokudai.ac.jp

of the skin, which is the most common site of extranodal FL, has a Bcl-2-negative immunophenotype and rarely shows IGH/BCL2 fusion (14).

In this study, we examined the clinical, morphological, immunohistochemical and genetic features of 26 cases of DFL at a single institution. We categorized the 26 DFLs for which staging data were available into two groups: 14 primary DFLs and 12 systemic DFLs, and compared them with previously described cases of nodal FL.

PATIENTS AND METHODS

Patients

Twenty-six consecutive cases with a histologic diagnosis of DFL between April 1997 and October 2005 were retrieved from the archival pathology files of the National Cancer Center Hospital, Tokyo, Japan. All of the 26 DFLs were confirmed on the basis of morphologic and immunohistochemical features, and if available, the genetic features of FL. Clinical information was obtained from the medical records. We categorized the 26 DFLs into two groups on the basis of clinical stage: 14 primary DFLs and 12 systemic DFLs. For staging, the Ann Arbor staging system was used. The 26 patients were examined for staging by bone marrow aspiration or biopsy and computed tomography, and optionally, gallium scintigraphy. More recently, positron emission tomography and/or panendoscopy have been advocated as a tool for staging optionally, but were not performed in this series.

Histologic Examination

Biopsy materials were fixed with 10% buffered formalin overnight, then 4 μm-thick sections were made from paraffin blocks and stained with haematoxylin and eosin (HE) for routine diagnosis. Each HE specimen was histologically reviewed by three of the authors (KS, AMM and YM). Following the World Health Organization classification (15), each case was graded based on the number of centroblasts/high-power field (HPF), and patterns of follicular and/or diffuse growth were judged semi-quantitatively. Specifically, FL grade 1 showed 1–5 centroblasts/HPF; grade 2, 6–15 centroblasts/HPF and grade 3, >15 centroblasts/HPF. The growth patterns were recorded as follicular when >75% of the tumour showed follicularity, follicular and diffuse when 25–75% of the tumour showed follicularity and focally follicular when <25% of the tumour showed follicularity. *Helicobacter (H.) pylori* infection was assessed by histologic examination using Giemsa staining, serologic testing or rapid urease test and was defined as positive if any of these tests gave a positive result.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using a panel of antibodies. Sections 4 μm thick were cut from each paraffin block, deparaffinized and incubated at 121°C in pH 6.0 citrate buffer for 10 min for antigen retrieval. Antibodies included those against the following antigens: CD3 (PS1, Novocastra, Newcastle-upon-Tyne, UK: Polymer method), CD20 [L26, DAKO, Glostrup, Denmark: labelled streptavidin-biotin method (LSAB)], CD5 (4C7 Novocastra: Polymer method), CD10 (56C6, Novocastra: Polymer method), Bcl-2 (124, DAKO: LSAB) and cyclin D1 (DSC-6, Novocastra: Polymer method). Positive and negative controls were used for each antibody and for each case. CD20 and Bcl-2 were stained using a Biogenex autostainer™, and CD3, CD5, CD10 and cyclin D1 were stained using a DAKO autostainer plus™. Immunoreactivity was judged positive if 30% or more of the tumour cells were stained.

Fluorescence *In Situ* Hybridization Analysis

The tissue fluorescence *in situ* hybridization (FISH) procedure was performed on formalin-fixed paraffin sections in accordance with the procedure described by Sekiguchi et al. (13). A dual-colour LSI IgH Spectrum Green/LSI BCL2 Spectrum Orange Dual Fusion Translocation Probe (Vysis, Downers Grove, IL, USA) was used to detect t (14;18): IGH/BCL2 fusion. To analyse the hybridization, a total of 100–200 nuclei per case were judged, and if fusion signals were detected in 7% or more nuclei, IGH/BCL2 fusion was judged to be positive.

RESULTS

The characteristics of the patients are shown in Table 1. The median age was 54 years (range 36–72 years), and there were 13 males and 13 females. The distribution according to the Ann Arbor staging system was stage I, 10 cases (38%); II, four cases (15%); III, one case (4%); IV, 11 cases (42%). Fourteen cases (stage I or II) were classified as primary DFL and 12 cases (stage III or IV) as systemic DFL. Seventy-nine per cent (11 of 14) of primary DFLs were found at routine medical check-ups for gastric cancer screening, and the remaining 21% (three of 14) of patients complained of digestive symptoms. Seventy-five per cent (nine of 12) of patients with systemic DFL complained of lymph node enlargement, weight loss or digestive symptoms, and duodenal involvement was found during the staging procedure at initial presentation. The remaining 25% (three of 12) of systemic DFLs were found at routine medical check-ups.

All of the 25 cases for which endoscopic reports were available for review involved the second portion of the duodenum, and in 10 cases (40%), lesions were found in the vicinity of the Vater's papilla. Fourteen cases had multiple polypoid lesions and 11 cases had a single elevated lesion.

The results of histologic and immunohistochemical analyses are shown in Table 2. The histologic grades of DFLs were as follows: in primary DFL, grade 1, 13 cases (93%); grade 2, one case (7%), in systemic DFL, grade 1, nine cases (75%); grade 2, three cases (25%). Two of the primary DFLs had been diagnosed as MALT lymphoma initially, and