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Appendix

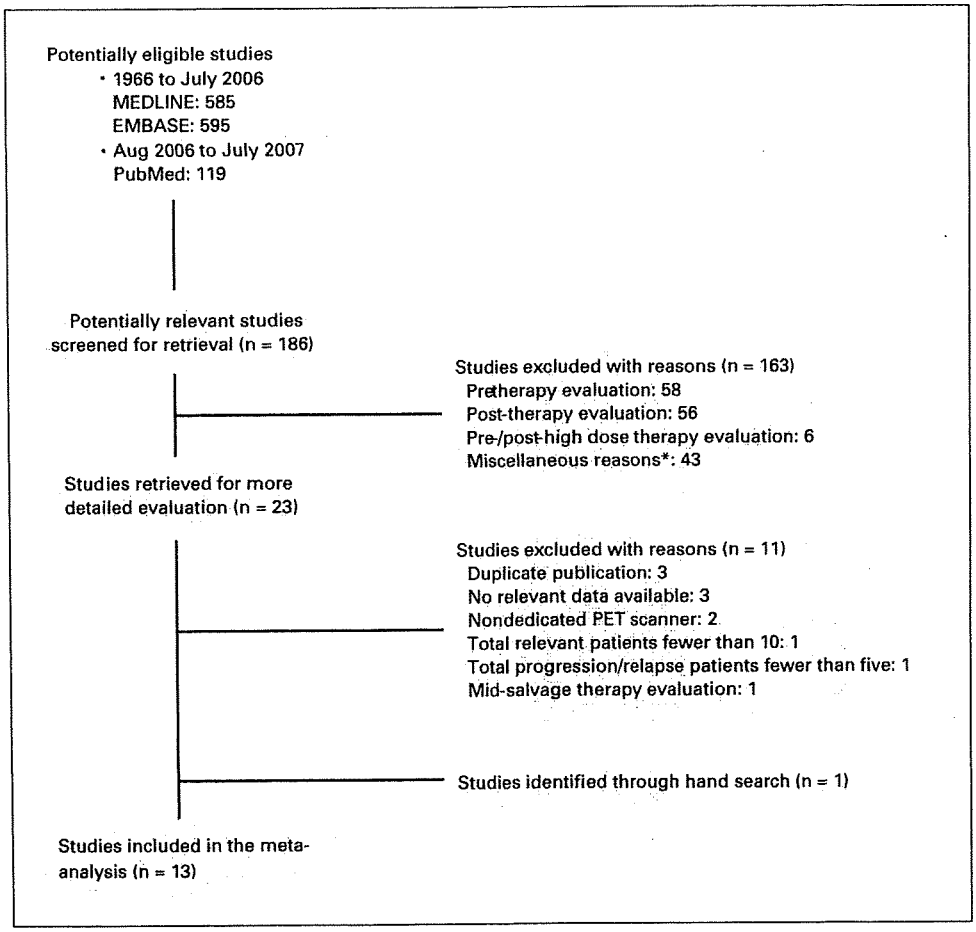

Fig A1. Article selection process. (*) Miscellaneous reasons include studies on staging evaluation at multiple different timings and contexts (n = 10), studies focusing on lymphoma involvement in a specific organ or anatomic region (n = 7), review articles (n = 5), studies focusing exclusively on positive positron emission tomography (PET) findings (n = 3), letters or comments (n = 3), studies on post-therapy follow-up (n = 3), studies on glucose metabolism (n = 3), and others (n = 9).

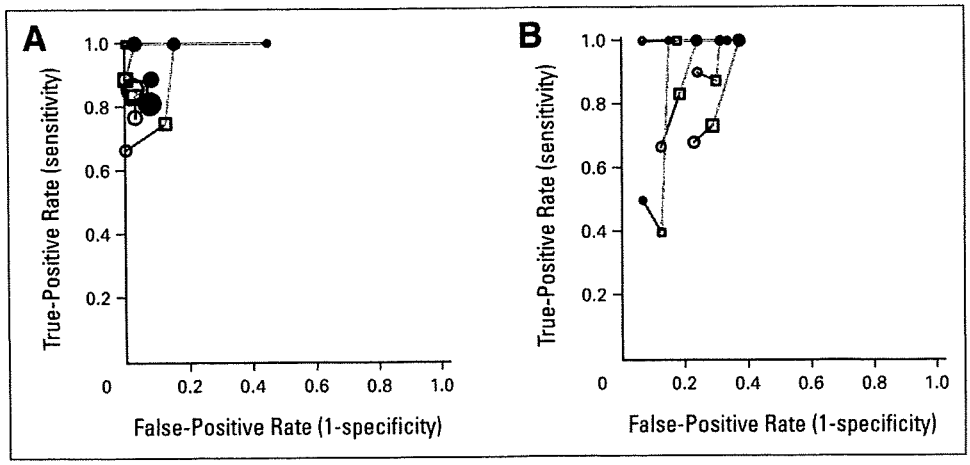

Fig A2. Receiver operating characteristic plotting for (A) advanced-stage Hodgkin's lymphoma and (B) diffuse large B-cell lymphoma. Individual study estimates of sensitivity and 1 − specificity are shown based on the duration of clinical follow-up: all treatment failures (open circles), early treatment failures (less than 12 months; open squares), very early treatment failures (less than 6 months; closed circles).

Table A1. Search Strategy

Ovid MEDLINE Search	EMBASE Search	PubMed Search
#1 exp Tomography, emission computed/	#1 exp computer assisted emission tomography/or exp positron emission tomography/or exp whole body tomography/	#1 tomography, emission computed [MeSH terms]
#2 positron emission tomography.ti,ab,rw,sh.	#2 positron emission tomography.ti,ab,hw,tn,mf.	#2 positron emission tomography
#3 pet$.ti,ab,rw,sh.	#3 pet$.ti,ab,hw,tn,mf.	#3 pet
#4 animal not (human and animal) sh.	#4 (animal not (human and animal)).ti,ab,hw,tn,mf.	#4 pet*
#5 #3 not #4	#5 3 not 4	#5 #1 OR #2 OR #3 OR #4
#6 #1 or #2 or #5	#6 1 or 2 or 5	#6 deoxyglucose [MeSH Terms]
#7 exp Deoxyglucose/	#7 exp Deoxyglucose/	#7 deoxyglucose
#8 deoxyglucose.ti,ab,rw,sh.	#8 deoxyglucose.ti,ab,hw,tn,mf.	#8 deoxy-glucose
#9 deoxy-glucose.ti,ab,rw,sh.	#9 deoxy-glucose.ti,ab,hw,tn,mf.	#9 fluorodeoxyglucose
#10 fluorodeoxyglucose.ti,ab,rw,sh.	#10 fluorodeoxyglucose.ti,ab,hw,tn,mf.	#10 18fluorodeoxyglucose
#11 18fluorodeoxyglucose.ti,ab,rw,sh.	#11 18fluorodeoxyglucose.ti,ab,hw,tn,mf.	#11 fludeoxyglucose
#12 fludeoxyglucose.ti,ab,rw,sh.	#12 fludeoxyglucose.ti,ab,hw,tn,mf.	#12 fdg
#13 fdg$.ti,ab,rw,sh.	#13 fdg$.ti,ab,hw,tn,mf.	#13 fdg*
#14 18fdg.ti,ab,rw,sh.	#14 18fdg.ti,ab,hw,tn,mf.	#14 18fdg
#15 f-18-dg.ti,ab,rw,sh.	#15 f-18-dg.ti,ab,hw,tn,mf.	#15 f-18-dg
#16 fluoro-2-deoxy-*d*-glucose.ti,ab,rw,sh.	#16 fluoro-2-deoxy-*d*-glucose.ti,ab,hw,tn,mf.	#16 fluoro-2-deoxy-*d*-glucose
#17 2fluoro-2deoxyglucose.ti,ab,rw,sh.	#17 2fluoro-2deoxyglucose.ti,ab,hw,tn,mf.	#17 2fluoro-2deoxyglucose
#18 fluoro-*d*-glucose.ti,ab,rw,sh.	#18 fluoro-*d*-glucose.ti,ab,hw,tn,mf.	#18 fluoro-*d*-glucose
#19 or/#8-#18	#19 or/#8-18	#19 #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18
#20 #7 or #19	#20 #7 or #19	#20 lymphoma
#21 #6 and #20	#21 #6 and #20	#21 lymphom*
#22 exp sensitivity-and-specificity or predict$ or diagnos$ or di.fs. or du.fs. or accura$	#22 sensitiv$ or detect$ or accura$ or specific$ or reliab$ or positive or negative diagnos$ or di.fs.	#22 Hodgkin*
#23 #21 and #22	#23 #21 and #22	#23 #20 OR #21 OR #22
#24 exp Lymphoma/	#24 exp Lymphoma/	#24 #5 AND #19 AND #23
#25 lymphoma.ti,ab,rw,sh.	#25 lymphoma.ti,ab,rw,sh.	
#26 lymphom$.ti,ab,rw,sh.	#26 lymphom$.ti,ab,rw,sh.	
#27 hodgkin$.ti,ab,rw,sh.	#27 hodgkin$.ti,ab,rw,sh.	
#28 or/#24-#27	#28 or/#24-#27	
#29 #23 and #28	#29 #23 and #28	

Table A2. Quality Assessment of Studies of Positron Emission Tomography for Interim Response Assessment of Malignant Lymphoma

Item No.	Bias or Issue Addressed	Question	How Scored
1	Avoidance of spectrum bias	Was the spectrum of patients representative of the patients who will receive the test in practice?	Scored as "yes" if patients were enrolled onto a study prospectively and consecutively based on the predefined inclusion criteria
2	Provision of a clear definition of the inclusion (and exclusion) criteria	Were selection criteria clearly described?	Scored as "yes" if a study reported clear inclusion criteria
3	Appropriateness of the reference standard	Is the reference standard likely to correctly classify the target condition?	Scored as "yes" if a study employed clinical follow-up with or without biopsy as the reference standard*
4	Avoidance of partial verification bias	Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?	Scored as "yes" if the whole patients of a study received disease verification through clinical follow-up with or without biopsy
5	Avoidance of differential verification bias	Did patients receive the same reference standard regardless of the index test result?	Scored as "yes" if the whole patients of a study received disease verification through clinical follow-up with or without biopsy regardless of the interim PET results
6	Avoidance of incorporation bias	Was the reference standard independent of the index test (i.e., the index test did not form part of the reference standard)?	Scored as "yes" as long as the ultimate diagnosis was made through predefined reference standard (ie, conventional response assessment with or without biopsy during clinical follow-up for disease progression or relapse, or sufficiently long follow-up for continuing remission) even if interim PET results were available to clinicians; scored as "no" only if the mid-therapy results were specifically used to determine the final clinical outcome
7	Replicativeness of the index test	Was the execution of the index test described in sufficient detail to permit replication of the test?	Scored as "yes" if a study reported sufficient details on the procedure and diagnostic criteria of interim PET
8	Replicativeness of the reference standard	Was the execution of the reference standard described in sufficient detail to permit its replication?	Scored as "yes" if a study evaluated disease status and followed up patients following the recommended standard guidelines*
9	Avoidance of test review bias	Were the index test results interpreted without knowledge of the results of the reference standard?	Scored as "yes" if interim PET was interpreted without knowledge of the clinical information on patients; scored as "no" if PET interpreters read the scan results in the presence of any clinical data including conventional imaging tests, laboratory test, and physical examinations obtained after the initiation of treatment, which could have contained the information on the response assessment or disease status of patients
10	Avoidance of diagnosis review bias	Were the reference standard results interpreted without knowledge of the results of the index test?	Scored as "yes" if the clinicians treated and followed up patients without knowledge of interim PET results
11	Availability of clinical data to test interpreters	Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?	Scored as "yes" if interpreters read interim PET scan in the presence of clinical information excluding baseline pre-therapy PET scan
12	Reporting of uninterpretable or intermediate results	Were uninterpretable/intermediate test results reported?	Scored as "yes" if a study reported the number of patients with minimal residual uptake
13	Provision of the information on withdrawals from a study	Were withdrawals from the study explained?	Scored as "yes" if a study clearly reported the number of patients satisfying the inclusion criteria that did or did not undergo interim PET and/or clinical follow-up with or without biopsy; scored as "no" if a study included exclusively patients who underwent interim PET and did not report the information on patients who satisfied the same inclusion criteria but did not undergo interim PET

Abbreviation: PET, positron emission tomography.
*Alternative more stringent criterion was also employed as follows: Scored as "yes" if a study explicitly stated that investigators assessed disease status according to the standard guidelines (Cheson BD, Horning SJ, Coiffier B, et al: J Clin Oncol 17:1244-1253, 1999; Lister TA, Crowther D, Sutcliffe SB, et al: J Clin Oncol 7:1630-1636, 1989) and followed up patients in remission including negative biopsy of lesions suspected of treatment failure (progression or relapse) for at least 1 year.

Table A3. Diagnostic Criteria and Interpreters of PET for Interim Response Assessment of Malignant Lymphoma

Study	Year	Interpretation Method	Qualitative Diagnostic Criteria			Reading Condition: Availability of Pretherapy PET to Interpreters of Interim PET	Interpreter	
			Positive	Negative	Minimal Residual Uptake		No.	Experience
Advanced-stage HL + DLBCL								
Kostakoglu et al (Kostakoglu L, Goldsmith SJ, Leonard JP, et al: Cancer 107:2678-2687, 2006)	2006	Qualitative analysis	Presence of FDG uptake that exceeded the uptake seen on the contralateral site or in the background in a location incompatible with normal anatomy or physiologic variants	No pathologically increased FDG uptake at any site compared with the uptake on the contralateral site or the background	Not specified	Yes	2	Expert
		Semi-quantitative analysis (complemental): SUV_{max} measured for only measurable nodal sites (lesion-based ROC analysis)	Increased FDG uptake in contralateral and asymmetrical sites compared with background activity to be compared with: general, the highest activity excluding pathological and physiologic sites of uptake; head and neck, within the jugular vessels; chest, in the mediastinum around the aortic arch region; abdomen/pelvis, in the mesentery or abdominal vessels, whichever had the higher activity					
Advanced-stage HL								
Friedberg et al (Friedberg JW, Fischman A, Neuberg D, et al: Leuk Lymphoma 45:85-92, 2004)	2004	Qualitative analysis	Nodal involvement: FDG avidity above mediastinal blood pool activity	Not specified	Not specified	No	2	Expert
Hutchings et al (Hutchings M, Mikhaeel NG, Fields PA, et al: Ann Oncol 16: 1160-1168, 2005)	2005	Qualitative analysis	Increased uptake suspicious for malignant disease, which does not have a benign explanation	No evidence of disease	Low-grade uptake of FDG (just above background) in a focus within an area of previously noted disease, not likely representing malignancy	Yes*	2	Expert
Gallamini et al (Gallamini A, Rigacci L, Merli F, et al: Haematologica 91:475-481, 2006)	2006	Qualitative analysis Semi-quantitative analysis (complemental): SUV_{max} measured for regions of interest (patient-based % of SUV_{max} reduction from baseline)	Presence of a focal concentration of FDG outside the areas of physiological uptake, with a value increased relative to background	No pathological FDG uptake at any site, including all sites of previously increased pathological uptake	Not specified	Yes	2	Expert

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Table A3. Diagnostic Criteria and Interpreters of PET for Interim Response Assessment of Malignant Lymphoma (continued)

Study	Year	Interpretation Method	Qualitative Diagnostic Criteria: Positive	Qualitative Diagnostic Criteria: Negative	Qualitative Diagnostic Criteria: Minimal Residual Uptake	Reading Condition: Availability of Pretherapy PET to Interpreters of Interim PET	Interpreter: No.	Interpreter: Experience
Hutchings et al (Hutchings M, Loft A, Hansen M, et al: Blood 107:52-59, 2006)	2006	Qualitative analysis Semi-quantiitative analysis (complemental): SUV_{max} measured for regions of interest (patient-based distribution of SUV_{max})	Focal FDG concentration outside the physiological uptake areas, with clearly increased activity relative to the background	No pathologic FDG uptake at any site, including all sites of previously increased pathologic uptake	Low-grade FDG-uptake with avidity smaller than, equal to, or only slightly higher than the uptake in the mediastinal blood pool structures	Yes	2	Expert
Zinzani et al (Zinzani PL, Tani M, Fanti S, et al: Ann Oncol 17:1296-1300, 2006)	2006	Qualitative analysis	Areas of focal uptake other than the sites of known accumulation, including the kidney, bladder, and gastrointestinal tract	No evidence of disease Skeletal areas showing symmetric joint uptake, especially within the shoulder (considered arthritis)	Low-grade uptake of FDG (just above background) in a focus within an area of previously noted disease	Yes	3	Expert
Gallamini et al (Gallamini A, Hutchings M, Rigacci L, et al: J Clin Oncol 25: 3746-3752, 2007)	2007	Qualitative analysis Semi-quantitative analysis (complemental): SUV_{max} was measured for regions of interest	Presence of a focal FDG concentration outside the physiological uptake areas, with clearly increased activity relative to the background	No pathologic FDG uptake at any site, including all sites of previously increased pathologic uptake	Low-grade FDG-uptake with avidity smaller than, equal to, or only slightly higher than the uptake in the mediastinal blood pool structures A SUV of 2.0 to 3.5	Yes	2	Expert
DLBCL								
Spaepen et al (Spaepen K, Stroobants S, Dupont P, et al: Ann Oncol 13: 1356-1363, 2002)	2002	Qualitative analysis	Any focal or diffuse area of increased activity in a location incompatible with normal anatomy and suspect for residual disease and/or new localizations	No evidence of disease	Not specified	Unclear	2	Expert
Haioun et al (Haioun C, Itti E, Rahmouni A, et al: Blood 106: 1376-1381, 2005)	2005	Qualitative analysis†	At least one residual with a low extent and moderate intensity of abnormal FDG uptake Two or more residual sites with any extent and intensity of abnormal FDG uptake	No residual abnormal FDG uptake A unique residual site with a low extent and low intensity of FDG uptake, with all the other previously hypermetabolic sites extinguished	Not specified	Yes	2	Expert
Mikhaeel et al (Mikhaeel NG, Hutchings M, Fields PA, et al: Ann Oncol 16: 1514-1523, 2005)	2005	Qualitative analysis	Persistence or appearance of new areas of increased uptake, thought to be lymphoma-related	Disappearance of all abnormal disease-related uptake	Low-grade uptake of FDG in a focus within an area of previously noted disease, likely to represent inflammation, where small volume malignancy could not be excluded	Yes*	2	Expert

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Table A3. Diagnostic Criteria and Interpreters of PET for Interim Response Assessment of Malignant Lymphoma (continued)

Study	Year	Interpretation Method	Qualitative Diagnostic Criteria			Reading Condition: Availability of Pretherapy PET to Interpreters of Interim PET	Interpreter	
			Positive	Negative	Minimal Residual Uptake		No.	Experience
Fruchart et al (Fruchart C, Reman O, Le Stang N, et al: Leuk Lymphoma 47:2547-2557, 2006)	2006	Qualitative analysis	At least one site of residual uptake	No significant residual uptake in suspected sites of lymphoma before treatment	Not specified	Yes	1	Expert
Querellou et al (Querellou S, Valette F, Bodet-Milin C, et al: Ann Hematol 85: 759-767, 2006)	2006	Qualitative analysis	Any focus of increased FDG uptake over background not located in areas of normal FDG uptake and/or FDG excretion Any focal or diffuse area of increased activity in a location suspect for residual disease	No evidence of disease, i.e., no abnormal residual uptake in previously involved sites resulting in a complete normalization	Not specified	Yes	2	Expert
Ng et al (Ng AP, Wirth A, Seymour JF, et al: Leuk Lymphoma 48: 596-600, 2007)	2007	Qualitative analysis	Increased FDG-avidity above a baseline level, as subjectively characterized by FDG-avidity of the liver parenchyma, in a region of lymphoma, documented clinically or radiologically, at diagnosis	No residual uptake in suspected sites of lymphoma before treatment	Not specified	Yes*	3	Expert

Abbreviations: CT, computed tomography; DLBCL, diffuse large B-cell lymphoma; FDG, fluorodeoxyglucose; HL, Hodgkin's lymphoma; PET, positron emission tomography; ROC, receiver operating characteristics; SUV_{MAX}, maximum standard uptake value in region(s) of interest.
*Mid-therapy PET was interpreted without pre-therapy baseline scan in some patients.
†Only visual interpretations were taken into account.

Table A4. Quality Assessment of Studies of Positron Emission Tomography for Interim Response Assessment of Malignant Lymphoma

Study	Year	QUADAS 1: Avoidance of Spectrum Bias	QUADAS 2: Reporting of Inclusion Criteria	QUADAS 3: Appropriate Reference Standard	QUADAS 4: Avoidance of Partial Verification Bias	QUADAS 5: Avoidance of Differential Verification Bias	QUADAS 6: Avoidance of Incorporation Bias
Advanced-stage HL + DLBCL							
Kostakoglu et al (Kostakoglu L, Goldsmith SJ, Leonard JP, et al: Cancer 107:2678-2687, 2006)	2006	No	Yes	Yes	Yes	Yes	Yes
Advanced-stage HL							
Friedberg et al (Friedberg JW, Fischman A, Neuberg D, et al: Leuk Lymphoma 45:85-92, 2004)	2004	Yes	Yes	Yes*	Yes	Yes	Yes
Hutchings et al (Hutchings M, Mikhaeel NG, Fields PA, et al: Ann Oncol 16:1160-1168, 2005)	2005	No	Yes	Yes*	Yes	Yes	Yes
Gallamini et al (Gallamini A, Rigacci L, Merli F, et al: Haematologica 91:475-481, 2006)	2006	Yes	Yes	Yes*	Yes	Yes	No‡
Hutchings et al (Hutchings M, Loft A, Hansen M, et al: Blood 107:52-59, 2006)	2006	Yes	Yes	Yes†	Yes	Yes	Yes
Zinzani et al (Zinzani PL, Tani M, Fanti S, et al: Ann Oncol 17:1296-1300, 2006)	2006	Yes	Yes	Yes	Yes	Yes	Yes
Gallamini et al (Gallamini A, Hutchings M, Rigacci L, et al: J Clin Oncol 25:3746-3752, 2007)	2007	Yes	Yes	Yes†	Yes	Yes	Yes
DLBCL							
Spaepen et al (Spaepen K, Stroobants S, Dupont P, et al: Ann Oncol 13:1356-1363, 2002)	2002	Yes	Yes	Yes*	Yes	Yes	Yes
Haioun et al (Haioun C, Itti E, Rahmouni A, et al: Blood 106:1376-1381, 2005)	2005	Yes	Yes	Yes*	Yes	Yes	Yes
Mikhaeel et al (Mikhaeel NG, Hutchings M, Fields PA, et al: Ann Oncol 16:1514-1523, 2005)	2005	No	Yes	Yes*	Yes	Yes	Yes
Fruchart et al (Fruchart C, Reman O, Le Stang N, et al: Leuk Lymphoma 47:2547-2557, 2006)	2006	Yes	Yes	Yes*	Yes	Yes	Yes
Querellou et al (Querellou S, Valette F, Bodet-Milin C, et al: Ann Hematol 85:759-767, 2006)	2006	No	Yes	Yes*	Yes	Yes	Yes
Ng et al (Ng AP, Wirth A, Seymour JF, et al: Leuk Lymphoma 48:596-600, 2007)	2007	No	Yes	Yes*	Yes	Yes	Yes

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Table A4. Quality Assessment of Studies of Positron Emission Tomography for Interim Response Assessment of Malignant Lymphoma (continued)

Study	QUADAS						
	7: Replicativeness of Index Test	8: Replicativeness of Reference Standard	9: Avoidance of Test Review Bias	10: Avoidance of Diagnosis Review Bias	11: Availability of Clinical Data to Test Interpreters	12: Reporting of Uninterpretable or Intermediate Results	13: Reporting of Withdrawals
Advanced-stage HL + DLBCL							
Kostakoglu et al (Kostakoglu L, Goldsmith SJ, Leonard JP, et al: Cancer 107:2678-2687, 2006)	No	Yes	Yes	No¶	No	No	No
Advanced-stage HL							
Friedberg et al (Friedberg JW, Fischman A, Neuberg D, et al: Leuk Lymphoma 45:85-92, 2004)	Yes	Yes	Yes	No	No	No	Unclear
Hutchings et al (Hutchings M, Mikhaeel NG, Fields PA, et al: Ann Oncol 16:1160-1168, 2005)	Yes	No§	Unclear	Unclear‖	Unclear	Yes	No
Gallamini et al (Gallamini A, Rigacci L, Merli F, et al: Haematologica 91:475-481, 2006)	Yes	Yes	No	No‡¶	Yes	No	Unclear
Hutchings et al (Hutchings M, Loft A, Hansen M, et al: Blood 107:52-59, 2006)	Yes	Yes	Yes	Yes#	No	No	Yes
Zinzani et al (Zinzani PL, Tani M, Fanti S, et al: Ann Oncol 17:1296-1300, 2006)	Yes	Yes	Unclear	Unclear¶	Unclear	Yes	Unclear
Gallamini et al (Gallamini A, Hutchings M, Rigacci L, et al: J Clin Oncol 25:3746-3752, 2007)	Yes	Yes	No	Yes#	Yes	No	Unclear
DLBCL							
Spaepen et al (Spaepen K, Stroobants S, Dupont P, et al: Ann Oncol 13:1356-1363, 2002)	Yes	Yes	Yes	Unclear	No	No	Unclear
Haioun et al (Haioun C, Itti E, Rahmouni A, et al: Blood 106:1376-1381, 2005)	Yes	Yes	Yes	Unclear¶	No	No	Unclear
Mikhaeel et al (Mikhaeel NG, Hutchings M, Fields PA, et al: Ann Oncol 16:1514-1523, 2005)	Yes	No§	Yes	Unclear‖	No	Yes	No
Fruchart et al (Fruchart C, Reman O, Le Stang N, et al: Leuk Lymphoma 47:2547-2557, 2006)	Yes	Yes	No	Yes#	Yes	No	Yes
Querellou et al (Querellou S, Valette F, Bodet-Milin C, et al: Ann Hematol 85:759-767, 2006)	Yes	Yes	Yes	Unclear‖¶	No	No	Unclear
Ng et al (Ng AP, Wirth A, Seymour JF, et al: Leuk Lymphoma 48:596-600, 2007)	No	No§	Unclear	Unclear‖	Unclear	No	No

Abbreviations: DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; QUADAS, quality assessment tool of diagnostic accuracy studies.

*Scored as "unclear" if alternative criterion was applied; none of these studies specified minimum follow-up period for continuous remission or reported the data on censoring within a year.

†Scored as "no" if alternative criterion was applied; all these studies explicitly reported at least one patient without treatment failure censored within a year.

‡One interim PET scan result was used to determine final clinical outcome because biopsy could not be performed.

§All these studies did not explicitly report the use of the standard guidelines; however, they actually employed them per unpublished data.

‖All these studies did not explicitly report the blinding of clinicians to interim PET scan; however, the scan results were timely made available to treating physicians per unpublished data.

¶Interim PET scan was not used at least to alter the preplanned treatment strategy including adjuvant involved-field radiation or high-dose chemotherapy although the blinding of treating physicians to the results was either unclear or unemployed.

#Interim PET scan results were explicitly excluded from clinical data with which treating physicians made clinical decision.

Table A5. Study Results of PET for Interim Response Assessment of Malignant Lymphoma

Study	Year	Total No.	Crude Cummulative Incidence of Treatment Failure (%)			Crude Incidence of Loss to Follow-Up	Treatment Failure (No.)									Continuing Remission (No.)			No. of Censored Patients ≤ 1 Year		
							PET Positive			PET MRU			PET Negative								
								Post-Therapy*			Post-Therapy*			Post-Therapy*							
			During Therapy	≤ 1 Year	Entire Follow-Up	≤ 1 Year (%)	During Therapy	Early	Late	During Therapy	Early	Late	During Therapy	Early	Late	PET Positive	PET MRU	PET Negative	PET Positive	PET MRU	PET Negative
Advanced-stage HL																					
Friedberg et al (Friedberg JW, Fischman A, Neuberg D, et al: Leuk Lymphoma 45:85-92, 2004)	2004	22	NR	NR	23	NR	NR	NR	4†	—	—	—	NR	NR	1†	1	—	16	NR	—	NR
Hutchings et al (Hutchings M, Mikhaeel NG, Fields PA, et al: Ann Oncol 16: 1160-1168, 2005)	2005	28	7	14	32	11	2	1	3	0	1	0	0	0	2	0	6	13	0	1	2
Gallamini et al (Gallamini A, Rigacci L, Merli F, et al: Haematologica 91:475-481, 2006)	2006	108	15	19	19	NR	13	4	1	—	—	—	3	0	0	2	—	85	NR	—	NR
Hutchings et al (Hutchings M, Loft A, Hansen M, et al: Blood 107:52-59, 2006)	2006	46	20	26	28	9	8	2	0	—	—	—	1	1	1	1	—	32	0	—	4
Kostakoglu et al (Kostakoglu L, Goldsmith SJ, Leonard JP, et al: Cancer 107: 2678-2687, 2006)	2006	10	10	50	50	0	1	4	0	—	—	—	0	0	0	0	—	5	0	—	0
Zinzani et al (Zinzani PL, Tani M, Fanti S, et al: Ann Oncol 17:1296-1300, 2006)	2006	40	18	23	23	0	7	1	0	0	1	0	0	0	0	0	3	28	0	0	0
Gallamini et al (Gallamini A, Hutchings M, Rigacci L, et al: J Clin Oncol 25: 3746-3752, 2007)	2007	106	—	10	18	NR	NR	9†	6	—	—	—	NR	2†	2	4	—	83	NR	—	NR
DLBCL																					
Spaepen et al (Spaepen K, Stroobants S, Dupont P, et al: Ann Oncol 13: 1356-1363, 2002)	2002	47	NR	NR	47	NR	NR	NR	20†	—	—	—	NR	NR	2†	0	—	25	NR	—	NR
Haioun et al (Haioun C, Itti E, Rahmouni A, et al: Blood 106: 1376-1381, 2005)	2005	83	NR	NR	39	NR	NR	NR	20†	—	—	—	NR	NR	12†	14	—	37	NR	—	NR

(continued on following page)

Table A5. Study Results of PET for Interim Response Assessment of Malignant Lymphoma (continued)

Study	Year	Total No.	Crude Cummulative Incidence of Treatment Failure (%)			Crude Incidence of Loss to Follow-Up	Treatment Failure (No.)									Continuing Remission (No.)			No. of Censored Patients ≤ 1 Year		
							PET Positive			PET MRU			PET Negative								
								Post-Therapy*			Post-Therapy*			Post-Therapy*							
			During Therapy	≤ 1 Year	Entire Follow-Up	≤ 1 Year (%)	During Therapy	Early	Late	During Therapy	Early	Late	During Therapy	Early	Late	PET Positive	PET MRU	PET Negative	PET Positive	PET MRU	PET Negative
Mikhaeel et al (Mikhaeel NG, Hutchings M, Fields PA, et al: Ann Oncol 16: 1514-1523, 2005)	2005	57	5	26	38	14	3	8	4	0	2	1	0	2	2	8	5	22	2	1	5
Fruchart et al (Fruchart C, Reman O, Le Stang N, et al: Leuk Lymphoma 47:2547-2557, 2006)	2006	35	17	23	29	3	6	1	2	—	—	—	0	1	0	6	—	19	0	—	1
Kostakoglu et al (Kostakoglu L, Goldsmith SJ, Leonard JP, et al: Cancer 107: 2678-2687, 2006)	2006	24	13	29	38	0	3	4	2	—	—	—	0	0	0	1	—	14	0	—	0
Querellou et al (Querellou S, Valette F, Bodet-Milin C, et al: Ann Hematol 85:759-767, 2006)	2006	21	5	24	29	5	1	1	1	—	—	—	0	3	0	1	—	14	0	—	1
Ng et al (Ng AP, Wirth A, Seymour JF, et al: Leuk Lymphoma 48: 596-600, 2007)	2007	44	5	14	27	2	2	3	3	—	—	—	0	1	3	4	—	28	0	—	2

Abbreviations: DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; MRU, minimal residual uptake; NR, not reported; PET, positron emission tomography.

*Treatment failures after completing first-line therapy were divided into two groups: early progression or relapse (within a year from the start of therapy) and late relapse (after a year).

†Data were reported as the total number of treatment failures through the entire follow-up period.

‡Data were reported as the total number of treatment failures within a year from the start of first-line therapy.

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Phase II study of oral fludarabine in combination with rituximab for relapsed indolent B-cell non-Hodgkin lymphoma

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Oral fludarabine is more convenient than intravenous fludarabine in an outpatient setting. To assess the efficacy and toxicity of oral fludarabine in combination with rituximab in patients with relapsed indolent B-cell non-Hodgkin lymphoma (B-NHL), we conducted a multicenter phase II study. Patients with relapsed indolent B-NHL with two or fewer prior regimens and up to 16 doses of rituximab were eligible. Patients received 375 mg/m² rituximab on day 1, and 40 mg/m² oral fludarabine once daily on days 1 through 5 every 28 days for up to six cycles. The primary endpoint was the overall response rate. Forty-one patients were enrolled, including 38 (93%) with follicular lymphoma. Thirty-four patients (83%) had received rituximab as prior therapy. Twenty-seven patients (66%) completed the planned six cycles. Dose reduction of oral fludarabine was required in 17 patients (41%). The overall response rate was 76% (31 of 41 patients; 95% confidence interval, 60–88%) with a complete response rate of 68% (28 of 41 patients; 95% confidence interval, 52–82%). Median progression-free survival for the 41 patients was 19.7 months (95% confidence interval, 12.3–26.5 months). Hematological toxicities, including grade 4 neutropenia (68%), were the most frequent toxicities. Non-hematological toxicities were mild, except for one patient who died of *Pneumocystis jiroveci* pneumonia 4 months after the protocol treatment. In conclusion, oral fludarabine in combination with rituximab is a highly effective and convenient therapy for patients with relapsed indolent B-NHL who have mostly been pretreated with rituximab. (ClinicalTrials.gov number, NCT00311129.) (*Cancer Sci* 2009; 100: 1951–1956)

Indolent B-cell non-Hodgkin lymphoma (B-NHL), mainly consisting of follicular lymphoma, has a long course of disease, usually requiring multiple treatment regimens.[1] Although a recent meta-analysis of seven randomized studies for previously untreated and treated indolent B-NHL confirmed a survival benefit associated with the addition of rituximab to chemotherapy,[2] no standard chemotherapy regimens have been established. Among various combination chemotherapy regimens, cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy, and cyclophosphamide, vincristine, and prednisone (CVP) are frequently used regimens in combination with rituximab;[3–6] however, they have certain limitations, including peripheral neuropathy, alopecia, and cardiac toxicity. In addition, recent reports regarding the long-term follow-up results of CVP and CHOP in combination with rituximab for indolent B-NHL revealed that these regimens are not satisfactory in view of long-term response durability.[6,7] Thus, novel combinations with highly effective and less-toxic profiles are required. Most patients with indolent B-NHL have a good performance status, which makes outpatient treatment desirable. Effective oral treatment in combination with rituximab may decrease the number of clinic visits and reduce the consumption of medical resources.

Fludarabine phosphate is one of the most effective drugs in the treatment of indolent B-NHL,[8,9] and exhibits a better progression-free survival (PFS) than CVP.[10] To improve the efficacy of fludarabine as a single agent, a variety of combination therapies has been reported, including fludarabine plus cyclophosphamide,[11] and fludarabine plus mitoxantrone and dexamethasone.[12,13] Despite the excellent antitumor activities of these combinations, long-lasting myelosuppression and increased risk of opportunistic infections are clinically significant problems.[14–16]

Several preclinical studies suggested the potential synergism between fludarabine and rituximab.[17–19] In addition, rituximab has been shown *in vitro* to sensitize drug-resistant lymphoma cell lines to cytotoxic chemotherapy.[20] Intravenous fludarabine in combination with rituximab was reported to be effective in the treatment of treatment-naïve or relapsed patients with indolent B-NHL, with 90% overall response rate (ORR).[21] Although the combination is very effective, intravenous administration of fludarabine for 5 consecutive days is inconvenient in an outpatient setting. We reported the results of phase I and II studies of oral fludarabine in relapsed indolent B-NHL previously.[22,23] As a single agent for relapsed patients who have been mostly pretreated with rituximab, it showed the ORR and complete response (CR) rates of 65 and 30%, respectively.[23] To further enhance the effectiveness of oral fludarabine, we conducted a multicenter phase II study to evaluate the efficacy and safety of oral fludarabine in combination with rituximab for relapsed indolent B-NHL.

Patients and Methods

Patient selection. Patients aged 20–74 years, with relapsed or refractory, histologically confirmed CD20-positive, indolent B-NHL (World Health Organization classification: small lymphocytic lymphoma, lymphoplasmacytic lymphoma, splenic marginal zone B-cell lymphoma, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type, nodal marginal zone

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B-cell lymphoma, and follicular lymphoma) were eligible.[24] Patients with mantle cell lymphoma were excluded. Patients were to have measurable disease, an Eastern Cooperative Oncology Group performance status of 0–1,[25] an expected survival of more than 3 months, and no major organ dysfunctions (neutrophil counts $\geq 1500/\mu L$, peripheral blood tumor cells $< 5000/\mu L$, platelet counts $\geq 7.5 \times 10^4/\mu L$, aspartate aminotransferase and alanine transaminase (ALT) $< 2.5 \times$ upper limit of normal, total bilirubin $< 1.5 \times$ upper limit of normal, serum creatinine $< 1.5 \times$ upper limit of normal, and no electrocardiogram (ECG) abnormalities requiring treatment). The number of prior chemotherapy regimens was limited to two or fewer, and prior rituximab treatments up to 16 times were allowed, but the duration from the last treatment had to be at least 4 weeks for chemotherapy and 6 months for rituximab. The following exclusion criteria were applied: positivity of hepatitis B virus surface antigen, hepatitis C virus antibody, or human immunodeficiency virus antibody, infectious disease, other serious complications, history of autoimmune hemolytic anemia, prior hematopoietic stem cell transplantation, and prior therapy with fludarabine, pentostatin, or cladribine. Patients with active other malignancies requiring treatment were also excluded. Pregnant or lactating women were not eligible, and all patients were required to use contraception as necessary. The study protocol was approved by the institutional review board of each participating institution before the patients were enrolled in the study. All patients gave written informed consent before they entered the study.

Study design and protocol treatment. This was a non-randomized phase II study. Rituximab (provided by Zenyaku Kogyo, Tokyo, Japan) at 375 mg/m^2 was intravenously administered on day 1 of each treatment cycle along with oral dosing of 40 mg/m^2 fludarabine phosphate tablets once daily from day 1 through 5 every 28 days for up to six cycles. Rituximab dosage was fixed throughout the course, and was infused according to the package insert. At the end of each cycle, adverse events were assessed, and the start of the next cycle and the dosage of fludarabine phosphate tablet were determined. If a patient developed toxicities on certain treatment days, the treatment was postponed, or the dosage of oral fludarabine was reduced to 30 mg/m^2 daily for all subsequent cycles. In addition, the dosage of oral fludarabine was reduced to 30 mg/m^2 daily if severe toxicities were observed during the treatment cycles. If postponement lasted longer than 14 days, the protocol treatment was terminated. Prophylactic use of trimethoprim and sulfamethoxazole and acyclovir was recommended, and granulocyte colony-stimulating factor (G-CSF) was given as needed.

Patient monitoring. Patients were admitted for the first infusion of rituximab to observe its infusion-related toxicities, but thereafter they could be treated as outpatients. The following evaluations were carried out during the pretreatment screening period: vital signs, ECG, laboratory studies, human antichimera antibody, bone marrow aspiration or biopsy with nested PCR for immunoglobulin heavy-chain and *bcl-2* gene (IgH/*bcl-2*) rearrangement, and computed tomography (CT) imaging. During the treatment, patients were observed by physical examination, complete blood counts, and serum chemistry every week. CT scan and bone marrow aspiration or biopsy were carried out 4 weeks after the start of the third and sixth courses in patients who showed positive lymphomatous lesions before enrolment. If baseline bone marrow IgH/*bcl-2* gene rearrangement was positive and the patient achieved a partial or better response, bone marrow PCR for IgH/*bcl-2* gene rearrangement was repeated. Patients were observed until 12 weeks after completion of the protocol treatment or until the assessment of progressive disease (PD); thereafter, the patients were followed up regularly until PD or death.

Data analysis. Responses were assessed according to the International Workshop Criteria for non-Hodgkin lymphoma as follows.[26] CR required the complete disappearance of all lesions and radiological or biological abnormalities and the absence of new lesions. CR unconfirmed described patients who met the criteria of CR but who had an indeterminate bone marrow assessment or a more than 75% decrease from baseline in the sum of the products of the greatest perpendicular diameters (SPD) of all measurable lesions but with a residual mass. Partial response was defined as a more than 50% decrease from baseline in the SPD of all measured lesions, no increase in the size of any other lesions, and no new lesions. Stable disease was defined as neither a 50% decrease nor a 50% increase in the SPD of measured lesions, and PD was defined as the appearance of any new lesion or a more than 50% increase in the SPD from nadir. Confirmation of response by repeat measurement 28 or more days later was not required. In addition to the efficacy evaluation at each participating institute, an independent, third-party panel of three radiologists (T.T., S.N., and M.M.) carried out a central evaluation using the collected CT films. The primary efficacy variable was best ORR (the relative frequency of responders showing CR, CR unconfirmed, or partial response). Secondary efficacy variables included CR rate, PFS (defined as the time from the date of enrollment to the date of PD assessment, the date of death of any cause, or the date necessitating other antilymphoma treatment), and overall survival (defined as the period from the date of registration to the date of death due to any cause).

Safety variables for the study were the types, incidence, severity, and reversibility of adverse events and adverse drug reactions, changes in laboratory tests, and the proportion of dose reduction. Toxicity was graded according to the Common Terminology Criteria for Adverse Event v3.0. Follicular Lymphoma International Prognostic Index (FLIPI) scores were calculated by summing the number of risk factors (age > 60 years, Ann Arbor stage III or IV, hemoglobin < 12 g/dL, elevated lactate dehydrogenase, and >4 nodal areas).[27] The following three risk groups were defined: low (none or one risk factor), intermediate (two risk factors), and poor risk (three to five risk factors).

Statistical methods. The study was designed to detect an expected ORR of 75%, assuming a threshold ORR of 50%. The level of significance was set at 2.5% (one-tailed) and the required sample size to attain statistical power of 80% was 35 patients. The target number of patients for registration was set at 40 patients, assuming that up to 15% of the enrolled patients might be judged unassessable. The Kaplan–Meier method was used to analyze survival probabilities.

Results

Patient characteristics. Between December 2005 and October 2006, 41 patients with relapsed or refractory indolent B-NHL were enrolled from seven institutions. Table 1 summarizes the patient characteristics. The majority of patients (93%) had follicular lymphoma, and 61% of patients had advanced-stage diseases upon entering the study. FLIPI risk groups were low in 25 (61%), intermediate in 11 (27%), and high in five patients (12%). Thirty-four patients (83%) had received rituximab before entering the study.

Protocol treatment. Of the 41 patients enrolled, 27 (66%) completed the planned six cycles of protocol treatment. The protocol treatment was terminated because of progression of the primary disease in two patients (5%), and due to adverse events in 12 (29%; neutropenia in five, neutropenia and thrombocytopenia in two, thrombocytopenia in three, hemolytic anemia in one, and withdrawal of consent due to anorexia in one). The dose reduction of oral fludarabine to 30 mg/m^2/day according to the protocol-defined criteria was required in 17 patients (41%). The dose was reduced during the second course in one, third in six, fourth in three, fifth in four, and sixth in three patients.

Efficacy. The efficacy results are summarized in Table 2. The ORR and CR rates were 76% (95% confidence interval [CI], 60–88%) and 68% (95% CI, 52–82%), respectively; and 25 (74%) of the 34 patients who had received prior rituximab

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responded to the combination. The CR rates correlated with the risk groups according to the FLIPI. The median PFS for all 41 patients was 19.7 months (95% CI, 12.3–26.5 months), and the median PFS for the 31 responders was 20.4 months (95% CI, 16.1–26.5 months) (Fig. 1a). The PFS correlated well with the risk groups according to the FLIPI (Fig. 1b). Sixteen of the 41 patients examined (39%) had positive IgH/*bcl-2* gene rearrangement by PCR in bone marrow at baseline. Of the 16 patients, 14 (88%) became negative by the sixth course, but one became positive again during the follow-up period.

Table 1. Patient baseline clinical characteristics

Characteristic	No. patients	%
All	41	100
Sex		
Male	17	41
Female	24	59
Age (years)		
Median (range)	57 (40–73)	
≤44	4	10
45–64	28	68
≥65	9	22
ECOG performance status†		
0	35	85
1	6	15
Histology (WHO Classification)		
Small lymphocytic lymphoma	1	2
Follicular lymphoma	38	93
Extranodal marginal zone B-cell lymphoma, MALT	2	5
Ann Arbor stage†		
IA	3	7
IIA	13	32
IIIA	10	24
IVA	15	37
FLIPI*		
Low	25	61
Intermediate	11	27
High	5	12
Prior therapy		
Rituximab monotherapy	9	22
Chemotherapy with rituximab	25	61
Chemotherapy without rituximab	7	17

ECOG, Eastern Cooperative Oncology Group; FLIPI, Follicular Lymphoma International Prognostic Index; MALT, mucosa-associated lymphoid tissue; WHO, World Health Organization.
†At study entry.

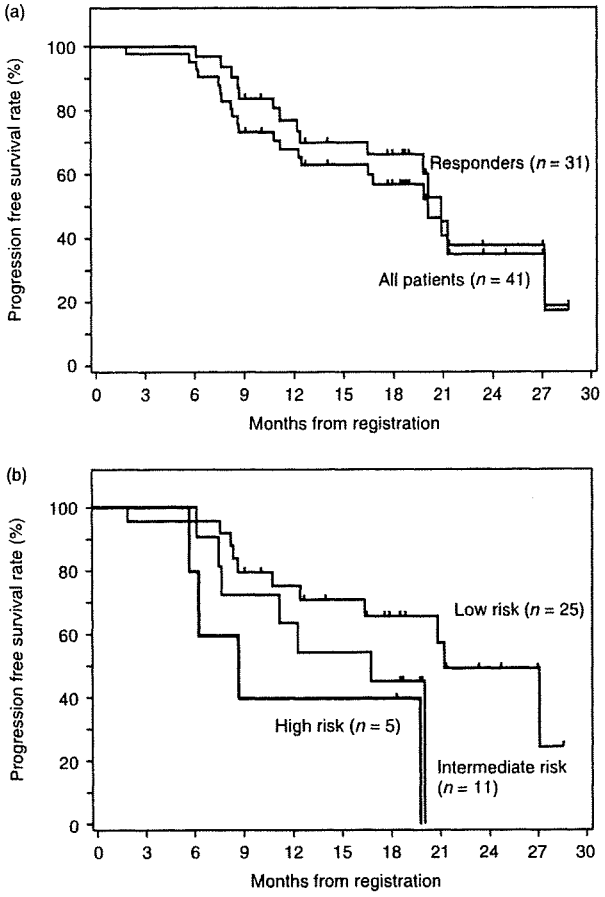

Fig. 1. Progression-free survival (PFS) of patients with relapsed indolent B-cell non-Hodgkin lymphoma who were treated with oral fludarabine and rituximab. (a) PFS of 41 patients and 31 responders. (b) PFS according to Follicular Lymphoma International Prognostic Index (FLIPI): low risk (n = 25), intermediate risk (n = 11), and high risk (n = 5).

Table 2. Antitumor effect of oral fludarabine and rituximab

Treatment group	No. patients	No. patients						ORR		CR rate	
		CR	CRu	PR	SD	PD	NE	%	95% CI	%	95% CI
All	41	22	6	3	6	2	2	76	60–88	68	52–82
FL	38	21	6	1	6	2	2	74		71	
MALT	2	0	0	2	0	0	0	100		0	
SLL	1	1	0	0	0	0	0	100		100	
Prior rituximab†											
+	34	17	6	2	6	2	1	74		68	
–	7	5	0	1	0	0	1	86		71	
FLIPI											
Low	25	15	5	0	3	1	1	80		80	
Intermediate	11	6	0	3	1	1	0	82		55	
High	5	1	1	0	2	0	1	40		40	

Responses were assessed according to the International Workshop Response Criteria for Non-Hodgkin Lymphoma.[26]
CR, complete response; CRu, complete response unconfirmed; FL, follicular lymphoma; FLIPI, Follicular Lymphoma International Prognostic Index; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; ORR, overall response rate; PD, progressive disease; PR, partial response; SD, stable disease; SLL, small lymphocytic lymphoma.
†+ and – indicate the presence and absence of prior rituximab treatment, respectively.

Table 3. Incidence of hematological and non-hematological toxicities observed in 20% or higher of 41 patients

Toxicity	Any grade		Grade 3		Grade 4	
	No. patients	%	No. patients	%	No. patients	%
Leukopenia	41	100	21	51	17	41
Neutropenia	40	98	11	27	27	66
Lymphopenia	41	100	1	2	40	98
Erythropenia	34	83	4	10	1	2
Thrombocytopenia	28	68	6	15	3	7
Anemia	27	66	5	12	2	5
Monocytopenia	27	66	0	0	0	0
LDH elevation	29	71	0	0	0	0
AST elevation	20	49	2	5	0	0
ALT elevation	16	39	3	7	0	0
IgM decrease	15	37	1	2	1	2
IgA decrease	10	24	1	2	1	2
IgG decrease	10	24	1	2	1	2
Hypoproteinemia	10	24	1	2	0	0
Creatinine elevation	9	22	1	2	0	0
Hyperbilirubinemia	9	22	0	0	0	0
Nausea	25	61	0	0	0	0
Anorexia	17	41	0	0	0	0
Stomatitis	13	32	0	0	1	2
Malaise	13	32	0	0	0	0
Pyrexia	13	32	0	0	0	0
Vomiting	12	29	2	5	0	0
Diarrhoea	11	27	1	2	0	0
Headache	11	27	0	0	0	0
Nasopharyngitis	11	27	0	0	0	0
Back pain	9	22	0	0	0	0
Chills	9	22	0	0	0	0
Constipation	9	22	0	0	0	0

ALT, alanine transaminase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. Hematological and non-hematologic toxicities were graded according to the Common Terminology Criteria for Adverse Event v3.0.

Adverse events. Hematological and non-hematological adverse events that were encountered in 20% or more of treated patients are listed in Table 3. Hematological toxicity was common, although it was manageable mostly in the outpatient setting. Grade 4 neutropenia was observed in 27 patients (66%), and grade 4 thrombocytopenia in three (7%). G-CSF of 50–100 μg was subcutaneously given to 10% (4/41) of patients at cycle 1, 24% (10/41) at cycle 2, 33% (13/39) at cycle 3, 36% (13/36) at cycle 4, 45% (15/33) at cycle 5, and 26% (7/27) at cycle 6. The duration of G-CSF administration was 1–3 days in most patients. There were four episodes of grade 3 infection (two with febrile neutropenia, one with cystitis, and one with influenza), with no grade 4 infections during the study. Non-hematological toxicities were generally mild. All non-hematological adverse events of grade 3 or greater were seen in 5% or fewer patients except for ALT elevation (7%). Grade 4 non-hematological toxicities were observed in only two patients (5%; one with stomatitis and immunoglobulin decrease, and one with hyperuricemia).

Eleven serious adverse events (SAE) were observed in nine patients during the study: neutropenia (grade 4, two patients), leukopenia (grade 4, one patient), pancytopenia (grade 4, one patient), hemolytic anemia (grade 2, one patient), febrile neutropenia (grade 3, one patient), creatinine elevation (grade 3, one patient), stomatitis (grade 4, one patient), cystitis (grade 2, one patient), deep vein thrombosis (grade 3, one patient), and gastric cancer (grade 3, one patient). Two SAE in two patients (one with pancytopenia and one with leukopenia) did not recover, and the study was terminated in three patients due to SAE (one due to leukopenia and neutropenia, one due to stomatitis, and one due to hemolytic anemia). In one patient, early gastric cancer was detected during cycle 6, which was successfully treated by endoscopic mucosal resection. After the study, three SAE were reported: one patient each for herpes zoster, pneumonia, and *Pneumocystis jiroveci* pneumonia. In all SAE, a causal relation with the study medications was not ruled out, except for gastric cancer, which was judged as not related to the study treatment.

There were no deaths during the study period; however, three deaths were reported after the completion of the study. Two were from lymphoma progression, but one patient died of *Pneumocystis jiroveci* pneumonia. A 71-year-old woman with follicular lymphoma, previously treated with rituximab plus CHOP chemotherapy, received six courses of oral fludarabine and rituximab. After the sixth course, lymphopenia and pancytopenia persisted. Four months later she developed *Pneumocystis jiroveci* pneumonia and died of respiratory failure. She was on prophylactic trimethoprim and sulfamethoxazole (1 Tab; 80 mg/400 mg daily) when she developed *Pneumocystis jiroveci* pneumonia. The event was considered to be associated with immune suppression caused by the study medications.

There was another episode of *Pneumocystis jiroveci* pneumonia 4 months after completion of six cycles of treatment. The patient was not on prophylactic trimethoprim/sulfamethoxazole after completion of the protocol treatment. This patient was successfully treated with trimethoprim/sulfamethoxazole, along with other supportive medications.

Discussion

This is the first study to document the high efficacy of oral fludarabine in combination with rituximab in patients with relapsed indolent B-NHL. The ORR of 76% and CR rate of 68% are higher than the 65% and 30%, respectively, in our previous phase II study of oral fludarabine alone,[23] although this is not a direct comparison. The efficacy rates by the combination are considered very high, although all patients enrolled in this study had relapsed disease, and the majority of patients had prior treatment with rituximab, either alone or in combination with chemotherapy. In the phase II study by Czuczman *et al.* using intravenous fludarabine plus rituximab, the ORR and CR rate were reported to be 90 and 80%, respectively.[21] In their study, however, the target population was mostly untreated patients (68%), and patients having a history of prior rituximab therapy were excluded.

In addition, the median PFS (19.7 months) obtained with this combination in the present study is promising, comparing with the reported median PFS by other agents in relapsed indolent B-NHL patients, including 8 months with cladribine,[28] 8.6 months with oral fludarabine alone,[23] 8–9 months with rituximab,[29,30] 11.2 months with yttrium-90-labeled ibritumomab tiuxetan,[31] and 23–24 months with bendamustine plus rituximab,[32,33] although the target population is somewhat different among studies. The high efficacy and long PFS suggest that oral fludarabine plus rituximab is a reasonable option in the current treatment of relapsed indolent B-NHL. In addition, considering the favorable efficacy results by the combination of intravenous fludarabine and rituximab for mostly untreated patients with indolent B-NHL,[21] it may deserve to conduct a prospective study of oral fludarabine plus rituximab for untreated indolent B-NHL. To elucidate the exact role of this combination in the treatment of untreated and relapsed indolent B-NHL, further carefully designed studies are required.

In the present study, 14 of the 16 patients (88%) whose bone marrow aspirates showed positive IgH/*bcl-2* gene rearrangement by PCR became negative by oral fludarabine plus rituximab therapy. It is likely that these results are at least equivalent to the 88% (14/16) in the phase II study of intravenous fludarabine plus rituximab for mostly untreated indolent B-NHL patients.[21]

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The most common toxicities were hematological, with grade 4 neutropenia occurring in 66% of patients, which was higher than the 37% with oral fludarabine alone.[23] Intravenous fludarabine in combination with rituximab was reported to have an increased risk of developing grade 3 or greater neutropenia.[21] Although neutropenia was also common in the present study, only four episodes of grade 3 infection were seen, with no grade 4 infections during the study period. In the study of intravenous fludarabine with rituximab,[21] the initial 10 patients showed frequent severe neutropenia, necessitating modifications to reduce the dose in case of prolonged hematological toxicity and omitting prophylactic trimethoprim/sulfamethoxazole. Our study used an approach to reduce the dose if severe hematological toxicities were observed, and allowed the liberal use of G-CSF, while using prophylactic trimethoprim/sulfamethoxazole and acyclovir. It is likely that this approach was effective in reducing infectious episodes. Although the exact mechanism of the higher incidence of neutropenia by the combined use of rituximab and fludarabine than that with fludarabine alone remains unknown, it may be partly associated with the hematological toxicity of rituximab alone[30] and late-onset neutropenia in patients pretreated with rituximab, especially with rituximab-containing chemotherapy.[34] The non-hematological toxicities were infrequent, mild, and mostly manageable.

Of note was that one death from *Pneumocystis jiroveci* pneumonia occurred 4 months after completion of the protocol treatment. Another patient developed *Pneumocystis jiroveci* pneumonia 4 months after completing the sixth course, but recovered with anti-*Pneumocystis* therapy. Fludarabine is known to cause prolonged immunosuppressive effects in view of decreased CD4-positive T-cell counts and decreased serum IgG levels,[16] and rituximab causes prolonged B-lymphocyte suppression.[29,30] These results suggest that prolonged prophylaxis for *Pneumocystis jiroveci* infection is recommended in patients who receive fludarabine plus rituximab, in addition to the careful monitoring of absolute lymphocyte counts and CD4-positive T-cell counts.

In this multicenter phase II study, a central pathology review was not carried out. As the diagnosis of only one of the 52 enrolled patients was changed in the central pathology review in our previous multicenter phase II study of oral fludarabine alone for relapsed indolent B-NHL,[23] we did not consider the central pathology review in this study to be essential.

In conclusion, oral fludarabine in combination with rituximab is a highly effective and convenient treatment for relapsed indolent B-NHL, in view of the high ORR, CR rate, long PFS, and the convenience of administering it in an outpatient setting. Although hematological toxicities appeared to be somewhat higher than with oral fludarabine alone, and dose reduction of oral fludarabine was necessary in a fraction of patients, non-hematological toxicities were generally mild; however, prolonged prophylaxis for *Pneumocystis jiroveci* pneumonia is recommended when using this combination. The encouraging results warrant further investigations of this combination in the treatment of indolent B-NHL, including frontline therapy.

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Disclosure Statement

Masaki Hayashi was an employee of Bayer HealthCare, Osaka, Japan. The remaining authors report no potential conflicts of interest.

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LETTERS

Frequent inactivation of A20 in B-cell lymphomas

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A20 is a negative regulator of the NF-κB pathway and was initially identified as being rapidly induced after tumour-necrosis factor-α stimulation[1]. It has a pivotal role in regulation of the immune response and prevents excessive activation of NF-κB in response to a variety of external stimuli[2–7]; recent genetic studies have disclosed putative associations of polymorphic *A20* (also called *TNFAIP3*) alleles with autoimmune disease risk[8,9]. However, the involvement of A20 in the development of human cancers is unknown. Here we show, using a genome-wide analysis of genetic lesions in 238 B-cell lymphomas, that *A20* is a common genetic target in B-lineage lymphomas. A20 is frequently inactivated by somatic mutations and/or deletions in mucosa-associated tissue lymphoma (18 out of 87; 21.8%) and Hodgkin's lymphoma of nodular sclerosis histology (5 out of 15; 33.3%), and, to a lesser extent, in other B-lineage lymphomas. When re-expressed in a lymphoma-derived cell line with no functional *A20* alleles, wild-type A20, but not mutant A20, resulted in suppression of cell growth and induction of apoptosis, accompanied by downregulation of NF-κB activation. The A20-deficient cells stably generated tumours in immunodeficient mice, whereas the tumorigenicity was effectively suppressed by re-expression of A20. In A20-deficient cells, suppression of both cell growth and NF-κB activity due to re-expression of A20 depended, at least partly, on cell-surface-receptor signalling, including the tumour-necrosis factor receptor. Considering the physiological function of A20 in the negative modulation of NF-κB activation induced by multiple upstream stimuli, our findings indicate that uncontrolled signalling of NF-κB caused by loss of A20 function is involved in the pathogenesis of subsets of B-lineage lymphomas.

Malignant lymphomas of B-cell lineages are mature lymphoid neoplasms that arise from various lymphoid tissues[10,11]. To obtain a comprehensive registry of genetic lesions in B-lineage lymphomas, we performed a single nucleotide polymorphism (SNP) array analysis of 238 primary B-cell lymphoma specimens of different histologies, including 64 samples of diffuse large B-cell lymphomas (DLBCLs), 52 follicular lymphomas, 35 mantle cell lymphomas (MCLs), and 87 mucosa-associated tissue (MALT) lymphomas (Supplementary Table 1). Three Hodgkin's-lymphoma-derived cell lines were also analysed. Interrogating more than 250,000 SNP sites, this platform permitted the identification of copy number changes at an average resolution of less than 12 kilobases (kb). The use of large numbers of SNP-specific probes is a unique feature of this platform, and combined with the CNAG/AsCNAR software, enabled accurate determination of 'allele-specific' copy numbers, and thus allowed for sensitive detection of loss of heterozygosity (LOH) even without apparent copy-number reduction, in the presence of up to 70–80% normal cell contamination[12,13].

Lymphoma genomes underwent a wide range of genetic changes, including numerical chromosomal abnormalities and segmental gains and losses of chromosomal material (Supplementary Fig. 1), as well as copy-number-neutral LOH, or uniparental disomy (Supplementary Fig. 2). Each histology type had a unique genomic signature, indicating a distinctive underlying molecular pathogenesis for different histology types (Fig. 1a and Supplementary Fig. 3). On the basis of the genomic signatures, the initial pathological diagnosis of MCL was re-evaluated and corrected to DLBCL in two cases. Although most copy number changes involved large chromosomal segments, a number of regions showed focal gains and deletions, accelerating identification of their candidate gene targets. After excluding known copy number variations, we identified 46 loci showing focal gains (19 loci) or deletions (27 loci) (Supplementary Tables 2 and 3 and Supplementary Fig. 4).

Genetic lesions on the NF-κB pathway were common in B-cell lymphomas and found in approximately 40% of the cases (Supplementary Table 1), underpinning the importance of aberrant NF-κB activation in lymphomagenesis[11,14] in a genome-wide fashion. They included focal gain/amplification at the *REL* locus (16.4%) (Fig. 1b) and *TRAF6* locus (5.9%), as well as focal deletions at the *PTEN* locus (5.5%) (Supplementary Figs 1 and 4). However, the most striking finding was the common deletion at 6q23.3 involving a 143-kb segment. It exclusively contained the *A20* gene (also called *TNFAIP3*), a negative regulator of NF-κB activation[3–7,15] (Fig. 1b), which was previously reported as a candidate target of 6q23 deletions in ocular lymphoma[16]. LOH involving the *A20* locus was found in 50 cases, of which 12 showed homozygous deletions as determined by the loss of both alleles in an allele-specific copy number analysis (Fig. 1b, Table 1 and Supplementary Table 4). On the basis of this finding, we searched for possible tumour-specific mutations of *A20* by genomic DNA sequencing of entire coding exons of the gene in the same series of lymphoma samples (Supplementary Fig. 5). Because two out of the three Hodgkin's-lymphoma-derived cell lines had biallelic *A20* deletions/mutations (Supplementary Fig. 6), 24 primary samples from Hodgkin's lymphoma were also analysed for mutations, where

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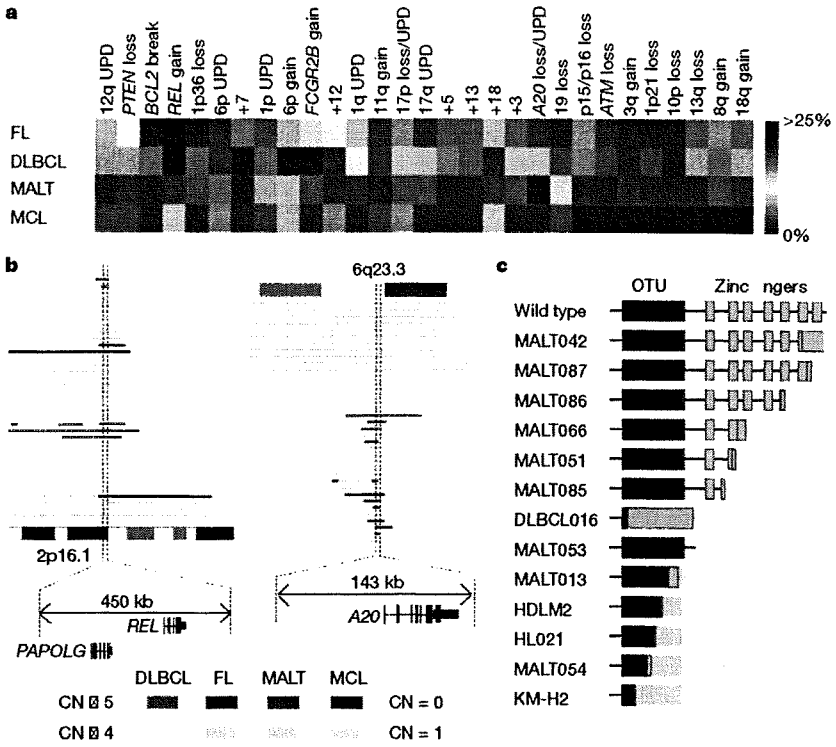

Figure 1 | Genomic signatures of different B-cell lymphomas and common genetic lesions at 2p16-15 and 6q23.3 involving NF-κB pathway genes. **a**, Twenty-nine genetic lesions were found in more than 10% in at least one histology and used for clustering four distinct histology types of B-lineage lymphomas. The frequency of each genetic lesion in each histology type is colour-coded. FL, follicular lymphoma; UPD, uniparental disomy. **b**, Recurrent genetic changes are depicted based on CNAG output of the SNP array analysis of 238 B-lineage lymphoma samples, which include gains at the *REL* locus on 2p16-15 (left panel) and the *A20* locus on 6q23.3 (right panel). Regions showing copy number gain or loss are indicated by horizontal lines. Four histology types are indicated by different colours, where high-grade amplifications and homozygous deletions are shown by darker shades to discriminate from simple gains (copy number ≤4) and losses (copy number = 1) (lighter shades). **c**, Point mutations and small nucleotide insertions and deletions in the *A20* (*TNFAIP3*) gene caused premature truncation of A20 in most cases. Altered amino acids caused by frame shifts are indicated by green bars.

genomic DNA was extracted from 150 microdissected CD30-positive tumour cells (Reed–Sternberg cells) for each sample. *A20* mutations were found in 18 out of 265 lymphoma samples (6.8%) (Table 1), among which 13 mutations, including nonsense mutations (3 cases), frame-shift insertions/deletions (9 cases), and a splicing donor site mutation (1 case) were thought to result in premature termination of translation (Fig. 1c). Four missense mutations and one intronic mutation were identified in five microdissected Hodgkin's lymphoma samples. They were not found in the surrounding normal tissues, and thus, were considered as tumour-specific somatic changes.

In total, biallelic *A20* lesions were found in 31 out of 265 lymphoma samples including 3 Hodgkin's lymphoma cell lines. Quantitative analysis of SNP array data suggested that these *A20* lesions were present in the major tumour fraction within the samples (Supplementary Fig. 7). Inactivation of *A20* was most frequent in MALT lymphoma (18 out of 87) and Hodgkin's lymphoma (7 out of 27), although it was also found in DLBCL (5 out of 64) and follicular lymphoma (1 out of 52) at lower frequencies. In MALT lymphoma, biallelic *A20* lesions were confirmed in 18 out of 24 cases (75.0%) with LOH involving the 6q23.3 segment (Supplementary Fig. 8). Considering the limitation in detecting very small homozygous deletions, *A20* was thought to be the target of 6q23 LOH in MALT lymphoma. On the other hand, the 6q23 LOHs in other histology types tended to be extended into more centromeric regions and less frequently accompanied biallelic *A20* lesions (Supplementary Fig. 8 and Supplementary Table 4), indicating that they might be more heterogeneous with regard to their gene targets. We were unable to analyse Hodgkin's lymphoma samples using SNP arrays owing to insufficient genomic DNA obtained from microdissected samples, and were likely to underestimate the frequency of *A20* inactivation in Hodgkin's lymphoma because we might fail to detect a substantial proportion of cases with homozygous deletions, which explained 50% (12 out of 24) of *A20* inactivation in other histology types. *A20* mutations in Hodgkin's lymphoma were exclusively found in nodular sclerosis classical Hodgkin's lymphoma (5 out of 15) but not in other histology types (0 out of 9), although the possible association requires further confirmation in additional cases.

A20 is a key regulator of NF-κB signalling, negatively modulating NF-κB activation through a wide variety of cell surface receptors and viral proteins, including tumour-necrosis factor (TNF) receptors, toll-like receptors, CD40, as well as Epstein–Barr-virus-associated LMP1 protein[2,5,17,18]. To investigate the role of A20 inactivation in lymphomagenesis, we re-expressed wild-type A20 under a *Tet*-inducible promoter in a lymphoma-derived cell line (KM-H2) that had no functional *A20* alleles (Supplementary Fig. 6), and examined the effect of A20 re-expression on cell proliferation, survival and downstream NF-κB signalling pathways. As shown in Fig. 2a–c and Supplementary Fig. 9, re-expression of wild-type A20 resulted in the suppression of cell proliferation and enhanced apoptosis, and in the concomitant accumulation of IκBβ and IκBε, and downregulation of NF-κB activity. In contrast, re-expression of two lymphoma-derived A20 mutants, $A20^{532Stop}$ or $A20^{750Stop}$, failed to show growth suppression, induction of apoptosis, accumulation of IκBβ and IκBε or downregulation of

Table 1 | Inactivation of A20 in B-lineage lymphomas

Histology	Tissue	Sample	Allele	Uniparental disomy	Exon	Mutation	Biallelic inactivation
DLBCL							5 out of 64 (7.8%)
	Lymph node	DLBCL008	−/−	No	–	–	
	Lymph node	DLBCL016	+/−	No	Ex2	329insA	
	Lymph node	DLBCL022	−/−	No	–	–	
	Lymph node	DLBCL028	−/−	Yes	–	–	
	Lymph node	MCL008*	−/−	Yes	–	–	
Follicular lymphoma							1 out of 52 (1.9%)
	Lymph node	FL024	−/−	No	–	–	
MCL							0 out of 35 (0%)
MALT							18 out of 87 (21.8%)
Stomach							3 out of 23 (13.0%)
	Gastric mucosa	MALT013	+/+	Yes	Ex5	705insG	
	Gastric mucosa	MALT014	+/+	Yes	Ex3	Ex3 donor site>A	
	Gastric mucosa	MALT036	+/−	No	Ex7	delintron6–Ex7†	
Eye							13 out of 43 (30.2%)
	Ocular adnexa	MALT008	−/−	No	–	–	
	Ocular adnexa	MALT017	−/−	No	–	–	
	Ocular adnexa	MALT051	+/−	No	Ex7	1943delTG	
	Ocular adnexa	MALT053	+/+	Yes	Ex6	1016G>A(stop)	
	Ocular adnexa	MALT054	+/−	No	Ex3	502delTC	
	Ocular adnexa	MALT055	−/−	No	–	–	
	Ocular adnexa	MALT066	+/−	No	Ex7	1581insA	
	Ocular adnexa	MALT067	−/−	No	–	–	
	Ocular adnexa	MALT082	−/−	Yes	–	–	
	Ocular adnexa	MALT084	−/−	Yes	–	–	
	Ocular adnexa	MALT085	+/+	Yes	Ex7	1435insG	
	Ocular adnexa	MALT086	+/+	Yes	Ex6	878C>T(stop)	
	Ocular adnexa	MALT087	+/+	Yes	Ex9	2304delGG	
Lung							2 out of 12 (16.7%)
	Lung	MALT042	−/−	No	–	–	
	Lung	MALT047	+/+	Yes	Ex9	2281insT	
Other‡							0 out of 9 (0%)
Hodgkin's lymphoma							7 out of 27 (26.0%)
NSHL	Lymph node	HL10	ND	ND	Ex7	1777G>A(V571I)	
NSHL	Lymph node	HL12	ND	ND	Ex7	1156A>G(R364G)	
NSHL	Lymph node	HL21	ND	ND	Ex4	569G>A(stop)	
NSHL	Lymph node	HL24	ND	ND	Ex3	1487C>A(T474N)	
NSHL	Lymph node	HL23	ND	ND	–	Intron 3§	
	Cell line	KM-H2	−/−	No	–		
	Cell line	HDLM2	+/−	No	Ex4	616ins29bp‖	
Total							31 out of 265 (11.7%)

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; MCL, mantle cell lymphoma; ND, not determined because SNP array analysis was not performed; NSHL, nodular sclerosis classical Hodgkin's lymphoma.
* Diagnosis was changed based on the genomic data, which was confirmed by re-examination of pathology.
† Deletion including the boundary of intron 6 and exon 7 (see also Supplementary Fig. 5b).
‡ Including 1 parotid gland, 1 salivary gland, 2 colon and 5 thyroid cases.
§ Insertion of CTC at −19 bases from the beginning of exon 3.
‖ Insertion of TGGCTTCCACAGACACACCCATGGCCCGA.

NF-κB activity (Fig. 2a–c), indicating that these were actually loss-of-function mutations. To investigate the role of A20 inactivation in lymphomagenesis *in vivo*, *A20*- and mock-transduced KM-H2 cells were transplanted in NOD/SCID/γ_c^{null} (NOG) mice[19], and their tumour formation status was examined for 5 weeks with or without induction of wild-type A20 by tetracycline administration. As shown in Fig. 2d, mock-transduced cells developed tumours at the injected sites, whereas the *Tet*-inducible *A20*-transduced cells generated tumours only in the absence of A20 induction (Supplementary Table 5), further supporting the tumour suppressor role of A20 in lymphoma development.

Given the mode of negative regulation of NF-κB signalling, we next investigated the origins of NF-κB activity that was deregulated by A20 loss in KM-H2 cells. The conditioned medium prepared from a 48-h serum-free KM-H2 culture had increased NF-κB upregulatory activity compared with fresh serum-free medium, which was inhibited by re-expression of A20 (Fig. 3a). KM-H2 cells secreted two known ligands for TNF receptor—TNF-α and lymphotoxin-α (Supplementary Fig. 10)[20]—and adding neutralizing antibodies against these cytokines into cultures significantly suppressed their cell growth and NF-κB activity without affecting the levels of their overall suppression after A20 induction (Fig. 3b, d). In addition, recombinant TNF-α and/or lymphotoxin-α added to fresh serum-free medium promoted cell growth and NF-κB activation in KM-H2 culture, which were again suppressed by re-expression of A20 (Fig. 3c, e). Although our data in Fig. 3 also show the presence of factors other than TNF-α and lymphotoxin-α in the KM-H2-conditioned medium—as well as some intrinsic pathways in the cell (Fig. 3a)—that were responsible for the A20-dependent NF-κB activation, these results indicate that both cell growth and NF-κB activity that were upregulated by A20 inactivation depend at least partly on the upstream stimuli that evoked the NF-κB-activating signals.

Aberrant activation of the NF-κB pathway is a hallmark of several subtypes of B-lineage lymphomas, including Hodgkin's lymphoma, MALT lymphoma, and a subset of DLBCL, as well as other lymphoid neoplasms[11,14], where a number of genetic alterations of NF-κB signalling pathway genes[21–25], as well as some viral proteins[26,27], have been implicated in the aberrant activation of the NF-κB pathway[14]. Thus, frequent inactivation of A20 in Hodgkin's lymphoma and MALT and other lymphomas provides a novel insight into the molecular pathogenesis of these subtypes of B-lineage lymphomas through deregulated NF-κB activation. Because A20 provides a