original article

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Statin-independent prognosis of patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP therapy

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Background: A recent laboratory study indicated that statins impaired the antitumor effects of rituximab by inducing conformational changes in CD20. Although these findings raised significant concerns about statin use during rituximab treatment, their clinical significance is unclear.

Patients and methods: We conducted a retrospective study investigating the effects of statins on the prognosis of diffuse large B-cell lymphoma (DLBCL) treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (RCHOP). Newly diagnosed DLBCL patients were analyzed ($n = 256$), including 35 patients taking statins.

Results: The 3-year progression-free survival rates were 84% and 73% ($P = 0.38$), while the overall survival rates were 89% and 78% ($P = 0.28$) for those patients treated with and without statins, respectively. After adjusting for the International Prognostic Index and serum cholesterol level, statin use was not associated with prognosis.

Conclusions: These results indicate that statins do not influence the clinical prognosis of DLBCL treated with RCHOP. Further studies with larger numbers of patients are warranted to confirm the prognostic significance of statins for patients with DLBCL receiving rituximab-containing chemotherapy.

Key words: diffuse large B-cell lymphoma, rituximab, statin

introduction

Rituximab, a chimeric anti-CD20 antibody, is highly effective in the treatment of various types of CD20-positive B-cell lymphomas [1, 2]. It has been reported that complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and the induction of apoptosis are the major mechanisms of action for rituximab [3, 4]. A significantly improved outcome has been obtained in young and elderly patients by combining cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) with rituximab [5–7].

3-Hydroxy-3-methylgutaryl coenzyme A reductase inhibitors, commonly known as statins, inhibit the rate-limiting step of the mevalonate pathway, which is essential for the biosynthesis of various compounds, including cholesterol [8]. The activity of rituximab may be reduced by conformational changes in CD20 induced by the depletion of serum cholesterol, which impairs rituximab binding and thereby significantly decreases rituximab-mediated CDC and ADCC against B-cell lymphoma cells [9].

Several authors have cited the same laboratory study [10–12] and these *in vitro* data have led some to recommend that statins might not be used when patients are being treated with rituximab. However, it is unclear whether statins actually affect the prognosis of diffuse large B-cell lymphoma (DLBCL) patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (RCHOP). The aim of this study was to assess the influence of statin use on the outcome of patients with RCHOP-treated DLBCL.

patients and methods

patient characteristics

We reviewed the medical records of patients with DLBCL who received RCHOP as a first-line therapy at the Cancer Institute Hospital and Okayama University Hospital between April of 2004 and May of 2008 and were followed up until January of 2009. In this period, the patients with

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statin use and the patients without statin use treated in the same time frame as the patients with statin-use group were analyzed. The definition of the patients with statin use was the patients who had been taking statins for at least 2 months at the start of RCHOP. The study protocol for this retrospective analysis was approved by the institutional review boards of both hospitals. Patients were analyzed if they were older than 18 years and had a performance status (PS) of zero to three according to the criteria of the Eastern Cooperative Oncology Group. Patients were excluded if they were positive for antibodies against human immunodeficiency virus-1 or -2. Patients with primary mediastinal large B-cell lymphoma, primary central nervous system lymphoma, or primary testicular lymphoma were also excluded from this study.

The disease stage was evaluated according to the Ann Arbor staging system. All patients had undergone staging investigations, including physical examinations, blood and serum analyses, bone marrow aspiration and biopsy, and computed tomography (CT) of the neck, chest, abdomen, and pelvis. Magnetic resonance imaging was used to evaluate involved organs in the head and neck. Some patients underwent positron emission tomography (PET)/CT with 2-[fluorine-18]fluoro-2-deoxy-D-glucose for staging evaluation and assessing treatment response. Chemotherapy sensitivity was defined according to standard volume criteria using CT and PET/CT imaging [13, 14].

The following clinical and laboratory information was available at the time of diagnosis: history of statin treatment; age; sex; PS; stage; number of extranodal sites; and serum levels of lactate dehydrogenase, total cholesterol (T-cho), triglycerides, and high-density lipoprotein cholesterol. This information allowed the determination of International Prognostic Index (IPI) scores for the included patients. Patients were categorized into either low-risk (IPI score 0–2) or high-risk (IPI score 3–5) groups. The serum cholesterol level was considered 'high' when higher than the median serum T-cho and 'low' when lower than the median serum T-cho.

pathological studies

Biopsied samples collected before treatment were fixed in formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin for morphological analysis. All diagnoses were made according to the World Health Organization classification [15]. Cases involving transformed low-grade/indolent B-cell lymphoma were excluded. All the samples were reviewed by expert hematopathologists (KT and TY).

treatment

For patients with stage IB–IV, rituximab was administered at the standard dose of 375 mg/m^2 in 8-week cycles during the first and second cycles of CHOP or six triweekly cycles concurrent with each of the six cycles of triweekly CHOP, as described previously [16]. Patients with stage IA were treated with three cycles of CHOP with subsequent radiotherapy; rituximab was administered in 8-week cycles during the first and second cycles of CHOP or three triweekly cycles concurrent with each of the three cycles of triweekly CHOP.

statistical analysis

Progression-free survival (PFS) was calculated from the date of RCHOP initiation to the date of documented disease progression, relapse, or the end date of the study. Overall survival (OS) was calculated from the date of RCHOP initiation until death from any cause or the last follow-up. If the stop date was not reached, the data were censored at the date of the last follow-up evaluation. Survival curves were created by the Kaplan–Meier method; overall differences were compared by the log-rank test. Cox multivariate analysis was carried out to estimate the prognostic impacts of statin use, cholesterol profile, and IPI on PFS and OS. Comparisons of the basic characteristics of each group (with and without statin use) were made using Fisher's exact test, the chi-square test, and the Mann–Whitney *U* test. The data were analyzed using SPSS software (version 11.0 for Windows; SPSS, Chicago, IL).

results

Of the 256 patients who met the inclusion criteria, CD20 expression was confirmed in all DLBCL patients by immunohistochemical staining and flow cytometry. The characteristics of the patients are listed in Table 1. A total of 35 patients (14%) received statins (atorvastatin in 17 cases, pravastatin in nine cases, simvastatin in five cases, and pivastatin and rosuvastatin in two cases each). In the statin group, all patients started statin therapy before RCHOP initiation, and the median duration from initiating statin therapy to RCHOP therapy was 38 months (range 3–63 months). In the no-statin group, patients did not intake any statins after RCHOP initiation. Most patients took statins once

Table 1. Patient characteristics

Clinical parameter	Statin use		*P*
	Yes, no. (%)	No, no. (%)	
All	35	221	
Sex			0.02
Male	13 (37)	130 (59)	
Female	22 (63)	91 (41)	
Age, years			0.08
Median (range)	68 (20–86)	64 (23–88)	
≤60	6 (17)	71 (32)	
>60	29 (83)	150 (68)	
Stage			0.46
1–2	18 (51)	131 (59)	
3–4	17 (49)	90 (41)	
PS			1.00
0–1	33 (94)	204 (92)	
2–4	2 (6)	17 (8)	
LDH			0.36
Normal	16 (46)	121 (55)	
High	19 (54)	100 (45)	
No. of extranodal sites			1.00
0–1	28 (80)	172 (78)	
2–	7 (20)	49 (22)	
IPI score			0.44
0–2	22 (63)	153 (69)	
3–5	13 (37)	68 (31)	
Time from diagnosis to treatment, days (range)	19 (2–34)	18 (0–94)	1.00
T-cho			
Median (range)	210 (141–318)	187 (101–296)	0.16
TG			
Median (range)	156 (48–534)	112 (40–790)	0.37
HDL-C			
Median (range)	49 (25–92)	48 (13–124)	0.86

PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase, U/l; IPI, International Prognostic Index; T-cho, total cholesterol, mg/dl (normal range 135–219); TG, triglyceride mg/dl (normal range 0–150); HDL-C, high-density lipoprotein cholesterol, mg/dl (normal range 40–80).

daily every day. The serum T-cho level tended to be higher for those patients taking statins than for those patients not using statins; however, the difference was not statistically significant. No significant difference was found between the two groups in terms of their basic characteristics except for sex: females were the dominant statin users.

The complete remission rate was not significantly different between the patients with (74%) and without (73%) statin use. With a median follow-up of 32 months (range 7–57 months), the Kaplan–Meier method revealed that the 3-year PFS rates were 84% and 73%, while the OS rates were 89% and 78% for the groups with and without statin use, respectively. Although both survival rates were superior in the statin group, the differences were not statistically significant ($P = 0.38$ and 0.28, respectively; Figure 1A and B). Similarly, the PFS and OS rates at 3 years were 75% and 87%, respectively, for the low-cholesterol group and 85% and 90%, respectively, for the high-cholesterol group; no significant differences in these parameters were observed between the two groups ($P = 0.39$ and $P = 0.28$, respectively). On the other hand, the 3-year PFS rates were 60% and 81% ($P = 0.005$), while the OS rates were 69% and 85% ($P = 0.004$) for the high- and low-IPI groups, respectively, and this result was not affected after adjusting for statin use (data not shown). There were no significant differences in PFS and OS between longer and shorter statin users who had statins for more and less than median duration, respectively ($P = 0.58$ and $P = 0.94$, respectively). To adjust for the prognostic value of serum cholesterol level and IPI, Cox multivariate analysis was carried out. As shown in Table 2, statin use did not have a significant prognostic value either for PFS or OS after adjusting for serum cholesterol level. Of 26 deaths, 21 were caused by progressive disease, while two were caused by interstitial pneumonia, two by bacterial infection, and one by suicide; no patient died of a cardiovascular event.

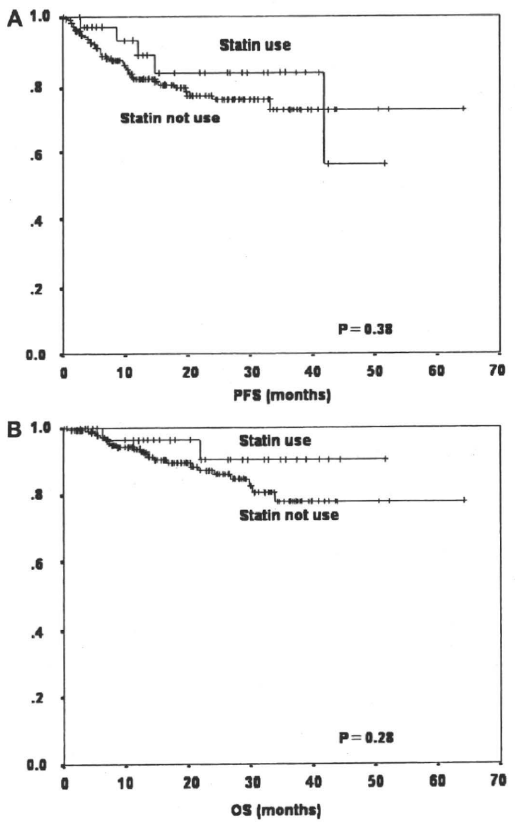

Figure 1. Progression-free survival (PFS) and overall survival (OS) curves for diffuse large B-cell lymphoma patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone according to statin use. PFS (A) and OS (B) curves after statin use ($n = 35$) compared with no statin use ($n = 221$).

discussion

Our data indicate no significant influence of statin use on prognosis in patients with DLBCL. Indeed, there is currently no report confirming the clinical significance of statins in patients treated with rituximab.

Our results do not support the previous laboratory data, and several explanations can be offered for these discrepancies. First, the cholesterol levels of the patients may not have been adequately lowered by the statin doses given. However, in this study, the general outcome was not affected even in the low-cholesterol group. Thus, it is important to determine the *in vivo* threshold level of cholesterol, if any, that affects the CD20 conformation reportedly required for rituximab-mediated CDC and ADCC [9]. Second, accumulating evidence indicates that statins have anticancer [17, 18] and antilymphoma activity [19, 20]. Therefore, statin use may have favorably influenced the RCHOP-treated DLBCL patients in this study. Third, several prognostic markers have been assessed and identified in patients with DLBCL treated with rituximab-combined chemotherapy [21–24]. Consistent with recent findings [21, 22, 25], the IPI score also had a prognostic impact on outcome in our cohort of patients who received immunochemotherapy; thus, biochemical markers and clinical prognostic factors may have greater predictive power than statin use.

Similar to statin use and serum cholesterol in this study, no prognostic marker other than IPI has been found to predict

Table 2. Cox multivariate analysis for PFS and OS

Variable	HR	95% CI	P
PFS			
Statin use	0.85	0.31–1.51	0.35
Cholesterol level	0.01	0.01–0.12	0.98
IPI	2.69	0.88–4.08	0.10
OS			
Statin use	0.52	00.29–1.90	0.52
Cholesterol level	0.001	0.00–0.00	0.98
IPI	6.55	1.34–8.89	0.01

HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; IPI, International Prognostic Index; OS, overall survival.

prognosis after rituximab was introduced into clinical practice. Nevertheless, a number of prognostic markers have been identified in patients with DLBCL treated with chemotherapy alone [26–29], some of which have been reassessed and shown to be unassociated with prognosis in patients treated with rituximab-combined chemotherapy [21–23]. BCL2 overexpression was reportedly associated with poorer survival in patients treated with CHOP-like regimens [26, 27], but several studies failed to confirm its prognostic value in patients treated with rituximab-combined chemotherapy, indicating that the addition of rituximab overcomes the negative influence of BCL2 overexpression [23]. BCL6, a marker of germinal center derivation, has been identified as a marker of a favorable outcome in DLBCL [28], but outcomes for patients treated with immunochemotherapy were not influenced by BCL6 status [21]. Similarly, no correlation was observed between the immunohistochemically defined GC phenotype and survival rate in patients receiving immunochemotherapy [29], which is in contrast to previous findings of inferior outcomes in non-GC patients relative to GC patients in the pre-rituximab era [22].

Although our study was not a randomized, prospective study and was possibly biased by factors other than statin use, the distribution of baseline characteristics, including IPI factors, was similar between the groups. Unfortunately, the sample size of statin users was likely too small to reach any definitive conclusions. In this study HRs of statin use for PFS and OS were 0.85 and 0.52 without statistical significance. Post hoc statistical power calculation revealed that the statistical power for these HRs were 5.9% and 35.6%, respectively. Therefore, one may say that we need more studies to draw more solid conclusion. Furthermore, our analysis is based solely upon patients with DLBCL receiving RCHOP therapy and may not be generalizable to patients with other types of B-cell lymphoma or patients receiving rituximab alone.

In conclusion, we investigated the effects of statin use on clinical outcome in DLBCL patients receiving RCHOP and found that statin use did not influence their prognosis. To confirm this conclusion, larger scale, prospective studies of DLBCL patients are required.

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Author contributions: DE designed the study, treated the patients, collected clinical data, and wrote the paper; HA designed the study, treated the patients, and collected clinical data; KT and YT scored the immunohistochemical staining, designed the study, and wrote the paper; MY treated the patients, assisted in designing the study, and writing the paper; YM, KS, and YT treated the patients and supervised the paper; KM made the statistical analysis and wrote the paper; and KI, KH, and M.T. designed the study, supervised all aspects of the research and analyses, and wrote the paper.

disclosure

The authors declare no conflict of interests and funding sources.

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Research Article

The therapeutic effect of rituximab on CD5-positive and CD5-negative diffuse large B-cell lymphoma

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Abstract

The prognosis of diffuse large B-cell lymphoma (DLBCL) has improved markedly in recent years of rituximab era. The prognosis of *de novo* CD5-positive DLBCL is reported to be poor, but the effect of rituximab on this type of lymphoma remains unclear. To investigate the effect of rituximab on CD5-positive DLBCL, we collected DLBCL patients and analysed prognostic factors. A total of 157 patients with DLBCL who were immunophenotyped with flow-cytometry (FCM) and treated with chemotherapy were subjected to analysis. Those treated with radiotherapy alone or with supportive therapy only were not included. Patients diagnosed in 2003 or later were treated with rituximab combined chemotherapy. There were 95 males and 62 females. Their age ranged from 20 to 91 years old, and the median was 65 years. Nineteen patients were diagnosed as having *de novo* CD5-positive DLBCL. Rituximab was given alongside chemotherapy in 85 patients. Of these, 11 were positive for CD5 and 74 were negative. The addition of rituximab improved the overall survival (OS) of DLBCL patients (2-year OS: 82% vs. 70%, $p = 0.01$). For CD5-negative DLBCL, patients treated with rituximab showed 2-year OS of 84%, which was significantly better than those treated without rituximab (70%, $p = 0.008$). However, for CD5-positive DLBCL, the prognosis was not statistically different between the patients treated with and without rituximab (59% vs. 50%, $p = 0.72$). Although rituximab improved the prognosis of DLBCL, such improvement was restricted to the CD5-negative group. Further investigation is required to improve the prognosis of patients with CD5-positive DLBCL.

Keywords: **rituximab; CD5; B-cell; lymphoma; BCL-2; prognosis**

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the largest category of malignant lymphoma and is now considered to be made up of several heterogeneous subgroups. [1,2] *De novo* CD5-positive DLBCL is a distinct subtype of DLBCL that is characterized by elderly onset, female predominance, an advanced stage of disease at diagnosis, the presence of B symptoms, a high lactate dehydrogenase (LDH) level, frequent extranodal involvement and poor prognosis. [3–8] Immunoglobulin mutation analysis of *de novo* CD5-positive DLBCL has identified that this lymphoma is derived from the post germinal centre (GC) stage of somatically mutated B-cells. [3,4,9] Genetic analyses using comparative genomic hybridization (CGH) and microarray technologies have further shown that *de novo* CD5-positive DLBCL is distinct from CD5-negative DLBCL and mantle cell lymphoma (MCL). [10–12]

Patients with advanced stage DLBCL used to be treated with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) based chemotherapy. [13,14] The prognosis of DLBCL has improved markedly in recent years of rituximab era. [15–19] However, the effect of rituximab-containing chemotherapy on *de novo* CD5-positive DLBCL remains unclear. To investigate the effect of rituximab on this type of lymphoma, we collected patients that were suffering from DLBCL and analysed prognostic factors.

Methods

Patient selection

From April 1998 to October 2006, a total of 424 patients were newly diagnosed with *de novo* DLBCL in the Yokohama City University School of Medicine and

collaborating institutes. Of these, 157 were analysed for surface markers with flow-cytometry (FCM) and received chemotherapy. Patients diagnosed before 2003 were previously reported. [20] Those treated with radiotherapy alone or with supportive therapy only were excluded from this study. Patient therapeutic records were retrospectively analysed. The study was approved by the Institutional Review Board from Yokohama City University Medical Center, and complied with the Helsinki Declaration.

Morphological and immunophenotypical analyses

CD5-positive DLBCL was diagnosed when the tumour cells were positive for CD5 according to FCM. All of the CD5-positive DLBCL specimens were re-examined histopathologically. Tissue was fixed in 10% formalin and embedded in paraffin. The sections (4 μm thick) were stained with hematoxylin and eosin, and immunostained for CD5 (4C7, Novocastra, Newcastle-upon-Tyne, UK), CD10 (56C6, Novocastra), CD20 (L26, Dako, Glostup, Denmark), BCL2, (124, Dako), BCL6 (PG-B6p, Dako), MUM1 (MUM1p, Dako) and cyclinD1 (P2D11F11, Novocastra) using the dextran-polymer method (EnVision+; Dako, Glostup, Denmark). For immunohistochemistry, heat-induced antigen retrieval pretreatment using Target Retrieval Solution, pH 9 (Dako) was carried out. Histologic specimens from all of the patients with CD5-positive DLBCL were reviewed by hematopathologists, including one of the authors (K.T.), according to the WHO classification. [2] All patients were diagnosed as having DLBCL. Patients diagnosed with intravascular lymphoma (IVL) were not included for this study. One patient diagnosed with DLBCL with an intravascular pattern was included because the area of DLBCL was the most prominent.

In immunohistochemical evaluation, BCL6, MUM1 and BCL2 were defined as positive when the proportion of stained lymphoma cells was 30% or higher. CD5 and CD10 were considered to be immunohistochemically positive when at least a small population of the neoplastic cells was positive. Subtype of GC or non-GC phenotype was judged using CD10, BCL6 and MUM1 according to the Hans's criteria. [21]

Treatment of patients

All patients diagnosed before 2003 were treated with chemotherapy with standard CHOP or CHOP-like anthracycline-containing regimens. In 2003 or later, patients were treated with rituximab containing chemotherapy (CHOP-R or others with rituximab). Fourteen patients were treated with additional radiotherapy (10 patients without rituximab and four patients with rituximab) and nine patients received autologous transplantation (seven patients without rituximab and two patients with rituximab). All but one patient who received additional therapy was diagnosed as having CD5-negative DLBCL.

Statistical analysis

Correlations between the two groups were examined with the χ^2 test, the Fisher exact test, and the Mann–Whitney *U*-test. Patient survival data were analysed with the Kaplan-Meier method and were compared by means of the log-rank test. Overall survival (OS) was defined as the time from diagnosis to the date of death or last contact. Progression free survival (PFS) was defined as the time from diagnosis to the date of progression, relapse, death or last contact. Univariate and multivariate analyses were performed with the Cox proportional hazard regression model. Variables were selected with the step-wise method. Data were analysed with Fisher (Nakayama-Shoten, Tokyo, Japan) and STATA statistical software (College Station, TX).

RH contributed to the design of the study, collected clinical data, analysed the data and wrote the manuscript. NT designed the study and collected clinical data. KT designed the study and wrote the manuscript. TA, AF, HK, CH, ST, JT, RS, HF, SF, KO and SM collected clinical data. RS wrote the manuscript. YI supervised the study and gave critical advice.

Results

Patient characteristics

There were 95 males and 62 females. Their ages ranged from 20 to 91 years old, and the median age was 65 years. The median follow-up of the surviving patients was 2.2 years. Nineteen patients were diagnosed as having *de novo* CD5-positive DLBCL (Table 1). Of these, the expression of CD5 by FCM was bright in 16 patients and dim in three. In comparison with CD5-negative DLBCL, the CD5-positive group had high serum lactate dehydrogenase (LDH) (95%, $p = 0.02$) and soluble IL-2 receptor levels (63%, $p = 0.0001$), advanced stage (84%, $p = 0.01$), a high International Prognostic Index (IPI) score (79%, $p = 0.01$) and also displayed B-symptoms (68%, $p = 0.002$). Rituximab was added to the chemotherapy in 85 patients. Of these, 11 were positive for CD5 and 74 were negative (Table 1).

In the patients treated with rituximab, there were 55 males and 30 females. Eleven patients were diagnosed as having *de novo* CD5-positive DLBCL (M/F = 5:6). In comparison with the CD5-negative DLBCL group, the CD5-positive group had a high soluble IL-2 receptor level (73%, $p = 0.001$), advanced stage disease (91%, $p = 0.01$), a high IPI score (91%, $p = 0.006$) and also displayed B symptoms (73%, $p = 0.006$).

Immunohistologic features

Among the 19 CD5-positive DLBCL patients, CD5 was positive in 14 patients (74%) according to immunohistochemistry. The expression was weak or focal in nine of the 14 patients. All of the 19 CD5-positive patients were negative for Cyclin D1 and positive for CD20. Only two of the patients were positive for CD10. Another two patients that were negative for CD10 were positive for BCL6 and

Table 1. Patient characteristics of CD5-positive and CD5-negative DLBCLS

Characteristics	CD5 + DLBCL ($n = 19$)	CD5 − DLBCL ($n = 138$)	p-value
Sex (male/female)	8/11	87/51	0.08
Age: median	66	64	
Range	30–91	20–88	
>60 years	14 (74)	83 (60)	0.25
Performance status >1	9 (47)	42 (30)	0.13
LDH: elevated	18 (95)	99 (72)	0.02
Advanced stage	16 (84)	76 (55)	0.01
No. of extranodal involvement >1	10 (53)	46 (33)	0.09
IPI: high-intermediate/high	15 (79)	69 (50)	0.01
B symptoms: present	13 (68)	45 (33)	0.002
Bulky mass: present	2 (11)	42 (30)	0.05
Soluble IL-2 receptor >5000	12 (63)	32 (23)	0.0001
Rituximab (−/+)	8/11	64/74	0.91

Values in parentheses indicate the percentage of patients.
DLBCL, diffuse large B-cell lymphoma; LDH, lactate dehydrogenase; IPI, International Prognostic index; IL-2, interleukin-2.

negative for MUM1. Therefore, a total of four patients with CD5-positive DLBCL showed the GC phenotype (21%). All but one of the CD5-positive patients were positive for BCL-2 (95%). Ki67-positive cells ranged from 50 to 70% except in one patient who had an extremely low positivity of 10%.

Therapeutic response and prognosis

The patients who were treated with rituximab showed significantly better OS that those treated without rituximab. Two-year OS was 82% for the former and 70% for the latter ($p = 0.01$, Figure 1A), and the respective 2-year PFSs were 66 and 53% ($p = 0.04$, Figure 1B). For CD5-negative DLBCL, the addition of rituximab significantly improved patient prognosis. Patients treated with rituximab showed 2-year OS of 84%, which was significantly better than that for patients treated without rituximab (70%, $p = 0.008$, Figure 2A). However, for CD5-positive DLBCL, the prognosis was not statistically different between the patients treated with and without rituximab (59% vs. 50%, $p = 0.70$, Figure 2B). Although rituximab improved the prognosis of DLBCL, such improvement was restricted to the CD5-negative group.

Univariate Cox analysis identified the following prognostic factors for all patients: performance status (PS), serum LDH level, clinical stage, extranodal involvement at

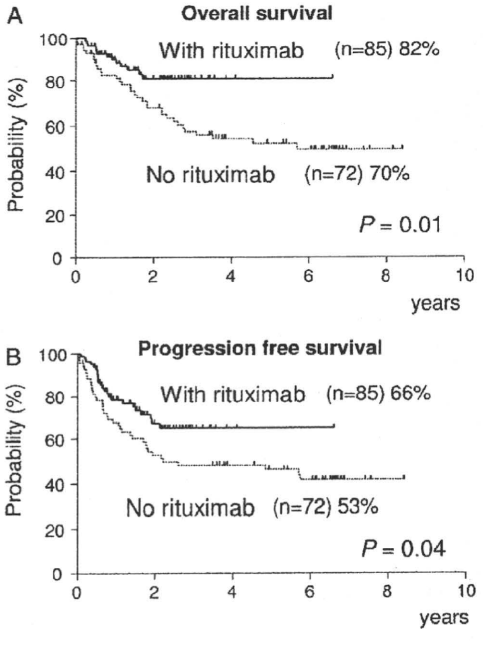

Figure 1. Survival curves of DLBCL patients treated with or without rituximab. The patients treated who received rituximab showed significantly better overall survival (A) and progression free survival (B) than those treated without rituximab

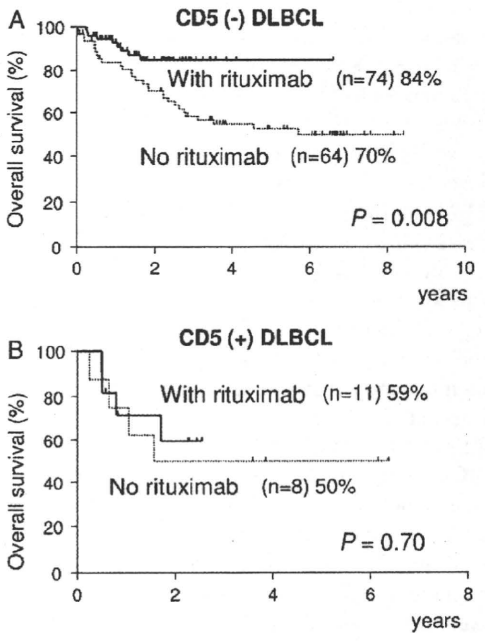

Figure 2. Overall survival curves of CD5-positive and -negative DLBCL patients treated with or without rituximab. (A) The prognosis of CD5-negative DLBCL patients was significantly improved by the use of rituximab. (B) In contrast, no beneficial change was observed for CD5-positive DLBCL patients after the introduction of rituximab

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Table 2. Prognostic factors affecting overall survival

Variables	Unfavourable factors	Univariate			Multivariate		
		HR	95%CI	*p*-value	HR	95%CI	*p*-value
CD5 status	Positive	—	NS				
Age	>60 years	—	NS				
PS	>1	3.7	(2.1–6.7)	<0.0001	2.3	(1.2–4.2)	0.007
LDH	> normal	9.8	(2.3–40.8)	0.001	8.7	(2.0–36.9)	0.003
Stage	III/IV	2.4	(1.2–4.8)	0.01	—	NS	
Extranodal sites	>1	2.3	(1.3–4.2)	0.004	—	NS	
B symptoms	Present	2.7	(1.4–4.9)	0.001	2.2	(1.1–4.1)	0.01
Bulky mass	Present	2.1	(1.1–3.9)	0.01	—	NS	
Rituximab	Not used	2.4	(1.2–5.0)	0.01	2.5	(1.3–5.0)	0.006

PS, performance status; LDH, lactate dehydrogenase; HR, hazard ratio; CI, confidence interval.

more than one site, the presence of B symptoms, the presence of a bulky mass and the induction of rituximab (Table 2). Multivariate analysis showed that PS, serum LDH level, the presence of B symptoms and the induction of rituximab were independent prognostic factors (Table 2). For patients treated with rituximab, univariate analysis identified that PS (hazard ratio (HR): 6.1, 95% confidence interval (CI): 1.8–20.0, $p=0.002$) and the presence of B symptoms (HR: 3.5, 95%CI: 1.1–10.9, $p=0.02$) were prognostic factors. Multivariate analysis showed PS was the only independent prognostic factor (HR: 5.0, 95%CI: 1.2–21.1, $p=0.02$). CD5 status was not identified as significantly prognostic by univariate or multivariate analysis either in all or rituximab-treated patients.

Discussion

The addition of rituximab to anthracycline-containing chemotherapy has improved the prognosis of B-cell lymphomas including DLBCL. [15–19] Our current study shows that this effect of rituximab is only seen in CD5-negative DLBCL patients.

Several factors are known to be prognostic for DLBCL, [2,22,23] and the most notable clinical factor is the IPI score. [24] In recent rituximab era, however, the prognostic model of IPI has been reclassified into a novel three prognostic groups, which is termed the revised-IPI (R-IPI). [25] In addition, the concept of a 'biological prognostic factor' has emerged, [26] and gene expression profiling using microarray technologies has significantly altered the assessment of biomarkers. [27–29] Gene expression profiling using microarray technology has resulted in DLBCL being divided into two major subgroups: one similar to normal germinal centre B cells (GCB) and the other similar to activated peripheral blood B cells (ABC). Hans *et al.* [21] proposed a model using the presence of three proteins (CD10, BCL6 and MUM1) to discriminate between GCB-type and ABC-type DLBCL. Lossos *et al.* [30] proposed another prognostic model using the expression of six predictive genes (*LMO2, BCL6, FN1, CCND2, SCYA3* and *BCL2)*. Other investigators have also claimed that BCL2 expression predicts a poor prognosis. [31–33] Recently, the introduction of rituximab has been reported to improve the poor prognosis of BCL2-positive DLBCL. [34–37] Therefore, rituximab is believed to have the potential to affect prognostic factors.

CD5-positive DLBCL accounts for 5–10% of all DLBCLs and is associated with female predominance, an advanced stage of disease at diagnosis, and an aggressive disease course, and it is reported that these characteristics cause poor prognosis. [8,38] However, CD5 status was not shown to be an independent prognostic factor by multivariate analysis. [8] Because CD5 expression is closely correlated with each of the factors that make up the IPI score, CD5 expression in DLBCL represents a biological feature of aggressiveness, which is also detectable by means of these clinical parameters. The present results are consistent with the previous findings in the literature. Notably, these features are concordant with GCB/ABC categorization since most CD5-positive DLBCLs are included in ABC-type DLBCLs.[39] Our study showed CD5-positive DLBCL frequently expresses BCL2, which is consistent with the literature findings that the expression of BCL2 is significantly more frequent in CD5-positive DLBCL than in CD5-negative DLBCL. [40,41] Because the induction of rituximab improves the poor prognosis of BCL2-positive DLBCL, [34–37] we initially postulated that the improvement in prognosis would be more prominent in CD5-positive DLBCL. However, the prognosis of CD5-positive patients did not improve after supplementation with rituximab. These findings suggest that the favourable outcome of rituximab containing chemotherapy is not mediated by BCL2. Our findings as well as those of Ennishi, *et al.* [41] showed the prognosis of CD5-positive patients was poor with rituximab treatment. Further studies are required to clarify the reasons for the poor prognosis of CD5-positive DLBCL patients.

In the literature, CD5-positive DLBCL has been described to have a close correlation with intravascular large B-cell lymphoma (IVL). [8,40] Approximately 30–40% of CD5-positive DLBCL shows a focal intravascular or intrasinusoidal pattern of lymphoma infiltration. On the other hand, varying degrees (22–75%) of CD5 expression have been reported for IVL. [42–46] In the current study, we excluded patients who were diagnosed with IVL so we could strictly focus on CD5-positive DLBCL. Recently, the addition of rituximab to anthracycline containing chemotherapy has been reported to improve the prognosis of

IVL.[47] Our current results for CD5-positive DLBCL are different from those of IVL, suggesting a need for careful differentiation of IVL from *de novo* CD5-positive DLBCL.

Investigation of appropriate treatment strategies for CD5-positive DLBCL is urgently required because the prognostic differences between CD5-positive and -negative DLBCL are expanding. One of the candidates for this is a dose-adjusted EPOCH and rituximab regimen that is more effective in patients with poor prognostic DLBCL. [48,49] Another candidate is a combination of rituximab and ACVBP chemotherapy that is reported to be effective for central nervous prophylaxis. [50] Prospective studies of these novel regimens are thus warranted.

In conclusion, rituximab improves the prognosis of CD5-negative DLBCL patients when combined with standard chemotherapy, but does not do the same for CD5-positive DLBCL patients.

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JB Review

Alternative lengthening of telomeres pathway: Recombination-mediated telomere maintenance mechanism in human cells

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Unlimitedly proliferating cells need to acquire the telomere DNA maintenance mechanism, to counteract possible shortening through multiple rounds of replication and segregation of linear chromosomes. Most human cancer cells express telomerase whereas the other cells utilize the alternative lengthening of telomeres (ALT) pathway to elongate telomere DNA. It is suggested that ALT depends on the recombination between telomere repetitive DNAs. However, the molecular details remain unknown. Recent studies have provided evidence of special structures of telomere DNA and genes essential for the phenotypes of ALT cells. The molecular models of the ALT pathway should be validated to elucidate recombination-mediated telomere maintenance and promote the applications to anti-cancer therapy.

Keywords: alternative lengthening of telomeres (ALT)/extrachromosomal telomeric repeat (ECTR)/PML body/recombination/telomere.

Abbreviations: ALT, alternative lengthening of telomeres; BIR, break-induced replication; CDK, cyclin-dependent kinase; DDR, DNA damage response; DSB, double-stranded DNA break; DSBR, double-stranded DNA break repair; HR, homologous recombination; RCR, rolling-circle replication.

Telomeres are specific chromatin structures at the linear chromosome ends of eukaryotic cells. Each telomere contains an end of a double-stranded (ds) DNA, which is protected from degradation and repair processes that can cause chromosome instability. This end protection requires functional telomere chromatin that includes specialized DNA (telomere DNA) and its binding proteins (telomere binding proteins) (Fig. 1A and B). The telomere DNA of most eukaryotes is repetitive DNA containing a guanine-rich (in human, 5′-TTAGGG-3′) strand and its complementary strand called G- and C-strands, respectively. The 3′-end of G-strand extends to form a single-stranded (ss) structure called the G-tail (*1*). The G-tail is thought to invade and hybridize with the proximal C-strand to form a special structure called the t-loop (*2*). Shelterin is a conserved protein complex on telomeres, and shelterin in mammalian cells is composed of one ss (POT1) and two ds (TRF1 and TRF2) telomere DNA binding proteins as well as specific proteins to connect those DNA binding proteins (*3*, *4*). This complex serves as the functional framework of telomere chromatin.

Telomere DNA in dividing cells is subject to possible shortening or the so-called end-replication problem (Fig. 1C) (*5*, *6*). Semi-conservative replication cannot complete the syntheses of the very ends of linear DNA. Thus after multiple rounds of cell division, human somatic cells have shortened telomere DNA, which results in irreversible cell growth arrest, namely, replicative senescence (*7*, *8*). On the other hand, cancer cells express certain mechanisms to counteract the shortening of telomere DNA and acquire immortality. Telomerase is a specific reverse transcriptase that elongates the G-strand telomere DNA (*9*). Approximately 90% of cancer cells maintain telomeres in a telomerase-dependent manner; however, some telomerase-negative human cell lines have been established from cancer cells and *in vitro* immortalized cells (*10*). Thus, it is imperative to understand both telomerase- and telomerase-independent pathways to inhibit the immortality of cancer cells.

In yeast and mouse, telomerase-negative cells have been directly isolated from 'survivors' after artificial disruption of the gene encoding the telomerase RNA component (telomere RNA: TR) or the catalytic subunit (telomere reverse transcriptase: TERT). Survivors in fission yeast *Schizosaccharomyces pombe* occasionally develop self-circularized chromosomes to circumvent the end-replication problem (*11*, *12*), whereas survivors in other species generally acquire the lengthening mechanism of telomere DNA at linear chromosome ends (Fig. 1D) (*13–15*). Phenotypic variations among telomerase-negative cells gave birth to the idea that there may be more than one telomerase-independent pathway. One of the most significant examples is the alternative lengthening of telomeres (ALT) pathway of human cells. Confusingly, 'ALT' is sometimes used for various telomerase-independent pathways in human and other species. The ALT we describe here is confined to the pathway found in a group of human telomerase-negative cells (hereafter, ALT cells) showing two distinctive telomere

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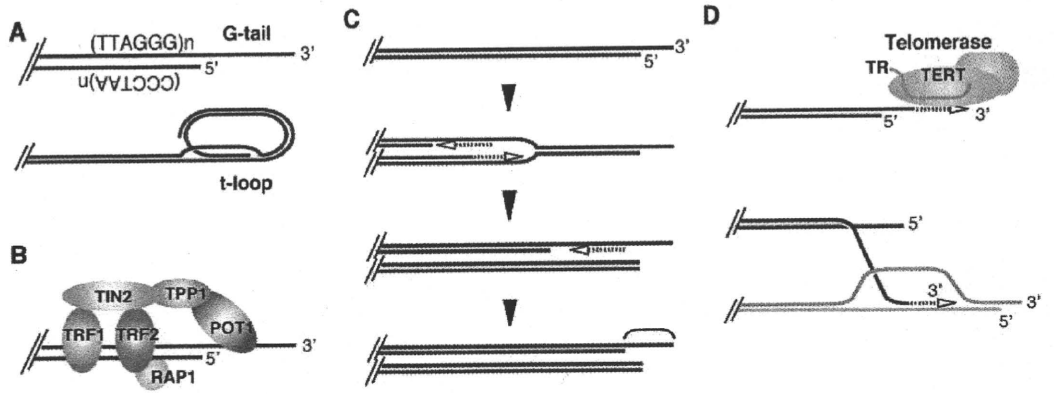

Fig. 1 Telomere structure and lengthening. (A) Vertebrate telomere DNA. (B) Shelterin complex in human cells. (C) End-replication problem. The G-strand (red) is completely replicated to the end. However, the C-strand (blue) of telomere DNA is synthesized as a lagging strand and thus the copy of the very end cannot be accomplished. This causes the gradual shortening of telomere DNA through rounds of cell divisions. (D) Two types of telomere lengthening mechanism. The G-strand is primarily elongated in the telomerase pathway (top). Telomerase-independent pathway (bottom) is generally recombination-mediated.

phenotypes; heterogeneous and long telomere DNA and the formation of ALT-associated promyelocytic leukaemia (PML) body (APB). These phenotypes are widely used as convenient markers for ALT cell lines. In this review, we will summarize the characteristics and the most recent findings of the human ALT pathway. We will also discuss the molecular mechanism of ALT and its relevance to diverse telomerase-independent pathways.

Telomere DNA length and recombination in ALT cells

Telomere DNA length is generally analysed by the Southern hybridization of genomic DNA treated with appropriate restriction enzymes. The mean length of telomere in human telomerase-positive cancer cell lines is usually <10 kb. In contrast, ALT cells have longer and more heterogeneous telomeres; the mean length is ~20 kb (*16*). This suggests that ALT is a distinct lengthening mechanism from the telomerase pathway and telomere length analysis is regarded as one of the vital tests for ALT. A fluorescent *in situ* hybridization (FISH) experiment of metaphase chromosomes also demonstrated the remarkable heterogeneity of telomere lengths in ALT cells. The signal strengths of the telomere foci in ALT cells varied markedly, whereas those in telomerase-positive cells were comparable between chromosome ends (*17*). It is unknown how the heterogeneity of telomere lengths developed; however, it may be related to telomere metabolism specific to ALT cells.

The dynamic behaviour of telomere length in ALT cells was described in a study that used cells with artificially tagged telomeres (*18*). A tag sequence inserted near the telomere repeats of a defined chromosome end was used as a probe to measure the length of this telomere DNA specifically. The telomere in an ALT cell line was drastically elongated during cell division, which differed from that in telomerase-positive cells. The telomeres also showed gradual shortening similar to normal somatic cells and sometimes exhibited rapid deletion. The rapid changes in the telomere DNA length of this ALT cell line suggest homologous recombination (HR) between telomeres. Another study demonstrated that a tag sequence within the telomere repeats of one chromosome was duplicated to other chromosome ends through cell division in ALT cells but not in telomerase-positive cells (*19*). This recombination event may reflect the ALT pathway in which telomeres are elongated by making a copy of the telomere DNAs of different chromosomes.

It is possible that ALT cells represent elevated levels of HR at other loci as well as telomere repeats. The instability of the repetitive sequences of minisatellites was more frequently noted in ALT cell lines than in telomerase-positive cell lines (*20*, *21*). However, ALT-specific hyper-recombination was not observed when the frequencies of intrachromosomal recombination were analysed by using a reporter construct integrated into a certain chromosome locus (*22*). Therefore, ALT cells may have the feature of elevated recombination only in the loci with repetitive sequences, such as telomeres and minisatellites.

To visualize *de novo* synthesized telomeric G- or C-strand specifically, chromosome orientation (CO)-FISH analysis was applied to telomeres (Fig. 2). In metaphase chromosomes, the CO-FISH signal was typically observed at the telomere of either one of the sister chromatids. The frequencies of telomere-sister-chromatid exchange (T-SCE) were calculated as the percentage of chromosome ends positive for CO-FISH signals on both sister telomeres. The high level of T-SCE was generally found in ALT but not in telomerase-positive cells (*23–25*) and therefore, hyper-recombination between sister telomeres was recognized as one of the features of ALT cells. However, T-SCE or the reciprocal exchange of sister telomeres by itself does not accompany massive DNA synthesis and could not account for the net elongation of telomeres or the ALT pathway.

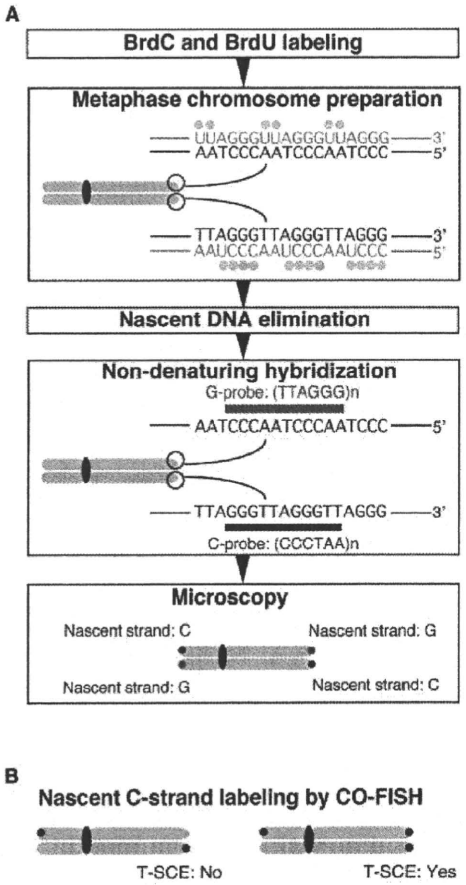

Fig. 2 CO-FISH analysis and T-SCE. (A) Outline of CO-FISH method. The nascent DNA strands (pale blue) are labelled with BrdU and BrdC and they can be specifically degraded. The template strands are exposed and can be hybridized with the florescent G- or C-probe (green or red bar). The signal of each probe can be observed at telomere of each one of sister chromatids.
(B) Observation of T-SCE by CO-FISH. When T-SCE is occurred after DNA replication, the signals of CO-FISH can be observed at the both sister telomeres.

Particular structures of telomere DNA in ALT cells

The first evidence of unusual telomere DNA in ALT cells is the extrachromosomal telomere repeat (ECTR). FISH analyses of metaphase chromosomes of ALT cells suggested the presence of significant telomere DNA repeats other than the chromosome ends (*26*). ALT cells harbour small ds linear telomere DNA in the soluble fractions of the cell extract, which can be separated from bulk chromatin (*27*). Circular DNA molecules were occasionally found in ALT cells by electron microscopy and these are thought to correspond to the circular form of telomere DNA (t-circle) determined by two-dimensional (2D) gel electrophoresis (*28*, *29*). T-circles are now regarded as a marker for ALT cells, although they can be observed in telomerase-positive cells that have a defect in TRF2 or that contain extensively elongated telomere DNA (*29*, *30*). T-circles can be produced by the intrachromosomal recombination of telomere repeats (Fig. 3A). ALT cells may acquire a feature that induces such recombination events.

ALT cells harbour in particular ss structures of telomere DNA (*31*). The average lengths of ss telomere DNA are shorter than those of ds telomere DNA, suggesting that telomere in ALT cells contains nicked and/or gapped structures. Different forms of ss telomere DNA were resolved by 2D gel electrophoresis. The small sized ones of G- and C-strands were separated in the electrophoresis and showed the different sensitivities to structure-specific nucleases: the single G-strand structure (ss-G) was concluded to be linear DNA whereas the single C-strand structure (ss-C) was circular DNA. A sensitive method to detect circular C-strands was developed and applied to examine various cell lines (*32*). Interestingly, ALT cell lines were positive for the circular C-strands whereas telomerase-positive cells were negative. This suggests that this 'C-circle (CC) assay' is useful to determine whether the tested cancer cells are ALT or not. In addition, branched molecules of telomere DNA were found in the ss telomere structures of ALT cells. These remarkable structures of telomere DNA, t-circles and various ss structures, must be specific intermediates or crucial substrates for telomere metabolism in the ALT pathway.

The ALT pathway must be mediated by some HR-based mechanism where the nascent telomere DNA is synthesized efficiently. That is, the ALT pathway could induce pairing of the G- and C-strands in *trans* by HR to form the primer and the template. DNA synthesis should follow to elongate telomere DNA from the primer. One possible mechanism is break-induced replication (BIR). In BIR, the 3′-end of ss DNA invades a region with a homologous sequence at first, and then DNA synthesis is initiated at the 3′-end by using the paired strand as template. It has been suggested that BIR must operate in the telomere lengthening in ALT cells (Fig. 3B). When the 3′-end of the G-tail is paired with the C-strand of other telomeres, a nascent G-strand can be synthesized and elongated up to the end of the template C-strand. BIR readily accounts for the high frequency of copy of a tag in telomere DNA (*19*), although the function of circular telomere DNA specifically found in ALT cells (t-circle and ss-C) is unclear.

The integration of t-circles into the telomere repeats at chromosome ends will result in telomere elongation (Fig. 3C). However, it does not increase the net amount of telomere DNA in the cells and cannot complete telomere maintenance through multiple divisions of ALT cells. Furthermore, circular forms of DNA can be involved in the rapid elongation of DNA ends in rolling-circle replication (RCR) (Fig. 3D). When the 3′-end of the G-tail is paired with ss-C and/or t-circles in ALT cells, the synthesis of the G-strand can be induced on this circular DNA as a template. RCR is fundamentally a continuous process and can accomplish very efficient lengthening even with small templates. Interestingly, the ss-G structure in ALT cells is similar to the linear ss intermediates of RCR in yeast mitochondria DNA (*33*). BIR and RCR are not exclusive of each other and both require invasion

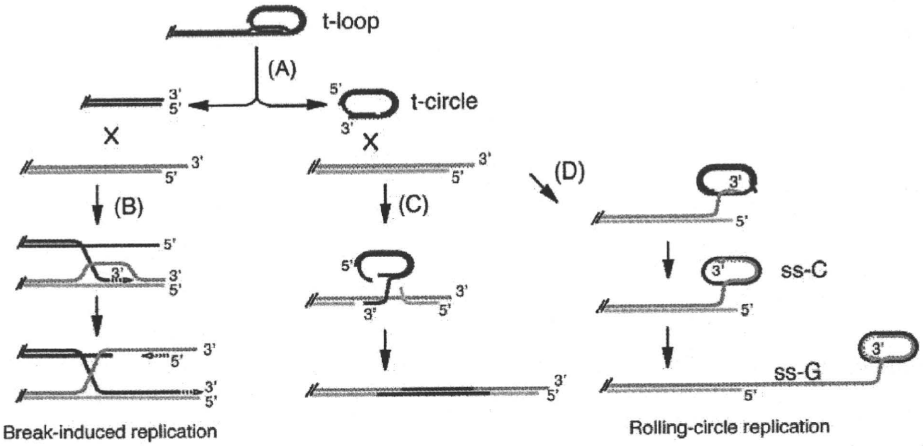

Fig. 3 Telomere DNA metabolism in ALT cells. Four types of recombination events result in particular DNA metabolisms and unique products. (A) Recombination within t-loop causes rapid telomere shortening and t-circle formation. (B) Recombination between telomeres of chromosome ends initiates break-induced replication. (C) Transfer of a strand of t-circle into telomeres at chromosomal DNA initiates elongation of the repetitive DNA. (D) Invasion of t-circle by G-tail triggers rolling-circle replication.

of the 3′-end of the G-tail with the C-strand and the initiation of DNA synthesis at the pairing sites. This implies that both mechanisms may contribute to the ALT pathway and be regulated by common molecular mechanisms.

ALT-associated PML body

PML body is a nuclear aggregate of PML and other proteins and is insensitive to low concentration of non-ionic detergent. PML bodies are present in many types of cells, *e.g.* in both telomerase-positive and ALT cell lines. It has been suggested that PML body functions in various cellular processes, including tumour formation, cellular senescence, stress response, DNA repair, etc. However, its molecular function(s) remains unsolved. Although PML bodies are usually unrelated to telomere, many telomerase-negative cell lines commonly have ALT-associated PML body (APB), a special form of PML body that includes telomere DNA and chromatin (*34*). This feature, namely, the colocalization of both markers for telomere and PML body, can be readily observed and thus, the formation of APB has been used as a marker for ALT cells. Although it is unknown how APBs are related to the ALT pathway, it is noteworthy that APBs possess a variety of proteins working in DNA metabolism and cell growth regulation (Table I). The localization of recombination proteins at APBs supports the idea that HR may be associated with ALT.

The incorporation of the thymidine analogue bromodeoxyuridine (BrdU) was observed in a fraction of APBs (*35*). This nascent DNA synthesis at APBs was suppressed by inhibitors of ATM and ATR (*36*), which are members of phosphoinositide 3-kinase related kinase (PIKK) crucial for the activation of DNA-damage response (DDR). This suggests that the ALT pathway may be regulated by damage signalling. Telomeres of ALT cells are colocalized with the markers for DDR, for example, γ-H2AX, 53BP1, the RAD9-RAD1-HUS1 (9-1-1) complex, and its loader RAD17 (*36*, *37*). However, it is unknown whether DDR of telomere in ALT cells is activated by particular ss structures of telomere DNA. Two reports suggested that APBs have a special form of telomere DNA. The major telomere DNA components in APB are chromosome ends; thus the chromosome ends may cluster at APBs to enhance HR between telomeres (*38*). In contrast, purification study suggests that APBs predominantly contain linear ECTRs (*39*). Therefore, the specific structures of telomere DNA in APBs and their relevance to the ALT pathway have still to be unraveled.

Proteins required by ALT cells

To elucidate the molecular mechanism of the ALT pathway, phenotypes of ALT cells were examined when the function of proteins and/or the expression of their coding genes were suppressed or induced (Table I). The candidate proteins of interest were the components of telomere chromatin, APB and DNA metabolisms including HR. Among the phenotypes, changes in telomere length were particularly notable in the analyses. The erosion of telomere DNA from ALT cells may be due to two reasons: one is a defect in the ALT pathway or the lengthening mechanism of telomere DNA. This will gradually reduce telomere lengths as the end-replication problem emerges. The other is the imperfect protection of telomere. As telomere DNA in ALT cells contains remarkable ss and/or branched structures, ALT cells may be sensitive to even small damages in the telomere DNA caused by certain gene mutations or overexpression. The defect may cause the sudden loss of telomere. In both cases, a shortened telomere DNA is expected to induce defects in cell growth and/or cellular senescence. In addition, ALT-specific phenotypes, for example, the formation of APB and the frequency of T-SCE, were also examined.

Table I. Proteins related to ALT pathway.

Name	Tests[a]	Remarks	References
53BP1	LK	DDR	(37,43)
ATM	L	DDR; PIKK	(46)
ATR	LK	DDR; PIKK	(57)
BLM	LK	DSBR; RecQ-like helicase	(47,48,70)
BRCA1	L	HR; breast cancer 1	(71)
Cdk2	L	Cell cycle; CDK	(58)
COUP-TF1	L	Unknown; orphan nuclear receptor	(59)
COUP-TF2	L	Unknown; orphan nuclear receptor	(59)
ERCC1	L	Excision repair; endonuclease	(72)
FANCA	K	DSBR	(57)
FANCD2	LK	DSBR	(57)
FANCJ	L	DSBR; structure-specific DNA helicase	(59)
FANCL	K	DSBR; E3 ubiquitin ligase	(57)
FEN1	K	Replication; flap endonuclease	(55)
γ-H2AX	L	DSB marker; phosphorylated H2AX	(36,37)
hnRNP A2	L	Regulator of splicing; hnRNP	(73)
HUS1	L	DDR; 9-1-1 PCNA-like clamp	(36)
HP1α	LK	Heterochromatin	(58)
HP1β	LK	Heterochromatin	(58)
HP1γ	LK	Heterochromatin	(58)
HSP90	L	Heat shock protein	(47)
MDC1	L	DDR	(37)
MMS21	LK	DDR; E3 SUMO ligase in SMC5/6	(41)
MRE11	LK	HR; MRN complex subunit	(42,43,74)
MUS81	LK	HR; structure-specific endonuclease	(53)
NBS1	LK	HR; MRN complex subunit	(42,43,74)
NXP2	L	PML body	(59)
p21	LK	Regulator of growth; CDK inhibitor	(58)
p53	LE[b]	Tumour suppression; transcription factor	(46,58)
PARP2	L	DSBR; poly (ADP-ribose) polymerase	(75)
PCNA	LK	Replication; clamp	(58)
PML	LK	PML body	(34,43)
POT1	L	Shelterin; ss telomere DNA binding	(48)
RAD1	L	DDR; 9-1-1 PCNA-like clamp	(36)
RAD9	L	DDR; 9-1-1 PCNA-like clamp	(36)
RAD17	L	DDR; chromatin loader for 9-1-1 clamp	(36)
RAD50	LK	HR; MRN complex subunit	(41–43,74)
RAD51	LK	HR; RecA-like, strand exchange protein	(34, 41)
RAD51D	LK	HR; resolution of Holliday junction	(54)
RAD52	L	HR; DNA binding protein	(34)
RAP1	LK	Shelterin; TRF2-interacting	(43,71)
RIF1	L	DDR	(76)
RIP140	L	Transcription; co-regulator	(59)
RPA32	LK	Replication and HR; ss DNA binding	(56)
RPA70	LK	Replication and HR; ss DNA binding	(56)
SMC5	LK	DDR; structural maintenance complex	(41)
SMC6	L	DDR; structural maintenance complex	(41)
Sp-100	LKE	PML body	(40,43,71)
STN1	L	Telomere; ss DNA binding CST complex	(77)
TEP1	L	Telomere	(47)
TF4	L	Unknown; orphan nuclear receptor	(59)
TIN2	LK	Shelterin	(43,48)
TopoIIα	L	DNA structure; topoisomerase, type II	(47)
TopoIIIα	LK	DNA structure; topoisomerase, type IA	(48)
TRF1	LK	Shelterin; ds telomere DNA binding	(34,43)
TRF2	LK	Shelterin; ds telomere DNA binding	(34,43,46)
WRN	LK	HR; RecQ-like helicase and nuclease	(78,79)
XPF	L	Excision repair; endonuclease	(72)
XRCC3	K	HR, resolution of Holliday junction	(44)

[a]L, localization at telomeres in ALT cells; K, knockdown of gene; E, ectopic expression of protein.
[b]Activation of p53 by RNAi of SV40 large T-antigen.

MRN complex

The MRN complex, which includes MRE11, RAD50 and NBS1, functions in the early steps of HR and ds DNA break (DSB) repair (DSBR). Interestingly, this complex constitutes APB and the homologous complex in budding yeast plays essential roles in the telomerase-independent telomere lengthening mechanism (see below). Overexpression of Sp-100, a component of PML body that can interact with NBS1, inhibited the proper localization of NBS1 to APBs. This sequestration of NBS1 from APBs or the knockdown of each of MRN gene induced the shortening of telomere DNA in ALT cells (*40–42*). MRN was also required for APB formation (*43*). The knockdown of the gene encoding RAD50 or the overexpression of Sp-100 caused a significant reduction of t-circles (*32*, *44*), suggesting the function of MRN in the maintenance of ALT-specific telomere DNA.

TRF2 and shelterin

TRF2 is a ds telomere DNA binding protein in the shelterin complex, which is essential for end protection. A defect in TRF2 stimulates chromosome fusion between telomeres in telomerase-positive cells (*45*). U2-OS is an ALT cell line established from human osteosarcoma, which is expressing wild-type p53 and Rb, but lacking p16. The phenotypes of U2-OS were examined after inhibition of TRF2 by siRNA or the expression of the dominant-negative form of the protein (*46*). Telomere DNA of TRF2-defecient U2-OS cells was shortened and the cells expressed β-galactosidase activity similar to senescent cells. The significant activation of p53 and p21 observed in those cells was dependent on PML protein, a major component of APB. The formation of APB was not affected in TRF2-deficient cells, although another report showed that four of six shelterin components, TRF1, TRF2, TIN2 and RAP1, were required for APB formation (*43*).

RecQ-like DNA helicases

DNA helicases of the RecQ family act on atypical DNA structures, such as intermediates of HR, stalled

sites of replication forks and the G-quartet structure of telomere DNA. This function is important for genome integrity; mutations in either one of the three genes encoding RecQ-like DNA helicases in human, *BLM* (Bloom syndrome), *WRN* (Werner syndrome) or *RECQL4* (Rothmund–Thomson's syndrome, RTS), cause chromosome abnormality and premature aging. BLM is an APB protein and the knockdown of the gene induces rapid shortening of telomere DNA and inhibition of ALT cell growth (*47*, *48*). BLM forms a functional complex with topoisomerase IIIα (TopoIIIα) to suppress HR (*49*). Knockdown of the TopoIIIα gene in ALT cells also resulted in the loss of the G-tail, chromosome bridges or fusions of sister telomeres at anaphase, and cell growth retardation (*48*). The phenotypes of TopoIIIα-knockdown cells could be due to indirect effects as the amounts of BLM and TRF2 were concurrently decreased.

WRN is also localized at APB, although it seems to be dispensable for ALT cells. When fibroblasts mutated in *WRN* from a Werner syndrome patient were immortalized with SV40, a cell line that utilizes the ALT pathway to maintain the telomere DNA was established (*50*, *51*). In mouse, the survivors of *Wrn* (encodes WRN homologue) *Terc* (TR) double knockout were obtained and they showed elevated levels of T-SCE as in human ALT cells (*52*). These results suggest that ALT (human) and a telomerase-independent lengthening mechanism (mouse) can be activated without the function of WRN.

Resolution of Holliday junction

A striking intermediate of HR is the Holliday junction, a cruciform structure formed by two ds DNAs (Fig. 4). ALT cells show elevated T-SCE and contain branched structures in telomere DNA, so that they may require efficient resolution of the junction. MUS81 and its interacting protein MMS4 together constitute endonuclease specific to unpaired ds DNA, for example, the Holliday junction. The knockdown of *MUS81* in ALT cells caused growth arrest, loss of telomeres on metaphase chromosomes, and reduced frequency of T-SCE (*53*). The ectopic expression of TERT complemented the suppression of cell growth, but not the low T-SCE frequency. Interestingly, the length of telomere DNA and the amount of t-circles from Southern analyses were not altered in *MUS81* knockdown cells. It is unknown how telomere loss from chromosome ends is induced without a significant reduction in telomere length.

XRCC3, a RAD51-paralog that is presumably involved in the resolution of the Holliday junction, is required for the maintenance of t-circles (*44*). RAD51D, another RAD51-paralogue for the resolution, is an APB protein and its inhibition led to shortened telomere DNA and chromosome bridges (*54*). These RAD51-paralogues must be required for HR of telomere DNA in ALT cells. RAD51, which plays a central role in strand transfer in HR rather than the resolution of the Holliday junction, was not crucial for APB formation (*41*). It remains unknown whether RAD51 functions in the HR of telomere DNA and the ALT pathway.

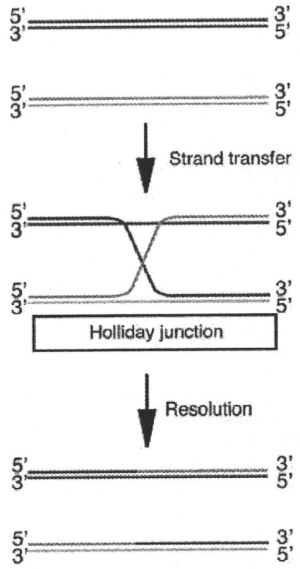

Fig. 4 Formation and resolution of the Holliday junction. The reciprocal exchange between two ds DNA (top, red/blue and pink/pale blue) is shown. The Holliday junction (middle) is a cruciform DNA formed in HR, and the resolution (bottom) requires cut and ligation at the junction.

Other proteins in DNA metabolism

FEN1 is a flap endonuclease that processes the 5′-end of Okazaki fragment to assist the completion of the lagging-strand synthesis. The homologue of FEN1 in budding yeast plays a role in the processing of the C-strand of telomere DNA. FEN1-deficient ALT cells showed elevated levels of DDR at telomeres (*55*). This response was suppressed by the ectopic expression of TERT. RPA is a conserved trimeric protein complex essential for replication and recombination. Two subunits of RPA, RPA32 and RPA70, are localized at APB, and the knockdown of either one of them in ALT cells resulted in ss G-strand accumulation and cell growth arrest (*56*). It needs to be clarified how crucial these replication proteins are for the telomere maintenance in ALT cells.

Sumoylation and ubiqutination

The PML body generally concentrates small ubiquitin-like modifier (SUMO) and proteins required for sumoylation. The first evidence of the function of sumoylation in ALT and APB was provided by a work on the SMC5/6 complex (*41*). SMC5/6, which is one of the structural maintenance of chromosomes (SMC) complexes in eukaryotes, plays important roles in DNA repair and DDR. The complex includes MMS21, a specific E3 SUMO ligase, as a non-SMC subunit. SMC5/6 and MMS21 are APB components and the knockdown of each of the genes caused reduced numbers of APBs in a cell. The knockdown of *SMC5* or *MMS21* in ALT cells induced telomere DNA shortening, telomere DNA loss at chromosome termini, cell growth suppression and β-galactosidase activity expression as in senescent cells. Meanwhile,

telomere DNA shortening and cell growth inhibition were not observed in SMC5- or MMS21-deficient telomerase-positive cells, suggesting their specific function in telomere maintenance and cell growth in ALT cells. MMS21 was able to sumoylate at least four shelterin proteins, TRF1, TRF2, TIN2 and RAP1. Mutated TRF1 at its sumoylated site abolished the localization at APB, suggesting that the sumoylation of the telomere binding proteins regulates APB formation.

FANC proteins were originally identified from mutated genes in patients with the genetic disease Fanconi anemia, whose cells commonly show chromosomal abnormality. The localization of FANCD2 at APBs required the function of FANCA and FANCL, both of which are essential for the monoubiquitination of FANCD2 (*57*). The knockdown of the gene encoding FANCD2 or FANCA induced telomere loss and T-SCE reduction in ALT cells. In DDR, the monoubiquitination of FANCD2 and the formation of FANCD2 nuclear foci required ATR kinase. Interestingly, the localization of FANCD2 at APB was also dependent on ATR. These suggest that FANCD2 may be regulated by ATR-dependent monoubiquitination for its proper localization and function on telomere in ALT cells.

Other factors related to ALT pathway

Tumour suppressor p53 is a crucial regulator of genome stability. Human p53 is localized at APB in ALT cells; however, it is not necessary for APB formation (*46*). In an ALT cell line established by the transformation with the T antigen of SV40, p53 was activated by the knockdown of the T antigen gene. APBs of those cells were enlarged (*58*), suggesting that p53 is a positive regulator of APB formation. This effect is dependent on the function of p21 and heterochromatin protein HP1, both of which are also APB components.

Several novel APB proteins were identified using a unique purification method to concentrate chromatin proteins associated with telomere DNA (*59*). The inhibition of COUP-TF2, a novel APB protein and an orphan nuclear receptor, caused a defect in APB formation and the shortening of telomere DNA. The molecular function of those novel APB proteins in the ALT pathway remains to be elucidated.

The phenotypes of ALT cells were suppressed in hybrid cell lines of ALT and telomerase-positive cells (*60*). This suggests that ALT cells may have some recessive mutations in gene(s) required for the inhibition of the ALT pathway. The genes proposed in that study have not been identified; nevertheless, they are expected to provide some hints for the ALT regulation mechanism. The coexistence of ALT and the lengthening by telomerase was apparently observed (*17*), indicating that telomerase activity alone is not sufficient to suppress the ALT phenotypes. This also suggests that some telomerase-positive cancer cells may activate the ALT pathway at the same time.

Similarities in telomerase-independent lengthening mechanisms

Cells that develop telomerase-independent lengthening mechanisms have been providing valuable hints for the ALT pathway. In the case of budding yeast *Saccharomyces cerevisiae* and its related species, survivors were recovered from cells with a disrupted TR gene and classified into two groups: type I cells have amplified subtelomeric DNA and depend on Rad51, and type II cells have elongated telomere repeats and require the function of MRX (MRN homologue) (*61*, *62*). As type II yeast cells and ALT cells have elongated telomere repeats, it is thought that these pathways depend on a conserved mechanism. It is significant that both human RAD51 and MRN are localized at APBs, suggesting that both of them are related to the ALT pathway. It is unknown whether the ALT pathway is made up of subpathways, as in types I and II cells of budding yeast.

As in human ALT cells, budding yeast type II cells harbour t-circles. When a model circular telomere DNA was introduced into type II cells, this DNA was copied to chromosome ends to form a tandem array (*63–65*). These results can be explained by RCR, in which the model circular DNA is utilized as a template for amplification. RCR may also play a crucial role in the replication of the linear mitochondrial DNA of the yeast *Candida parapsilosis*. Replication intermediates of the ends of mitochondrial DNA include a strand-specific ss DNA structure (*33*) that is commonly observed in RCR. These findings imply that RCR may be a conserved and general mechanism for the telomerase-independent pathway in eukaryotes.

The mouse knockout mutant of any genes of interest is one of the best model systems for human biology. Survivor cells from *Terc* $(TR)^{-/-}$ apparently maintained their telomere DNA in a telomerase-independent manner. In a cell line established from $Terc^{-/-}$ embryonic stem (ES) cells, the tandem arrays of telomeric and non-telomeric sequences were observed at the chromosome ends (*66*). Interestingly, most of the chromosome ends contained the same arrays. This suggests that these cells can amplify the arrays to maintain the chromosome ends. An array of this unique sequence was possibly built at one chromosome end at first, and then it expanded to the other chromosome ends. In an example of a human telomerase-negative cell line, the array of telomeric and ectopic DNA sequences (derived from SV40 DNA) was attached to most of the chromosome ends (*50*, *51*). These cells harboured circular extrachromosomal DNA that includes the similar sequence to the array at the chromosome ends, suggesting that the circular DNAs may be analogous to t-circles in ALT cells. It is interesting to examine whether the circular episome is required for the lengthening of the chromosome ends in these cells. Another survivor cell line from $Terc^{-/-}$ mouse embryonic fibroblasts showed heterogeneous telomere DNA lengths and the formation of APBs (*67*). This would serve as an invaluable

model system for the human ALT pathway and cancer development.

The ES cells from knockout mice mutated in DNA methyltransferase genes showed frequent T-SCE and APB formation, even though the cell lines were positive for telomerase activity (*68*). DNA hypomethylation at the subtelomere region was observed in those cells. This suggests that the epigenetic state of the cells may affect the frequency of T-SCE and APB formation. In human ALT cells, on the other hand, the relationship between the methylation status at the subtelomere region and T-SCE frequency was less clear (*69*).

Conclusions

Several lines of evidence strongly suggest that the ALT pathway is dependent on HR. First, a DNA fragment in the telomere repeats of chromosome ends can be copied into the ends of different chromosomes in ALT cells. Second, several kinds of proteins essential for HR are localized at APB. Third, a defect in some HR proteins induces erosion of telomere DNA in ALT cells. Lastly, the unique structures of telomere DNA found in ALT cells, such as ss telomere DNA and circular ECTR, may represent the intermediates and/or substrates of the elongation mechanisms based on HR. Importantly, the similar telomere DNA structures are also observed in cells that utilize HR-mediated telomere maintenance in other organisms. For the further elucidation of the ALT pathway, it is essential to establish a model experiment system in order to acquire direct evidence of the mechanism of HR-dependent telomere elongation.

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Conflict of interest

None declared.

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