厚生労働科学研究委託費

革新的がん医療実用化研究事業

悪性リンパ腫の腫瘍細胞と微小環境構成細胞の比較解析と微小環境構成細胞による腫瘍支持機構を標的とする新規治療法の開発

平成２６年度　委託業務成果報告書

業務主任者　島田　和之

(名古屋大学)

平成２７年（２０１５）年　３月

本報告書は、厚生労働省の厚生労働科学研究委託事業による委託業務として、国立大学法人名古屋大学が実施した平成２６年度「悪性リンパ腫の腫瘍細胞と微小環境構成細胞の比較解析と微小環境構成細胞による腫瘍支持機構を標的とする新規治療法の開発」の成果を取りまとめたものです。

《目　次》

I.委託業務成果報告（総括）

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（総括）

悪性リンパ腫の腫瘍細胞と微小環境構成細胞の比較解析と微小環境構成細胞による腫瘍支持機構を標的とする新規治療法の開発

業務主任者　島田　和之　名古屋大学　高等研究院　特任講師

研究要旨：
複数の悪性リンパ腫患者リンパ節生検検体よりリンパ節の微小環境構成細胞である線維芽細胞が単離可能で、腫瘍細胞のみならず単離された一部の線維芽細胞においてもゲノムコピー数異常が観察された。リンパ節生検検体由来悪性リンパ腫細胞は線維芽細胞との共培養下においてその生存が支持され、その支持効果は症例によって異なることが示唆された。線維芽細胞依存的に生存する悪性リンパ腫細胞を免疫不全マウスに異種移植することにより、腫瘍細胞の生着が得られ、線維芽細胞依存性悪性リンパ腫マウス異種移植モデルの作製が可能であり、今後の治療開発への応用が期待できる。

坂本　明彦・名古屋大学大学院医学系研究科
血液・腫瘍内科学　研究員

A. 研究目的
悪性リンパ腫の腫瘍細胞及び微小環境構成細胞の両者に着目し、遺伝子異常を含む生物学的特徴と微小環境依存性の臨床的意義の解明、微小環境依存性を標的とする新規治療法の開発を通して、現在難治性とされる悪性リンパ腫病型に対する有効な治療法の開発を目指す。

B. 研究方法
悪性リンパ腫患者のリンパ節生検検体の初代培養系より微小環境構成細胞である線維芽細胞を単離し、他の悪性リンパ腫患者リンパ節生検検体と共培養することにより、線維芽細胞の悪性リンパ腫細胞に対する生存支持効果を検討した。腫瘍細胞と線維芽細胞のゲノムコピー数を、アレイ CGH 法を用いて評価した。線維芽細胞に生存が支持される悪性リンパ腫細胞を用いて、腫瘍細胞支持機構の分子メカニズムを解析した。線維芽細胞による腫瘍細胞支持機構を標的とする新規治療法の開発のために、線維芽細胞に生存が支持される悪性リンパ腫患者由来腫瘍細胞のマウス異種移植モデルを作製した。

（倫理面への配慮）
患者の人権擁護上の配慮から、研究目的の利用について文書による同意が得られた臨床検体のみを本研究に使用した。臨床検体の使用に関しては、血液・腫瘍内科学教室において「造血器疾患の発症原因及び治療効果に影響を与える因子を解析するための基礎研究」（承認番号 1357）、「造血器疾患の発症、病勢進行、薬剤感受性を規定する分子病態の解析研究」（承認番号；2014-0081）として本学生命倫理審査委員会の承認を得ている。また異種移植モデルの作製を含む実験動物の使用に関しては、本学の承認を受け（平成 26 年度承認番号；26176）、名古屋大学における動物実験等に関する取扱規程に基づき、適切な動物実験の実施に努めている。

C. 研究結果
悪性リンパ腫を含む血液疾患関連患者のリンパ節生検検体より 6 例の線維芽細胞が単離された。6 例の疾患の内訳は、濾胞性リンパ腫 3 例、びまん性大細胞型 B 細胞リンパ腫 1 例、Castleman 病 1 例、反応性リンパ節病変 1 例であった。初期に得られた 2 例の濾胞性リンパ腫由来の腫瘍細胞と線維芽細胞を各々アレイ CGH 法にてゲノムコピー数を解析すると、腫瘍細胞は多様なゲノム領域のコピー数異常が認められたのに対し、線維芽細胞においても 2 例中 1 例において生存関連遺伝子及びサイトカイン分泌関連遺伝子を含むゲノム領域においてコピー数異常が認められた。さらにこの 2 例の線維芽細胞を用いて、他の悪性リンパ腫患者リンパ節生検由来腫瘍細胞を共培養すると、共培養下において腫瘍細胞の生存が支持され、症例毎で支持される効果が異なることが観察された。マウス線維芽細胞様細網細胞株にて生存が支持される悪性リンパ腫患者リンパ節生検検体由来腫瘍細胞を免疫不全マウスに異種移植することにより、マウス内にて腫瘍細胞の生着が得られ、同腫瘍細胞は継代移植が可能であった。

D. 考察

複数のリンパ節生検検体より線維芽細胞が単離され、共培養下にて生存が支持されていることより、悪性リンパ腫のリンパ節微小環境においても線維芽細胞が腫瘍形成に関与していることが示唆される。腫瘍細胞によって線維芽細胞による支持効果が異なることも興味深く、薬剤感受性への関与や治療成績への影響について興味が持たれる。今後評価症例数を蓄積し、微小環境の支持効果の臨床的意義を明らかにしていくとともに、分子機構を解明することで新たな治療法の開発につながることが期待される。

E. 結論

リンパ節微小環境において、線維芽細胞が腫瘍細胞の生存に関与していることが示唆される。線維芽細胞による腫瘍細胞支持機構を解明することで今後の新たな治療法の開発につながることが期待される。

F. 健康危険情報

特記すべき事項なし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）
1. 特許取得
特になし

2. 実用新案登録
特になし

3. その他
特になし

Ⅱ．委託業務成果報告（業務項目）

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告書

悪性リンパ腫の腫瘍細胞と微小環境構成細胞の比較解析と微小環境構成細胞による腫瘍支持機構を標的とする新規治療法の開発

悪性リンパ腫細胞と線維芽細胞様細網細胞の生物学的特徴の解明、悪性リンパ腫の微小環境依存性の臨床的意義の解明

業務分担者　坂本 明彦　名古屋大学 大学院医学系研究科 研究員

研究要旨：
複数の血液関連疾患患者のリンパ節で線維芽細胞が単離された。濾胞性リンパ腫患者由来の線維芽細胞 2 例中 1 例では、ゲノムコピー数の異常が散在していた。いずれの線維芽細胞も生存関連のサイトカインやケモカインを分泌することで、リンパ節細胞の生存を支持することが示唆された。また、線維芽細胞に依存して増殖が認められた難治性 Burkitt 様 B 細胞リンパ腫患者の B 細胞では、mTOR/PI3K 経路が活性化されることが分かった。

A. 研究目的
悪性リンパ腫の間質細胞について遺伝子異常の有無を網羅的に解析し、悪性リンパ腫への寄与を明らかにする。また、難治性 Burkitt 様 B 細胞リンパ腫を例に間質細胞による腫瘍支持機構を明らかにし、新規治療法の開発に応用する。

B. 研究方法
血液関連疾患患者のリンパ節生検検体を汎用培地中で培養し、間質細胞を単離した。研究の初期に得られた 2 例の患者由来間質細胞の性状を解明するため、表面抗原の発現とゲノムのコピー数を調べた。また、得られた間質細胞の悪性リンパ腫微小環境としての機能を評価するため、リンパ節生検時に得られた血液関連疾患患者のリンパ節細胞を共培養し、生存促進の有無を調べた。間質細胞に依存して増殖が認められた B 細胞リンパ腫患者の B 細胞を用いて、腫瘍支持機構を調べた。

（倫理面への配慮）
患者の人権擁護上の配慮から、研究目的の利用について文書による同意が得られた臨床検体のみを本研究に使用した。当教室では「造血器疾患の発症原因及び治療効果に影響を与える因子を解析するための基礎研究」（承認番号 1357）、「造血器疾患の発症、病勢進行、薬剤感受性を規定する分子病態の解析研究」（承認番号；2014-0081）として本学生命倫理審査委員会の承認を得た上で、臨床検体を使用している。

C. 研究結果
血液関連疾患患者のリンパ節生検検体のうち下記 6 例で、間質細胞が単離された。
・濾胞性リンパ腫：3 例
・びまん性大細胞型 B 細胞リンパ腫：1 例
・Castleman 病：1 例
・反応性リンパ節病変：1 例
また現在、濾胞性リンパ腫、血管免疫芽球型 T 細胞リンパ腫各 1 例で、間質細胞が増殖している。

マウスのリンパ節の微小環境は、線維芽細胞、濾胞樹状細胞、血管内皮細胞の 3 つで構成されていて、表面抗原の発現で分類できる。研究の初期に得られた 2 例の濾胞性リンパ腫患者の間質細胞の表面抗原を調べた結果、いずれも線維芽細胞であることが分かった。

腫瘍細胞では、腫瘍の進展に伴ってゲノムのコピー数が変化することが知られている。本研究では、微小環境を構成する間質細胞についても着目し、濾胞性リンパ腫患者由来の 2 例の線維芽細胞について、アレイ CGH 法でゲノムのコピー数を解析した。1 例はコピー数に異常が認められなかったが、もう 1 例は性染色体を除く 6 つの染色体中の 8 つの遺伝子で、コピー数の異常が認められた。多くは生存関連の遺伝子だったが、サイトカイン分泌関連の遺伝子も含まれた。また、コピー数の異常が認められたゲノム領域は、リンパ球を主とする腫瘍部位で異常が認められたゲノム領域とはほとんど重複しなかった。

次に、線維芽細胞の微小環境としての機能を評価するため、生検時に得られた血液関連疾患患者約 10 例のリンパ節細胞を線維芽細胞と共培養し、リンパ節細胞の生存促進を評価した。リンパ節細胞の生存率は、線維芽細胞と共培養することで有意に増加した。また、良性病変の患者のリンパ節細胞より悪性リンパ腫患者のリンパ節細胞の方が、線維芽細胞による生存促進の効果が大きかった。リンパ節中の T 細胞も、微小環境構成細胞として腫瘍を支持することが知られている。そこで、B 細胞リンパ腫患者 3 例のリンパ節生検検体から分離した B 細胞を線維芽細胞と共培養することで、線維芽細胞の微小環境としての機能を評価した。B 細胞の生存率は線維芽細胞と共培養することで有意に増加したが、患者によって生存促進の程度に差が認められた。

最後に、2 例の B 細胞リンパ腫患者のリンパ節生検検体由来の B 細胞において、線維芽細胞に依存して B 細胞を増殖させることが可能であった。線維芽細胞の培養上清で B 細胞を培養した場合も、B 細胞の生存率が有意に増加し、これらの B 細胞では mTOR/PI3K 経路が活性化されていた。さらに、培養上清中のサイトカインやケモカインを抗体アレイで調べた結果、2 例の線維芽細胞いずれも、生存に関連した複数のサイトカインやケモカインを分泌することが分かった。

D. 考察

複数の血液関連疾患患者のリンパ節で間質細胞が単離され、濾胞性リンパ腫患者検体より単離された 1 例の線維芽細胞ではゲノムコピー数の異常が認められた。悪性リンパ腫のリンパ節病変では、リンパ球だけでなく間質細胞にも何らかの異常を来している可能性が示唆される。また、リンパ節由来の線維芽細胞が B 細胞リンパ腫患者の B 細胞の生存を支持していた。この分子機構として、線維芽細胞が生存関連のサイトカインやケモカインを分泌することで、B 細胞の mTOR/PI3K 経路などのシグナル経路を活性化するモデルが想定される。今後は間質細胞の寄与を疾患間や患者間で比較し、予後や治療効果との関連を明らかにしたい。

E. 結論

複数の血液関連疾患患者のリンパ節で間質細胞が単離された。濾胞性リンパ腫患者由来の 2 例の線維芽細胞のうち 1 例では、ゲノムコピー数の異常が散在していた。いずれの線維芽細胞も生存関連のサイトカインやケモカインを分泌することで、リンパ節細胞の生存を支持することが示唆された。また、線維芽細胞に依存して増殖が認められた難治性 Burkitt 様 B 細胞リンパ腫患者の B 細胞では、mTOR/PI3K 経路が活性化されることが分かった。

F. 研究発表

1. 論文発表

特になし

2. 学会発表

1. 第 73 回日本癌学会学術総会 口演 J-3054
2. 第 4 回生理学研究所名古屋大学医学系研究科合同シンポジウム ポスター P-43

G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

Ⅲ．学会等発表実績

様式第１９

学 会 等 発 表 実 績

委託業務題目：「悪性リンパ腫の腫瘍細胞と微小環境構成細胞の比較解析と微小環境構成細胞による腫瘍支持機構を標的とする新規治療法の開発」

機　関　名：国立大学法人名古屋大学

1. 学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
骨髄スメア標本とパイロシークエンス法を用いたB細胞性腫瘍におけるMYD88 L265P変異解析（口頭）	鈴木康裕、冨田章裕、入山智沙子、島田和之、山本絵里奈、金田典雄、清井仁	第12回日本臨床腫瘍学会学術集会	2014/7/17-7/19	国内
Utilization of peripheral blood cell-free DNA for genetic analyses in MDS（口頭）	Suzuki Y, Tomita A, Iriyama C, Shirahata M, Shimada K, Kiyoi H	The 35th XXXV World Congress International Soceity of Hematology. Beijing.	2014/9/4-9/7	国外
末梢血cell-free DNAを用いたB細胞リンパ腫における遺伝子変異解析（ポスター）	鈴木康裕、冨田章裕、吉田健一、島田和之、入山智沙子、真田昌、白石友一、千葉健一、田中洋子、宮野悟、小川誠司、清井仁	第73回日本癌学会学術総会	2014/9/25-9/27	国内
一分子イメージングで明らかになった骨髄増殖性腫瘍関連MPL変異体の動的な二量体化（口頭）	坂本明彦、加藤尚志、島田和之、冨田章裕、清井仁、船津高志	第73回日本癌学会学術総会	2014/9/25-9/27	国内
Mutational analysis of MYD88 L265P in WM/LPL using preserved bone marrow smears by pyrosequencing（ポスター）	Suzuki Y, Tomita A, Iriyama C, Shimada K, Hibi Y, Nakamura F, Yamamoto E, Kaneda N, Kiyoi H	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Detection of genetic mutations in B-cell lymphomas using peripheral blood cell-free-DNA（口頭）	Suzuki Y, Tomita K, Iriyama C, Shimada K, Yoshida K, Ogawa S, Kiyoi H	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Prognostic significance of pleural/pericardial effusion, and the optimal treatment in PMBL（口頭）	Aoki T, Shimada K, Suzuki R, Izutsu K, Tomita A, Nakaseko C, Sasaki M, Arima H, Takizawa J, Mitani K, Igarashi T, Maeda Y, Fukuhara N, ishida F, Niitsu N, Ohmachi K, Takasaki H, Nakamura N, Kinoshita T, Nakamura S, Ogura M	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Anti-CD20 chimeric antigen receptor+ T cells can recognize remarkably low target antigen expression（口頭）	Watanabe K, Terakura S, Imai M, Sakemura R, Goto T, Hanajiri R, Imahashi N, Shimada K, Tomita A, Nishida T, Murata M, Naoe T	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Detection of Fcγ receptor single nucleotide polymorphisms important for anti-CD20 immunotherapy（口頭）	Iriyama C, Hargreaves C, Rose-Zerilli M, Strefford J, Cragg M, Shimada K, Naoe T, Tomita A, Kiyoi H	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Intravascular large B-cell lymphoma-from bench to bedside（口頭）	Shimada K	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Biological and genetic analyses of intravascular large B-cell lymphoma using xenograft mouse models（口頭）	Shimada K, Shimada S, Sugimoto K, Hayakawa F, Katayama M, Hirakawa A, Nakamura S, Seto M, Naoe T, Tomita A, Kiyoi H.	第76回日本血液学会学術集会	2014/10/31-11/2	国内
Clinical and Molecular Significance of Peripheral Blood Cell-Free DNA in B-Cell Lymphomas for Detection of Genetic Mutations and Correlation with Disease Status（ポスター）	Suzuki Y, Tomita K, Yoshida K, Shimada K, Iriyama C, Sanada M, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Ogawa S, Kiyoi H.	The 56th Annual Meeting of the American Society of Hematology. San Francisco, USA	2014年12月	国外
Development and Analysis of Novel Intravascular Large B-Cell Lymphoma NOD/Shi-Scid IL2Rγnull Mouse Xenograft Model（口演）	Shimada K, Shimada S, Sugimoto K, Hayakawa F, Katayama M, Hirakawa A, Takagi Y, Nakamura S, Seto M, Naoe T, Tomita A, Kiyoi H.	The 56th Annual Meeting of the American Society of Hematology. San Francisco, USA	2014年12月	国外

2. 学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Efficacy of ofatumumab against rituximab-resistant B-CLL/SLL cells with low CD20 protein expression.	Shimada K, Tomita A, Saito S, Kiyoi H.	Br J Haematol	2014 Aug;166(3):455-7.	国外
Prognostic significance of pleural or pericardial effusion and the implication of optimal treatment in primary mediastinal large B-cell lymphoma: a multicenter retrospectivestudy in Japan.	Aoki T, Izutsu K, Suzuki R, Nakaseko C, Arima H, Shimada K, Tomita A, Sasaki M, Takizawa J, Mitani K, Igarashi T, Maeda Y, Fukuhara N, Ishida F, Niitsu N, Ohmachi K, Takasaki H, Nakamura N, Kinoshita T, Nakamura S, Ogura M.	Haematologica	2014 Dec;99(12):1817-25	国外
Target antigen density governs the efficacy of anti-CD20-CD2-CD3ζ chimeric antigen receptor-modified effector CD8+ T cells	Watanabe K, Terakura S, Martens AC, van Meerten T, Uchiyama S, Imai M, Sakemura R, Goto T, Hanajiri R, Imahashi N, Shimada K, Tomita A, Kiyoi H, Nishida T, Naoe T, Murata M	J Immunol	2015 Feb;194(3):911-20	国外

Ⅳ. 研究成果の刊行物・別刷

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Efficacy of ofatumumab against rituximab-resistant B-CLL/SLL cells with low CD20 protein expression

The anti-CD20 monoclonal antibody (mAb), rituximab, has improved the clinical outcomes of patients with B-cell lymphoma (Coiffier *et al*, 2010). However, a sizable proportion of patients subsequently develop rituximab resistance (Smith, 2003; Hiraga *et al*, 2009; Tokunaga *et al*, 2014). It has been demonstrated that lower levels of CD20 in chronic lymphocytic leukaemia (CLL) cells predict poor efficacy for rituximab (Golay *et al*, 2001). Ofatumumab is a type I mAb that, according to *in vitro* data, considerably enhanced complement-dependent cytotoxicity (CDC), particularly with CLL cells (Teeling *et al*, 2004; Beurskens *et al*, 2012). Although ofatumumab has a higher level of efficacy than rituximab and showed clinical efficacy as a single agent in CLL (Teeling *et al*, 2004; Cheson, 2010; Wierda *et al*, 2011; Beurskens *et al*, 2012), there is relatively little clinical data regarding its efficacy in patients with rituximab resistance. The present report describes a patient with refractory B-CLL/small lymphocytic lymphoma (SLL) and clinical rituximab resistance in whom the partial efficacy of ofatumumab was confirmed by both *in vitro* and *in vivo* data.

A 78-year-old female was diagnosed with B-CLL/SLL in 2006 and underwent repetitive rituximab-containing chemotherapy for 5 years. She then received rituximab monotherapy for almost 2 years, and partial sensitivity was observed (Fig 1A). In early 2013, rituximab resistance was confirmed clinically with a persistently high white blood cell (WBC) count, mostly CD20+/CD19+/CD5+/λ+ B-lymphocytes, even after rituximab administration (Fig 1A). Flow-cytometry (FCM) analysis of the patients' peripheral blood cells indicated a weak positive expression of CD20, which had not significantly altered throughout her clinical course (Fig 1A, a–c). We concluded that the CLL cells had acquired clinical resistance to rituximab despite partial CD20 expression.

In June 2013, the patient was treated with ofatumumab. An *in vitro* CDC assay (Tokunaga *et al*, 2014) was performed (Fig 1B–D) using tumour cells harvested from her peripheral blood. Comparison of CDC activity using rituximab and ofatumumab revealed that the cytotoxic activity of ofatumumab was significantly higher than that of rituximab ($P = 0{\cdot}0091$ at 2 μg/ml and $P = 0{\cdot}0263$ at 10 μg/ml, P-value was evaluated by unpaired t test) (Fig 1C). Cytotoxicity activity for CD20-positive Raji cells was confirmed in both mAbs, and the effectiveness was significantly higher in ofatumumab than that in rituximab (Fig 1B). After eight cycles of treatment with ofatumumab, the WBC count gradually decreased (from 21·0 to 6·5 × 10^9/l), haemoglobin level increased (85–1·8 g/l), and both the enlarged spleen and post-peritoneal tumour mass decreased in size (Fig 1A, Pre and Post). FCM analysis after ofatumumab treatment revealed the elimination of the CD20-positive fraction of tumour cells (Fig 1A, d), indicating that ofatumumab was effective *in vivo* against weakly CD20-positive rituximab-resistant cells. The cytotoxic activity of ofatumumab was no longer confirmed in the *in vitro* CDC assay when using the CLL cells harvested after treatment with four cycles of ofatumumab (Fig 1D).

This present case demonstrates the following: (i) rituximab resistance occurs even if the CD20 expression can be confirmed by FCM; (ii) ofatumumab appears relatively more effective than rituximab in eliminating CD20-positive cells in

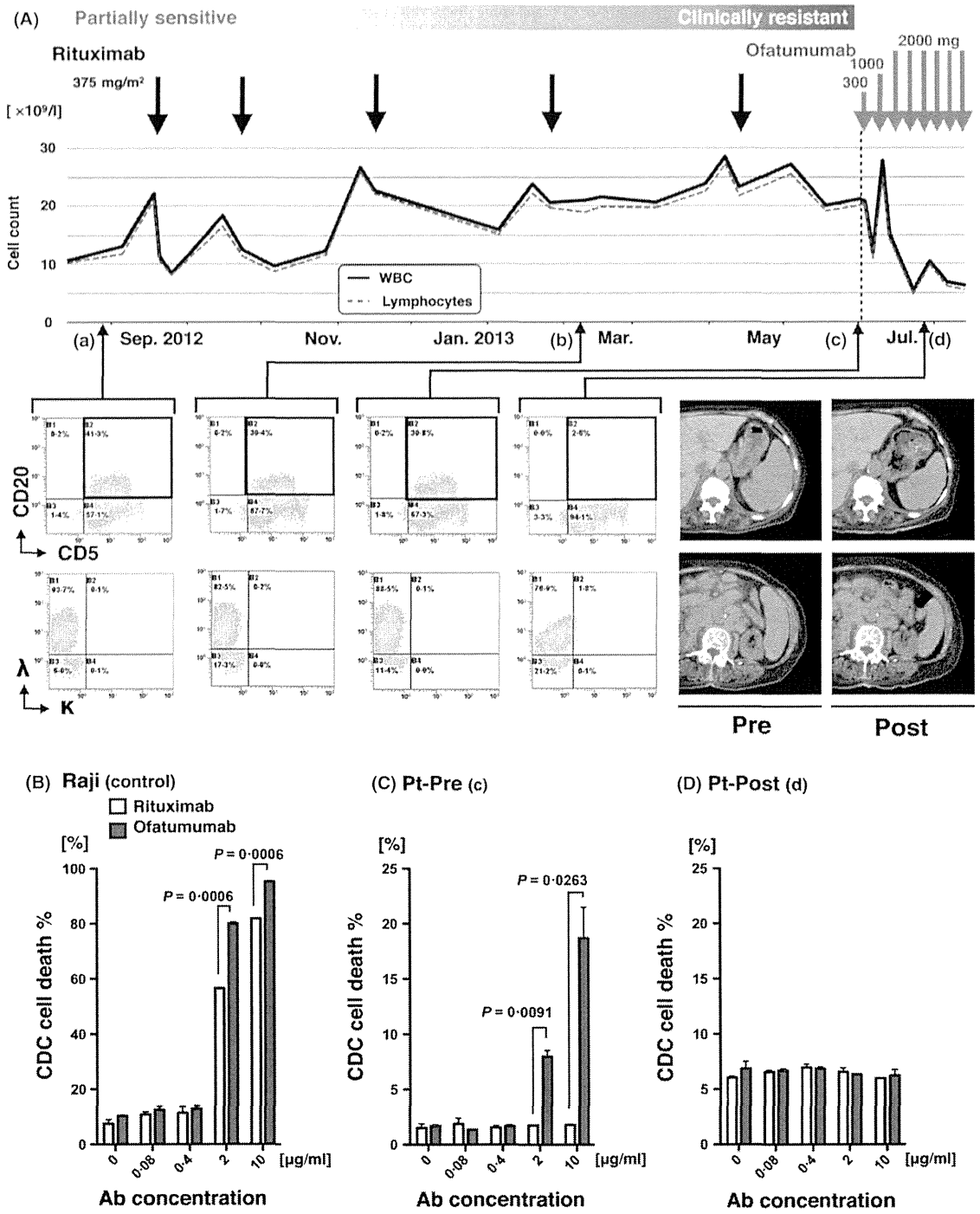

Fig 1. Clinical efficacy of ofatumumab in a patient with rituximab resistant B-CLL/SLL. (A) Clinical course of a B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma (B-CLL/SLL) patient with acquired clinical rituximab resistance. Partial rituximab sensitivity was observed until November 2012. After that, rituximab resistance was clinically confirmed with persistently high WBC counts even after rituximab administration (black arrows). After starting ofatumumab (grey arrows), the WBC counts markedly decreased. Flow cytometric analyses were performed using peripheral blood CLL cells at the time points indicated (a, b, c and d). Note that CD20 expression intensity on the CLL cells had not significantly changed despite differences in the clinical efficacy of rituximab (time-point a, b and c). Ofatumumab almost completely eliminated rituximab-resistant CLL cells that were weakly CD20-positive; however, CD20-negative CD5+/λ+ cells remained at time-pont (d). Computerized tomography was performed both before (Pre) and after (Post) ofatumumab administration. The enlarged spleen and the tumour mass in the post-peritoneal space both decreased in size in response to ofatumumab. (B) *In vitro* CDC assay against CD20-positive Raji cells using rituximab and ofatumumab. The same assay was carried out using peripheral blood CLL cells of the patient harvested before (C) and after (D) treatment with ofatumumab. Differences in CDC activity by rituximab and ofatumumab were analysed with unpaired *t*-test using PRISM version 5 software (Graph Pad Software, Inc., La Jolla, CA, USA). The *P* values were two-tailed, and were considered statistically significant when <0·05.

patients who show rituximab resistance; and (iii) CD20-negative cells in FCM are resistant to ofatumumab, as well as rituximab, as previously reported (Teeling *et al*, 2006). These findings suggest both that molecular analysis of rituximab resistance in patients with the CD20-positive phenotype might be meaningful to help direct therapy, and that there is a clinical need for these newer generation mAbs with cytotoxicity, even against tumour cells that very weakly express CD20, and/or novel molecular targeting drugs that can be utilized in combination with mAb therapeutics. Further analyses using CD20-positive rituximab-resistant cells from a larger cohort of patients are required.

Acknowledgements

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Author contributions

K.S. and A.T. designed the experiments, performed the research, analysed data, and wrote the manuscript; A.T. and S.S. cared for the patient and prepared the samples. A.T. and H.K. interpreted the data and supervised the experiments.

Conflicts of interest

H. K. research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., LTD. and Kyowa Hakko Kirin Co., LTD. The other authors have no relevant conflicts to disclose.

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Prognostic significance of pleural or pericardial effusion and the implication of optimal treatment in primary mediastinal large B-cell lymphoma: a multicenter retrospective study in Japan

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ABSTRACT

The prognosis of patients with primary mediastinal large B-cell lymphoma has improved over recent years. However, the optimal treatment strategy including the role of radiotherapy remains unknown. We retrospectively analyzed the clinical outcomes of 345 patients with newly diagnosed primary mediastinal large B-cell lymphoma in Japan. With a median follow up of 48 months, the overall survival at four years for patients treated with R-CHOP (n=187), CHOP (n=44), DA-EPOCH-R (n=9), 2nd- or 3rd-generation regimens, and chemotherapy followed by autologous stem cell transplantation were 90%, 67%, 100%, 91% and 92%, respectively. Focusing on patients treated with R-CHOP, a higher International Prognostic Index score and the presence of pleural or pericardial effusion were identified as adverse prognostic factors for overall survival in patients treated with R-CHOP without consolidative radiotherapy (IPI: hazard ratio 4.23, 95% confidence interval 1.48-12.13, P=0.007; effusion: hazard ratio 4.93, 95% confidence interval 1.37-17.69, P=0.015). Combined with the International Prognostic Index score and the presence of pleural or pericardial effusion for the stratification of patients treated with R-CHOP without radiotherapy, patients with lower International Prognostic Index score and the absence of effusion comprised approximately one-half of these patients and could be identified as curable patients (95% overall survival at 4 years). The DA-EPOCH-R regimen might overcome the effect of these adverse prognostic factors. Our simple indicators of International Prognostic Index score and the presence of pleural or pericardial effusion could stratify patients with primary mediastinal large B-cell lymphoma and help guide selection of treatment.

Introduction

Primary mediastinal large B-cell lymphoma (PMBL) is characterized by distinct clinical, pathological and genetic features and comprises a subtype of diffuse large B-cell lymphoma (DLBCL) according to the current World Health Organization (WHO) classification.[1] The disease is more common in younger females and often presents with bulky mediastinal mass without extrathoracic spread and pleural or pericardial effusion.[2-5]

Prior to the introduction of rituximab, the outcomes of patients treated with anthracycline-containing chemotherapies, including cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP), had a suboptimal progression-free survival (PFS) of 38%-52%.[5,6] Several retrospective analyses revealed that the outcomes of the 2nd- and 3rd-generation chemotherapeutic regimens, such as methotrexate, leucovorin, doxorubicin, cyclophosphamide, vincristine, bleomycin and prednisolone (MACOP-B), might be superior to those of CHOP regimens.[5,7-10] High-dose chemotherapy followed by autologous stem cell transplantation (HDT/ASCT) was also associated with encouraging results (PFS >75% for newly diagnosed PMBL patients).[5,11,12] These reports indicate that intensive regimens might be beneficial in a certain proportion of PMBL patients.

In the rituximab era, the combination of rituximab and

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chemotherapy has improved outcomes in various subtypes of B-cell lymphoma.[13-22] In the literature, more than 80% of patients with PMBL receiving immunochemotherapy with or without radiotherapy (RT) also achieved long-term overall survival (OS).[17-22] Despite the outstanding advances with R-CHOP, 20%-30% of patients still experience progression or relapse and have poor outcomes. Moreover, approximately 80% of long-term survivors treated with R-CHOP required consolidative RT for residual mediastinal disease.[20-23] Considering late adverse events induced by the mediastinal RT, namely the increased risk of secondary breast cancer and cardiac toxicity, the risk of RT should be minimized, especially for younger patients.[24-26]

Recently, Dunleavy *et al.* reported excellent outcomes for dose-adjusted etoposide, cyclophosphamide, doxorubicin, vincristine, prednisolone and rituximab (DA-EPOCH-R) when restricting candidates for RT according to the results of positron-emission tomography/computed tomography (PET/CT).[27] Although outcomes were reported from a phase II trial, the regimen might be a promising treatment strategy to reduce the risk of RT. Meanwhile, the DA-EPOCH-R regimen is somewhat complicated and expensive, requiring continuous infusion for 96 h in each cycle and frequent evaluation of complete blood counts. Considering R-CHOP-based regimens without RT could provide curative potential for a significant proportion of PMBL patients without hospitalization,[19,21] it would, therefore, be beneficial to identify the subset of patients that could be cured with this treatment strategy.

The goal of the present multicenter co-operative retrospective study in Japan was to investigate the optimal treatment strategy for PMBL patients by evaluating the clinical outcomes in response to various treatments and to assess a risk-stratified treatment strategy to minimize the risk of late adverse events in PMBL patients.

Methods

Patients

A total of 363 patients with PMBL newly diagnosed between May 1986 and September 2012 at one of any of the 65 participating hospitals in Japan were retrospectively analyzed. We registered consecutive patients who were diagnosed with PMBL at each institution in accordance with the WHO classification.[1] The time period during which we could collect the clinical data from each institution varied due to the differences in the length of time medical records are kept there. Medical record data since the 1980s were collected from three institutions, while data since the 1990s and 2000s were available from 10 and 65 institutions, respectively. In this study, PMBL was defined as patients with a dominant mass within the anterior mediastinum, irrespective of the tumor size. In addition, a central pathological review was performed by a hematopathologist (SN) for 196 patients for whom histological paraffin-embedded tissue materials could be provided. Eighteen of the 363 patients were excluded from analysis due to disease other than PMBL (n=10) by central pathological review or due to the absence of important clinical information (n=8). For the remaining patients who were not available for the central review, the histological diagnosis of PMBL was re-confirmed by a pathologist at each institution, according to the current WHO classification. Therefore, 345 patients were finally analyzed for the present study. Patients were treated according to each institution's treatment standards. The study protocol was approved by the institutional review boards of Nagoya Daini Red Cross Hospital where this study was organized and of each participating hospital. The study complied with all the provisions of the Declaration of Helsinki.

Immunohistochemistry

Immunohistochemistry was performed using formalin-fixed, paraffin-embedded tissue sections using the avidin-biotin peroxidase complex method. Monoclonal antibodies targeting the following proteins were used: CD20, CD30, CD3, CD10, BCL6, MUM1 and CD15 (Dako). In addition, programmed cell death ligand-1 (PDL1) was evaluated, as previously described.[28] To evaluate PDL1, we used a polyclonal rabbit antibody for CD274 (ab82059; Abcam) according to the manufacturer's instructions. The cut-off values for these markers were 20% for CD30, and 30% for Bcl-6, MUM1 and PDL1.[29-31]

Treatment

Initial treatments were performed based on the physicians' decisions at each institution, as there had been no uniform treatment guidelines for PMBL in Japan. Patients who received CHOP or a CHOP-like regimen, with or without rituximab, were categorized and analyzed as the R-CHOP or CHOP group, respectively. Patients who received 2nd-/3rd-generation treatments were categorized and analyzed as the 2nd-/3rd-generation regimen group, irrespective of the use of rituximab. Patients who received the DA-EPOCH-R regimen[27] were analyzed as the DA-EPOCH-R group. Patients who underwent consolidative HDT/ASCT after initial therapy were analyzed as the HDT/ASCT group, irrespective of the use of rituximab. CHOP- or R-CHOP-based regimens were mainly selected in 46 institutions. Physicians at six institutions selected 2nd-/3rd-generation chemotherapeutic regimens other than CHOP- or R-CHOP-based regimens as the first-line treatment. HDT/ASCT as the first-line treatment was performed at 13 institutions. Consolidative RT was performed according to the treatment strategy used at each institution.

Response assessment

Clinical data were collected from case report forms. In principle, an effusion was evaluated by CT and/or echocardiography, as per the usual pre-treatment evaluation. Responses were evaluated by each investigator in accordance with the 1999 International Workshop Criteria.[32]

Statistical analysis

Overall survival was defined as the period from diagnosis to death or last follow up. Progression-free survival (PFS) was defined as the period from diagnosis to disease progression, relapse, death from any cause, or last date of follow up. Patients who did not achieve a complete remission (CR) or partial response (PR) were considered to have primary refractory disease. Early relapse was defined as relapse occurring less than 12 months after diagnosis. PFS and OS were analyzed using Kaplan-Meier methods and results were compared using the log rank test. Univariate and multivariate Cox regression analyses were performed to assess the effects of prognostic factors. Multivariate analysis was built with a forward/backward, step-wise method using threshold values for removal from and addition to the model of P=0.20 and P=0.05, respectively. The individual factors of IPI were entered into the model in multivariate analysis. All probability values were two-sided and had an overall significance level of 0.05. Statistical analyses were performed with Stata SE 12 software (StataCorp LP, College Station, TX, USA).

Results

Patients' characteristics

Patients' characteristics are summarized in Table 1. Median age was 32 years (range 17-83 years) and females were predominant (58%). The median diameter of mediastinal mass was 10 cm (range 3-32 cm). Stage I/II disease, low-risk disease according to the International Prognostic Index (IPI), and performance status (PS) 0/1 were also predominant (67%, 52% and 75%, respectively). The presence of pleural or pericardial effusion, elevated lactate dehydrogenase (LDH) level and more than one extranodal lesion were observed in 46%, 80% and 9% of patients, respectively. For the patients who had extra-nodal involvement, major extra-nodal sites were lung (n=44), effusion (n=49) and cardiac (n=28). Pathological features are listed in Table 1. Lymphoma cells in all patients expressed CD20. Further, CD30, BCL6, and MUM1 expression was detected in 71%, 61%, and 96%, respectively. PDL1 was expressed in 62% of 110 evaluable patients.

Table 1. Patients' characteristics.

Characteristic	All		CHOP		R-CHOP		DA-EPOCH-R		2nd/3rd generation		HDT/ASCT	
	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%
Median follow up (months)	48		118		36		19		48		101	
Patient number	345		44		187		9		45		57	
Age at diagnosis (years)												
Median	32		31.5		33.5		30		31		27	
Range	17-83		17-77		17-83		24-64		18-76		17-63	
>60 years	47	14	10	23	30	16	1	11	3	7	3	5
Gender, male	146	42	18	41	85	45	4	44	12	27	27	47
PS, ≥2	84/338	25	12/42	29	40/182	22	3	33	8	18	20	3
Extranodal sites, >1	64/334	19	7/40	17	31/181	18	0	0	11	24	15/56	27
Stage, I/II	230/342	67	27	61	133/184	72	7	78	31	69	31	54
LDH at diagnosis, ≥ULN	270/337	80	35/41	85	134/183	73	8	89	37	82	54/56	96
B symptoms, present	90/337	27	15/42	36	40/183	22	2	22	11	24	22/55	40
IPI												
Low	175/334	52	19/40	48	103/181	57	5	56	26	58	21/56	38
Low-intermediate	84/334	25	11/40	28	44/181	24	3	33	9	20	16/56	29
High-intermediate	43/334	13	4/40	10	21/181	12	0	0	5	11	12/56	21
High	32/334	10	6/40	15	13/181	7	1	11	5	11	7/56	13
Bulky tumor size												
Median	10		10		9.2		12.6		10.5		10	
≥10 cm	166/324	51	20/36	56	84/180	47	6	67	26	59	30/56	58
s-IL2R after first-line therapy, ≥1000 U/mL	141/305	46	20/30	67	91/175	52	2/8	25	17/40	43	33/49	67
Presence of pleural or pericardial effusion	159/343	46	15/43	35	83/186	43	5	56	23	51	31	54
WBC, >10×10⁹/L	23/339	7	2/42	5	12/184	7	0	0	5	11	3/56	5
Hemoglobin, ≤12 g/dL	119/329	36	16/39	41	57/81	31	3	33	21	47	19/52	37
Platelet count, <150×10⁹/L	20/331	6	2/40	5	16/182	9	0	0	0	0	2/52	4
ALC at diagnosis, <0.5×10⁹/L	62/321	19	2/33	6	29/180	16	5	56	12	27	13/52	25
IHC staining, positive												
CD20	152/152	100	15/15	100	99/99	100	5/5	100	8/8	100	25/25	100
CD10	4/129	3	1/11	9	2/85	2	0/5	0	0/7	0	1/21	5
CD30	100/140	71	9/13	69	62/85	70	5/5	100	5/8	63	18/25	72
BCL6	72/116	61	8/11	73	46/75	61	2/5	40	4/6	67	12/19	63
MUM1	105/109	96	10/11	91	67/68	99	4/5	80	6/6	100	18/19	95
PDL-1	68/110	62	7/11	64	44/68	65	2/5	40	1/5	20	14/21	67
Treatment												
Administration of rituximab	267	77	0	0	187	100	9	100	28	62	43	75
Consolidation RT	145	42	21	48	64	34	4	44	30	67	24	42
Late adverse event												
Secondary cancer	7	2	1	2	4	2	0	0	0	0	2	4
Cardiac toxicity	10	3	0	0	9	5	0	0	0	0	1	2

CHOP: cyclophosphamide, adriamycin, vincristine and prednisone; R: rituximab; DA-EPOCH-R: dose-adjusted etoposide, cyclophosphosphamide, doxorubicin, vincristine, prednisolone and rituximab; HDT/ASCT: high-dose chemotherapy followed by autologous stem cell transplantation; PS: performance status; LDH: lactate dehydrogenase; ULN: upper limit of normal; IPI: international prognostic index; s-IL2R: soluble interleukin-2 receptor; WBC: white blood cell count; ALC: absolute lymphocyte count; IHC: immunohistochemical staining; RT: radiation therapy.

Treatment regimen

In all, 267 patients received rituximab-containing chemotherapy. CHOP and R-CHOP chemotherapy groups consisted of 44 and 187 patients, respectively. DA-EPOCH-R chemotherapy was administered to 9 patients. In the 2nd-/3rd-generation regimen group (n=45), 28 patients received MACOP-B with (n=18) or without (n=10) rituximab, 15 patients received cyclophosphamide, vincristine, bleomycin, etoposide, doxorubicin and prednisolone (CyclOBEAP)[33] with (n=12) or without (n=3) rituximab, and 2 patients received vincristine, cyclophosphamide, doxorubicin, ranimustine, vindesine, etoposide carboplatin and prednisone (JCOG-LSG15 study regimen).[34] In the HDT/ASCT group (n=57), 43 patients received rituximab-containing chemotherapy as the initial chemotherapy. Consolidative RT was given to 42% of all patients. After approval of the use of rituximab for DLBCL in Japan in 2003, the use of rituximab-containing regimens rapidly increased, as shown in *Online Supplementary Table S1*. There was a moderate decrease in the use of HDT/ASCT and radiation therapy after initial treatment. The DA-EPOCH-R regimen was selected in the latest period.

Clinical outcomes

With a median follow up of 48 months in surviving patients, the OS and PFS at four years were 87% and 70%, respectively (Figure 1A and B). The OS and PFS of patients treated with rituximab-containing chemotherapy were superior to those of patients receiving chemotherapy without rituximab (4-year OS: 91% *vs.* 77%, $P<0.001$; 4-year PFS: 75% *vs.* 54%, respectively, $P<0.001$). There was no difference in the risk of central nervous system (CNS) relapse between patients treated with and patients treated without rituximab as first-line treatment (3.8% *vs.* 1.3%; $P=0.251$). The OS at four years for patients treated with CHOP, R-CHOP, DA-EPOCH-R, the 2nd-/3rd-generation regimens, and HDT/ASCT was 67%, 90%, 100%, 91% and 92%, respectively, with median follow-up durations of 118 months, 36 months, 19 months, 48 months and 101 months, respectively ($P<0.001$) (Figure 1C); PFS at four years was 40%, 71%, 100%, 83% and 76%, respectively ($P<0.001$) (Figure 1D).

Secondary malignancies and cardiac toxicity developed after treatment in 7 and 10 patients, respectively. The median age of these 17 patients was 62 years. Seven of 17

Table 2. Risk factors for overall survival, progression-free survival and early relapse for patients treated with R-CHOP without consolidative radium therapy.

	OS				PFS					Early relapse					
	univariate analysis			multivariate analysis			univariate analysis			multivariate analysis					
Variables	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Effusion present	4.93	1.37-17.69	0.015	4.67	2.28-9.57	<0.001	3.53	1.69-7.40	0.001	6.45	2.45-16.98	<0.001	6.11	2.30-16.24	<0.001
Age > 60 years	2.23	0.75-6.68	0.150	0.62	0.26-1.48	0.282	–	–	–	0.14	0.019-1.05	0.056	–	–	–
Sex Male	1.35	0.47-3.89	0.584	0.93	0.50-1.73	0.821	–	–	–	0.67	0.31-1.43	0.299	–	–	–
PS > 1	4.50	1.56-12.97	0.005	2.85	1.49-5.47	0.002	–	–	–	2.68	1.25-5.73	0.011	–	–	–
Extranodal sites > 1	2.47	0.83-7.37	0.106	2.28	1.16-4.51	0.017	–	–	–	2.38	1.08-5.27	0.032	1.75	0.79-3.91	0.169
Stage III/IV	1.75	0.61-5.06	0.300	2.76	1.47-5.18	0.002	2.16	1.14-4.11	0.018	2.89	1.37-6.09	0.005	–	–	–
LDH > ULN	1.80	0.50-6.46	0.369	3.72	1.45-9.53	0.006	2.28	0.86-6.00	0.096	3.02	1.05-8.71	0.041	–	–	–
B symptoms present	0.74	0.17-3.32	0.697	1.08	0.49-2.35	0.853	–	–	–	1.55	0.68-3.53	0.292	–	–	–
IPI ≥ 3	4.23	1.48-12.13	0.007	2.94	1.55-5.57	0.001	–	–	–	2.95	1.40-6.25	0.005	–	–	–
Tumor diameter ≥ 10 cm	1.31	0.44-3.90	0.150	2.40	1.26-4.60	0.088	–	–	–	3.69	1.61-8.43	0.002	–	–	–
s-IL2R > 1000 U/L	1.88	0.57-6.25	0.302	2.40	1.18-4.90	0.016	–	–	–	1.93	0.85-4.37	0.115	–	–	–
Serum albumin < 3.5 g/dL	1.82	0.56-5.89	0.322	1.46	0.69-3.10	0.321	–	–	–	1.80	0.79-4.10	0.159	–	–	–
ALC < 0.5×10⁹/L	1.15	0.26-5.15	0.855	1.17	0.49-2.79	0.728	–	–	–	1.33	0.50-3.50	0.566	–	–	–
Hemoglobin <12 g/dL	1.86	0.64-5.37	0.253	1.17	0.60-2.29	0.643	–	–	–	1.20	0.55-2.59	0.651	–	–	–
Platelet count <150×10⁹/L	2.15	0.48-9.65	0.316	1.82	0.71-4.67	0.316	–	–	–	0.93	0.22-3.91	0.919	–	–	–

OS: overall survival; PFS: progression-free survival; R-CHOP: rituximab, cyclophosphamide, adriamycin, vincristine and prednisone; RT: radiation therapy; HR: hazard ratio; CI: confidence interval; Effusion: pleural or pericardial effusion; PS: performance status; LDH: lactate dehydrogenase; ULN: upper limit of normal; IPI: international prognostic index; s-IL2R: soluble interleukin-2 receptor; ALC: absolute lymphocyte count.