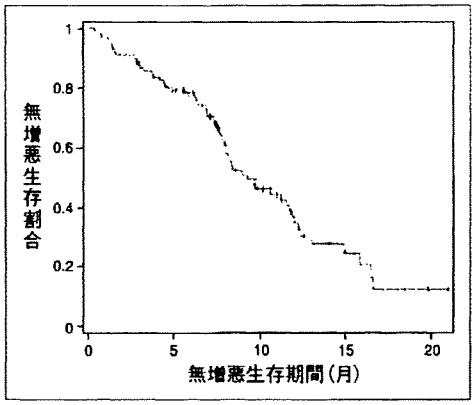

図2　ベンダムスチン単剤第III相試験の無増悪生存曲線　(文献[4]より引用改変)

マブ抵抗性の低悪性度B細胞性リンパ腫に対してベンダムスチンを承認した.

有害事象では，感染症が全グレードで69%，グレード3以上が21%と，これまでより高い頻度が報告された．注目されるのは，帯状疱疹12エピソード，CMV感染5エピソードと免疫抑制が強い可能性が示唆されたことである．原疾患，濃厚な治療歴の影響もあるが，ベンダムスチン投与後の免疫抑制に関しては，今後検討が必要な点である.

2．リツキシマブ併用の治療成績

ベンダムスチン単剤での高い有効性，動物モデルでリツキシマブ併用時に相乗効果が認められたことより[5]，リツキシマブとベンダムスチンの併用(B-R)療法が検討された.

(1)ドイツでの第II相試験[6]

対象：リツキシマブ未使用の，再発，治療抵抗性低悪性度リンパ腫，マントル細胞リンパ腫で，1～3レジメンの化学療法の既往がある症例．かつ，造血不全(Hb＜11g/dlあるいは好中球＜1,500/μlあるいは血小板数＜10万/μl)，B症状の存在，リンパ腫関連症状のため治療が必要な症例.

治療法：Day 1にリツキシマブ375mg/m²，day 2, 3にベンダムスチン90mg/m²投与．これを4週間ごとに4コース繰り返し，1コース目の1週間前，4コース終了後4週間後にリツキシマブ375mg/m²を各1回投与するデザインで実施された.

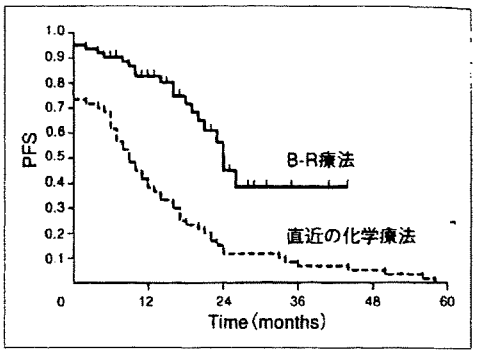

図3　B-R療法と直近の化学療法のPFSの比較　(文献[6]より引用改変)

結果：63名がエントリー．年齢の中央値64歳(40～81歳)．前治療歴1, 2, 3レジメンは，それぞれ43人，12人，8人．30%が直前の治療に抵抗性．マントル細胞リンパ腫16人中7人が前治療抵抗性.

63人中59人が予定の4コース完遂．2人が2コース(白血球減少1名，患者希望1名)，2人が3コース(1名が治療効果なし，1名が原因不明)で中止となった.

全奏効率90%，完全寛解率60%．マントル細胞リンパ腫に関しては，全奏効率75%，完全寛解率50%．PFSの中央値は24か月で，各症例の直前の治療後のPFSの中央値9か月より有意に(P＜0.0001)延長していた(図3)．マントル細胞リンパ腫では，PFSの中央値が18か月で，解析時点で6名が最長22か月寛解を維持していた．全症例の48か月の時点でのOSは55%.

毒性に関しては血液毒性が中心で，グレード3(WHO)以上の白血球減少が16%認められたが，グレード3以上の血小板減少，貧血はそれぞれ3%，1%であった．非血液毒性は全般的に軽度で，悪心/嘔吐が43%に認められたが，グレード2以下であった．感染症に関しては，細菌性肺炎2例，帯状疱疹2例，口唇ヘルペスが2エピソード認められた.

コメント：リツキシマブとベンダムスチンの併用の最初の報告である．高い有効性が示され，リツキシマブを併用したにもかかわらず治療完遂率が高く，懸念された感染症が単剤より低頻

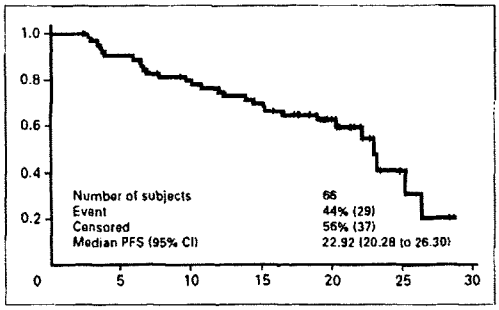

図4 米国第II相試験の無増悪生存曲線
（文献[7]より引用改変）

度であったことは，採用された投与法の妥当性を示したものである．またマントル細胞リンパ腫に関しても，少数例の検討ではあるが高い有効性が示され，その毒性がこれまでのマントル細胞リンパ腫で広く実施されているhyper-CVAD療法などと比較して軽度であることが注目される．

(2)米国の第II相試験[7]

対象：リツキシマブ抵抗性(リツキシマブを含む化学療法でPR未満，あるいは終了後6か月以内にPD)ではないCD20陽性再発低悪性度リンパ腫，マントル細胞リンパ腫．前治療は最大3レジメンまでで，放射免疫療法の既往がある症例は除く．

治療法：投与法はドイツの第II相試験と同様である．ただし腫瘍縮小が持続している場合は，最大6コースまで投与可能なデザインで実施された．

症例数：期待奏効率70%，閾値奏効率50%．検出力80%で症例数は60例．

結果：67名がエントリーしたが1名投与せず，解析対象は66名．年齢の中央値60歳(40～84歳)．前治療歴1，2，3レジメンは，それぞれ56%，33%，11%．リツキシマブの投与歴があるのは56%．アルキル化剤，プリンアナログ，アントラサイクリン系の使用歴はそれぞれ85%，23%，58%．

92%が4コース完遂．62%は6コースの投与を受けた．6例が4コース完遂できなかったが，その内訳は2例が有害事象，1例は原疾患の進行，2例が担当医の判断，1例が追跡不能のためであった．また計346サイクル実施され治療遅延12%であったが，そのうち74%は14日以内の遅延であった．Relative dose intensityの平均は，ベンダムスチン93%，リツキシマブ95%であった．

表3 リツキシマブ併用ベンダムスチン療法の治療効果

	ドイツの第II相試験	米国の第II相試験
全奏効率	90%	92%
CR率	60%	55%
PFS(中央値)	24か月	22.9か月

（文献[6][7]より引用改変）

全奏効率は92%，完全寛解率55%．観察期間の中央値が20か月で，治療奏効期間の中央値21か月，PFSの中央値が23か月(図4)．リツキシマブの投与歴の有無では，投与ありの場合，全奏効率87%，完全寛解率35%，投与なしの場合全奏効率100%，完全寛解率48%．マントル細胞リンパ腫12例に関しては，全奏効率92%，完全寛解率59%，治療奏効期間の中央値19か月であった．

有害事象は血液毒性が中心で，グレード3以上の好中球減少が36%，血小板減少が9%，貧血が2%認められた．非血液毒性では悪心70%，感染64%，倦怠感59%，便秘が44%認められたが大半はグレード2以下であった．グレード3以上は感染の10%が最多で，6例に10エピソード認められた．その内訳は，真菌感染2エピソード，ウイルス感染3エピソード，細菌感染，憩室炎，肺炎1エピソード，発熱性好中球減少2エピソードであった．グレード4のCMV感染が1エピソード認められた．

コメント：ドイツでの第II相試験で得られた高い治療効果(表3)と治療完遂率，単剤での第II相試験の結果と比較して低い感染症の頻度が再確認された試験である．

(3)ドイツでのB-R療法とR-CHOP療法の比較第III相試験[8]

対象：初発低悪性度リンパ腫，マントル細胞リンパ腫．

治療法：B-R療法(day 1にリツキシマブ375mg/m^2，day 1，2にベンダムスチン90mg/m^2)を28日ごと，または通常量のR-CHOP療法を21日

表4　B-R療法とCHOP-R療法の治療効果

	B-R療法	CHOP-R療法	
ORR	93.8%	93.5%	
CR率	40.1%	30.8%	$P=0.0323$
PFS(median)	54.8か月	34.8か月	$P=0.0002$
EFS	54か月	31か月	$P=0.0002$
TTNT	Not reached	40.7か月	$P=0.0002$

（文献[8]より引用改変）

ごとに最大6コース実施された．

患者背景：549名が登録．年齢の中央値は64歳(31～83歳)．組織型はB-R療法，R-CHOP療法それぞれ濾胞性リンパ腫55%，56%，マントル細胞リンパ腫18%，19%，他の低悪性度リンパ腫27%，24%．

結果：6コース完遂率はB-R療法82%，R-CHOP療法86%．全奏効率はB-R療法93.8%，R-CHOP療法63.5%．PFS, EFS, 次治療までの期間の中央値はB-R療法で54.8か月，54か月，中央値に達せず，R-CHOP療法で34.8か月，31か月，40.7か月と，いずれもB-R療法が上回った(表4)．

毒性に関しても，血液毒性ではグレード3以上の好中球減少はB-R療法で10.7%，R-CHOP療法で46.5%，グレード3以上の白血球減少がB-R療法で12.1%，R-CHOP療法で38.2%とB-R療法が軽度であった．非血液毒性に関しても脱毛，感染症，末梢神経障害，口内炎のいずれもB-R療法が軽度であった．薬剤関連の皮膚反応のみがB-R療法の方が高頻度であった．

コメント：現在低悪性度リンパ腫に対して広く実施されているR-CHOP療法とB-R療法のはじめての比較試験の結果である．有効性，安全性ともにB-R療法が上回っており，B-R療法が低悪性度リンパ腫初回治療の第一選択となる可能性を示唆したものとなった．

3．今後の検討課題

単剤投与での120mg/m² 2日間投与，1コース21日のスケジュールは，治療完遂率が低いこと，減量，遅延の頻度が高いこと，免疫抑制が強いことが推察され，一般臨床での使用では少し検討が必要と思われる．

また放射免疫療法実施の既往があると，血小板減少でベンダムスチン投与完遂が困難であることが示唆されたが，現在では放射免疫療法は再発早期の使用が推奨されているため，放射免疫療法との投与順序が今後問題となる．ベンダムスチン投与既往例における放射免疫療法実施後の血小板減少の程度も現時点で不明であり，ベンダムスチンのポジショニングに関しては，濾胞性リンパ腫の治療体系全体を考慮した検討が必要である．

マントル細胞リンパ腫に関しては，治癒を期待する強力な化学療法が実施できない症例，再発例に関しては，選択肢の1つとなるが，初回治療への組み込みに関しては今後の課題である．

慢性リンパ性白血病に対する有効性，安全性

1．塩酸ベンダムスチン単剤の治療成績

(1)再発例に対する第I/II相試験[9]

対象：クロラムブシルあるいはフルダラビンの治療歴のあるBinet B, C のCLL.

治療法：開始用量はベンダムスチン100mg/m²で，day 1, 2 に投与して3～4週ごとに繰り返す．用量制限毒性(DLT)の有無により10mg/m²刻みで増量，減量するデザインで実施された．DLTはグレード3以上の非血液毒性，グレード4の原疾患によらない血液毒性と定義された．

結果：16名がエントリーされた．年齢の中央値は67歳(57～83歳)で，Binet C は10名．前治療レジメン数の中央値は3レジメン．

高尿酸血症，感染症，血液毒性などのDLTが複数100mg/m²，90mg/m²，80mg/m²コホートで観察され，MTD, 推奨用量ともに70mg/m²に決定された．奏効率(CR+PR+SD)56%でCR 2例であった．

コメント：非ホジキンリンパ腫と異なりCLLでは，原疾患により骨髄機能が低下していること，また免疫抑制状態にあり易感染性であることより用量の再検討が必要であり，結果的に再発非ホジキンリンパ腫に対する用量の約6割が推奨用量となった．

(2)初発例に対する第III相試験[10]

対象：未治療，CLL. Binet stage B, C.

治療法：ベンダムスチン投与群とクロラムブシル投与群に1：1に無作為化割り付け．ベンダムスチンは100mg/m²をday 1, 2 に投与．1コー

スを4週間として，治療効果により最大6コースまで投与．クロラムブシル群は0.8mg/kgをday 1, 15に投与．1コースを4週間として，同様に治療効果により最大6コースまで投与．

結果：ベンダムスチン群162例，クロラムブシル群157例の計319例が登録．Binet stage B, Cの割合は，ベンダムスチン群71.6%，28.4%．クロラムブシル群70.7%，29.3%．

全奏効率はベンダムスチン群68%(CR率31%)，クロラムブシル群31%(CR率2%)．無増悪生存率の中央値はベンダムスチン群21.6か月，クロラムブシル群8.3か月(図5)．

有害事象の頻度はベンダムスチン群89%，クロラムブシル79%．一般的なものは，好中球減少，発熱，血小板減少，悪心，貧血，白血球減少，嘔吐などで，ベンダムスチン群では9例が過敏反応で治療中止となった．

コメント：ベンダムスチンの有効性はクロラムブシルを上回り，その毒性は管理可能であること，またこれまでのフルダラビン単剤の報告に匹敵する有効性が示された．本試験の結果をもってFDAは2008年3月，CLLに対してベンダムスチンを承認した．

2．リツキシマブ併用の治療成績：初発CLLに対する第II相試験[11]

対象：治療を要する初発CLL．

治療法：ベンダムスチン90mg/m²をday 1, 2に投与し，28日ごとに最大6コース実施．リツキシマブは1コース目は375mg/m²，2コース目以降は500mg/m²投与．

結果：117名がエントリーされ，合計583コース実施された．Binet A, B, Cは，それぞれ11.1%，41.0%，47.9%．全奏効率90.9%，CR率32.7%．主な有害事象は骨髄抑制で，グレード3以上の頻度は，全実施コース数あたり貧血4.9%，白血球減少14.6%，好中球減少6.5%，血小板減少6.1%．グレード3以上の感染症は29エピソード，全実施コース数の5.1%に認められた．

コメント：B-R療法の未治療CLLの有効性を明らかにしたもので，有害事象も懸念された感染症を含めて許容範囲であった．この結果を受けてドイツではB-R療法とFCR療法の比較試験を実施中である．

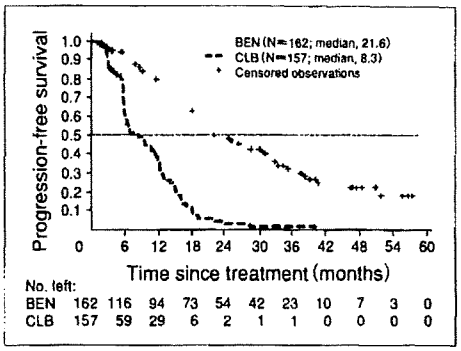

図5 クロラムブシル，ベンダムスチン群の無増悪生存曲線 (文献[10]より引用改変)

3．今後の検討課題

これまでの試験の成績をみる限り，既治療例に対してベンダムスチンが治療選択肢の1つとなるのは明らかである．初発例に関しては，第III相試験はクロラムブシルとの比較である点が議論を呼ぶところであり，B-R療法とFCR療法の比較試験の結果が待たれる．

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Biochemical and Biophysical Research Communications 390 (2009) 48–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Escape mechanisms from antibody therapy to lymphoma cells: Downregulation of *CD20* mRNA by recruitment of the HDAC complex and not by DNA methylation

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ARTICLE INFO

Article history:
Received 15 September 2009
Available online 19 September 2009

Keywords:
CD20
Rituximab
Epigenetics
Histone deacetylases
DNA methytransferases

ABSTRACT

Although rituximab is a critical monoclonal antibody therapy for CD20-positive B-cell lymphomas, rituximab resistance showing a CD20-negative phenotypic change has been a considerable clinical problem. Here we demonstrate that *CD20* mRNA and protein expression is repressed by recruitment of a histone deacetylase protein complex to the *MS4A1* (*CD20*) gene promoter in CD20-negative transformed cells after treatment with rituximab. *CD20* mRNA and protein expression were stimulated by decitabine (5-Aza-dC) in CD20-negative transformed cells, and was enhanced by trichostation A (TSA). Immunoblotting indicated that DNMT1 expression was first downregulated 1 day after treatment with 5-Aza-dC, but IRF4 and Pu.1, the transcriptional regulators of *MS4A1*, were still expressed with or without 5-Aza-dC. Interestingly, CpG methylation of the *MS4A1* promoter was not observed in CD20-negative transformed cells without 5-Aza-dC. A chromatin immunoprecipitation (ChIP) assay indicated that the Sin3A–HDAC1 co-repressor complex was recruited to the promoter and dissociated from the promoter with 5-Aza-dC and TSA, resulting in histone acetylation. Under these conditions, IRF4 and Pu.1 were continually recruited to the promoter with or without 5-Aza-dC and TSA. These results suggest that recruitment of the Sin3A–HDAC1 complex is related to downregulation of CD20 expression in CD20-negative B-cells after treatment with rituximab.

Introduction

Rituximab is the first therapeutic monoclonal antibody targeting human malignant tumors, and is now an indispensable molecular-targeting drug for CD20-positive B-cell lymphomas [1–3]. Although the effectiveness is significant, resistance to rituximab has also become a considerable problem [4].

Several mechanisms of the resistance have been suggested, including loss of CD20 protein expression after rituximab use [5–12] and CD20 gene mutations [13]. Furthermore, other mechanisms have also been suggested [4] such as internalization of CD20 protein [14], interference with accessibility of rituximab to CD20 protein by inhibitory factors, rapid metabolism of the antibody, abnormalities in B-cell signaling in tumor cells [15], abnormalities of apoptosis [16], antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) [17].

Very recently, we reported observation of downregulation of CD20 protein expression in CD20-positive B-cell lymphoma patients after treatment with rituximab-containing combination chemotherapies [6,7]. In those cases, it was strongly suggested that aberrant downregulation of *MS4A1* expression was closely related to the loss of CD20 protein expression, and that expression of CD20 and rituximab sensitivity were partially restored by some molecular-targeting drugs [6,7]. Although these findings suggest that epigenetic mechanisms, in part, contribute to the downregulation of CD20 expression, the molecular mechanisms are still not clear. Furthermore, a recent report indicated that reduced CD20 protein expression in *de novo* diffuse large B-cell lymphoma is associated with a poor survival rate [18]. Thus, understanding the mechanisms of downmodulation of CD20 protein expression is likely to be very important from both basic research and clinical viewpoints.

In this report, we show that the recruitment of a histone deacetylase (HDAC) co-repressor complex to the *MS4A1* promoter

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doi:10.1016/j.bbrc.2009.09.059

region, but not DNA methylation [19], is involved in CD20-negative phenotypic changes in B-cell lymphoma cells after treatment with rituximab. We show that the complex dissociated from the promoter in the presence of a DNA methyltransferase (DNMT) inhibitor and a HDAC inhibitor [20], resulting in partial restoration of CD20 expression.

Materials and methods

Cell culture conditions and treatment with epigenetic drugs. RRBL1 [6], Raji, and NALM6 cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) with 10% fetal calf serum. Five-Aza-dC (5-aza-2′-deoxycytidine; Sigma, St. Louis, MO) and TSA (Sigma) at final concentrations of 100 μM and 100 nM, respectively, were added directly to the culture medium.

Immunoblotting. Cells (~5×10^5) were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.5% NP-40, 300 mM NaCl, 0.2 mM PMSF, 1 mM DTT, and a complete mini protease inhibitor tablet (Roche)). After centrifugation at 10,000 *g* for 10 min, the supernatants were placed in new tubes, and 100 μl of 2× SDS sample buffer was added. After boiling for 5 min, samples were separated with SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting was carried out as described previously [21,22] using anti-CD20, -IRF4, -Pu.1, -GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-DNMT1 antibody (Abcam, Cambridge, MA, USA).

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA from cell lines (1×10^5 cells) was obtained using Trizol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was prepared as reported previously [7,22].

For RT-PCR, the following primers were designed: CD20-U; 5′-ATGAAAGGCCCTATTGCTATG-3′, CD20-L; 5′-GCTGGTTCACAGTTGTATATG-3′, β-actin-U; 5′-TCACTCATGAAGATCCTCA-3′, and β-actin-L; 5′-TTCGTGGATGCCACAGGAC-3′. Semi-quantitative RT-PCR with AmpliTaq Gold was performed as described previously [6].

Methylation status of the MS4A1 promoter. To examine the methylation status, bisulfite sequencing was performed. Genomic DNA was prepared with a QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA). Bisulfite treatment was performed using EpiTect Bisulfite kits (Qiagen). After bisulfite treatment, PCR of the *MS4A1* promoter was performed using the specific primers as follows, MS4A1-pro-MSPU; 5′-GGTAGTATGAGTATGTTAGGTAGTT-3′, MS4A1-pro-MSPL; 5′-TTTTCCTTACCTAAATCTCCAAAA-3′. PCR fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Flow cytometry (FCM) analysis. Cell surface antigens of RRBL1 with or without 5-Aza-dC and TSA treatment were analyzed using a BD FACSCalibur Flow Cytometer (BD Bioscience, Franklin Lakes, NJ, USA) with anti-CD20 antibody (Leu-16 PE, BD) and mouse IgG1 κ isotype control (PE-Cy7, BD).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed as described previously [22,23]. For immunoprecipitation (IP), the following antibodies were used; anti-Pu.1, -IRF4 (Santa Cruz Biotechnology), -acetylated H4 (Millipore, Billerica, MA, USA), -Sin3A, and anti-HDAC1 (Abcam) antibodies. Immunoprecipitated DNA was used for semi-quantitative PCR using LA-Taq polymerase (TAKARA, Ohtsu, Japan). The following primers for the *MS4A1* promoter and 3′-intron sequence (negative control) were used; CD20pro-U; 5′-CTAAAAGTGAAGCCAGAAGG-3′, CD20pro-L; 5′-GGAGGGTGAGTGGTGTAGT-3′, CD20-3′U; 5′-GCTGACCTCACATAACTCCT-3′, CD20-3′L; 5′-GAAATCCCTCAGACTCAGAC-3′.

Immunoprecipitation (IP) assay. The IP assay was carried out as described previously [22]. Whole cell lysate was obtained from RRBL1 cells (1×10^7) using 800 μl of lysis buffer. After adding 800 μl of lysis buffer without NP-40 and NaCl, the lysate was divided into four tubes (400 μl each) and IP using anti-IRF4, -Sin3A, and -HDAC1 antibodies was performed. The precipitated samples were applied to SDS–PAGE followed by immunoblotting. For the pre-IP samples, 5% of the whole cell lysate was used.

Results

CD20 protein and mRNA expression were stimulated by treatment with 5-Aza-dC in CD20-negative transformed cells

As we reported previously [6,7], the downregulation of CD20 protein and mRNA expression has been observed in some CD20-positive B-cell lymphoma patients after treatment with rituximab-containing chemotherapies. We also reported that the downregulation of CD20 expression was partially stimulated by treatment with the epigenetic drugs 5-Aza-dC and TSA. RRBL1 cells were established from a patient with B-cell lymphoma who showed a CD20-negative phenotypic change after treatment with rituximab [6]. To examine the mechanisms of stimulation of CD20 expression by 5-Aza-dC, we examined the protein expression pattern that may affect CD20 gene transcription in RRBL1 cells. RRBL1 cells were treated with 5-Aza-dC for 24 h, and were then washed and incubated for up to 7 days (Fig. 1A). During this procedure, the cells were harvested several times as indicated and analyzed using semi-quantitative RT-PCR and immunoblotting (IB) (Fig. 1B). *CD20* mRNA and protein expression were stimulated by 5-Aza-dC, and the peak of expression was observed around day 3 after treatment with 5-Aza-dC (lane 6). After day 5, CD20 protein expression had gradually decreased. DNMT1 depletion was confirmed at 24 h after treatment with 5-Aza-dC (lane 4) as reported previously [24]. IRF4 and Pu.1 are transcription factors that interact with the *MS4A1* promoter and regulate CD20 expression [25]. IRF4/Pu.1 was almost constantly expressed throughout the 5-Aza-dC treatment duration (lanes 3–8), but only a modest upregulation was observed after treatment with 5-Aza-dC around day 2 (lane 5). These results suggested that DNMT1 depletion by 5-Aza-dC may be related to stimulation of *MS4A1* expression.

DNA methylation status of the MS4A1 promoter

To explain the activation of *CD20* mRNA and protein expression after treatment with 5-Aza-dC in RRBL1 cells, we next examined the CpG methylation status of the *MS4A1* promoter (Fig. 1C). Interestingly, CpG islands were not observed on the promoter region located ~5 kb upstream from the transcription start site, and only four CG sites were found on the promoter from the −1000 to +100 region. Bisulfite sequencing was carried out to confirm methylated CpG. As shown in Fig. 1C, no CpG methylation was observed on the three CpG sites around the transcription start site in RRBL1 cells. In NALM6 cells, a CD20-negative lymphoblastic leukemia cell line, several methylated CpGs were observed. Furthermore, the same analysis was performed using primary tumor cells from a patient suffering from CD20-negative transformed B-cell lymphoma after treatment with rituximab-containing combination chemotherapies. (Detailed information about this patient is described in our previous paper as UPN3 [7]). The three CpG sites were not methylated, as observed in RRBL1 cells (Fig. 1C, UPN3). These results suggest that transcriptional activation of *MS4A1* by 5-Aza-dC may not be regulated by its promoter CpG demethylation in RRBL1 cells.

Histone deacetylase inhibitor TSA enhances CD20 expression by 5-Aza-dC in CD20-negative transformed cells

Next, we analyzed the effect of a HDAC inhibitor in addition to 5-Aza-dC on *MS4A1* expression in RRBL1 cells. CD20 protein expression in RRBL1 cells was confirmed using immunoblotting

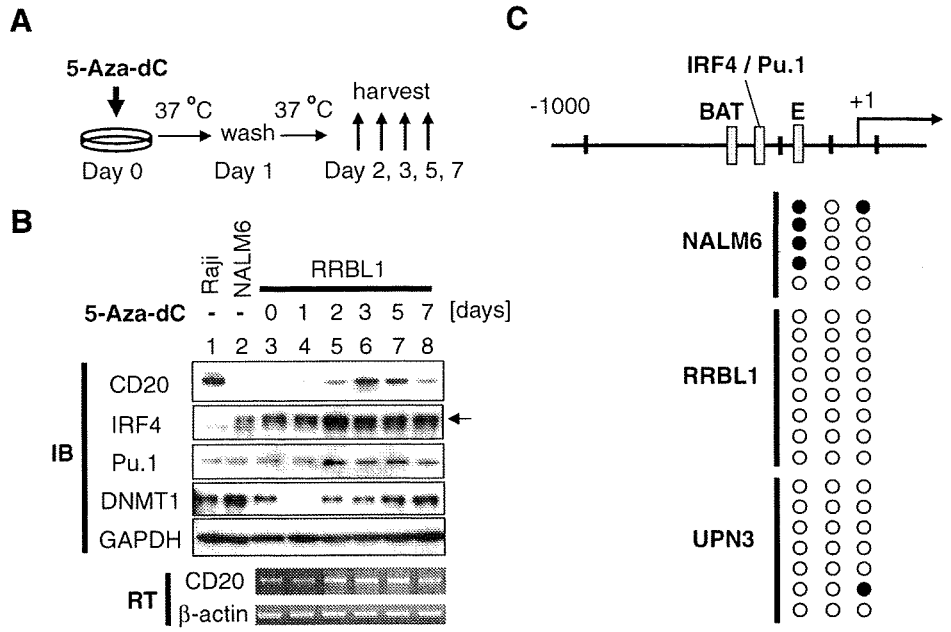

Fig. 1. CD20 protein and mRNA expression were transiently stimulated by treatment with a DNMT inhibitor. (A) Schematic representation of 5-Aza-dC treatment of the CD20-negative transformed B-lymphoma cells. RRBL1 cells were incubated at 37 °C for 24 h, and then washed twice with RPMI medium with 10% FCS without 5-Aza-dC. Cells were further incubated for up to 7 days, and were harvested at days 1, 2, 3, 5, and 7. (B) Protein expression was examined using immunoblotting (IB) with the indicated antibodies. The mRNA expression level was determined using semi-quantitative RT-PCR (RT). The black arrow indicates the band for IRF4. Raji and NALM6 cells were used as positive and negative controls, respectively. GAPDH and β-actin were measured as internal controls. (C) The structure of the *MS4A1* promoter near the transcription start site (from −1000 to +100) is depicted. The BAT-box, IRF4/Pu.1 binding sites, and E-box are shown as shaded boxes. Only four CpG sites, which are putative methylation sites, were found and are shown as black vertical bars. The methylation status of the three CpG sites around the transcription start site in NALM6, RRBL1, and primary B-lymphoma cells that show CD20-negative transformation was analyzed with bisulfite sequencing. Five to eight clones were analyzed from each sample. Black and open circles indicate methylated and non-methylated CpGs, respectively.

and flow cytometry (FCM) (Fig. 2A and B) following treatment with 5-Aza-dC and/or TSA. When RRBL1 cells were treated with 5-Aza-dC or TSA alone, minimal activation of CD20 protein expression was observed using immunoblotting (Fig. 2A, lanes 4 and 5) and FCM (Fig. 2B, 5-Aza-dC). In the presence of 5-Aza-dC and TSA, CD20 protein expression was significantly increased (Fig. 2A, lane 6, and B, 5-Aza-dC + TSA). These results suggested that *MS4A1* expression is, in part, regulated by epigenetic mechanisms such as histone modification including lysine acetylation, rather than DNA CpG methylation of the *MS4A1* promoter.

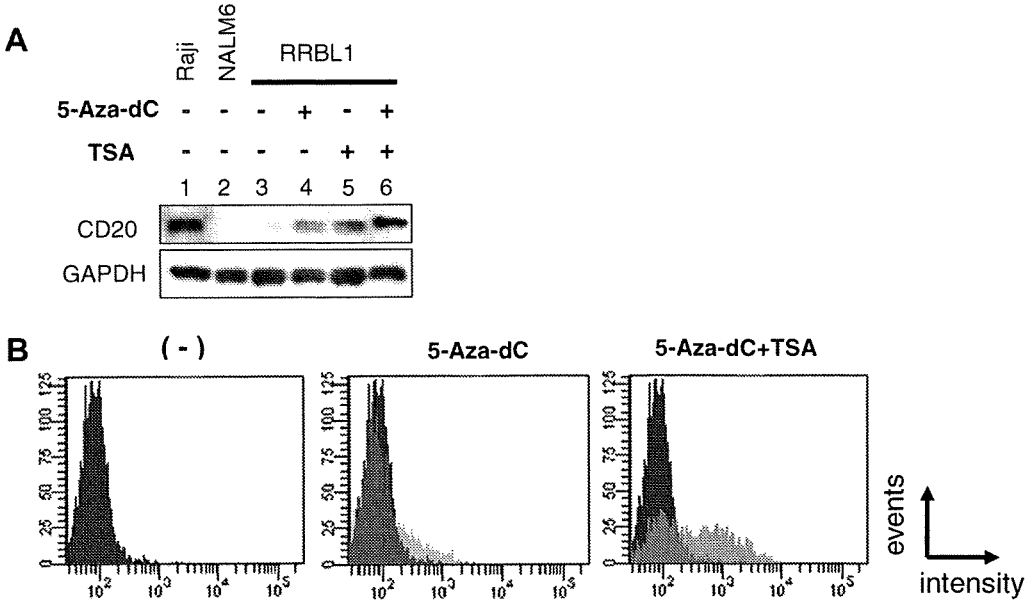

Fig. 2. CD20 protein expression by 5-Aza-dC was enhanced by TSA. CD20 protein expression was shown with IB (A) and FCM (B) with or without epigenetic drugs. For 5-Aza-dC treatment, RRBL1 cells were incubated with 5-Aza-dC for 24 h followed by washing and two additional days of incubation. TSA was added at the start of day 3, and cells were incubated for 24 h. If cells were not treated with 5-Aza-dC, washing was also carried out at day 1 to adjust the incubation conditions. All the cells were harvested at day 4 and utilized for IB and FCM analyses. The untreated and treated cells with the epigenetic drugs were depicted as black and gray areas, respectively (B).

Recruitment of co-repressor proteins and histone deacetylation on the MS4A1 promoter in the absence of epigenetic drugs

To study the molecular mechanisms of transcriptional repression of *MS4A1*, a ChIP assay was performed. RRBL1 cells were incubated with or without 5-Aza-dC and TSA, and a ChIP assay was carried out using anti-Pu.1, -IRF4, -Sin3A, -HDAC1, and -acetylated-histone H4 antibodies. After IP, precipitated genomic DNA was utilized in semi-quantitative PCR using primers for the *MS4A1* promoter (Fig. 3A) and the 3′-intron sequences as a negative control. IRF4 and Pu.1 interactions were consistently observed on the promoter region, but not on the 3′-intron region (Fig. 3B, lanes 5–8). Sin3A and HDAC1, which form a transcription repressor protein complex [26], interacted with the promoter region only in the absence of 5-Aza-dC and TSA (Fig. 3B, lanes 11 and 13). Acetylated-histone H4 was observed at the promoter region with 5-Aza-dC and TSA, but the acetylation was decreased in the absence of the two drugs (lane 9). In the 3′-intron region, histone acetylation was consistently observed with or without 5-Aza-dC and TSA. These results strongly suggest that the Sin3A–HDAC1 co-repressor complex may be recruited to the *MS4A1* promoter through some transcription factors in the absence of epigenetic drugs, resulting in histone deacetylation and transcriptional repression. In addition, the recruitment may be dissociated from the promoter by adding 5-Aza-dC and TSA, resulting in histone acetylation and transcription activation.

The Sin3A–HDAC1 co-repressor complex is found in RRBL1 cells with or without epigenetic drugs

To show that loss of Sin3A–HDAC1 interaction with the *MS4A1* promoter was due to protein complex dissociation and not degradation, we confirmed the protein expression in RRBL1 cells with and without epigenetic drugs using IB. As shown in Fig. 4A, HDAC1 and Sin3A protein expression levels did not change in the presence of epigenetic drugs. Next, we performed an IP assay using anti-IRF4, -Sin3A, and -HDAC1 antibody to confirm that Sin3A and HDAC1 exist as a protein complex, and to examine whether the Sin3A–HDAC1 co-repressor complex was recruited by IRF4 in the absence of epigenetic drugs. The Sin3A–HDAC1 interaction was confirmed with an IP assay using anti-Sin3A and -HDAC1 antibodies (Fig. 4B, lanes 4 and 5), but interaction of IRF4 with this complex was not observed (lane 3). These results indicate that the Sin3A–HDAC1 complex exists in RRBL1 cells with or without 5-Aza-dC and TSA, and that the recruitment of the complex to the *MS4A1* promoter may not involve a direct interaction with IRF4.

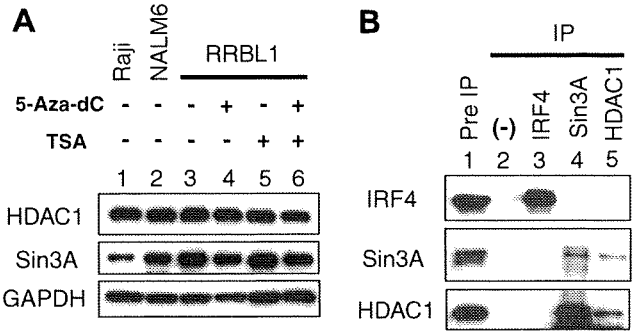

Fig. 4. The Sin3A–HDAC1 co-repressor complex is stably expressed in RRBL1 cells with or without 5-Aza-dC and/or TSA. (A) IB was performed using the RRBL1 lysate after treatment with 5-Aza-dC and/or TSA. Raji and NALM6 cells were used as expression controls. Similar levels of expression of HDAC1 and Sin3A were observed in each sample. (B) Whole cell lysate of RRBL1 cells was obtained using lysis buffer. Lysates were divided into four samples and used for IP using anti-IRF4, -Sin3A, and -HDAC1 antibodies. Five percent of the whole cell lysate was used for the pre-IP samples (lane 1). As a negative control, antibodies for IP were omitted (lane 2). IB indicated that endogenous Sin3A–HDAC1 interacted in RRBL1 cells without epigenetic drugs, but significant interaction with IRF4 was not observed in this assay system.

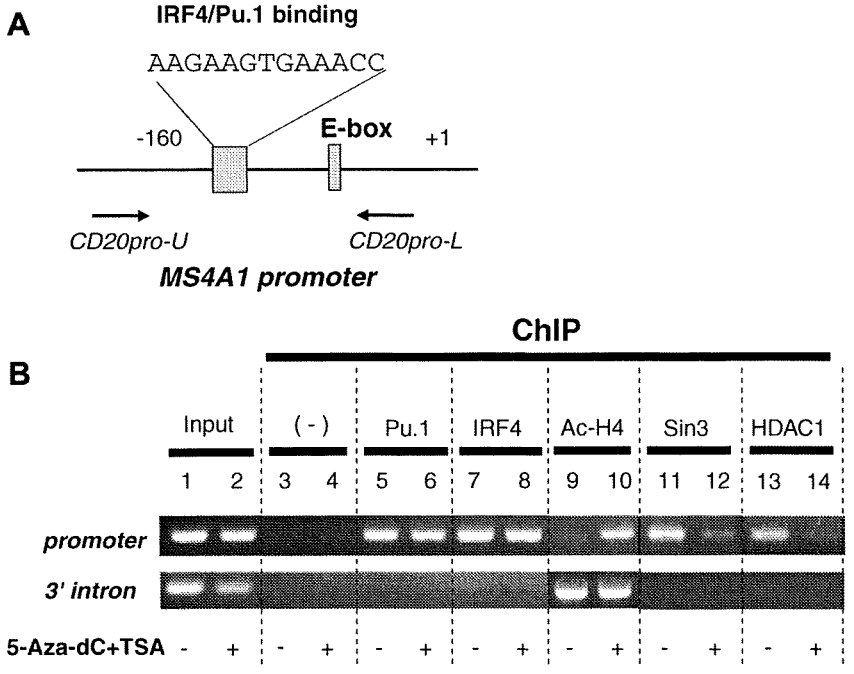

Fig. 3. ChIP assay of the *MS4A1* promoter. The primer set used for amplification of the *MS4A1* promoter (−160 to +1) is shown in (A). The positions of the upper and lower primers are indicated as black arrows. (B) A ChIP assay using anti-Pu.1, -IRF4, -Sin3A, -HDAC1, and -acetylated-histone H4 was performed using the cell lysate from cells treated with or without 5-Aza-dC and TSA. Semi-quantitative PCR was performed, and the amplified DNA fragments were visualized by 1.5% agarose gel electrophoresis. As a positive control, lysate without the IP step was used (input). A ChIP sample without antibodies was used as a negative control (−). PCR using the primers for the 3′-intron region of *MS4A1* was also used as a control. Sin3A and HDAC1 recruitment to the promoter was observed in lanes 11 and 13, and accumulation of histone deacetylation was seen in lane 9.

Discussion

In clinical practice, CD20 expression abnormalities have been reported. Johnson et al. [18] reported that 43 out of 272 (16%) patients with diffuse large B-cell lymphoma (DLBCL) showed reduced CD20 expression using FCM analysis at the time of initial diagnosis, and that the survival rate of this phenotype was significantly lower than that of patients with CD20-positive phenotype. Furthermore, we previously reported that a CD20-negative phenotypic change after using rituximab resulted in resistance to salvage chemotherapies with or without rituximab [6,7]. We observed that all of these patients died of disease progression within 1 year after the diagnosis of CD20-negative transformation, suggesting that the CD20-negative phenotype may be related to the poor prognosis. From these findings, we realized the importance of investigating the mechanisms of downmodulation of CD20 expression to explore overcoming strategies including salvage combination chemotherapies with anti-CD20 antibodies.

In this study, we firstly investigated the effect of 5-Aza-dC on RRBL1 cells. DNMT1 protein reduction was observed 1 day after adding 5-Aza-dC, followed by temporal upregulation of CD20 protein expression (Fig. 1B). This phenomenon suggested that CpG demethylation of the *MS4A1* promoter region was a result of DNMT1 depletion. But interestingly, significant CpG islands were not located at the promoter, suggesting that *MS4A1* activation by 5-Aza-dC was not regulated directly by *MS4A1* promoter methylation.

The next hypothesis we investigated was that expression of transcription factors, which is critical for *MS4A1* expression, was regulated by the methylation status of the promoter DNA. We analyzed the protein expression level of IRF4/Pu.1, and only a modest upregulation was observed. Furthermore, the ChIP assay showed that IRF4/Pu.1 recruitment to the *MS4A1* promoter was fairly stable in the presence or absence of 5-Aza-dC and TSA (Fig. 3B). On the other hand, Sin3A–HDAC1 recruitment and histone deacetylation was observed in the absence of epigenetic drugs. Because previous reports have indicated that HDACs form large protein complexes, such as Sin3 [26], NuRD/Mi-2 [27], and N-CoR/SMRT co-repressor complexes [26,28], and are recruited to the specific promoter by transcription factors, we analyzed whether the Sin3A–HDAC1 complex interacts with IRF4 in RRBL1 cells. Using an IP assay, we observed that HDAC1 interacts with Sin3A but not with IRF4 (Fig. 4B). We also analyzed the recruitment of the proteins N-CoR, HDAC3, and TBLR1 (transducin β-like protein 1 relating protein), which are all expressed in the same co-repressor complex *in vivo* [21–23,26,28], to the promoter region using the ChIP assay. Significant recruitment of these proteins was not seen in this assay (data not shown).

Thus, these findings suggest that, (1) *MS4A1* repression is not directly regulated by methylation of its promoter and (2) transcription factors other than IRF4 recruit the Sin3A–HDAC1 co-repressor complex to the *MS4A1* promoter to repress transcription through histone deacetylation. Our previous report [6] showed that treatment with TSA without 5-Aza-dC upregulates CD20 expression in RRBL1 cells within 1 day, suggesting that the activity of HDAC may be more critical for *MS4A1* expression than the activity of DNMTs. One explanation for why 5-Aza-dC can stimulate *MS4A1* expression is that the expression of some transcription factors, whose expression is critical for CD20 expression, may be regulated by CpG methylation of the gene promoters. The maximal effect of 5-Aza-dC on CD20 protein expression was seen at 3 days after treatment with 5-Aza-dC, which is consistent with this hypothesis. The knockdown of endogenous DNMT1 using the siRNA technique may help explain the importance of DNMT1 for *MS4A1* repression. On the other hand, the possibility that CpG islands in *MS4A1* that affect its expression are in a location that is relatively remote (~5 kb) from the transcription start site cannot be excluded. Further investigation is needed.

In our study, the efficiency of stimulating CD20 protein expression in CD20-negative transformed cells using epigenetic drugs is not complete (Fig. 2B). As we showed previously [7], this efficiency may not be sufficient to overcome resistance to rituximab. Using the newer generation humanized-anti-CD20 monoclonal antibodies, such as ofatumumab [29], GA-101 [30], and others, which have higher antibody binding capacity with CD20 and/or a higher CDC/ADCC activity, may help overcome the resistance. We also anticipate the use of those therapies in combination with epigenetic drugs such as HDAC and/or DNMT inhibitors. Further investigation is still needed.

Acknowledgments

This work is supported in part by a Grant-in-Aid for Cancer Research (21-6-3) from the Ministry of Health, Labor and Welfare, a Grant-in-Aid from the National Institute of Biomedical Innovation, and a Grant-in-Aid in Scientific Research (20591116) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr. Shinsuke Iida for providing critical information about IRF4. We also thank Akihide Atsumi, Tomoka Wakamatsu, Yukie Konishi, Mari Otsuka, Eriko Ushida, and Chieko Kataoka for valuable laboratory assistance.

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Presentation and management of intravascular large B-cell lymphoma

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Intravascular large B-cell lymphoma (IVLBCL) is a rare disease entity of non-Hodgkin lymphoma according to the current WHO classification. This rare form of B-cell lymphoma is characterised by selective growth of tumour cells in the lumina of small vessels of various organs. Strange characteristics of IVLBCL, including the absence of marked lymphoadenopathy and the usually aggressive clinical behaviour, result in the delay of timely and accurate diagnosis and fatal complications. Thus, the prognosis of IVLBCL is extremely poor. The success achieved with the anti-CD20 chimeric monoclonal antibody, rituximab, represents an important milestone in the clinical practice of B-cell lymphoma. An advantage of adding rituximab to conventional chemotherapies has been shown, in the process of increasing our understanding of the clinical and pathological manifestations for IVLBCL. This Review describes the cutting edge of research on IVLBCL, and discusses the unsolved issues from biological and clinical perspectives to provide a better understanding of this rare lymphoma.

Lancet Oncol 2009; 10: 895–902

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Introduction

Intravascular large B-cell lymphoma (IVLBCL) is a rare disease entity of malignant lymphoma, characterised by the selective growth of lymphoma cells within the lumina of vessels. This type of lymphoma was first reported in 1959 by Pfleger and Tappeiner[1] as "angioendotheliomatosis proliferans systemisata" and was considered to be endothelial in origin.[2] In 1982, Ansell and colleagues[3] suggested a lymphoid origin by showing surface immunoglobulin on neoplastic cells. Leucocyte common antigen on neoplastic cells was subsequently reported in 1985,[4,5] and the lymphoid nature of this entity was confirmed in 1986 by Wick and co-workers.[6] Since the first description, other historical names for IVLBCL have included angioendotheliomatosis proliferans systemica, malignant angioendotheliomatosis, neoplastic angioendotheliosis, intravascular lymphomatosis, angioendotheliotropic (intravascular) lymphoma (Kiel classification), angiotropic large-cell lymphoma (Luke-Collins classification), and diffuse large B-cell lymphoma (Revised European American Lymphoma classification).[7] According to the last WHO classification,[8] IVLBCL was defined as a rare subtype of extranodal diffuse large B-cell lymphoma, and has been classified as an independent disease entity in the recent revision of classifications.[9]

Definition of IVLBCL

According to the current WHO classification, IVLBCL is defined as an extranodal B-cell lymphoma characterised by tumour involvement in the lumina of vessels, especially capillaries, with the exception of larger arteries and veins (figure 1).[9] Lymphoadenopathy is usually absent in IVLBCL. Peculiar characteristics of this lymphoma result in tumour cells involving all types of organs, such as bone marrow, the CNS, skin, lung, adrenal gland, liver, kidney, spleen, thyroid, pituitary gland, and gastrointestinal tract, among others, with insult to organs resulting from tumour infiltration.[10–18] The cells of origin for IVLBCL are not completely understood, but might be postgerminal-centre cells, on the basis of the presence of somatic mutations in immunoglobulin heavy chain variable region (VH) gene analyses.[19]

Morphology and immunophenotype

Neoplastic lymphoid cells mainly exist in the lumina of small or intermediate-sized vessels in various organs. Tumour cells are large with prominent nucleoli and frequent mitotic figures. According to the recent consensus meeting of expert haemopathologists and clinicians for IVLBCL, rare cases of this disease show cells with anaplastic features or of smaller size, and minimum extravascular location of neoplastic cells can sometimes be seen.[20] Sinusoidal involvement occurs in the liver, spleen, and bone marrow (figure 2).[21–23] Malignant cells are occasionally detected in peripheral blood.[24] Most IVLBCL tumour cells immunophenotypically express B-cell-associated antigens.[9] Several case

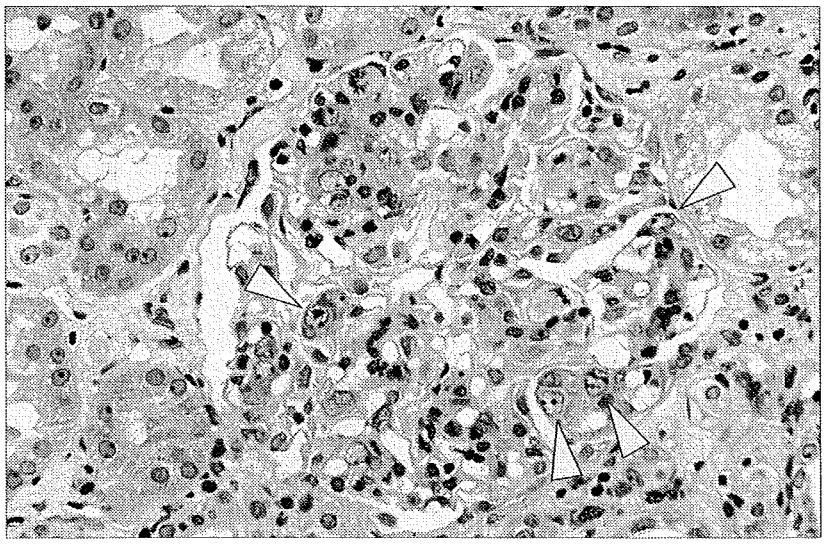

Figure 1: **Intravascular large B-cell lymphoma (IVLBCL)**
Arrows indicate IVLBCL tumour cells. Tumour cells with large nucleoli and mitosis involve small vessels in glomerular capillaries (haematoxylin and eosin stain, original magnification ×200).

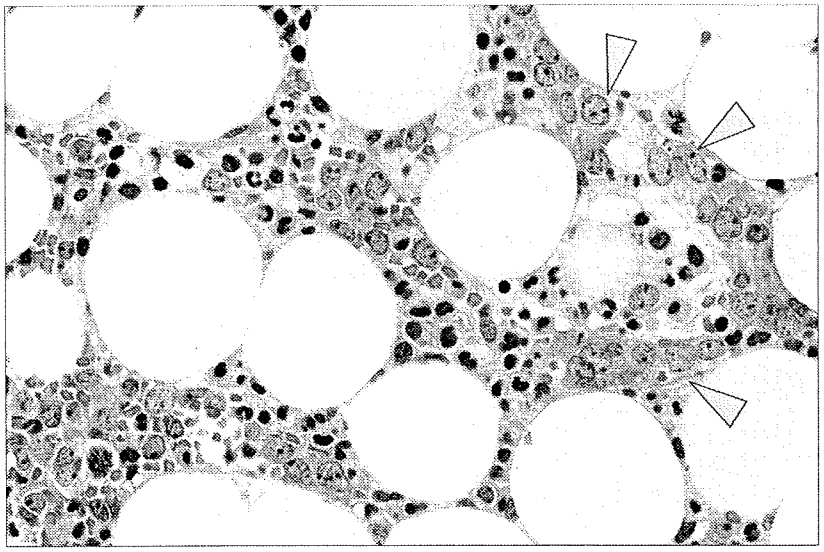

Figure 2: **Bone marrow specimen from a patient diagnosed with intravascular large B-cell lymphoma**
Arrows show tumour cells. Tumour cells show intrasinusoidal patterns (haematoxylin and eosin stain, original magnification ×200). Reproduced with permission from reference 20.

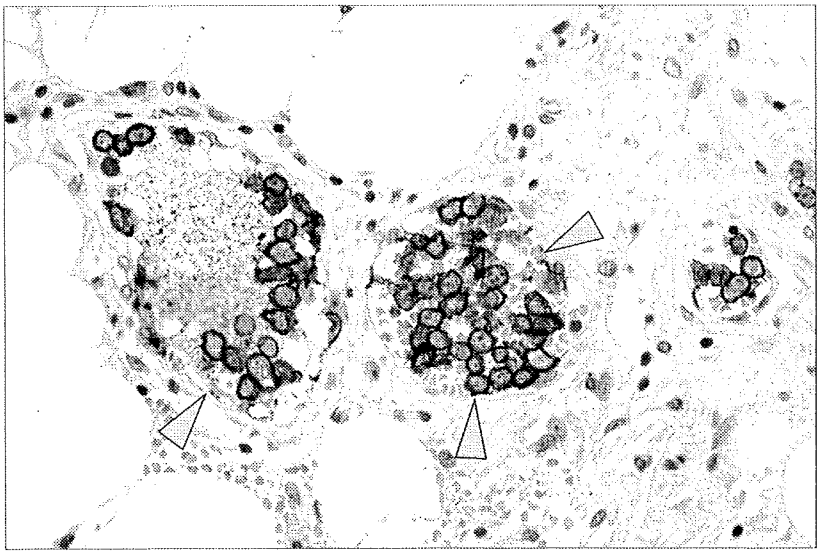

Figure 3: **Immunohistochemical staining for intravascular large B-cell lymphoma**
Arrows show CD20+ lymphoma cells. CD20+ lymphoma cells are increasing in lumina of small vessels in splenic hilar veins (CD20 immunostaining, original magnification ×200).

reports have shown intravascular T-cell or natural-killer-cell lymphoma confirmed by immunophenotypical analysis,[25–27] and some of these cases have been associated with Epstein–Barr virus infection.[28–30] Cases possessing T-cell or natural-killer-cell antigens are thought to represent different disease entities from IVLBCL. Almost all patients with IVLBCL express CD20 (figure 3), and CD5 expression has been shown to be positive in 36 of 96 patients (38%) and CD10 in 12 of 96 patients (13%) with IVLBCL.[17] CD5-positive cases of IVLBCL are accompanied by expression of MUM-1, and categorised as a non-germinal-centre type.[17,31] In a recent analysis of cases in Asian countries, all cases of CD10-negative IVLBCL were categorised as non-germinal-centre types.[17] By comparison, 20% of cases of IVLBCL were classified as germinal-centre B-type in an immunophenotypical analysis of cases in European countries.[21] These findings suggest that almost all cases of IVLBCL are classified as non-germinal-centre types. This finding is shared with other extranodal lymphomas, such as diffuse large B-cell lymphoma of the CNS,[32] but whether this is related to the strange biological behaviour of extranodal distributions remains to be clarified. This characteristic of IVLBCL leads us to postulate that the pathogenetic mechanism in IVLBCL is associated with adhesion molecules or homing receptors necessary for migration into extravascular regions. A small number of reports regarding adhesion molecules have been published. Ponzoni and colleagues[33] suggested that tumour cells of IVLBCL do not express CD29 (beta-1 integrin) or CD54 (intercellular adhesion molecule [ICAM]-1) on the basis of an immunohistochemical analysis of a small number of cases. Other groups have reported that CD11a was related to the presence of tumour cells in vessel lumina.[34,35] However, biological evidence for tumour cells lodging in the lumina of vessels remains insufficient. The difficulties of obtaining sufficient patient samples of IVLBCL and immunohistochemical staining for adhesion molecules have contributed to this paucity of information.

Cytogenetic and genetic features

Characteristics of the cytogenetic and genetic features of IVLBCL remain incompletely understood. Previous reports have suggested that immunoglobulin genes are clonally rearranged.[19,34] In a small case series, structural aberrations in chromosomes 1, 6, and 18, especially 1p (in four of six patients) and trisomy 18 (in four of six patients), were shown.[36] Another study showed a patient with chromosomal abnormalities including t(11;14)(q13;q32), indicating an abnormality of cyclin D1, which was confirmed by karyotype analysis of tumour cells and interphase fluorescence in-situ hybridisation (FISH).[37] In our recent cohort analysis, 48 of 84 (57%) available patients showed cytogenetic abnormalities.[24] Pathognomonic cytogenetic abnormalities have not been reported in IVLBCL until recently.

Clinical features

IVLBCL typically occurs in elderly patients. In our recent cohort, with clinical outcomes updated in December, 2007, the median age of the 106 patients was 67 years (range 34–84), and 76 of 106 patients (72%) were older than 60 years.[24] IVLBCL is equally common in men and women, with a male:female ratio of 1·3:1 (56% *vs* 44%). Tumour cells can involve any systemic organ, with various systemic symptoms, such as fever of unknown origin, general fatigue, marked deterioration in performance status, and neurological alteration. Identifying this disease in patients with such heterogeneous and

non-specific symptoms can be difficult for many clinicians. However, as a result of the recent developments in diagnostic procedures and the spread of knowledge about IVLBCL, the frequency of patients diagnosed with this disease antemortem is believed to be increasing.

Presenting symptoms at initial diagnosis in our cohort compared with those in a European series of patients with IVLBCL are summarised in table 1.[24,38] The clinical manifestations differ between these two distinct geographical areas. In Asian countries, IVLBCL predominantly accompanies a haemophagocytic syndrome known as Asian-variant IVLBCL.[24] In European countries, IVLBCL predominantly involves skin and the CNS, especially as a "cutaneous variant" limited to the skin.[38] In both cohorts, the most common symptom was fever (78 of 106 [74%] and 17 of 38 [45%], respectively), with general fatigue also noted in many patients.[24,38] Furthermore, loss of appetite was the most common symptom in patients with gastrointestinal symptoms in our cohort.[24] The fact that fever, general fatigue, and loss of appetite without lymphadenopathy, all of which are extremely common symptoms, are the most prevalent symptoms of IVLBCL directly contributes to the difficulty of accurate and timely diagnosis.

Neurological symptoms at initial diagnosis were noted in 25% of patients in our cohort.[24] Although this proportion is smaller than that noted in the European series,[12,38,39] neurological symptoms are important symptoms leading to accurate diagnosis. Neurological symptoms noted in patients with IVLBCL are heterogeneous, and alteration of consciousness, motor and sensory deficits, seizure, paresis, dementia, intentional tremor, disorientation, and gating disturbance were recorded in our cohort.[24] Patients with IVLBCL often present with more than one neurological symptom. Various causes of these neurological symptoms can be considered. Invasion of tumour cells into the CNS or peripheral nerves, which leads to impaired microcirculation of these nerves, might represent a major cause of these symptoms. High fever and metabolic aberration with disease progression might be considered as important causes of alterations of consciousness, especially in elderly patients with IVLBCL. However, the specificity of neurological testing is low.[40] In terms of the neuroimaging features of IVLBCL, radiological findings are non-specific, and features of IVLBCL and CNS vasculitis can be identical. Computed tomography (CT) is generally considered non-diagnostic and magnetic resonance imaging (MRI) can show non-specific hyperintensity of white-matter lesions suggestive of small-vessel ischaemic disease or demyelination.[41] However, imaging modalities detect CNS involvement in only half of patients with IVLBCL with neurological symptoms.[41] Therefore, suspecting the existence of IVLBCL on the basis of these neurological symptoms is difficult. Nevertheless, clinicians should consider IVLBCL as one of the differential diagnoses in patients with neurological symptoms and fever, and MRI should be done in patients with suspected IVLBCL.

	Asian cohort[24] (n=106), n (%)	European cohort[38] (n=38), n (%)
Fever	78 (74)	17 (45)
General fatigue	28 (26)	6 (16)
Gastrointestinal symptoms	21 (20)	2 (5)
Neurological symptoms	26 (25)	13 (34)
Dyspnoea	21 (20)	1 (3)
Oedema	11 (10)	2 (5)
Urinary tract symptoms	1 (1)	3 (8)
Skin eruptions	6 (6)	15 (39)

Table 1: **Presenting symptoms at initial diagnosis of intravascular large B-cell lymphoma**

Skin lesions differ substantially between the Asian and European series.[24,38] In European countries, 10 of 38 patients (26%) with IVLBCL were diagnosed with the cutaneous variant of the disease.[38] Skin lesions showed heterogeneous morphology and distribution, including painful indurate erythematous eruption, violaceous plaques, cellulitis, solitary plaques, and ulcerated nodules.[38] A few studies have reported generalised telangiectasia as a clinical manifestation of IVLBCL.[42,43] In our recent cohort, two patients showed skin eruption at initial onset of disease and an additional four patients developed skin eruptions before diagnosis.[24] Furthermore, 13 patients without skin eruptions were diagnosed with skin involvement of tumour cells on the basis of random skin biopsies. To accurately assess the incidence of skin involvement in Asian countries, further studies are needed in which random skin biopsies are used. Such a wide difference between Asian and European countries, in terms of neurological symptoms and skin eruptions, is deemed to reflect differences in clinical manifestations between the two groups.

As a characteristic of Asian-variant IVLBCL, haemophagocytic syndrome represents the most relevant clinical manifestation.[44,45] Haemophagocytic syndrome was noted in 63 of 106 (59%) of patients in our recent cohort.[24] However, haemophagocytosis was absent in patients with IVLBCL in a European series.[12] To our knowledge, only small numbers of patients with IVLBCL and haemophagocytic syndrome have been reported in USA and France.[22,46] This difference between distinct geographical areas is intriguing. Although the reason underlying the prevalence of haemophagocytosis in Asian series is unknown, this difference might exist as a result of ethnic differences associated with the production of inflammatory cytokines, including interferon-γ, tumour necrosis factor-α, and interleukin-1β, and soluble interleukin-2 receptor (sIL2R), leading to systemic inflammatory response.[47,48] In fact, sIL2R levels were significantly higher in patients with Asian-variant IVLBCL than in patients with non-Asian-variant IVLBCL in our series.[24]

	Number of patients, n (%)
Anaemia (Hb <110 g/L or RBC <350×10⁹/L)	72 (68)
Thrombocytopenia (platelet count <100×10⁹/L)	62 (58)
Leucocytopenia (WBC <4·0×10⁹/L)	29 (27)
Increased lactate dehydrogenase (>ULN)	104 (98)
Hypoalbuminaemia (<30 g/L)	62 of 102 (61)
Bilirubin (>15 mg/L)	18 of 102 (20)
Creatinine (>15 mg/L)	11 of 102 (13)
C-reactive protein (>50 mg/L)	61 of 104 (59)
Soluble interleukin-2 receptor (>5000 U/mL)	63 of 96 (66)

Hb=haemoglobin. RBC=red-blood-cell count. WBC=white-blood-cell count. ULN=upper limit of normal.

Table 2: **Abnormalities on blood examination in the Asian cohort (n=106)[24]**

Other organ involvement including liver, lung, and kidney should be mentioned. These organs might be potential targets for diagnostic biopsies. In our cohort, liver dysfunction (bilirubin >25·7 μmol/L) was noted in 18 of 102 patients (18%) and renal dysfunction (serum creatinine concentration >132·6 μmol/L) was noted in 11 of 102 patients (13%).[24] In fact, 14 of 81 patients (17%) were diagnosed with IVLBCL by liver biopsy.[17] Respiratory symptoms and lesions, including dyspnoea, hypoxia, and pulmonary involvement of tumour cells were noted in 35 of 106 patients (33%).[24] In a study from a single institute, hypoxia diagnosed by blood-gas analyses was noted in 11 of 12 patients, irrespective of the existence of clinical symptoms.[49] This finding suggests that potential pulmonary involvement of IVLBCL is more common than generally recognised. Furthermore, a few studies have reported endocrine dysfunction with IVLBCL.[50] A case of hypopituitarism with IVLBCL and gradual reversal of pituitary dysfunction after immunochemotherapy has been reported.[10]

Staging

Ferreri and colleagues[12] reported stage IV disease accounting for 76% of cases of prevailing cutaneous variant of IVLBCL in the European series.[12] In another report, 12 of 30 patients (40%) with in-vivo diagnosis of IVLBCL showed stage IE disease, according to the Ann Arbor staging system.[38] In our cohort, all patients with IVLBCL were classified with stage IV disease.[24] In view of the fact that autopsy cases showed disseminated disease and random skin biopsies showed tumour-cell involvement, despite an absence of apparent skin eruptions, IVLBCL can spread widely in the absence of apparent signs of involvement of various organs.

Abnormalities on blood examination

In our cohort, all patients with IVLBCL showed various abnormalities on blood examination (table 2).[24] The most common abnormality was increased concentration of serum lactate dehydrogenase, which was noted in 104 of 106 patients (98%) in our cohort and in 33 of 39 (69%) in the European series.[12,24] Haematological abnormalities are common; in our cohort, 72 of 106 patients (68%) had anaemia (haemoglobin concentration <110 g/L or red-blood-cell count <350×10⁹ per L), 62 of 106 (58%) had thrombocytopenia (platelet count <100×10⁹ per L), and 29 of 106 (27%) had leucocytopenia (white-blood-cell count <4·0×10⁹ per L).[24] In the European series, anaemia (haemoglobin concentration <120 g/L) and leucocytopenia (white-blood-cell count <4·0×10⁹ per L) have been noted to the same extent, but thrombocytopenia (platelet count <150×10⁹ per L) was less frequent, reflecting the existence of haemophagocytosis in the Asian cohort.[38] SIL2R level was substantially high (>5000 U/mL) in 63 of 96 patients (66%) in our series.[24] Hypoalbuminaemia (<30 g/L) is common in Asian series (62 of 102 patients [61%]), but not in the European series (six of 33 [18%]).[12,24] Substantially increased aminotransferase concentrations are also uncommon, with only 20% of patients showing increased aminotransferase concentrations above the upper limit of normal in our cohort.[24]

Diagnosis of IVLBCL

Organ biopsies are mandatory for the accurate diagnosis of IVLBCL. Timely and accurate diagnosis is extremely important for patients with this disease, because appropriate treatment can improve clinical outcomes.[24] However, no standard procedure for accurate diagnosis of IVLBCL has been established. In view of the fact that tumour involvement can occur in any organ and identification of tumour cells is relatively easy from pathological specimens, the organs selected by the physician for biopsy are key to accurate diagnosis, when physicians suspect the existence of this type of disease from symptoms such as fever or increased serum lactate dehydrogenase concentration. In Asian cohorts, the most relevant diagnostic site seems to be the bone marrow.[17] Tumour-cell involvement with intrasinusoidal patterns in bone-marrow biopsy specimens is common (figure 2). In fact, a previous report showed a role for repeated bone-marrow biopsies in accurate diagnosis.[11] Furthermore, reports on random skin biopsies are promising for prompt diagnosis.[42,51–53] The fact that tumour cells are identified from random skin biopsies, not only in European series in which the cutaneous variant is prevalent, but also in Asian series in which the cutaneous variant is rare, is remarkable. In our recent cohort, the increasing number of random skin biopsies has led to an increase in the number of patients who can be diagnosed with IVLBCL by this diagnostic procedure.[24] However, some patients present with negative findings from random skin biopsies. Further large-scale studies are needed to clarify the significance of random skin biopsies, because the number of studies on this new diagnostic procedure is currently too small to confirm its usefulness. Renal biopsies under CT guidance have also been reported.[54] However, the severe complication of intraperitoneal

haemorrhage on renal biopsy has occurred in patients with IVLBCL (Shimada K, unpublished data). In our cohort, disseminated intravascular coagulation had developed by the time of diagnosis in 18 of 72 patients (25%).[24] Physicians should therefore pay close attention to laboratory findings when organ biopsies are done. Regarding pulmonary investigations, 11 patients in our cohort received lung biopsies and were diagnosed with pulmonary involvement of tumour cells.[24] Transbronchial lung biopsies might be useful for diagnosis, especially in patients with respiratory symptoms.

In the assessment of patients with non-Hodgkin lymphoma, 18[F]-fluorodeoxyglucose (FDG)-PET has emerged as a powerful functional imaging tool.[55] Use of FDG-PET has been recommended for initial staging and post-treatment assessment in diffuse large B-cell lymphoma.[56] However, the role of FDG-PET in IVLBCL remains controversial. In view of the aggressive and progressive clinical behaviour with increased lactate dehydrogenase concentrations in almost all cases, rapid sugar consumption due to high cell turnover in IVLBCL would be expected.[57,58] FDG-PET in this disease is therefore expected to be useful, as it is in diffuse large B-cell lymphoma. In fact, a small number of case reports have shown the usefulness of FDG-PET for diagnosis of IVLBCL, when the disease was clinically suspected, especially due to fever of unknown origin.[59–61] However, we assessed the accuracy of FDG-PET in detecting disease involvement of IVLBCL.[57] In our study, FDG-PET was able to detect only two of seven pathologically confirmed lesions as positive FDG-PET findings and the number of tumour cells in pathological specimens tended to be high when FDG-PET and biopsy findings matched.[57] This finding suggests that the number of tumour cells per volume might be lower for IVLBCL, with selective growth of tumour cells in the lumina of small vessels, than for nodal lymphoma. Although diagnostic accuracy of FDG-PET in IVLBCL might be lower compared with nodal diffuse large B-cell lymphoma, FDG-PET could detect useful information leading to accurate diagnosis and prediction of severe complications, especially pulmonary complications as described in the toxicity section, which could not be obtained using conventional diagnostic methods.[62,63] Further studies are needed to establish the role of FDG-PET in this disease.

Treatment of IVLBCL

Clinical outcomes of IVLBCL were extremely dismal before the rituximab era. One of the important reasons for such dismal prognosis was the difficulty of timely and accurate diagnosis. In previous reports, about half of patients were diagnosed post mortem and the effect of steroid therapies was tentative.[64] In fact, we still occasionally see and hear of patients diagnosed with IVLBCL post mortem. In 1994, DiGiuseppe and colleagues[65] reported clinical outcomes for ten patients at Johns Hopkins Hospital (MD, USA). In their report, four

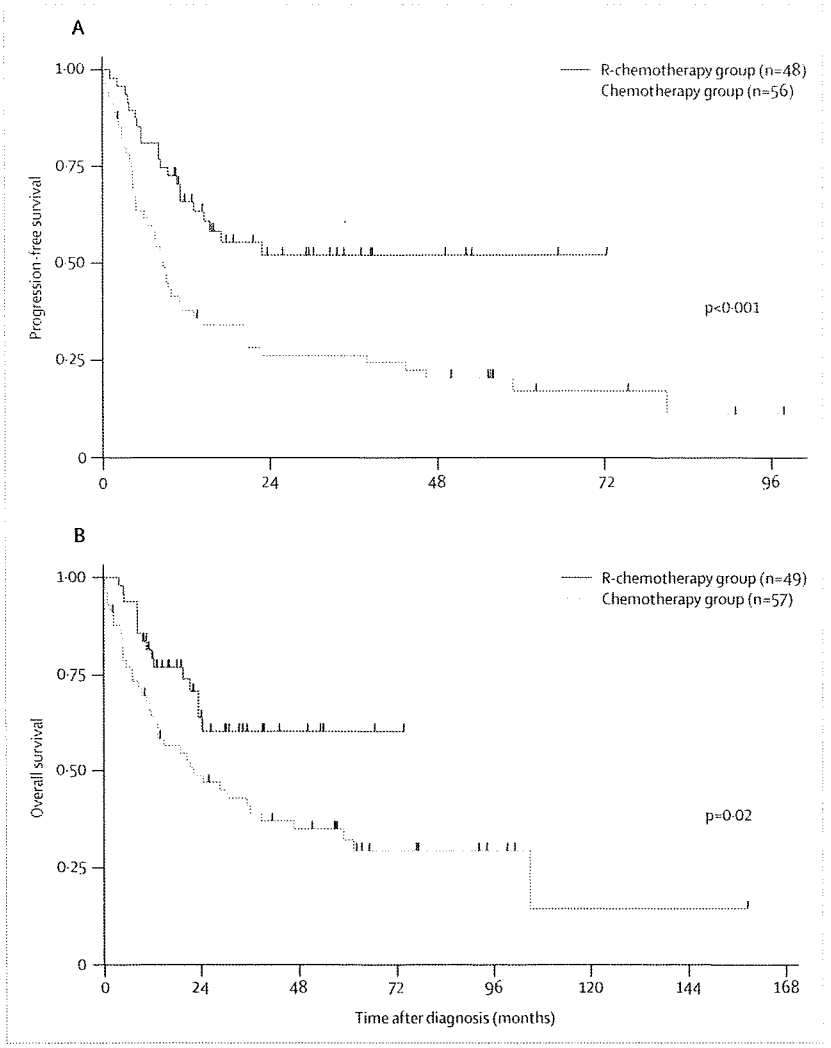

Figure 4: **Progression-free survival (A) and overall survival (B) for patients who received chemotherapy with rituximab (R-chemotherapy) or without rituximab (chemotherapy) in our recent cohort**

of the ten patients received chemotherapy for IVLBCL, and two of the four patients receiving chemotherapy survived for around 4 years after diagnosis. The median survival of the remaining six patients without chemotherapy was 3 months (range 1–19). Ferreri and co-workers[66] reported clinical outcomes for 22 patients who received chemotherapy in 2004. 3-year overall survival was 33%. In 2008, we reported a retrospective analysis of clinical outcomes for 106 patients receiving chemotherapy with or without rituximab.[24] We assessed the clinical outcomes of 57 patients receiving chemotherapy without rituximab and 49 patients receiving chemotherapy with rituximab. With a median follow-up of 18 months (range 1–95), progression-free survival (PFS) and overall survival at 2 years in patients receiving chemotherapy with rituximab were 56% and 66%, respectively.[24] In patients who received chemotherapy without rituximab, PFS and overall survival at 2 years were 27% and 46%, respectively.[24]

Search strategy and selection criteria

Data for this Review were identified by searches of PubMed using the following search terms: "lymphoma", "intravascular and large-cell lymphoma", "angiotrophic lymphoma", "angiotrophic lymphomatosis", "hemophagocytic syndrome", and "FDG-PET". Reports from correspondence in the *Journal of Clinical Oncology* were included only when related directly to previously published work. The definition of IVLBCL was referred to by "WHO Classification Tumours of Haematopoietic and Lymphoid Tissues". Except for papers regarding the history of IVLBCL, only reports published between January, 1984, and May, 2009, were included. Only articles published in English were used.

In the follow-up data,[24,67] with a median follow-up of 26 months (range 10–74) in patients receiving chemotherapies with rituximab, PFS and overall survival at 3 years were 53% and 60%, respectively (Kazuyuki Shimada [unpublished data]; figure 4). This finding suggests that clinical outcomes for rituximab-containing chemotherapy remain constant for more than 2 years of follow-up. Ferreri and colleagues[68,69] also showed improved clinical outcomes for patients with IVLBCL who received immunochemotherapies in European countries.[68,69] In their study, overall survival at 3 years was 81% in 33 patients who received chemoimmunotherapies.[68] Both of the retrospective studies from Asian and European countries suggested improvement of clinical outcomes of IVLBCL in the rituximab era.

Several reports have shown efficacy for high-dose therapy with autologous stem-cell support (ASCT) in patients with IVLBCL.[70–72] In our study, ten of 14 patients who received ASCT did so in the first remission.[24] Seven patients survived without relapse after ASCT until the end of the study.[24] In view of the fact that about 80% of patients with IVLBCL were classified into the high-risk group according to international prognostic index (IPI), ASCT in first remission might represent a useful treatment option.[73] In our cohort, median age at diagnosis in patients with IVLBCL was 67 years (range 34–84), and 55% of patients were more than 65 years of age.[24] This meant that most patients with IVLBCL were ineligible for ASCT.

Toxic effects of immunochemotherapy

Toxic effects associated with administration of rituximab should be mentioned. The characteristic of tumour-cell growth in the lumina of small vessels is deemed to have major ramifications for severe immunoreactions with rituximab infusion. In fact, severe pulmonary complications related to rituximab infusion as an initial treatment have been reported.[63] In our study, 14 (29%) of 49 patients developed adverse events related to rituximab infusion (fever [n=12]; hypotension [n=9]; hypoxia [n=5]; and chills [n=3]). Grade 3 hypoxia was noted in one patient (2%) who received rituximab infusion on the first day of treatment.[24] Mild adverse effects, including chills, fever, and hypotension were also noted in three of 34 patients (12%), and severe adverse events, including pulmonary failure and coma after the first course of treatment, were noted in two of 34 patients (6%) in a recent European cohort.[68] Therefore, delaying rituximab administration by several days in the first course of treatment might be a useful option to avoid severe infusion reactions to rituximab, especially in patients with severe organ damage, including hypoxia related to pulmonary involvement.

Prognostic factors

Ferreri and colleagues[66] reported favourable prognostic factors, including performance status 1, disease limited to the skin, stage I disease, and use of chemotherapy in European countries. Murase and colleagues[17] also reported use of chemotherapy as a favourable prognostic factor in an Asian series. Both cohorts included a small number of patients using immunochemotherapies, so these findings represent prognostic factors in the pre-rituximab era. In our study, use of rituximab was identified as a favourable prognostic factor for IVLBCL.[24] Because almost all patients with IVLBCL were categorised in the high-risk group according to IPI in our cohort,[24] the established prognostic index might not be useful. Up to now, predictive factors that are useful for risk-stratification of patients have not been established.

Conclusion and future perspective

With recent developments in the understanding of disease manifestation, the accuracy of diagnosis for IVLBCL seems to be increasing. Recent retrospective analyses of Asian and European cohorts suggest that clinical outcomes of IVLBCL have improved in the immunochemotherapy era.[24,67,68,69] Although the difficulty of obtaining tumour cells from patients is related to research difficulties, in terms of biological aspects, we expect further improvements in the understanding of this rare type of lymphoma. In terms of treatment strategy, whether the rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) regimen is a sufficient treatment strategy should be investigated. In view of the high incidence of CNS relapse and involvement at initial diagnosis, CNS-oriented therapy, including high-dose methotrexate, might be warranted.[68] Further investigations are mandatory to achieve better outcomes for patients with IVLBCL.

Contributors

KS contributed to the idea for the paper, literature search, and writing. TK helped with the idea, writing, financial support, and critical review. TN helped with writing, financial support, and critical review, and SN contributed to the writing and critical review.

Conflicts of interest

KS, TK, and SN have received honoraria from Chugai Pharmaceutical Ltd (Tokyo, Japan). TK has received a grant for research from Chugai Pharmaceutical Ltd and Zenyaku Kogyo (Tokyo, Japan). TN declared no conflicts of interest.

Acknowledgments

This work is supported in part by Grant-in-Aid for Cancer Research (19-8) from the Ministry of Health, Labour and Welfare, Tokyo, Japan, and Kitamura Memorial Fund, Nagoya, Japan.

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blood

2009 113: 4885-4893
Prepublished online Feb 26, 2009;
doi:10.1182/blood-2008-08-175208

Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.