表 1 Patients' characteristics

Stage	Platform	Source	No. samples	Female (%)	Age (mean ± SD)	Cancer types, *N*			
						Pancreatic	Lung	Bile duct	Others
GWAS									
ADR	Illumina HumanHap610-Quad	BioBank Japan	21	45.0	64.8 ± 10.9	12	6	1	2
non-ADR	Illumina HumanHap610-Quad	BioBank Japan	58	41.8	64.0 ± 8.7	23	19	10	1
Replication study									
ADR	Invader assay	BioBank Japan, Sapporo Medical University, Wakayama Medical University, Kure Kyosai Hospital	33	35.5	64.2 ± 9.9	28	3	4	3
non-ADR	Invader assay	BioBank Japan, Sapporo Medical University, Wakayama Medical University, Kure Kyosai Hospital	62	30.2	64.9 ± 9.0	36	7	17	2

ADR : adverse drug events

表 2 Summary of association results of GWAS and replication study

SNP	Chromosome	Chromosome location*	Gene	Allele 1/2 (risk)	Stage	ADR				non-ADR				*P* value			False discovery rate	Odds ratio (95% CI)†
						11	12	22	RAF	11	12	22	RAF	Allelic	Dominant	Recessive		
rs11141915	9	89425614	*DAPK1*	T/G (T)	GWAS	18	3	0	0.93	21	30	7	0.62	1.27×10^{-4}	1.04×10^{-4}	1.80×10^{-1}		7.94 (2.32-27.25)
					Follow up	22	11	0	0.83	23	31	8	0.62	2.77×10^{-3}	9.23×10^{-3}	4.73×10^{-2}	0.185	3.05 (1.45-6.41)
					Combined	40	14	0	0.87	44	61	15	0.62	1.27×10^{-6}	6.91×10^{-6}	6.11×10^{-3}		4.10 (2.21-7.62)
rs1901440	2	134154429	No gene	A/C (C)	GWAS	11	3	7	0.40	31	27	0	0.23	4.42×10^{-2}	1.00×10^{-0}	4.01×10^{-5}		60.52 (5.45-632.87)
					Follow up	20	8	5	0.27	42	19	1	0.17	1.30×10^{-1}	5.05×10^{-1}	1.82×10^{-2}	0.655	10.89 (1.22-97.64)
					Combined	31	11	12	0.32	73	46	1	0.20	1.44×10^{-2}	7.39×10^{-1}	3.11×10^{-6}		34.00 (4.29-269.48)
rs12046844	1	66010967	*PDE4B*	T/C (C)	GWAS	1	5	15	0.83	12	32	14	0.52	3.93×10^{-4}	1.95×10^{-4}	1.67×10^{-1}		7.86 (2.56-24.12)
					Follow up	4	10	19	0.73	7	34	21	0.61	1.50×10^{-1}	3.09×10^{-2}	1.00×10^{-0}	0.545	2.65 (1.11-6.31)
					Combined	5	15	34	0.77	19	66	35	0.57	3.05×10^{-4}	4.56×10^{-5}	3.43×10^{-1}		4.13 (2.10-8.14)
rs11719165	3	196067377	No gene	C/T (C)	GWAS	9	10	2	0.67	5	27	26	0.32	1.15×10^{-4}	3.49×10^{-3}	1.21×10^{-3}		4.27 (2.01-9.05)
					Follow up	9	16	8	0.52	7	31	24	0.36	4.61×10^{-2}	1.78×10^{-1}	8.12×10^{-2}	0.741	1.87 (1.02-3.42)
					Combined	18	26	10	0.57	12	58	50	0.34	5.98×10^{-5}	3.26×10^{-3}	3.66×10^{-4}		2.60 (1.63-4.14)

RAF, risk allele frequency : CI, confidence interval : GWAS, genome-wide association study.
*Based on NCBI 36 genome assembly.
†Odds ratios were shown for the model with minimum *P* values.

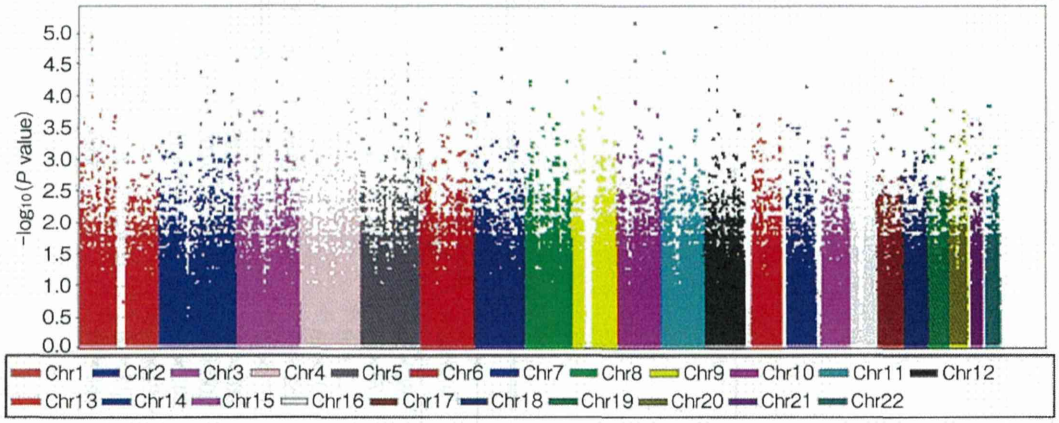

図 1 マンハッタンプロット

ゲノム全体のマーカー SNP（点）について各染色体を横軸に，ジェムシタビンによる骨髄抑制との関連の強さを縦軸に表示している。ほとんどの SNP（点）が下方に位置し関連が認められない一方で，いくつかの SNP は強い関連がある可能性が示されている。

表 3 Prediction scores of gemcitabine-induced sever leukopenia/neutropenia using rs11141915, rs1901440, rs12046844 and rs11719165

Score	ADR, *N*（%）(*N*=54)	non-ADR, *N*（%）(*N*=120)	Odds ratio（95% CI）*P* value	General control, *N*（%）(*N*=934)
0	4（7.4%）	50（41.7%）	1.00（reference）	271（29.0%）
1	9（16.7%）	50（41.7%）		423（45.3%）
2	28（51.9%）	18（15.0%）	11.97（5.23-27.37）6.25×10^{-10}	194（20.8%）
3	13（24.1%）	2（1.7%）	50.00（10.13-246.90）4.13×10^{-9}	46（4.9%）
		（trend test）	9.91（5.56-17.67）1.31×10^{-14}	

CI, confidence interval.

アを示すことが確認された（trend test $P=1.31\times10^{-14}$）。さらに日本人一般集団をこのスコアリングシステムにあてはめた場合の分布を検討した結果，0 点が 29.0%，1 点が 45.3%，2 点が 20.8%，3 点が 4.9%になることが示され，このスコアリングシステムをジェムシタビン治療開始前に応用することで骨髄抑制の危険性が少なくより安全かつ適切な治療選択に有用となる可能性が示された（図 2）。

V．考　察

われわれはゲノムワイド関連解析によりジェムシタビンによる骨髄抑制と深い関係があると考えられる遺伝領域として，9 番，2 番，1 番，3 番染色体上の遺伝子多型（SNP）rs11141915，rs1901440，rs12046844，and rs11719165 をそれぞれ同定した。さらにこの四つの遺伝子多型を組み合わせて解析することによりジェムシタビンによる骨髄抑制をより正確に予測できる可能性が示唆された。

本研究において rs11141915 は最もジェムシタビンによる骨髄抑制と強い関連（$P=0.00000127$，オッズ比 4.10），を示したが，この SNP は *DAPK1* 遺伝子の 3 番目のイントロン上に存在する。*DAPK1* 遺伝子はリン酸化酵素の一種で骨髄や末梢血細胞において発現していることが知られている。この遺伝子はジェムシタビンを含む抗癌剤に対する耐性と何らかの関係があることが指摘されており，機序は不明だがジェムシタビンによる骨髄抑制を引き起こす上で重要な役割を担っている可能性が高いものと考えられる[6]。

また，rs12046844 はジェムシタビンによる骨髄抑制との関連が $P=0.0000456$，オッズ比 4.13 であったが，このSNPを含む領域には*PDE4B*遺伝子が含まれていた。*PDE4B* 遺伝子は加水分解酵素の一種であるが，好中球や単球などで機能しており炎症細胞の活性調節を担っている。また肺癌においてジェムシタビン耐性に関係していることが指摘されていることも考慮する

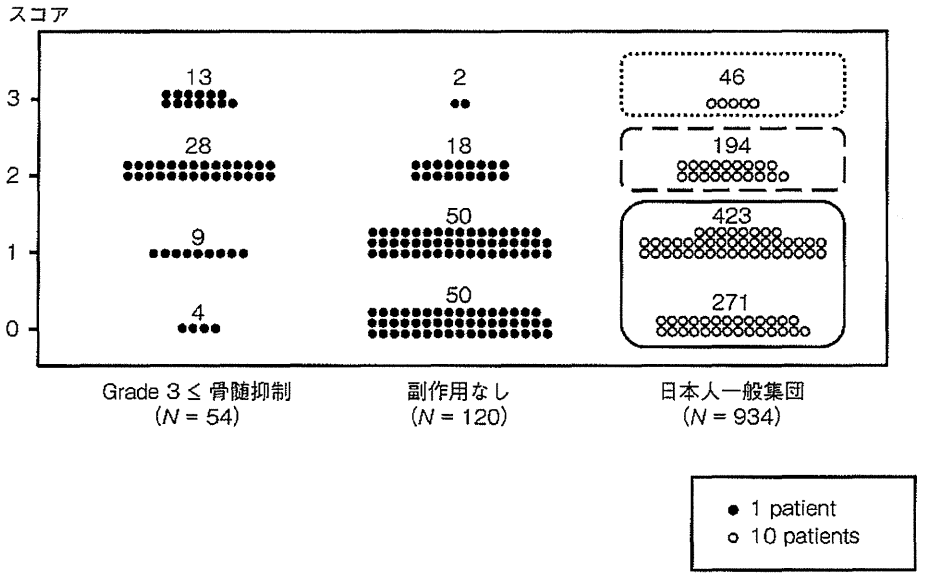

図 2　四つの遺伝情報を用いたジェムシタビン骨髄抑制予測診断システム
4 SNP について骨髄抑制リスクジェノタイプの合計数に応じて各症例をスコアリングした場合の分布図。

と，*PDE4B* はジェムシタビンによる骨髄抑制において重要な役割を担っている可能性が考えられる。

ジェムシタビンが体内に入り細胞に到達し細胞内で代謝を受ける過程においてCDA，dCK，SLC28A1，SLC28A3，SLC29A1 などの遺伝子が関係していることは知られているが[7~14]，今回のゲノムワイド関連解析の結果からは有意水準を超える強い関連を見いだすことはできなかった。つまり，ジェムシタビンによる骨髄抑制はこれまで知られていないメカニズムによって引き起こされている可能性を示唆するものではないかと考えられる。

最後に今回同定された四つの遺伝子多型を含む遺伝領域はジェムシタビンによる骨髄抑制と何らかの関連があることが示唆され，さらにこの四つの遺伝子多型情報を用いた骨髄抑制予測システムによりジェムシタビン治療を行う前に骨髄抑制のリスクを回避できる可能性が考えられる。このようなゲノム情報に基づいた適切かつ安全な治療は今後ジェムシタビンに限らず，多くの薬剤についても応用されていくものと考えられる。

参考文献

1) Mackey JR, Yao SY, Smith KM, et al.：Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst **91**：1876-1881, 1999.
2) Ritzel MW, Ng AM, Yao SY, et al.：Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem **276**：2914-2927, 2001.
3) Mackey JR, Mani RS, Selner M, et al.：Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res **58**：4349-4357, 1998.
4) Plunkett W, Huang P, Gandhi V：Preclinical characteristics of gemcitabine. Anticancer Drugs **6**：7-13, 1995.
5) Kiyotani K, Mushiroda T, Tsunoda T, et al.：A genome-wide association study identifies locus at 10q22 associated with clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients in Japanese. Hum Mol Genet **21**：1665-1672, 2012.
6) Zhang X, Yashiro M, Qiu H, et al.：Establishment and characterization of multidrug-resistant gastric cancer cell lines. Anticancer Res **30**：915-921, 2010.
7) Rha SY, Jeung HC, Choi YH, et al.：An association between RRM1 haplotype and gemcitabine-induced neutropenia in breast cancer patients. Oncologist **12**：622-630, 2007.
8) Sugiyama E, Kaniwa N, Kim SR, et al.：Pharmacokinetics of gemcitabine in Japanese cancer patients：the impact of a cytidine deaminase polymorphism. J Clin Oncol **25**：32-42, 2007.
9) Tanaka M, Javle M, Dong X, et al.：Gemcitabine metabolic and transporter gene polymorphisms are associated with drug toxicity and efficacy in patients with locally advanced pancreatic cancer. Cancer **116**：5325-5335, 2010.

10) Chew HK, Doroshow JH, Frankel P, et al. : Phase II studies of gemcitabine and cisplatin in heavily and minimally pretreated metastatic breast cancer. J Clin Oncol 27 : 2163-2169, 2009.
11) Tibaldi C, Giovannetti E, Vasile E, et al. : Correlation of CDA, ERCC1, and XPD polymorphisms with response and survival in gemcitabine/ cisplatin-treated advanced non-small cell lung cancer patients. Clin Cancer Res 14 : 1797-1803, 2008.
12) Okazaki T, Javle M, Tanaka M, et al. : Single nucleotide polymorphisms of gemcitabine metabolic genes and pancreatic cancer survival and drug toxicity. Clin Cancer Res 16 : 320-329, 2010.
13) Yonemori K, Ueno H, Okusaka T, et al. : Severe drug toxicity associated with a single-nucleotide polymorphism of the cytidine deaminase gene in a Japanese cancer patient treated with gemcitabine plus cisplatin. Clin Cancer Res 11 : 2620-2624, 2005.
14) Soo RA, Wang LZ, Ng SS, et al. : Distribution of gemcitabine pathway genotypes in ethnic Asians and their association with outcome in non-small cell lung cancer patients. Lung Cancer 63 : 121-127, 2009.

*　　*　　*

ELSEVIER

Experimental Hematology

Experimental Hematology 2015;■:■–■

Ribosomal protein L11- and retinol dehydrogenase 11-induced erythroid proliferation without erythropoietin in UT-7/Epo erythroleukemic cells

Q39 Tanawan Kummalue[a], Tomoko Inoue[b,c], Yoshie Miura[b], Megumi Narusawa[b], Hiroyuki Inoue[b,d], Norio Komatsu[e], Wanchai Wanachiwanawin[f], Daisuke Sugiyama[c], and Kenzaburo Tani[b,d]

[a]*Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand;* [b]*Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan;* [c]*Department of Research and Development of Next Generation Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan;* [d]*Department of Advanced Molecular and Cell Therapy, Kyushu University Hospital, Fukuoka, Japan;* [e]*Department of Hematology, School of Medicine, Juntendo University, Japan;* [f]*Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

(Received 17 May 2014; revised 8 January 2015; accepted 11 January 2015)

Q1 **Erythropoiesis is the process of proliferation, differentiation, and maturation of erythroid cells. Understanding these steps will help to elucidate the basis of specific diseases associated with abnormal production of red blood cells. In this study, we continued our efforts to identify genes involved in erythroid proliferation. Lentivirally transduced UT-7/Epo erythroleukemic cells expressing ribosomal protein L11 (RPL11) or retinol dehydrogenase 11 (RDH11) could proliferate in the absence of erythropoietin, and their cell-cycle profiles revealed G_0/G_1 prolongation and low percentages of apoptosis. RPL11-expressing cells proliferated more rapidly than the RDH11-expressing cells. The antiapoptotic proteins BCL-XL and BCL-2 were expressed in both cell lines. Unlike the parental UT-7/Epo cells, the expression of hemoglobins (Hbs) in the transduced cells had switched from adult to fetal type. Several signal transduction pathways, including STAT5, were highly activated in transduced cells; furthermore, expression of the downstream target genes of STAT5, such as *CCND1*, was upregulated in the transduced cells. Taken together, the data indicate that RPL11 and RDH11 accelerate erythroid cell proliferation by upregulating the STAT5 signaling pathway with phosphorylation of** Q2 **Lyn and CREB.**

Erythropoiesis, the process of production of red blood cells, consists of several stages that depend on various specific cytokines; these factors promote the differentiation and proliferation of hematopoietic stem cells into mature erythrocytes. The maturation process of erythrocytes involves many steps, including chromatin condensation, hemoglobinization, enucleation, and expulsion of certain organelles. Erythropoietin (Epo), the major growth factor in erythropoiesis, plays an essential role in proliferation and prevention of apoptosis, starting at the stage of the initial erythroid precursor.

Understanding erythroid proliferation and maturation will help to clarify the pathogenesis and prognosis of several hematologic diseases that are accompanied by anemia resulting from the abnormal production of erythroid cells. Such insights should lead to improvements in therapeutic approaches for these conditions. Most of these diseases, which include myelodysplastic syndrome and acute erythroleukemia, are still too difficult to manage, and specific treatments remain to be developed. This situation prompted us to elucidate the pivotal genes that control the growth and proliferation of erythroid cells.

To determine novel essential genes involved in this process, we performed studies using UT-7/Epo, an erythropoietin-dependent human erythroleukemic cell line [1]. Based on our previous research, we examined candidate genes with potential roles in erythroid growth and maturation by delivering genes from a human fetal

TK and TI contributed equally to this work.

Offprint requests to: Professor Dr. Kenzaburo Tani, Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; E-mail: taniken@bioreg.kyushu-u.ac.jp

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2015.01.006.

http://dx.doi.org/10.1016/j.exphem.2015.01.006

liver–derived Entry complementary DNA (cDNA) library into UT-7/Epo cells, using a lentiviral system [2,3]. After identifying eight candidate genes in a colony-forming assay, we focused on two potential candidate genes, ribosomal protein L11 (*RPL11*) and retinol dehydrogenase 11 (*RDH11*), in subsequent experiments. Here, we demonstrated that these lentivirally transduced cells could proliferate and produce fetal Hb (γ-globin) and adult Hb (β-globin) in a culture medium that lacked Epo. Moreover, during the proliferation of these erythropoietin-independent transduced cells, the STAT5 signaling pathway was significantly upregulated relative to the levels in parental UT-7/Epo cells.

Materials and methods

Cell culture conditions

The UT-7/Epo cell line [1] was cultured in IMDM (Gibco) supplemented with 10% fetal bovine serum and 1 U/mL human recombinant Epo (R&D Systems, Minneapolis, MN) at 37°C in 5% CO_2.

Screening for candidate genes in erythropoiesis

The process of screening candidate genes involved in erythropoiesis was performed as previously described [2]. In brief, 8 candidate genes with full-length insertions in transduced cells were selected from our previous report. cDNA from each gene was cloned into the pCSII-EF-RfA-IRES2-Venus lentiviral vector (kindly provided by H. Miyoshi, RIKEN, Tsukuba, Japan) using Gateway Clonase Enzyme Mix (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing. Specific lentiviral supernatant was produced from 293T cells and used to transduce UT-7/Epo cells. Cells transduced with each of the 8 lentiviruses were cultured in methylcellulose (Nacalai Tesque, Kyoto, Japan) without Epo for 1 month before analysis.

Hematopoietic colony formation assay

A total of 1×10^4 colony-derived cells were collected and seeded into 1 mL of methylcellulose using a 2.5-mL syringe and an 18G needle. The mixture of cells and methylcellulose was dispensed into 35 × 10 mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) at 1 mL per dish. Dishes were gently tilted and rotated to distribute the methylcellulose evenly, and then 3 mL of sterile water were added into an extra uncovered dish before incubation for 1 month at 37°C and 5% CO_2. Colonies in each dish were counted at day 30 and then picked, cytospun onto glass slides, and stained with May–Grunwald Giemsa solution for microscopic observation. Photographs of colonies were taken using a microscope equipped with the AxioVision software (Zeiss, Oberkochen, Germany).

Western blotting

Transduced cells, including UT-7/Epo cells, were collected at 24, 48, and 72 hours. Cells were lysed with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 1.0% NP-40. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Whole-cell extracts (5 μg/lane) were subjected to 12.5% SDS - polyacrylamide gels, and protein was transferred to PVDF membranes (Bio-Rad, Hercules, CA). The immunoreaction was performed by incubating the membrane for 1 hour at room temperature (RT) with primary antibodies as follows: mouse antihuman BCL-XL (Santa Cruz Biotechnology; dilution, 1:200), mouse anti-human BCL-2 (Santa Cruz Biotechnology; dilution, 1:200), or mouse anti–β-actin (C4, sc-47778, Santa Cruz Biotechnology; dilution, 1:1,000). Membranes were incubated at RT for 1 hour with HRP -conjugated secondary antibody: antimouse IgG Ab (sc-2005, Santa Cruz Biotechnology; dilution of 1:10,000). Antigen–antibody reactions were detected using the enhanced chemiluminescence assay (Amersham Biosciences, Piscataway, NJ). Western blots were analyzed on an LAS3000 (Fuji Film Co., Tokyo, Japan).

Gene expression analysis by quantitative RT-polymerase chain reaction (PCR)

To determine the expression of STAT5 regulated genes, RNAs were extracted from UT-7/Epo and RPL11- and RDH11-transduced cells at day 3 using the RNeasy Mini kit QIAGEN (QIAGEN, Hilden, Germany). Concentration of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) before proceeding to cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Expression of *PIM2* and *CCND1* was analyzed using the Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems/Life Technologies, Grand Island, NY). For detection of *PIM2*, the forward primer was 5′-TGGGCATCCTCCTCTATGAC-3′, and the reverse primer was 5′-GTACATCCTCGGCTGGTGTT-3′. For *CCND1*, the forward primer was 5′-GATCAAGTGTGACCCGGACT-3′, and the reverse primer was 5′-TCCTCCTCTTCCTCCTCCTC-3′. The PCR mixture was as follows: 10 μL Fast SYBR Green master mix (Applied Biosystems), 0.2 μL forward primer (10 μmol/L), 0.2 μL reverse primer (10 μmol/L), 1.0 μL cDNA, and 8.6 μL dH_2O. The PCR conditions were as follows: 95°C for 20 sec (holding stage); 40 cycles of 95°C for 3 sec and 60°C for 30 sec (cycling stage); and 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec (melting curve stage).

To confirm *Bcl-xL* gene expression in RPL11- and RDH11-transduced cells, quantitative RT-PCR was performed using the following primers: h*Bcl-xL* forward: 5′-CTGCCTCACTTCCTAC AAGAGC-3′ and h*Bcl-xL* reverse: 5′-CTGAGGTAGGGAAG ACCCTG-3′. In brief, RNAs were extracted from RPL11- and RDH11-transduced cells and UT7/Epo cells at 24, 48, and 72 hours before converting to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). PCR mixture was: 5 μL Fast SYBR Green master mix (Applied Biosystems), 0.1 μL Bcl-xL forward and reverse primers (10 μmol/L), or 0.1 μL GAPDH forward and reverse primers (5 μmol/L), 1.0 μL cDNA, and 3.8 μL dH_2O. The PCR was performed as above.

Cell proliferation assay

To determine the growth and proliferation of UT-7/Epo and RPL11- and RDH11-transduced cells, proliferation assays were performed using Cell Count Reagent SF (Nacalai Tesque). Briefly, each cell line was seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL culture medium, with or without Epo. After growth for 2, 4, and 6 days, 10 μL of Cell Count Reagent SF was added to each well and incubated for 1 hour at 37°C in 5% CO_2. Absorbance at 450 nm (ref. 650 nm) was recorded using a

microplate reader (Thermo Scientific). The experiments were performed in triplicate, and data were analyzed by plotting the corrected absorbance at 450 nm on the y axis and time points on the x axis.

For detection of growth factors produced in an autocrine manner, culture media from RPL11- and RDH11-transduced cells at 48 hours were collected and filtered through 0.22 μm syringe filter before used. The erythropoietin levels of these collected culture media were measured by LSI Medience Corporation (Tokyo, Japan). The UT7/Epo cells deprived of Epo were cultured with medium collected from RPL11- or RDH11-transduced cells for 2, 4, and 6 days before assessment of cell proliferation using Cell Count Reagent SF, as described above.

Determination of STAT5 signaling pathway involving in cell proliferation using STAT5 inhibitor

To determine whether STAT5 signaling pathway was involved in cell proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor (573108, Merck Millipore, Darmstadt, Germany) was added in culture medium for inhibition of cell growth [4]. Drug was dissolved with dimethyl sulfoxide (DMSO, Nacalai, Japan), diluted with medium, and used at the final concentrations of 100 and 200 μmol/L with 0.1% DMSO in cell proliferation assay.

UT7/Epo with Epo and RPL11- and RDH11-transduced cells were cultured in medium with STAT5 inhibitor at the final concentrations of 100 and 200 μmol/L for 12 hours. After washing the treated cells with PBS, cells were seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL drug-free medium. Untreated cells were used as the control group. Cells were cultured until days 2, 4, and 6 before analysis using Cell Count Reagent SF as mentioned earlier.

Flow-cytometry analysis for intracellular Hb expression

UT-7/Epo and RPL11- and RDH11-transduced cells were cultured in medium with or without Epo for 2 days before analysis of intracellular Hb expression. Cells were collected and fixed with cold 0.05% glutaraldehyde for 10 min at RT. After washing with PBS-0.1% BSA, cells were permeabilized for 5 min at RT with 0.1% Triton X-100 and then blocked with PBS-BSA. Cells were incubated at RT for 15 min with diluted primary antibody in 0.1% BSA in PBS: F-APC–conjugated mouse anti-Hb (Invitrogen; dilution, 1:17) or β-PerCP-Cy5.5–conjugated mouse anti-Hb (Santa Cruz Biotechnology; dilution, 1:200). Antibody-stained cells were analyzed on a FACSCalibur (BD Biosciences) using the CellQuest software.

Cell-cycle analysis

UT-7/Epo and RPL11- and RDH11-transduced cells were seeded in 12-well plates at 2×10^5 cells/well and incubated at 37°C for 24, 48, or 72 hours in medium with or without Epo. At each time point, cells were collected, washed with PBS, and fixed with cold 70% ethanol for 10–14 hours. Cells were incubated with FITC -conjugated anti-BrdU (BD Biosciences) for 30 min, and then treated with RNase A (Nacalai Tesque) and 7-AAD (BioLegend, San Diego, CA) to exclude nonviable cells. The cell-cycle profile (i.e., the proportions of cells at G_0/G_1, S, and G_2/M phases, as well as apoptotic cells) was analyzed on a FACSCalibur with the CellQuest software.

Phosphokinase array for analysis of signaling pathways in transduced cells

To identify the signal transduction pathways activated in transduced cells, samples were analyzed using the Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN). In brief, cells were cultured with and without Epo for 12 hours, and cell lysates were prepared using the lysis buffer provided in the kit. Then, the provided membranes were blocked with Array Buffer 1 prior to incubation with cell lysates. After overnight incubation at 2–8°C, membranes were washed, and specific kinases were detected using Detection Antibody Cocktail A and B, provided in the kit. Membranes were washed and probed with Streptavidin-HRP (BD Biosciences) before being analyzed using an LAS3000 (Fuji Film Co., Tokyo, Japan). Pixel densities were measured using a transmission-mode scanner and image analysis software.

To focus particularly on STAT5 signaling pathway involved in the growth and proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor at final concentration of 100 μmol/L was added into culture medium of all cell lines. After 12 hours, samples were prepared and assayed as above.

Immunocytochemical detection for CREB, Lyn, and JAK2 phosphorylation

To determine the phosphorylation of CREB, Lyn, and JAK2, cells were cultured with or without Epo. After 12 hours, RPL11- and RDH11-transduced cells and UT7/Epo cells were harvested, cytospun at 450 rpm for 5 min, and let dry for 2 hours at RT. Cells were fixed with 1% paraformaldehyde in PBS for 10 min at RT. After washing with ice cold PBS for 3 times, cells were permeabilized and blocked using 0.05 % Triton X-100 in 1% BSA/PBS for 30 min. Cells were then incubated with diluted primary antibody: mouse antihuman phospho-CREB (dilution 1:25, R&D Systems, UK), rabbit antihuman phospho-JAK2 (dilution 1:50, abcam, Cambridge, UK), mouse antihuman phospho-Lyn (dilution 1:25, R&D Systems, UK) in blocking buffer at 4°C overnight. After washing 3 times with PBS, cells were incubated with secondary antibody: Alexa Fluor 647 donkey antimouse (dilution 1:500; Life Technologies), Alexa Fluor 647 donkey antirabbit (dilution 1:500, Invitrogen) for 30 min at RT in the dark. Mounting and fixing were performed using VECTASHIELD with DAPI (Vector Laboratories, Inc., CA) before analysis, followed by the observation using fluorescence imaging with Olympus Ix81 Inverted Microscope ().

In addition, Hela cells treated with 200 nmol/L PMA (phorbol 12-myristate 13-acetate, Sigma) for 2 hours were used as positive control to detect CREB phosphorylation. One mmol/L of Pervanadate was prepared from Sodium orthovanadate (Sigma) and hydrogen peroxide (Nacalai Tesque) diluted with PBS as previously described [5]. Jurkat cells and Hela cells treated with 1 mmol/L Pervanadate were respectively used as positive control for JAK2 phosphorylation and Lyn phosphorylation.

Statistical analysis

Data are shown as means ± SEM. A p value <0.05 was considered to represent statistical significance.

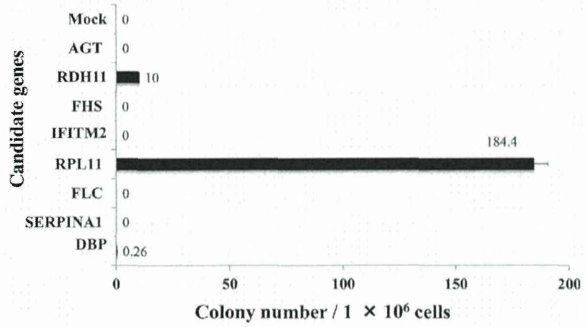

Figure 1. Identification of eight candidate genes involved in erythroid proliferation. From our screening, eight candidate genes with full-length insertions were detected. They were angiotensinogen (*AGT*), retinol dehydrogenase 11 (*RDH11*), ferritin heavy chain subunit (*FHS*), interferon-induced transmembrane protein 2 (*IFITM2*), ribosomal protein L11 (*RPL11*), ferritin light chain (*FLC*), serpin peptidase inhibitor clade A (*SERPINA1*), and D-site binding protein (*DBP*). In colony formation assays, RPL11-transduced cells yielded the highest average number of colonies (about 184). All colonies were cultured for 1 month in semisolid medium without Epo.

Results

Determination of candidate genes, and mechanisms involving in erythroid proliferation of RPL11- and RDH11-transduced cells

To identify candidate genes involved in human erythropoiesis, we first prepared lentiviruses expressing eight candidate genes, and used these viruses to transduce UT-7/Epo cells. These genes encoded angiotensinogen (AGT), ferritin heavy chain subunit (FHS), interferon-induced transmembrane protein 2 (IFITM2), ferritin light chain (FLC), ribosomal protein L11 (RPL11), retinol dehydrogenase 11 (RDH11), serpin peptidase inhibitor clade A (SERPINA1), and D-site (DBP) binding protein. After culture in semisolid medium without Epo for 1 month, we found that two of these candidate factors, RPL11 and RDH11, resulted in formation of a larger number of colonies than the other genes (RPL11, 184.4 ± 6.2; RDH11, 10.0 ± 0; Fig. 1). Colonies were positive for Venus expression (data not shown).

To further investigate cell proliferation, we next transferred the colonies derived from UT-7/Epo and RPL11- and RDH11-transduced cells into liquid culture and subjected them to proliferation assays at various time points. In the assay we used, higher absorbance at 450 nm reflected higher cell proliferation. UT-7/Epo cells incubated with Epo (■) proliferated most rapidly, whereas no proliferating cells could be detected in UT-7/Epo cells incubated without Epo (×), particularly on days 4 and 6 (Fig. 2A). In contrast to nontransduced cells, both of the RPL11- (▲) and RDH11- (●) transduced cells cultured in the absence of Epo increased cell proliferation. Compared to RDH11- (●) transduced cells, RPL11- (▲) transduced cells proliferated 2.35-, 2.67-, and 6.64-fold faster on days 2, 4, and 6, respectively; these differences were statistically significant. In addition, on day 6, RPL11- (▲) transduced cells exceeded the proliferation of UT-7/Epo cells (■) cultured in the presence of Epo. Even under the Epo-free condition, both RPL11- and RDH11-transduced cells maintained their proliferation, suggesting that the products of the transduced genes could substitute for Epo signaling in UT-7/Epo erythroleukemic cells.

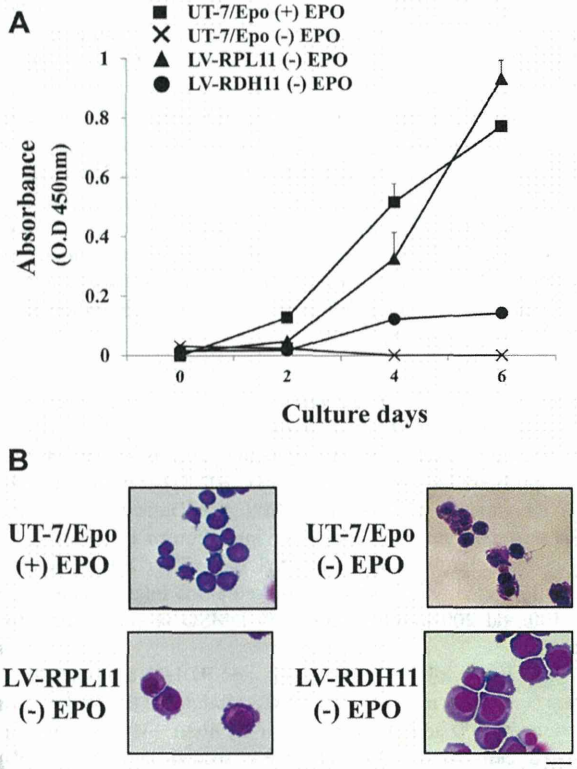

Figure 2. (**A**) Erythroid proliferation of transduced cells cultured without Epo. Cell proliferation assay of UT-7/Epo and RPL11- and RDH11-transduced cells in liquid culture. Without Epo, UT-7/Epo cells could not proliferate, whereas in the presence of Epo, these cells could proliferate very well, especially at days 2 and 4, with average ODs of 0.12 and 0.51, respectively. At day 6, RPL11-transduced cells without Epo yielded the highest cell number among these three groups, with an average OD of 0.93. (**B**) Cell morphology. UT-7/Epo cells in the presence of Epo (Upper left). UT-7/Epo cells, RPL11- and RDH11-transduced cells by lentiviruses (LV-RPL11, LV-RDH11), were cultured in the absence of Epo for 72 hours (upper right, lower left, and lower right, respectively). Cells were cytospun and subjected to May–Grunwald Giemsa staining. Scale bar = 10 um.

Morphological observation by May–Grunwald Giemsa staining indicated that by 72 hours, UT-7/Epo cells cultured without Epo had condensed nuclei and exhibited apoptotic features (Fig. 2B). On the other hand, relatively larger cells with less condensed nuclei were observed in both RPL11- and RDH11-transduced samples, compared with nontransduced cells, irrespective of the presence of Epo. This

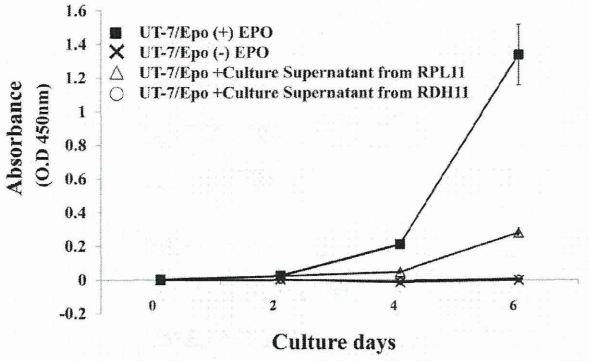

Figure 3. The proliferation of UT-7/Epo cells in the supernatant of RPL11- and RDH11-transduced cells. In order to investigate whether RPL11- and RDH11-transduced cells proliferated in an autocrine manner, UT-7/Epo cells were cultured in the absence of Epo with the supernatant of RPL11- and RDH11-transduced cells. The proliferation of UT-7/Epo cells cultured in the supernatant of RPL11- and RDH11-transduced cells was significantly decreased.

observation implies that RPL11- and RDH11-transduced cells proliferated in an immature state.

To investigate whether RPL11- and RDH11-transduced cells proliferated in autocrine manner, culture medium from respective transduced cells was used to culture UT-7/Epo without Epo. At days 4 and 6, the proliferation of UT-7/Epo cells was moderately suppressed by the culture medium from RPL11-transduced cells but completely suppressed by that from RDH11-transduced cells (Fig. 3). The Epo levels of culture supernatant of respective transduced cells were measured and were not detected, as observed with nontransduced UT-7/Epo without Epo (data not shown).

To evaluate differentiation stage, we used intracellular staining to assess Hb expression in transduced UT-7/Epo cells after 2 days of culture. Based on flow-cytometric analysis, 94.0% of UT-7/Epo cells cultured with Epo expressed β-globin, whereas only 1.2% of them expressed γ-globin. Similarly, UT-7/Epo cells cultured without Epo predominantly expressed β-globin. By contrast, both RPL11- and RDH11-transduced cells cultured without Epo expressed γ-globin (41.5% and 38.3% of cells, respectively), whereas ~30% of both types of transduced cells expressed β-globin (Supplementary Figure 1, online only, available at www.exphem.org). Taken together, these data indicate that transduction of RPL11 and RDH11 into UT-7/Epo cells induced and maintained their proliferation in an immature state.

Change of cell-cycle status in RPL11- and RDH11-transduced cells.

To investigate the mechanisms underlying proliferation, we performed cell-cycle analyses by BrdU and 7-AAD staining, followed by flow cytometry (Supplementary Figure 2, online only, available at www.exphem.org). UT-7/Epo cells cultured with Epo exhibited a prolonged S phase after 24, 48, and 72 hours of culture. On the other hand, UT-7/Epo cells cultured without Epo exhibited a reduction in the number of S-phase cells (35.0%, 17.7%, 8.2%), in accordance with increasing the number of apoptotic cells (0.5%, 5.9%, 14.8%). By contrast, both RPL11- and RDH11-transduced cells cultured without Epo exhibited a lower percentage of apoptotic cells at every time point than non-transduced cells did. UT-7/Epo cells cultured with Epo had the lowest percentage of apoptotic cells among these cell lines, whereas UT-7/Epo cultured without Epo had the highest percentage of apoptotic cells and G_2/M arrest, especially after 72 hours of culture (Fig. 4).

To clarify the mechanisms of inhibition of apoptosis in RPL11- and RDH11-transduced cells cultured without Epo, we evaluated the expression of two antiapoptotic proteins, BCL-XL and BCL-2. We found that both types of transduced cells expressed these proteins. By contrast, UT-7/Epo cultured without Epo did not express either anti-apoptotic protein, reflecting the higher percentage of apoptotic cells in this group. As previously reported [6], prominent BCL-XL expression and slight BCL-2 expression were detected in UT-7/Epo cells cultured in the presence of Epo (Fig. 5). Quantitative RT-PCR to detect BCL-XL expression also showed the same results (Fig. 6).

Signaling pathways of two transduced cell lines

To elucidate the signal transduction pathways involved in RPL11- and RDH11-driven proliferation, we performed phosphokinase array analysis after 12 hours of culture in the absence of Epo (Fig. 7A). The phosphorylation statuses of p53 (S392), Akt (T308), and AMPKa1 were almost the same among the four samples tested: UT-7/Epo cells cultured with or without Epo and RPL11- and RDH11-transduced cells cultured without Epo. The phosphorylation of p38 was the highest in UT-7/Epo cells cultured with Epo, and phosphorylation of p53 (S46) was the highest in RDH11-transduced cells. On the other hand, phosphorylation levels of both CREB and Lyn were higher in RPL11- and RDH11-transduced cells, and phosphorylated Chk-2 and AMPKa2 were upregulated in the Epo-free condition, regardless of gene transduction. Phosphorylated STAT5a (Y699) and HSP27 were downregulated in UT-7/Epo cells cultured without Epo relative to UT-7/Epo cells cultured with Epo; these phosphoproteins were upregulated in RPL11- and RDH11-transduced cells to the same level as in UT-7/Epo with Epo (Fig. 7B).

To ascertain that STAT5 signaling pathway was involved in the proliferation of RPL11- and RDH11-transduced cells, we conducted phosphokinase array and proliferation assay using these cells in the presence of STAT5 inhibitor. Our results from phosphokinase array confirmed that STAT5 phosphorylation was dramatically decreased in the presence of STAT5 inhibitor (Fig. 8A). Importantly, proliferation assay revealed that RDH11-transduced cells showed significantly decreased proliferation at any observed points in the presence of 100 and

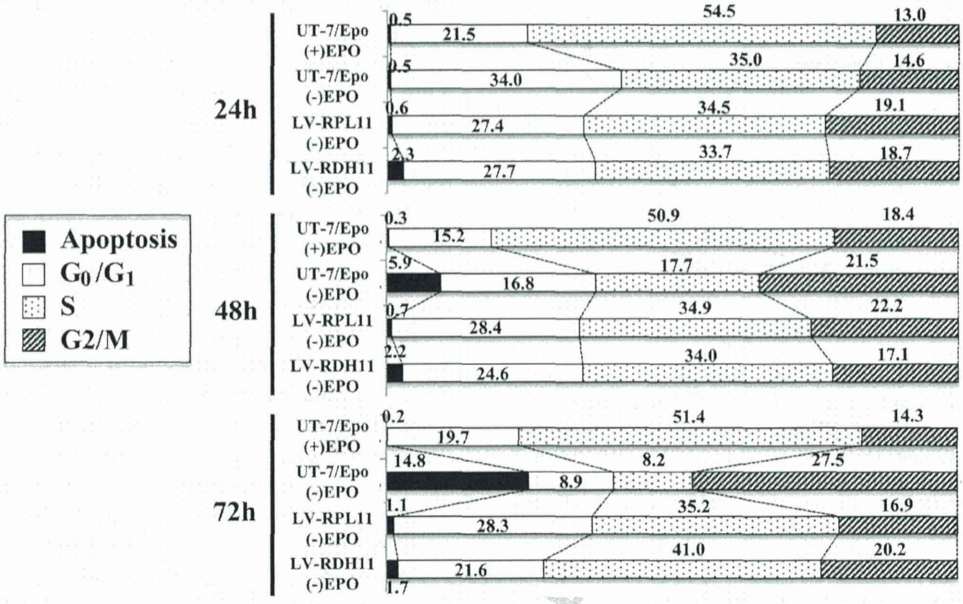

Figure 4. Cell-cycle determination of three cell lines. At 24, 48, and 72 hours after cultured, cells were collected and analyzed with flow cytometry. UT-7/Epo cells without Epo exhibited the highest apoptosis and G_2/M arrest at 72 hours (14.8% and 27.5% of cells, respectively). The lowest percentage of apoptosis and the highest percentage of S phase arrest at every time point were observed in UT-7/Epo cultured with Epo. Between the 2 types of transduced cells, RPL11-transduced cells exhibited the lower percentage of apoptosis than RDH11-transduced cells, especially at 24 and 48 hours. Q35

200 μmol/L STAT5 inhibitor, whereas RPL11-transduced cells showed significantly decreased proliferation only at day 2 in the presence of 200 μmol/L STAT5 inhibitor (Fig. 8B). CREB, Lyn, and JAK2 phosphorylation were also studied using immunocytochemistry, and the phosphorylation of both CREB and Lyn were observed (Fig. 8C). Of note, the phosphorylation of JAK2 could not be demonstrated in our study (data not shown).

To further examine STAT-5 regulated genes, we observed the expression of *PIM2* and *CCND1* by real-time PCR analysis [7,8]. The results showed that *PIM2* expressions were not different among the samples, but *CCND1* expression was elevated by 43.4-fold in RDH11-transduced cells and 2.5-fold in RPL11-transduced cells compared with those in the UT-7/Epo control (Fig. 9).

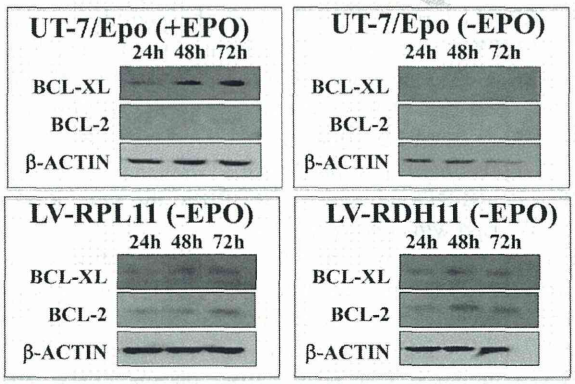

Figure 5. Expression of antiapoptotic proteins was demonstrated by Western blotting. Neither BCL-XL nor BCL-2 was detected in UT-7/Epo cultured without Epo, whereas the expression level of BCL-XL was higher than that of BCL-2 in UT-7/Epo cultured with Epo. Both types of transduced cells also expressed BCL-XL and BCL-2 at every time point. β-ACTIN was used as internal control.

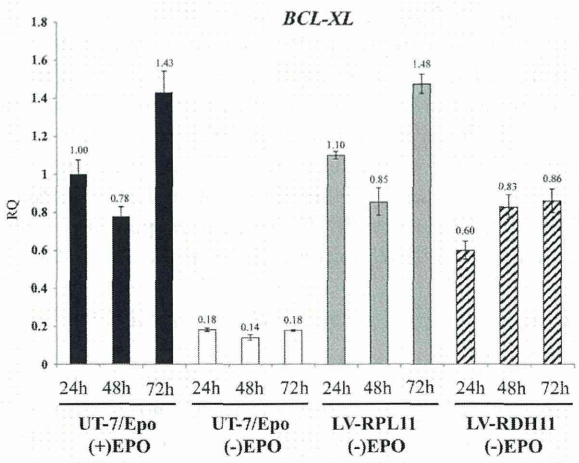

Figure 6. Quantitative RT-PCR of *Bcl-xL* gene. The expression of *Bcl-xL* gene of RPL11- and RDH11-transduced cells was demonstrated. The highest expression was detected in all cell lines at 72 hours. *RQ* = ■■■. Q36