CapG は gelsolin family に属し、アクチンの capping に関わる蛋白であり細胞形態の変化や細胞運動だけでなく、細胞の増殖やアポトーシスあるいは腫瘍化の制御にも関与していることが明らかにされている。ちなみに CapG ノックアウトマウスでは好中球遊走能の低下が認められている。以上より CapG は MDS の病態に深く関与している蛋白であることが示唆される。CapG はリン酸化を受け活性化する蛋白と推測されている。今回、MDS 好中球においてそのリン酸化状態および細胞内局在に異常があることが明らかになった。今後、これらの異常と病態との関連を明らかにする必要がある。
MDS に高発現していた蛋白の 1 つである Thiol-specific antioxident protein（TSA）は peroxiredoxin family に属する蛋白であり、活性酸素種（ROS）消去に重要な役割を担っている。MDS の血球はさまざまなサイトカインによる酸化ストレスにより、活性酸素種が増加していることが知られている。したがって，それらの酸化ストレスに対するフィードバック機構として TSA の発現が増加している可能性が考えられる。その遺伝子発現量は正常および MDS の好中球において有意差は認められず、発現量の増加は翻訳後調節によるものと推定される。
これらの MDS に異常を認める蛋白の機能解析を進めることは、MDS の診断あるいは治療の標的となる蛋白の同定につながるものと考える。

E. 結論

MDS 患者の好中球には正常人のそれとは異なる蛋白の異常発現が認められる。これらの蛋白の解析により，MDS の病因，病態が明らかになり，治療法の開発につながる可能性がある。

F. 健康危険情報

なし

G. 研究発表

Kazama H, Teramura M, Yoshinaga, K, Kato T, Motoji T, Mizoguchi H. Proteomics approach to identifying proteins abnormally expressed in neutrophils in patients with myelodysplastic syndrome. Leukemia Research, 29 (Suppl 1) S46, 2005

H. 知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

厚生労働科学研究費補助金　(難治性疾患克服研究事業)
分担研究報告

CGH アレイを用いた MDS の原因遺伝子の探索

分担研究者　小川誠司　東京大学医学部附属病院　造血再生医療講座　客員助教授

研究要旨
骨髄異形成症候群(MDS)は造血前駆細胞に生ずるゲノム変異に起因する難治性造血器腫瘍疾患である。本年度の研究では、MDS に対する有効な分子標的薬開発のためのターゲット分子を同定することを目的として、Affymetrix 社の SNP タイピング用オリゴヌクレオチドアレイを用いた高精度ゲノム解析システム(CNAG)を構築し、本症候群で生ずるゲノムコピー数の異常およびヘテロ接合性の消失(LOH)のゲノムワイドな解析を行うことにより、MDS の原因遺伝子の探索を行った。種々の病型を含む 150 例の MDS 症例の解析の結果、5q-, 7q-, 20q-など、従来 MDS で高頻度に報告されている染色体の増加・欠失の微細な構造が明らかになるとともに、その他の微細な領域におけるゲノムコピー数の異常と当該領域にコードされる遺伝子が多数同定された。また、MDS ではゲノムの特定の領域が胚細胞レベルで高頻度にホモ接合を生じており、MDS 発症における遺伝的背景の関与が示唆された。今後、アレイ解析で同定された MDS の標的遺伝子の検証と MDS の遺伝的リスクの遺伝子レベルでの解明を試みる。

A. 研究目的

骨髄異形成症候群(MDS)に対しては、現在、造血幹細胞移植以外には根治的治療法がなく、今後、副作用の少ない有効な治療法を確立するためには、本疾患の原因分子の同定とこれを標的とした分子標的薬の開発が重要である。MDS は造血前駆細胞におけるゲノム異常の蓄積によって発症すると考えられるが、RAS や AML1 など一部の例外を除いて、未だこうしたゲノム異常の標的となっている遺伝子に関する知見は限られている。本分担研究では、近年 Affymetrix 社により開発された高密度オリゴヌクレオチドアレイを用いて MDS ゲノムに生ずるゲノムコピー数の異常およびヘテロ接合性の消失(LOH)を網羅的に探索することにより、MDS の発症に関わる標的遺伝子の同定を試みた。

B. 研究方法

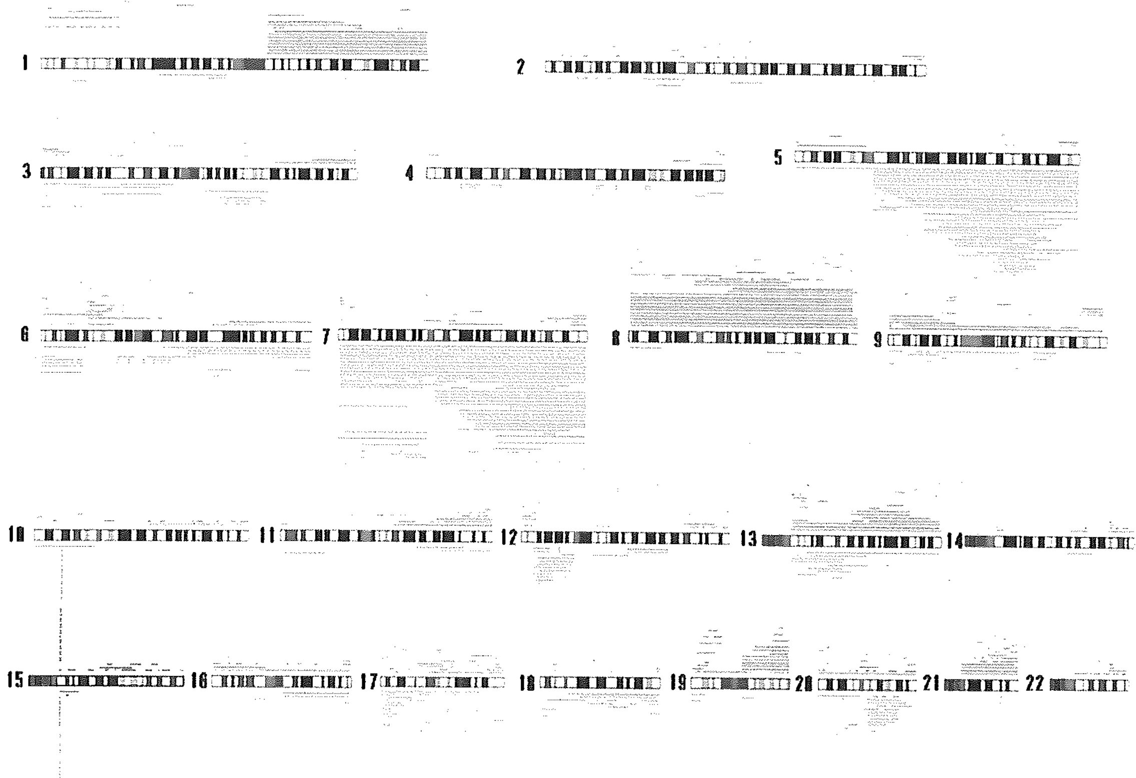
(1)GeneChip 100K/500K アレイを用いたゲノム解析システム(CNAG)の開発
Affymetrix GeneChip 100K/500K は大規模 SNP タイピング用に開発された高密度オリゴヌクレオチドアレイで、数百万個の SNP 特異的プローブセットを用いて、それぞれ 10 万ないし 50 万 SNPs のタイピングが可能であるが、アレイシグナルの定量的特性を利用することにより、ゲノムコピー数の解析に用いることができる。我々は、アレイシグナルに含まれる系統的誤差を統計的に処理することにより、GeneChip を用いた高精度のゲノムコピー数の解析を可能とする独自のアルゴリズムを含むゲノムの統合解析環境(CNAG)の開発を行った。

(2) CNAG を用いた MDS のゲノム解析
種々の病型を含む 150 例の MDS 検体について、ゲノム DNA を抽出し、GeneChip100K ないし 500K アレイによる解析をしたのち、(1)で開発した CNAG を用いて 10 万～50 万個の各プローブのシグナルを正常対照 DNA のアレイ解析で得られたシグナルと比較することにより、ゲノムコピー数の定量を行った。また、同時に得られる大量の SNP 情報に基づいてゲノムワイドな LOH の解析を行った。
(倫理面への配慮)
検討に用いた検体は、当該患者からインフォームドコンセントを得たのちに連結可能匿名化を施して検討に用いた。当院の倫理委員会の承認済みである。

C. 結果

GeneChip を用いたゲノムコピー数の高精度解析プログラム CNAG を独自に開発し、これを用いて MDS におけるゲノム異常の網羅的探索を行った。図 1 に 150 症例の MDS のゲノムコピー数の解析結果を示した。5q-, 7q-, +8, 12p-, 13q-, 17p-, 20q-など、従来の染色体分析により報告されている代表的な染色体異常が明瞭に描出されているのは当然であるが、本解析の特徴は、これらの異常の切断点を極めて高精度(～100Kb 程度)に同定していることである。その結果これらの染色体異常の微細なマッピングが可能となり、上記の染色体の内部で共通に異常を受ける領域を明確に同定することが可能となった。一例を挙げれば、20q の共通欠失領域は約 5 個程度の既知の構造遺伝子がコードされる 400Kb 程度の領域にマッピングされている。一方、これ

図1. Copy number abnormalities found in 150 MDS cases



らの染色体レベルでの異常に加えて、従来の解析手段では同定しえなかった微細な領域におけるコピー数の異常が多数同定された。これらの領域は極めて高い解像度で決定されており、多くの領域について、単一の責任遺伝子の候補を同定することが可能であった(既知の例では、21q の AML1, 12p の K-RAS, 17q の NF-1 など)。

一方、SNP タイピングデータを用いた解析により、多数の LOH を示す領域が同定された。これらの中には、コピー数の減少を伴わない LOH(UPD)が含まれ、それらは 11p など特定の染色体領域に集積する傾向が認められた。さらに、約 20%程度の症例では、アレルの特定の領域が胚細胞レベルで長距離にわたってホモ接合を生じていると考えられる領域が認められ、MDS の発症に遺伝的背景が関与している可能性が示唆された。

D. 考察

Affymetrix GeneChip では 10 万~50 万個の SNP 特異的プローブを用いることにより、全ゲノムについて平均解像度 23.4kb ~5.8kb でゲノムに生ずるコピー数の変化を解析することが可能であるが、CNAG を用いた解析により、元データに含まれる系統的誤差が有効に排除され、極めて S/N 比の高いゲノムコピー数の解析が可能となった。また、本システムでは、SNP 特異的プローブの使用によって、ゲノムワイドな LOH の解析やアレル別にコピー数の検出を行うことも可能となっている。本プログラムは、我々のホームページ (http://www.genome.umin.jp/) を通じて公開されており、MDS のみならず癌のゲノム解析の強力なツールとして世界的に高く評価されている。

GeneChip を用いた 150 例のゲノム解析では、従来の解析手段では同定不可能な、非常に微小な領域における異常が多数同定された。本システムでは、その高い解像度により、異常の境界領域を正確にマッピング可能なことが重要な特徴であって、多くの領域について異常の標的となっている可能性のある遺伝子がほぼピンポイントで同定されており、効率的な標的遺伝子の候補の同定が可能であった。

また、SNP 特異的プローブの使用によって、ゲノムワイドな高精度の LOH の解析が初めて可能となった。また、長距離に及ぶホモ接合が胚細胞レベルで生じている観察結果からは、本疾患の成因に遺伝的背景が関与しているという可能性が示唆され、今後の解析に興味が持たれる。

E. 結論

Affymetrix 社の高密度オリゴヌクレオチドアレイを用いた高精度なゲノムコピー数/LOH 解析を可能とする独自のゲノム解析システム CNAG を開発し、MDS におけるゲノム異常の網羅的な解析を行った。CNAG を用いた 150 例の MDS 試料の解析により、MDS で高頻度に認められる種々の染色体欠失の詳細なマッピングを行うとともに、多数の微細なゲノムの異常および MDS の発症に関わる可能性のある多数の候補遺伝子の同定を行った。さらに、MDS 発症に関して遺伝学的素因が関与する可能性を明らかにした。

F. 健康危険情報

なし

G. 研究発表

論文発表

1. Hosoya N, Sanada S, Nannya Y, Nakazaki K, Wang L, Hangaishi A, Kurokawa M, Chiba S, Ogawa S. Genome-wide screening of DNA copy number changes in chronic myelogenous leukemia with the use of high-resolution array-based comparative genomic hybridization. Genes Chromosomes and Cancer, in press
2. Hosoya N, Qiao Y, Hangaishi A, Wang L, Nannya Y, Sanada M, Kurokawa M, Chiba S, Hirai H, Ogawa S. Identification of a SRC-like tyrosine kinase gene, FRK, fused with ETV6 in a patient with acute myelogenous leukemia carrying a t(6;12)(q21;p13) translocation. Genes Chromosomes Cancer. 2005;42:269-279.
3. Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 65: 6071-6079, 2005

学会発表

真田昌，南谷泰仁，中崎久美，王莉莉，細谷紀子，半下石明，黒川峰夫，藤田和博，小峰光博，千葉滋，小川誠司。 超高密度オリゴアレイを用いた AML・MDS におけるゲノム異常の解析。
第 67 回日本血液学会・第 46 回臨床血液学会総会（平成 17 年 9 月、札幌）

中崎久美，南谷泰仁，真田昌，細谷紀子，王莉莉，半下石明，黒川峰夫，千葉滋，小川誠司。高密度オリゴヌクレオチドアレイを用いた癌ゲノム解析ツールの開発。

真田昌，南谷泰仁，中崎久美，半下石明，王莉莉，細谷紀子，千葉滋，山田修司，滝田順子，金井文彦，林泰秀，小俣政男，小川誠司。高密度オリゴアレイを用いた腫瘍ゲノムにおけるアレル不均衡の網羅的解析。
第 64 回日本癌学会総会(平成 17 年 9 月、横浜)

H. 知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他
 いずれも予定なし

III.　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Takahashi W, Sasaki K, Komatsu N, Mitani K.	TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM.	Cancer Sci	96	340-348	2005
Nakamura F, Nakamura Y, Maki K, Sato Y, Mitani K.	Cloning and characteri-zation of a novel chimeric gene TEL/PTPRR in acute myelogenous leu-kemia with inv(12)(p13q13).	Cancer Res	65	6612-6621	2005
Maki K, Yamagata T, Asai T, Yamazaki I, Oda H, Hirai H, Mitani K.	Dysplastic definitive hematopoiesis in AML1/Evi-1 knock-in embryos.	Blood	106	2147-2155	2005
Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Ueda R, Kinoshita T, Emi N, Naoe T.	Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia.	Blood	106	2854-2861	2005
Takeshita A, Shinjo K, Naito K, Matsui H, Sahara N, Shigeno K, Horii T, Shirai N, Maekawa M, Ohnishi K, Naoe T, Ohno R.	Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells.	Leukemia	19	1306-1311	2005
Iwai M, Kiyoi H, Ozeki K, Kinoshita T, Emi N, Ohno R, Naoe T.	Expression and methylation status of the FHIT gene in acute myeloid leukemia and myelodysplastic syndrome.	Leukemia	19	1367-1375	2005
Mitsuma A, Asano H, Kinoshita T, Murate T, Saito H, Stamatoyannopoulos G, Naoe T.	Transcriptional regulation of FKLF-2 (KLF13) gene in erythroid cells.	Biochim Biophys Acta	1727	125-133	2005
Kajiguchi T, Yamamoto K, Sawa M, Emi N, Naoe T.	Increased erythropoietin level and reticulocyte count during arsenic trioxide therapy.	Leukemia	19	674-676	2005
Ohyashiki K, Shay JW, Ohyashiki JH.	Lack of mutations of the human telomerase RAN gene (hTERC) in myelodysplastic syndrome.	Haematologica	90	691	2005
Ohyashiki K, Aota Y, Akahane D.	The JAK2 V617F tyrosine kinase mutation in myelodysplastic syndromes (MDS) developing myelofibrosis indicates the myeloproliferative nature in a subset of MDS patients.	Leukemia	103	2359-2360	2005

研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Ohyashiki JH, Hisatomi H, Ohyashiki K.	Quantitative relationship between functionally active telomerase and major telomerase components (hTERT and hTR) in acute leukaemia cells.	Br J Cancer	92	1942-1947	2005
Kazama H, Teramura M, Yoshinaga K, Kato T, Motoji T, Mizoguchi H.	Proteomics approach to identifying proteins abnormally expressed in neutrophils in patients with myelodysplastic syndrome.	Leukemia	29 Suppl 1	S46	2005
Inukai T, Inaba T, Dang J, Kuribara R, Ozawa K, Miyajima A, Wu W, Look AT, Arinobu Y, Iwasaki H, Akashi K, Kagami K, Goi K, Sugita K, Nakazawa S.	TEF, an anti-apoptotic bZIP transcription factor related to the oncogenic E2A-HLF chimera, inhibits cell growth by downregulating expression of the common b chain of cytokine receptors.	Blood	105	4437-4444	2005
Matsui H, Shinjyo T, Inaba T.	Structure of the human Bim gene and its transcriptional regulation in Baf-3, interleukin-3-dependent hematopoietic cells.	Mol Biol Rep	32	79-85	2005
Yamasaki M, Mishima KH, Yamashita H, Kashiwagi K, Murata K, Minamoto A, Inaba T.	Neuroprotective effects of erythropoietin on glutamate and nitric oxide toxicity in primary cultured retinal ganglion cells.	Brain Res	1050	15-26	2005
Hosoya N, Sanada S, Nannya Y, Nakazaki K, Wang L, Hangaishi A, Kurokawa M, Chiba S, Ogawa S.	Genome-wide screening of DNA copy number changes in chronic myelogenous leukemia with the use of high-resolution array-based comparative genomic hybridization.	Genes Chromosomes Cancer		(in press)	
Hosoya N, Qiao Y, Hangaishi A, Wang L, Nannya Y, Sanada M, Kurokawa M, Chiba S, Hirai H, Ogawa S.	Identification of a SRC-like tyrosine kinase gene, FRK, fused with ETV6 in a patient with acute myelogenous leukemia carrying a t(6;12)(q21;p13) translocation.	Genes Chromosomes Cancer	42	269-279	2005
Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S.	A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays.	Cancer Res	65	6071-6079	2005

Ⅳ．　研究成果の刊行物・別刷

TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM

Wataru Takahashi,[1] Ko Sasaki,[1] Norio Komatsu[2] and Kinuko Mitani[1,3]

[1]Department of Hematology, Dokkyo University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293; and [2]Division of Hematology, Yamanashi University, 1110 Shimokatou, Tamaho, Nakakoma, Yamanashi 409-3898, Japan

(Received March 2, 2005/Accepted March 24, 2005/Online publication June 15, 2005)

TEL/ETV6 accelerates erythroid differentiation in the murine erythroleukemia cell line. To clarify the effects of TEL on megakaryocytic maturation as well as erythroid differentiation, we chose the human leukemia cell line UT-7/GM that differentiates into the erythroid and megakaryocytic lineages by treatment with erythropoietin and thrombopoietin, respectively. Upon erythropoietin exposure, overexpressed TEL stimulated hemoglobin synthesis and accumulation of the erythroid differentiation-specific transcripts such as γ-globin, δ-aminolevulinic acid synthase-erythroid, and erythropoietin receptor. Moreover, the glycophorin A(+)/glycoprotein IIb(–) fraction appeared more rapidly in the TEL-overexpressing cells. Interestingly, overexpression of TEL was associated with lower levels of the megakaryocytic maturation-specific glycoprotein IIb and platelet factor 4 transcripts under the treatment with thrombopoietin. Consistently, the glycophorin A(–)/glycoprotein IIb(+) fraction increased more slowly in the TEL-overexpressing cells. Finally, expression of endogenous TEL proteins in UT-7/GM cells was down-regulated following erythropoietin and thrombopoietin exposure. All these data suggest that TEL may decide the fate of human erythrocyte/megakaryocyte common progenitors to differentiate towards the erythroid lineage and against the megakaryocytic lineage. (*Cancer Sci* 2005; 96: 340–348)

TEL (also known as ETV6) is a member of the E26 transformation-specific (ETS) family of transcription factors.[1] The highly conserved ETS domain is located at the C-terminal region, while a distinct domain with weak homology to the well-described helix-loop-helix (HLH) domain (also referred to as the pointed domain) is located at the N-terminal region. The former serves for DNA binding to the ETS-binding consensus site (EBS) (GGAA/T) and the latter for homodimerization and heterodimerization with other ETS family members.[2,3] Through interacting with relevant corepressors mSin3A, N-CoR and SMRT, and histone deacetylase-3,[4] TEL mediates transcriptional repression on its target genes such as *FLI-1*,[2] inhibitor of differentiation/DNA binding-1 (*Id-1*),[5] stromelysin-1[6] and *Bcl-X$_L$*.[7] Transcriptional activities of TEL are regulated through phosphorylation with mitogen-activated protein kinases[8,9] and small ubiquitin-like modifier conjugation.[10,11]

The *TEL* gene that is mapped to 12p13 is most frequently rearranged and fused to various partner genes by chromosomal translocations in human leukemias and myelodysplastic syndromes. The partners include receptor type or non-receptor type tyrosine kinases and transcription factors. Providing tyrosine kinases, such as platelet-derived growth factor receptor β (PDGFRβ) in t(5;12) (q33;p13),[12] ABL1 in t(9;12) (q34;p13),[13] ARG (ABL2) in t(1;12) (q25;p13),[14] JAK2 in t(9;12) (p24;p13)[15] and Syk in t(9;12) (q22;p13),[16] with the HLH domain, TEL homodimerizes them and thereby stimulates their kinase activities. In contrast, TEL gives corepressor-binding domains to a transcription factor AML1 in t(12;21) (p13;q22) and interferes with its transcriptional abilities.[17] Therefore, dysregulation of the partner proteins by TEL functional domains seems to cause leukemia in patients with 12p13 translocations. Moreover, inactivation of the *TEL* gene is speculated to be the second hit in t(12;21) (p13;q22) type leukemia, because the wild-type-*TEL* allele is deleted in the vast majority of the patients.[18,19] Thus, TEL appears to be a tumor suppressor. Consistent with its roles as a putative tumor suppressor, expression of TEL in Ras-transformed NIH3T3 cells inhibits cell growth in liquid and soft agar cultures,[6] and in serum-starved NIH3T3 cells induces apoptosis.[6]

TEL is required for mouse development as its inactivation by homologous recombination results in embryonic lethality at E10.5–11.5.[18] The knockout embryos show defects in yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, while they present normal yolk sac hematopoiesis. Analyzing chimeric mice with *TEL(–/–)* ES cells, an essential role of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow has been uncovered, although *TEL(–/–)* ES cells contributed to both primary and definitive fetal hematopoiesis.[19] As for lineage-specific roles in hematopoietic systems, we have reported that TEL accelerates erythroid differentiation of mouse erythroleukemia (MEL) cells induced by hexamethylene bisacetamide (HMBA) or dimethylsulfoxide (DMSO).[20] Because both erythroblasts and megakaryocytes arise from common progenitors, this observation prompted us to search for TEL's roles in lineage commitment of the bi-potential progenitors.

A human tri-factor dependent hematopoietic cell line UT-7/GM[21] is a subline of UT-7 that was originally established from a patient of acute megakaryoblastic leukemia.[22] UT-7/GM cells show absolute dependence for growth and survival on granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO) or thrombopoietin (TPO). They differentiate into the erythroid or megakaryocytic lineage in the presence of EPO or TPO, while they keep immature phenotypes and proliferate in the presence of GM-CSF.[21] Thus, UT-7/GM cells are considered to mimic erythrocyte/megakaryocyte common progenitors and differentiate along two distinct lineages in relatively physiological conditions. We employed this cell line and examined influences of TEL overexpression on erythroid differentiation and megakaryocytic maturation. As judged from higher percentages of benzidine positivity in TEL-overexpressing cells under treatment with EPO, TEL accelerated erythroid differentiation in UT-7/GM cells similar as in MEL cells. The TEL-overexpressing cells showed increased expression of the transcripts for γ-globin, δ-aminolevulinic acid synthase-erythroid (*ALAS-E*) and EPO receptor (*EPO-R*) during the erythroid differentiation. Moreover, accumulation of the glycophorin A(+)/glycoprotein (GP) IIb(–) fraction was more prompt in these cells. Interestingly, expression

[3]To whom correspondence should be addressed. E-mail: kinukom-tky@umin.ac.jp

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00052.x

levels of the transcripts for *GPIIb* and platelet factor 4 (*PF 4*) under the treatment with TPO were lower in the TEL-overexpressing cells. Consistent with this, accumulation of the glycophorin A(–)/GPIIb(+) fraction was delayed and appearance of platelet peroxidase (PPO)-positive cells was reduced in these cells. Endogenous TEL proteins disappeared after 14 and 21 days upon EPO and TPO exposure, respectively. We conclude that TEL stimulates erythroid differentiation while opposing megakaryocytic maturation in human hematopoietic system.

Materials and Methods

Cell culture. Parental UT-7/GM cells, the mock (M-1 and M-4) and the TEL-overexpressing (T-5 and T-6) clones were maintained in Isocove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 1 ng/mL of recombinant human (rh) GM-CSF. To physiologically induce erythroid or megakaryocytic differentiation, these cells were cultured in IMDM supplemented with 10% FCS, and 10 U/mL of rhEPO or 100 ng/mL of rhTPO. Light microscopic examination was performed on Wright-Giemsa-stained cytospin preparations. Erythroid differentiation was evaluated by counting percentages of benzidine-positive cells.

Isolation of stable transfectants. The expression of plasmid pCXN2-FLAG-TEL was described in a previous study.[20] To establish stable transfectants, 1×10^7 of UT-7/GM cells were electroporated with 20 μg of pCXN2-FLAG-TEL at 380 V and 975 μF using Gene Pulser (Bio-Rad, Hercules, CA). Transfected cells were selected with 0.8 mg/mL of G418 (Sigma-Aldrich, St. Louis, MO) and cloned by limiting dilution. Expression of FLAG tagged-TEL proteins was confirmed by the western analysis method using anti-FLAG antibody (Sigma-Aldrich).

Immunoprecipitation and western analysis. UT-7/GM cells were lyzed on ice in lysis buffer composed of 20 mM Tris pH 8.0, 50 mM sodium fluoride (NaF), 2 mM ethylenediamine-*N,N,N′,N′*-tetra-acetic acid (EDTA), 1% NP-40, 500 U/mL aprotinin, 1 mM sodium orthovanadate (Na_3VO_4), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Immunoprecipitation and western analysis were performed as described in a previous study,[20] using anti-TEL (N-19 for immunoprecipitation and H-214 for western analysis; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-FLAG monoclonal (Sigma-Aldrich) antibodies. The blots were visualized by ProtoBlot AP system (Promega, Madison, WI).

Northern analysis. Total RNA was extracted from the mock and the TEL-overexpressing cells using ISOGEN (Nippon Gene, Tokyo, Japan) under the manufacturer's instruction. Twenty μg of each RNA sample was resolved by electrophoresis on agarose formaldehyde gels, transferred to Hybond-N+ nylon membranes (Amersham, Piscataway, NJ) in 20 × standard sodium citrate (SSC) and hybridized to human cDNA fragments for *ALAS-E*, *EPO-R*, γ-globin, *GPIIb* and *PF 4* that were labeled with [α-^{32}P] dCTP using the Megaprime DNA labeling system (Amersham). Human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe was used as a control. After overnight incubation at 42°C in the presence of 50% formamide, membranes were washed with 0.1 × SSC containing 0.1% sodium dodecyl sulfate (SDS) at 42°C and autoradiographed using Fujix BAS2500 Bio-image Analyzer (Fuji Photo Film, Tokyo, Japan). Relative expression levels to the level at day 0 in each clone were quantified.

Fluorescence activated cell sorter (FACS) analysis. The mock and the TEL-overexpressing cells were incubated for 30 min at 4°C with appropriately diluted fluorescein-labeled antiglycophorin A and anti-GPIIb (CD41b) antibodies (Beckman Coulter, Fullerton, CA). After washing, cells were analyzed using Becton Dickinson FACS Calibur.

Electron microscopic analysis. Ultrastructural PPO activity was detected by a conventional method.[23]

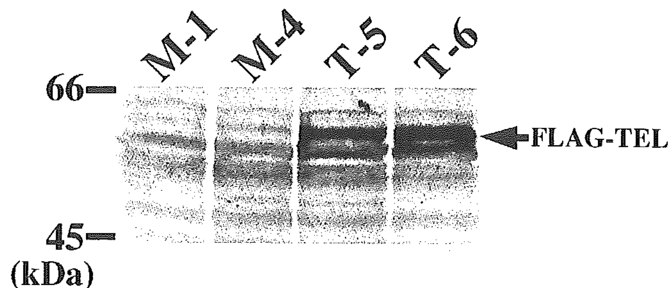


Fig. 1. Establishment of UT-7/GM sublines overexpressing FLAG-tagged TEL proteins. Clones T-5 and T-6 were obtained from UT-7/GM cells that were transfected with pCXN2-FLAG-TEL and selected by G418 resistance. Clones M-1 and M-4 were established from UT-7/GM cells that were transfected with the empty pCXN2 vector and selected by G418 resistance. Expression of FLAG-tagged TEL proteins was confirmed by western analysis with anti-FLAG antibody. An arrow indicates overexpressed FLAG-TEL proteins.

Results

TEL accelerates erythroid differentiation upon EPO treatment in UT-7/GM cells. Human leukemia UT-7/GM cells differentiate into either erythroblasts or megakaryocytes upon cytokine exposure. Thus, this cell line provides a useful tool to analyze the effects of TEL on erythroid differentiation and megakaryocytic maturation in human hematopoietic cells. We established UT-7/GM clones stably overexpressing FLAG-tagged TEL by electroporating the expression plasmid containing *TEL* cDNA and selecting cells with G418 resistance. Western analysis with anti-FLAG antibody demonstrated that representative clones T-5 and T-6 expressed TEL proteins at high levels (Fig. 1). Mock clones M-1 and M-4 were also isolated by introducing the empty expression plasmid. Overexpression of TEL slightly retarded growth of the cells under treatment with EPO or TPO, but did not influence proliferation of the cells maintained in GM-CSF (data not shown).

We previously reported that TEL acts as an accelerator of erythroid differentiation induced by chemical compounds such as HMBA and DMSO in MEL cells.[20] To confirm this effect of TEL under a more physiological condition in human hematopoietic cells, we treated the mock and the TEL-overexpressing clones with EPO. We observed no morphological differences between them, except a faint color difference in the cytoplasm. Figure 2

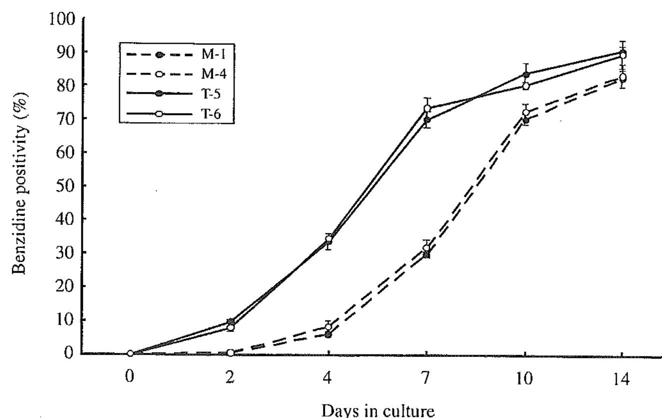


Fig. 2. TEL accelerates hemoglobin synthesis induced by treatment with erythropoietin (EPO) in the UT-7/GM clones. The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones were cultured in the presence of EPO (10 U/mL). Hemoglobin synthesis was evaluated by the proportions of benzidine-positive cells and their averages in three independent experiments were indicated with standard deviations.

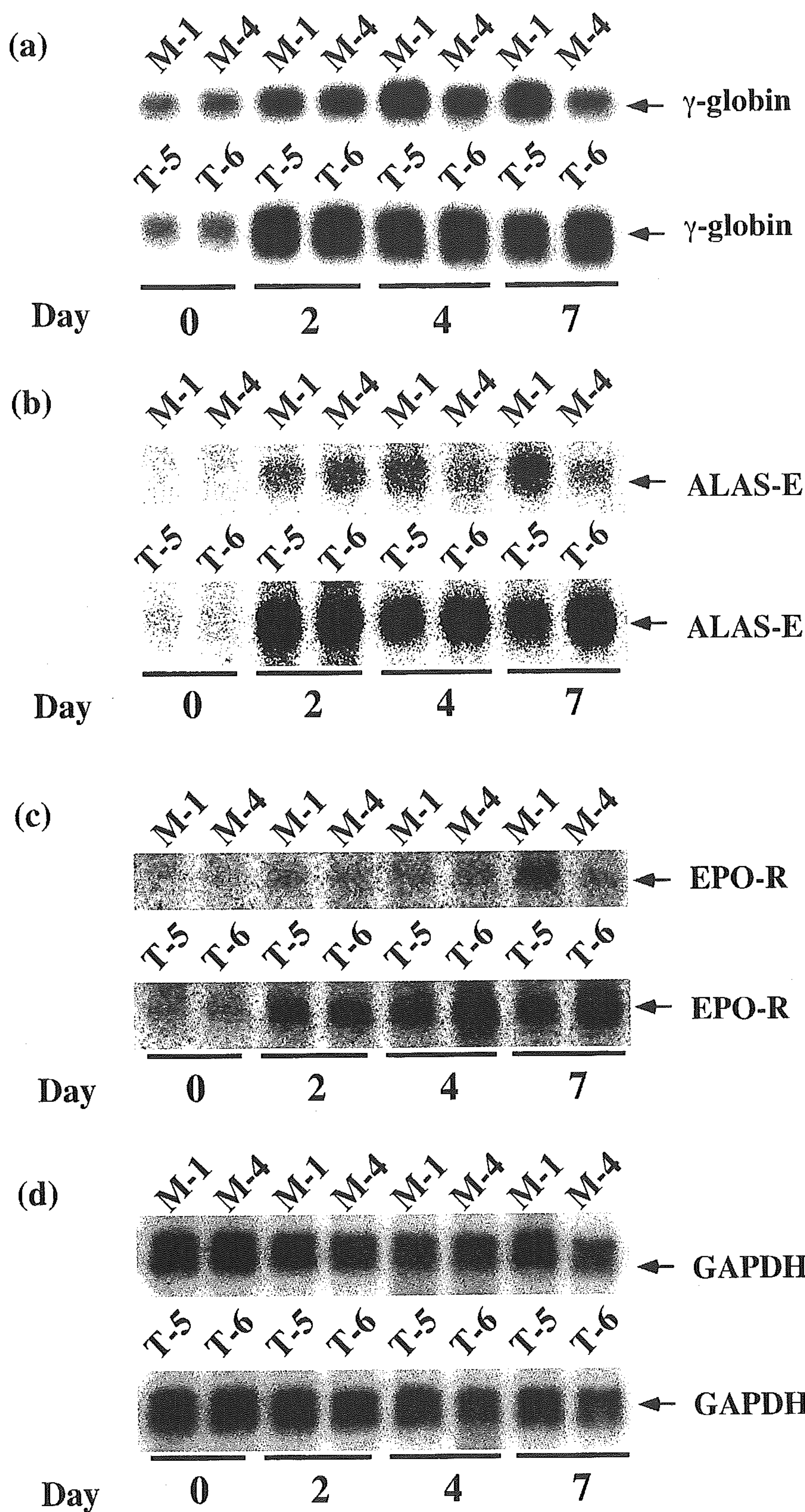


Fig. 3. Erythroid lineage-specific gene transcription in the UT-7/GM clones under treatment with erythropoietin (EPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of EPO (10 U/mL) were harvested at each time point indicated (days 0, 2, 4, 7). Total mRNA was extracted and subjected to northern analysis with *γ-globin* (A), *ALAS-E* (B), *EPO-R* (C) and *GAPDH* (D) probes.

indicates time courses of hemoglobin synthesis estimated by proportions of benzidine-positive cells in these UT-7/GM clones. In the mock clones, proportions of benzidine-positive cells reached to 80% within two weeks. Interestingly, the TEL-overexpressing clones showed rapid onset and higher saturation of benzidine positivity in comparison with the mock clones. Eighty percent of the cells became positive for benzidine staining after 10 days of culture and 90% after 14 days. We thus conclude that TEL is also an accelerator for erythroid differentiation upon cytokine stimulation in human hematopoietic cells.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00052.x

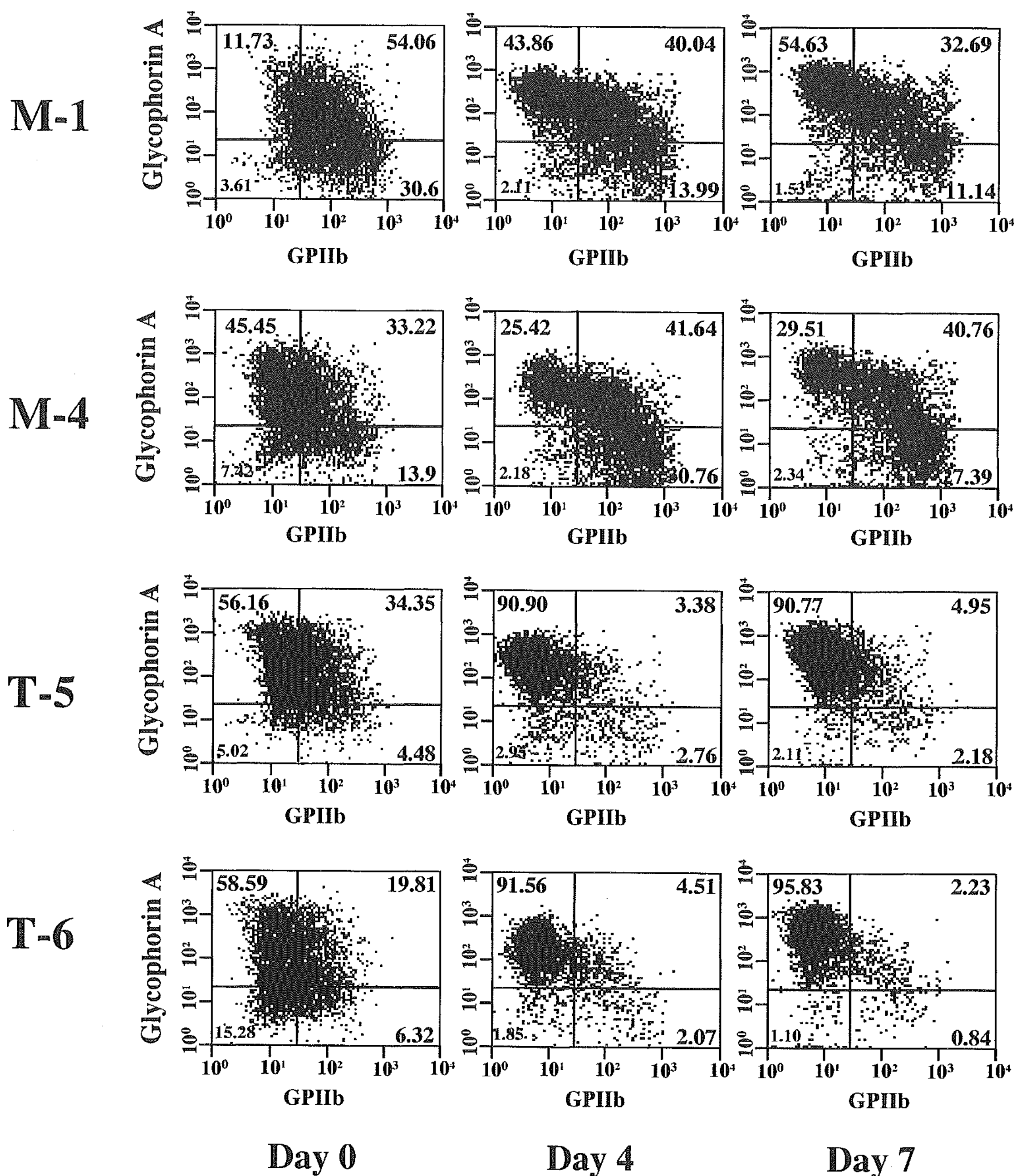


Fig. 4. Erythroid and megakaryocytic lineages-specific surface antigen expression in the UT-7/GM clones under the treatment with erythropoietin (EPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of EPO (10 U/mL) were harvested at each time point indicated (days 0, 4, 7) and subjected to flow-cytometric analysis. GPIIb on *X* axis and glycophorin A on *Y* axis were megakaryocyte- and erythrocyte-specific markers, respectively.

To further obtain evidence for erythroid differentiation exaggerated by TEL in UT-7/GM cells, erythroid differentiation-specific transcripts were analyzed using northern analysis. As shown in Fig. 3, transcripts for γ-globin, *ALAS-E* and *EPO-R* increased upon EPO exposure in both cell types. However, even before the treatment (at day 0), expression of these genes appeared to be stimulated by overexpressed TEL proteins. This tendency was maintained at all the time points examined. Next, we performed flow cytometric analysis to assess expression levels of erythrocyte-specific glycophorin A and megakaryocyte-specific GPIIb in the cell surface during the course of erythroid differentiation. Proportions of the glycophorin A(+)/GPIIb(–) fractions were significantly higher at days 4 and 7 in the TEL-overexpressing cells than in the mock cells (Fig. 4). The glycophorin A(–)/GPIIb(+) fractions disappeared more rapidly in the TEL-overexpressing cells. These results collectively confirm the TEL functions as an erythroid differentiation stimulator and indicate the possibility that TEL might concomitantly accelerate erythroid differentiation and repress megakaryocytic maturation.

TEL inhibits megakaryocytic maturation upon TPO treatment in UT-7/GM cells. To clarify the roles of TEL in megakaryocytic maturation of human hematopoietic cells, we induced megakaryocytic maturation by treatment with TPO in the mock and the TEL-overexpressing clones and first analyzed their morphological changes. Differing from the mock clones, the TEL-overexpressing clones hardly maturated into megakaryocyte-containing multilobulated nuclei even after 28 days of culture with TPO

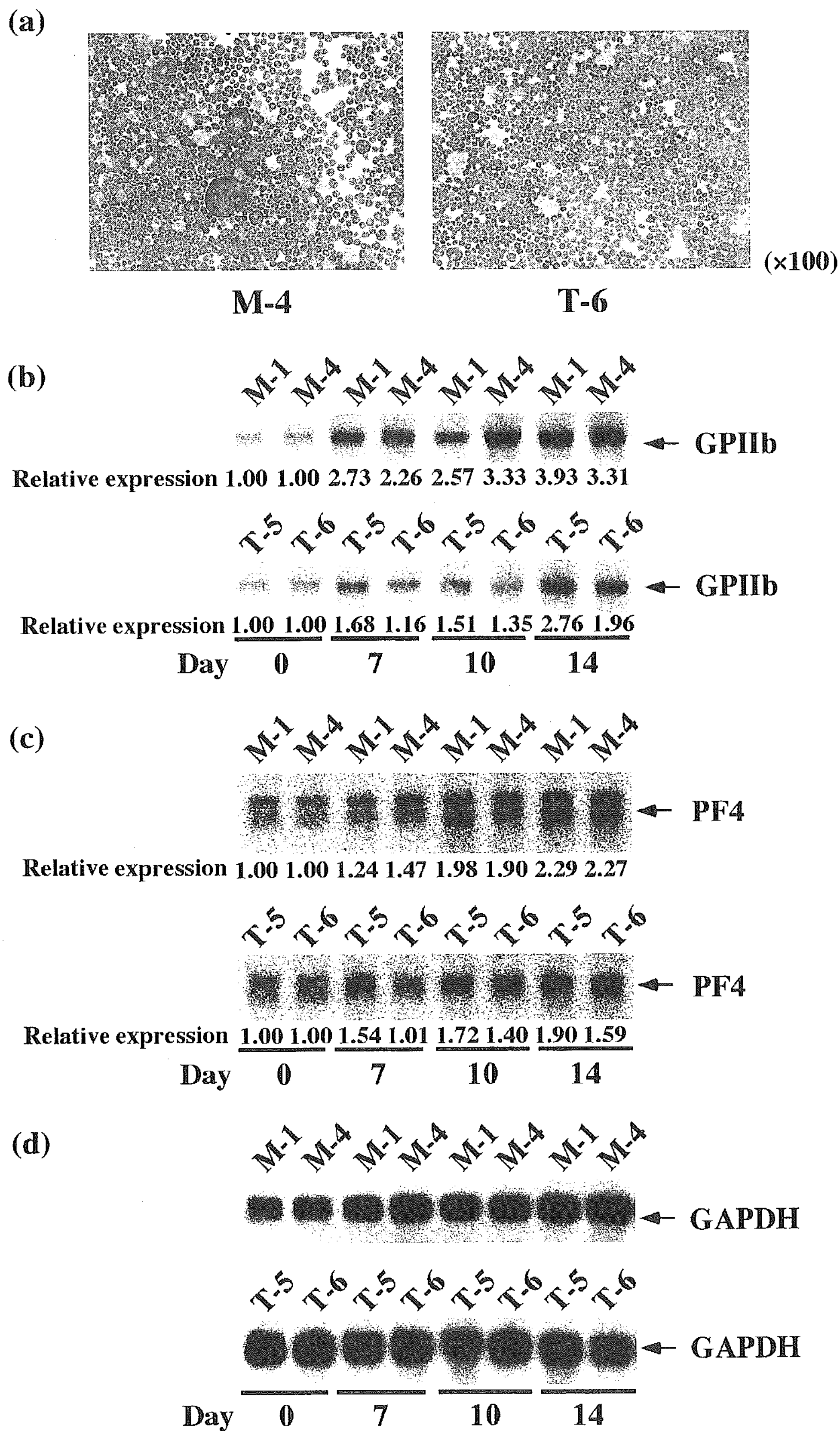


Fig. 5. Morphology and megakaryocytic lineage-specific gene transcription in the UT-7/GM clones under treatment with thrombopoietin (TPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of TPO (100 ng/mL) were harvested at each time point indicated (days 0, 7, 10, 14, 28). (a) Cytospin preparations of M-4 and T-5 at day 28. Wright-Giemsa staining, ×100. (b–d) Total mRNA was extracted and subjected to northern analysis with GPIIb (b), *PF 4* (c) and *GAPDH* (d) probes. Signal ratios between day 0 and the indicated time points were quantified and presented below each lane.

© Japanese Cancer Association
doi: 10.1111/j.1349-7006.2005.00052.x

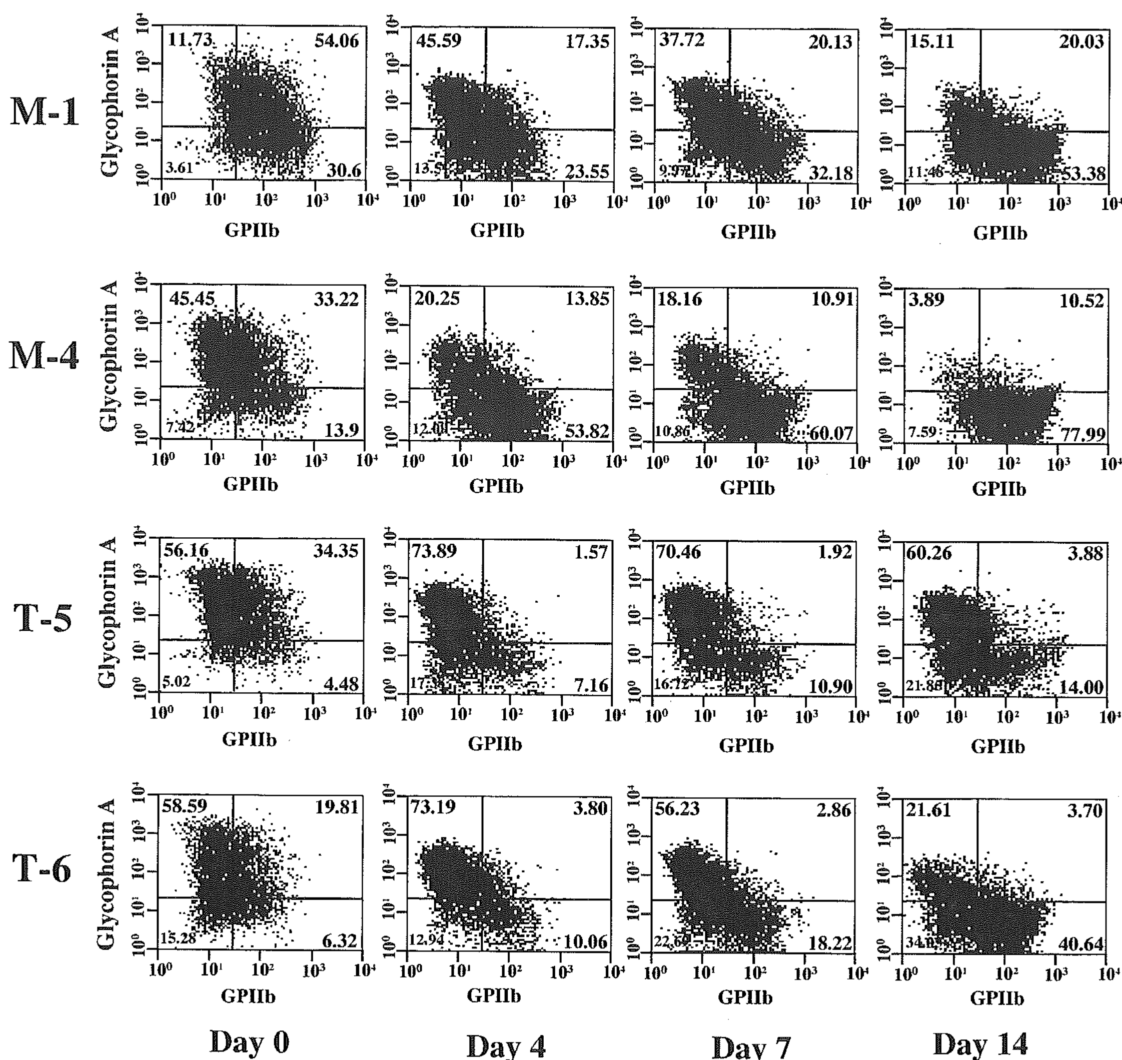


Fig. 6. Erythroid and megakaryocytic lineage-specific surface antigen expression in the UT-7/GM clones under the treatment with thrombopoietin (TPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of TPO (100 ng/mL) were harvested at each time point indicated (days 0, 4, 7, 14) and subjected to flow-cytometric analysis. GPIIb on *X* axis and glycophorin A on *Y* axis were megakaryocyte- and erythrocyte-specific markers, respectively.

(Fig. 5a). Expression of megakaryocytic maturation-specific genes such as *GPIIb* and *PF 4* was also examined using northern analysis. The TEL-overexpressing cells expressed these transcripts at almost comparable levels to mock cells before the treatment (Fig. 5b–d). As expected, they increased upon TPO exposure in both cell types. It is interesting to note that levels of these transcripts were lower in the TEL-overexpressing cells than in the mock cells at least until day 14. We again examined cell surface expression of glycophorin A and GPIIb during the course of megakaryocytic maturation. Proportions of the glycophorin A(–)/GPIIb(+) fractions were markedly lower until day 14 in the TEL-overexpressing cells than in the mock cells, whereas proportions of the glycophorin A(+)/GPIIb(–) fractions higher (Fig. 6). Furthermore, fewer percentages of the cells became positive for electron microscopic PPO in the TEL-overexpressing clones after 14 days treatment with TPO (Fig. 7). We hypothesize that TEL could prevent megakaryocytic maturation and maintain expression of erythroid markers in erythrocyte/megakaryocyte common progenitors even when induced towards the megakaryocytic lineage.

Expression of endogenous TEL proteins decreases upon both EPO and TPO treatments in UT-7/GM cells. Finally, we examined changes of endogenous TEL expression during both the courses of erythroid and megakaryocytic differentiation in parental UT-7/GM cells to further obtain findings for the physiological roles of TEL. Under the presence of GM-CSF, endogenous TEL proteins were detected at almost the same size as overexpressed TEL proteins in the T-5 clone (lane 4, Fig. 8a) using western analysis (lane 3) and self-immunoprecipitation assay (lane 1). When the cells were induced to erythroid differentiation by treatment with EPO, endogenous TEL proteins maintained steady expression until 3 days of culture and then began to decline (Fig. 8b). At day 14, endogenous TEL proteins almost completely disappeared. When induced to megakaryocytic maturation by treatment with TPO, UT-7/GM cells kept constant expression of endogenous TEL proteins until 14 days of culture and lost their expression at day 21 (Fig. 8c). These data suggest that endogenous TEL may work in the early phase of differentiation to either lineage and accelerate erythroid differentiation and actively repress megakaryocytic maturation.

Discussion

We demonstrated in the present study that TEL accelerates erythroid differentiation induced by a physiological cytokine

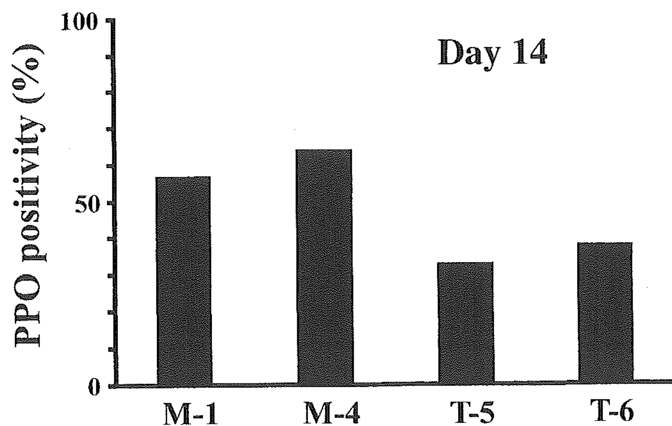


Fig. 7. TEL represses ultrastructural platelet peroxidase (PPO) reactions after 14 days of treatment with thrombopoietin (TPO) in the UT-7/GM clones. The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones were cultured in the presence of TPO (100 ng/mL) for 14 days. PPO reactions were evaluated by electron microscopic analysis.

EPO in human leukemia cell line UT-7/GM. Associated with increased expression of erythroid differentiation-specific transcripts γ-globin, *ALAS-E* and *EPO-R*, and a surface antigen glycophorin A, the TEL-overexpressing cells accumulate hemoglobin more rapidly than the mock cells. A megakaryocyte maturation-specific surface marker GPIIb disappears more quickly during the course of erythroid differentiation in the TEL-overexpressing cells. Importantly, morphological maturation towards megakaryocyte with multilobulated nuclei and induction of megakaryocyte maturation-specific transcripts *GPIIb* and *PF 4* after TPO treatment are weaker when TEL is overexpressed. Moreover, GPIIb accumulates and glycophorin A disappears more slowly in the cell surface of these cells. Electron microscopic PPO reaction is detected at fewer ratios. All these data collectively suggest that TEL might drive erythroid differentiation and suppress megakaryocytic maturation in erythrocyte/megakaryocyte common progenitors. Consistently, endogenous TEL proteins are expressed only in the early phase of either differentiation in which TEL is expected to function, and thereafter disappear. This paper is the first describing the unique role of TEL in the megakaryocytic lineage.

Because TEL is a transcriptional regulator for EBS-containing promoters, it is interesting to know whether the *cis*-regulatory elements actually exist in the erythrocyte or megakaryocyte-specific genes, the expression of which was found in this study to be altered by overexpressed TEL. Numerous megakaryocyte-specific genes contain EBS and GATA-1 binding sites in their promoters.[24] Of note, both ETS-1 and GATA-1 are reported essential for positive regulation of *GPIIb* and *PF 4* gene transcription.[25] Moreover, ETS-1 is demonstrated to directly bind to their promoters by chromatin precipitation assays.[26] Although it remains undetermined whether TEL binds to EBS in the promoters of *GPIIb* and *PF 4* genes, overexpressed TEL could repress it directly or indirectly. In the latter case, TEL may

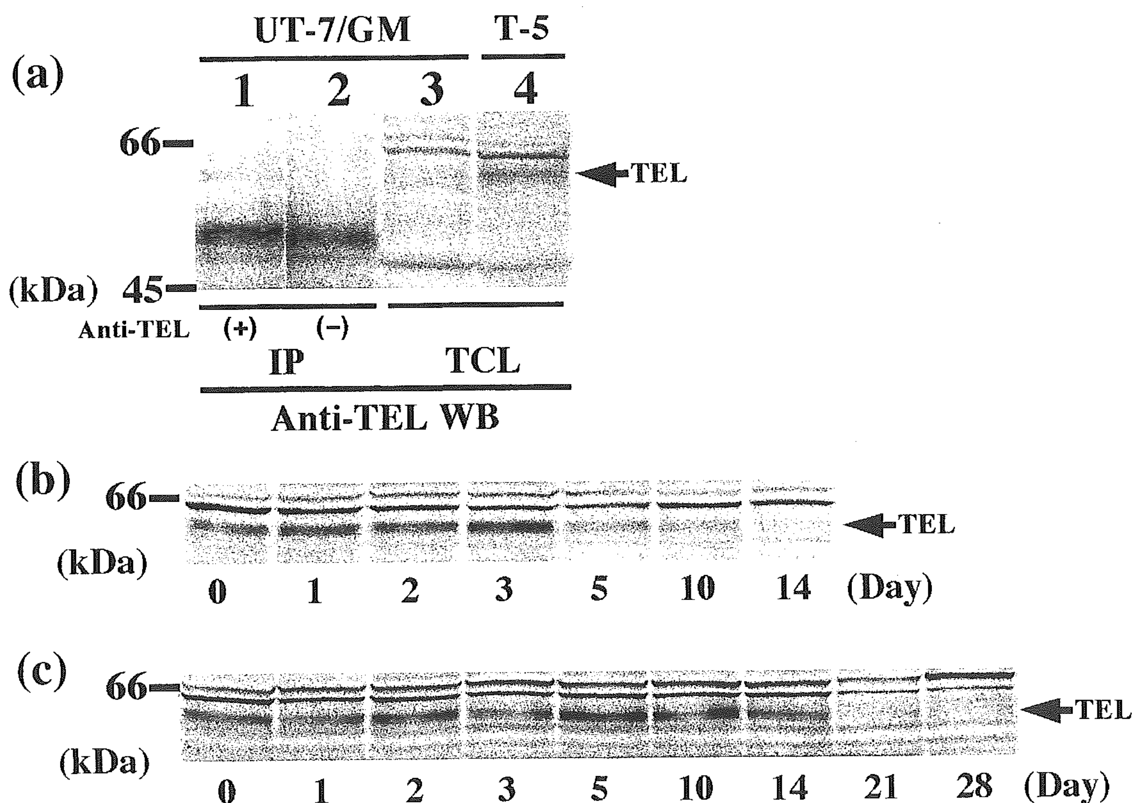


Fig. 8. Expression of endogenous TEL proteins in parental UT-7/GM cells. (a) Expression of endogenous TEL proteins in parental UT-7/GM cells was confirmed under the presence of GM-CSF (1 ng/mL) by western analysis (lane 3) or immunoprecipitation assay (lane 1) with anti-TEL antibody. Overexpressed FLAG-tagged TEL proteins in clone T-5 were shown in lane 4. An arrow indicates endogenous TEL or overexpressed FLAG-tagged TEL proteins; (b,c) Parental UT-7/GM cells cultured in the presence of erythropoietin (10 U/mL); (b) or thrombopoietin (100 ng/mL); (c) were harvested at each time point indicated (days 0, 1, 2, 3, 5, 10, 14, 21, 28). Cell lysates were extracted and subjected to western analysis with anti-TEL antibody. Arrows indicate endogenous TEL proteins.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00052.x

dominantly suppress functions of other ETS family members such as ETS-1 that show transactivation abilities on the promoters through heterodimerizing with them by the HLH domain. However, because EBS is not identified in the promoters of the erythrocyte-specific genes examined in this study, we have no ground to speculate that TEL could be involved in their transcriptional regulation.

We hypothesize that TEL could trigger erythroid differentiation and prevent megakaryocytic maturation through repressing transcription of its target genes that play key roles in hematopoietic differentiation. Among the known target genes of TEL, *FLI-1* and *Id-1* are shown to have functions in erythrocyte/megakaryocyte differentiation. The *FLI-1* gene was first isolated as a common site for retroviral integration in Friend virus-induced erythroleukemia cells,[27] and also encodes a member of the ETS family of transcription factors. FLI-1 suppresses erythroid differentiation partly through inhibiting transcription of the genes such as *GATA-1*,[28] *Rb*[29] and β-globin[30] that promote erythroid differentiation. Moreover, *FLI-1* knockout mice are embryonic lethal around mid-gestation and display a marked reduction of megakaryocytes in the fetal liver as well as a vascular developmental aberration,[31] suggesting a critical role of FLI-1 in megakaryocytic maturation. FLI-1 binds and transactivates the promoters from megakaryocyte-specific genes including *GPIX*,[32] *GPIIb*[32] and *TPO* receptor.[33] Therefore, FLI-1 appears to play opposite roles in erythroid differentiation and megakaryocytic maturation. We analyzed expression levels of FLI-1 proteins before and after induction of erythroid differentiation or megakaryocytic maturation in the mock and TEL-overexpressing UT-7/GM cells. However, overexpressed TEL proteins did not affect the expression of FLI-1 in UT-7/GM cells (data not shown). In spite of this, there still remains the possibility that TEL could repress molecular functions of FLI-1 in these cells, because TEL has been proved to exert a dominant-negative effect on FLI-1 in reporter assays.[2] The *Id-1* gene was initially cloned from MEL cells by virtue of homology to the helix 2 subdomain in c-myc, MyoD and myogenin, and codes for the first member of Id-family that has the HLH domain.[34] Id-1 has been reported to be functionally implicated in differentiation of specific hematopoietic lineages including erythroid,[35] myeloid,[36] and B cells[37] and negatively control erythroid differentiation. We observed that expression of Id-1 proteins slightly increased after induction of erythroid differentiation or megakaryocytic maturation in UT-7/GM cells, but that overexpressed TEL proteins did not influence its expression levels. Therefore, we failed to obtain evidence that *FLI-1* or *Id-1* could be targets of TEL-induced transcriptional repression in UT-7/GM cells. Identification of novel target genes for TEL that regulate erythroid and/or megakaryocytic differentiation should provide new insights into molecular mechanisms in hematopoietic cell differentiation. Studies to determine the target genes of TEL in differentiating MEL and UT-7/GM cells are now in progress in our laboratory.

Acknowledgments

TEL10/pcDNA3 and pCXN2 are generous gifts from Dr T.R. Golub (Dana-Farber Cancer Institute, Boston, USA) and Dr J. Miyazaki (University of Osaka, Osaka, Japan), respectively. rhGM-CSF, EPO and TPO were provided by KIRIN Brewery. We thank Dr M. Eguchi (Dokkyo University School of Medicine, Tochigi, Japan) for ultrastructural analysis of PPO. This work was financially supported in part by Grants-in-Aid from ministries in Japan of Education, Culture, Sports, Science and Technology, and Health, Labour and Welfare, The Japan Health Sciences Foundation, and Japanese Society for the Promotion of Science.

References

1. Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor β to a novel ets-like gene, *Tel*, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell* 1994; **77**: 307–316.
2. Kwiatkowski BA, Bastian LS, Bauer TR, Jr, Tsai S, Zielinska-Kwiatkowska AG, Hickstein DD. The *ets* family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. *J Biol Chem* 1998; **273**: 17525–30.
3. Potter MD, Buijs A, Kreider B, van Rompaey L, Grosveld GC. Identification and characterization of a new human ETS-family transcription factor, *TEL2*, that is expressed in hematopoietic tissues and can associate with *TEL1/ETV6*. *Blood* 2000; **95**: 3341–8.
4. Wang L, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. *Oncogene* 2001; **20**: 3716–25.
5. Martinez R, Golub TR. *Blood* 2000; **96**: 453a.
6. Fenrick R, Wang L, Nip J *et al*. TEL, a putative tumor suppressor, modulates cell growth and cell morphology of ras-transformed cells while repressing the transcription of *Stromelysin-1*. *Mol Cell Biol* 2000; **20**: 5828–39.
7. Irvin BJ, Wood LD, Wang L *et al*. TEL, a putative tumor suppressor, induces apoptosis and represses transcription of Bcl-X_L. *J Biol Chem* 2000; **278**: 46378–86.
8. Arai H, Maki K, Waga K *et al*. Functional regulation of TEL by p38-induced phosphorylation. *Biochem Biophys Res Commun* 2002; **299**: 116–25.
9. Maki K, Arai H, Waga K *et al*. Leukemia-related transcription factor TEL is negatively regulated through extracellular signal-regulated kinase-induced phosphorylation. *Mol Cell Biol* 2004; **24**: 3227–37.
10. Wood LD, Irvin BJ, Nucifora G, Luce KS, Hiebert SW. Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. *Proc Natl Acad Sci USA* 2003; **100**: 3257–62.
11. Chakrabarti SR, Sood R, Nandi S, Nucifora G. Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. *Proc Natl Acad Sci USA* 2000; **97**: 13281–5.
12. Jousset C, Carron C, Boureux A *et al*. A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFRβ oncoprotein. *EMBO J* 1997; **16**: 69–82.
13. Golub TR, Goga A, Barker GF *et al*. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol* 1996; **16**: 4107–16.
14. Cazzaniga G, Tosi S, Aloisi A *et al*. The tyrosine kinase *abl*-related gene *ARG* is fused to *ETV6* in an AML-M4Eo patient with a t(1;12) (q25;p13): molecular cloning of both reciprocal transcripts. *Blood* 1999: 94: 4370–3.
15. Lacronique V, Boureux A, Valle VD *et al*. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* 1997; **278**: 1309–12.
16. Kuno Y, Abe A, Emi N *et al*. Constitutive kinase activation of the *TEL-Syk* fusion gene in myelodysplastic syndrome with t(9;12) (q22;p12). *Blood* 2001: **97**: 1050–5.
17. Hiebert SW, Sun W, Davis JN *et al*. The t(12;21) translocation converts AML–1B from an activator to a repressor of transcription. *Mol Cell Biol* 1996: **16**: 1349–55.
18. Wang LC, Kuo F, Fujiwara Y, Gilliland DG, Golub TR, Orkin SH. Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *EMBO J* 1997; **16**: 4374–83.
19. Wang LC, Swat W, Fujiwara Y *et al*. The *TEL/ETV6* gene is required specifically for hematopoiesis in the bone marrow. *Genes Dev* 1998; **12**: 2392–402.
20. Waga K, Nakamura Y, Maki K *et al*. Leukemia-related transcription factor TEL accelerates differentiation of Friend erythroleukemia cells. *Oncogene* 2003; **22**: 59–68.
21. Komatsu N, Kirito K, Shimizu R *et al*. In vitro development of erythroid and megakaryocytic cells from a UT-7 subline, UT-7/GM. *Blood* 1997; **89**: 4021–33.
22. Komatsu N, Nakauchi H, Miwa A *et al*. Establishment and characterization of a human leukemic cell line with megakaryocytic features dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res* 1991; **51**: 341–8.
23. Breton-Gorius J, Guichard J. Ultrastructural localization of peroxidase activity in human platelets and megakaryocytes. *Am J Pathol* 1972; **66**: 277–93.
24. Zhang C, Thornton MA, Kowalska MA *et al*. Localization of distal regulatory domains in the megakaryocyte-specific platelet basic protein/platelet factor 4 gene locus. *Blood* 2001; **98**: 610–7.
25. Lemarchandel V, Ghysdael J, Mignotte V, Rahuel C, Romeo PH. GATA and Ets *cis*-acting sequences mediate megakaryocyte-specific expression. *Mol Cell Biol* 1993; **13**: 668–76.
26. Lu J, Pazin MJ, Ravid K. Properties of ets-1 binding to chromatin and its effect on platelet factor 4 gene expression. *Mol Cell Biol* 2004; **24**: 428–41.

27. Ben-David Y, Giddens EB, Letwin K, Bernstein A. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes Dev* 1991; **5**: 908–18.
28. Athanasiou M, Mavrothalassitis G, Sun-Hoffman L, Blair DG. FLI-1 is a suppressor of erythroid differentiation in human hematopoietic cells. *Leukemia* 2000; **14**: 439–45.
29. Tamir A, Howard J, Higgins RR *et al.* Fli-1, an Ets-related transcription factor, regulates erythropoietin-induced erythroid proliferation and differentiation: evidence for direct transcriptional repression of the *Rb* gene during differentiation. *Mol Cell Biol* 1999; **19**: 4452–64.
30. Starck J, Cohet N, Gonnet C *et al.* Functional cross-antagonism between transcription factors FLI-1 and EKLF. *Mol Cell Biol* 2003; **23**: 1390–402.
31. Hart A, Melet F, Grossfeld P *et al.* Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity* 2000; **13**: 167–77.
32. Bastian LS, Kwiatkowski BA, Breininger J, Danner S, Roth G. Regulation of the megakaryocytic glycoprotein IX promoter by the oncogenic Ets transcription factor Fli-1. *Blood* 1999; **93**: 2637–44.
33. Deveaux S, Filipe A, Lemarchandel V, Ghysdael J, Romeo PH, Mignotte V. Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. *Blood* 1996; **87**: 4678–85.
34. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 1990; **61**: 49–59.
35. Lister J, Forrester WC, Baron MH. Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. *J Biol Chem* 1995; **270**: 17939–46.
36. Kreider BL, Benezra R, Rovera G, Kadesch T. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science* 1992; **255**: 1700–2.
37. Pongubala JM, Atchison ML. Functional characterization of the developmentally controlled immunoglobulin kappa-3′ enhancer. regulation by Id, a repressor of helix-loop-helix transcription factors. *Mol Cell Biol* 1991; **11**: 1040–7.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00052.x

Cloning and Characterization of the Novel Chimeric Gene *TEL/PTPRR* in Acute Myelogenous Leukemia with inv(12)(p13q13)

Fumihiko Nakamura,[1,2] Yuichi Nakamura,[4] Kazuhiro Maki,[1] Yuko Sato,[3] and Kinuko Mitani[1]

[1]Department of Hematology, Dokkyo University School of Medicine, Tochigi, Japan; [2]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo; [3]Division of Molecular Cytogenetics, Department of Clinical Pathology, Research Institute, International Medical Center of Japan, Tokyo, Japan; and [4]Division of Hematology, Department of Medicine, Saitama Medical School, Saitama, Japan

Abstract

We have cloned a novel *TEL/protein tyrosine phosphatase receptor-type R* (*PTPRR*) chimeric gene generated by inv(12)(p13q13). PTPRR is the first protein tyrosine phosphatase identified as a fusion partner of TEL. The chimeric gene fused exon 4 of the *TEL* gene with exon 7 of the *PTPRR* gene, and produced 10 isoforms through alternative splicing. Two isoforms that were expressed at the highest level in the leukemic cells could have been translated into COOH-terminally truncated TEL protein possessing the helix-loop-helix domain (tTEL) and TEL/PTPRR chimeric protein linking the helix-loop-helix domain of TEL to the catalytic domain of PTPRR. These two mutant proteins exerted a dominant-negative effect over transcriptional repression mediated by wild-type TEL, although they themselves did not show any transcriptional activity. Heterodimerization with wild-type TEL might be an underlying mechanism in this effect. TEL/PTPRR did not exhibit any tyrosine phosphatase activity. Importantly, overexpression of TEL/PTPRR in granulocyte macrophage colony-stimulating factor–dependent UT7/GM cells resulted in their factor-independent proliferation, whereas overexpression of tTEL did not. After cytokine depletion, phosphorylated signal transducers and activators of transcription 3 (STAT3) significantly declined in mock cells, but remained in both tTEL- and TEL/PTPRR-overexpressing cells. Loss of tumor suppressive function of wild-type TEL and maintenance of STAT3-mediated signal could at least partly contribute to the leukemogenesis caused by inv(12)(p13q13). (Cancer Res 2005; 65(15): 6612-21)

Introduction

The 12p13 translocations are one of the most commonly observed chromosomal abnormalities in human leukemia and myelodysplastic syndrome and fuse the *TEL* gene on 12p13 with various partner genes. The *TEL* gene was originally cloned as a gene that was rearranged by t(5;12)(q33;p13) in chronic myelomonocytic leukemia, and encodes a member of the ETS family transcription factors (1). TEL shares with other ETS proteins an evolutionarily conserved ETS domain at the COOH terminus that is responsible for DNA binding to the ETS-binding consensus site (EBS; ref. 2). TEL also contains an NH_2-terminal domain that is referred to as the helix-loop-helix or pointed domain. The helix-loop-helix domain in TEL has the unique property of inducing its stable homodimerization or heterodimerization with other ETS family members (3–6). Associating with the relevant corepressors mSin3A and N-CoR as well as histone deacetylase-3 (7), TEL works as a transcriptional repressor. Known target genes of TEL are *FLI-1* (8), *Id1* (9), *stromelysin-1* (10), and *Bcl-X$_L$* (11).

Gene-engineered mice have highlighted critical roles of this transcription factor in the embryonic development and hematopoietic regulation. The ablation of the *TEL* gene by homologous recombination causes death in uteri between E10.5 and E11.5 (12). These knock-out embryos show defect in yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, although they present normal yolk sac hematopoiesis. Generating chimeric mice with TEL−/− embryonic stem cells, the pivotal function of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow has been clarified, whereas TEL−/− embryonic stem cells contributed to both primary and definitive fetal hematopoiesis (13). Moreover, a recent study shows that inactivation of TEL in adult mice leads to decrease of hematopoietic stem cells in bone marrow (14). On the other hand, TEL is believed to function as a tumor suppressor because its overexpression in NIH3T3 fibroblasts results in reduced cell growth in liquid and soft agar cultures (10, 15).

Molecular dissecting of the *TEL*-related chimeric genes has provided interesting clues to the pathogenesis of 12p13 translocation-type leukemia. In some translocations, receptor-type and nonreceptor-type tyrosine kinases are fused to the NH_2-terminal portion of TEL and are thus catalytically activated by homodimerization through the helix-loop-helix domain in the TEL moiety. Examples for the former include platelet-derived growth factor receptor β in t(5;12)(q33;p13) (refs. 1, 16, 17) and tyrosine kinase C in t(12;15)(p13;q25) (ref. 18), and those for the latter Abl in t(9;12)(q34;p13) (refs. 19, 20), Janus-activated kinase (JAK)-2 in t(9;12)(p24;p13) (refs. 21, 22), Syk in t(9;12)(q22;p13) (ref. 23), and Abl-related gene in t(1;12)(q25;p13) (ref. 24). In other translocations, transcription factors are structurally and functionally modified by fusing with the NH_2- or COOH-terminal part of TEL. Examples include acute myelogenous leukemia (AML)-1 in t(12;21)(p13;q22) (refs. 25–29), MN1 in t(12;22)(p13;q11) (refs. 30, 31), Evi-1 in t(3;12)(q26;p13) (ref. 32), PAX5 in t(9;12)(q11;p13) (ref. 33), and CDX2 in t(12;13)(p13;q12) (ref. 34). Thus, perturbation of original functions of the partner genes could be a mechanism in causing leukemia in patients with such translocations. Furthermore, disruption of tumor-suppressive function of wild-type TEL itself

Requests for reprints: Kinuko Mitani, Department of Hematology, Dokkyo University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293, Japan. Phone: 81-282-86-1111, ext. 2744; Fax: 81-282-86-5630; E-mail: kinukom-tky@umin.ac.jp.

©2005 American Association for Cancer Research.

seems to be another cause of leukemogenesis because some chimeric molecules such as TEL/AML1 are shown to dominantly interfere with function of wild-type TEL (35).

To obtain a new insight into the molecular mechanism in leukemogenesis by the 12p13 translocations, we cloned several species of novel chimeric cDNAs generated by inv(12)(p13q13) found in a patient with acute myelogenous leukemia [M2 according to the French-American-British (FAB) classification]. These cDNAs contained the NH_2-terminal *TEL* sequence followed by the COOH-terminal sequence from *protein tyrosine phosphatase receptor-type R* (*PTPRR*) and were expected to produce either truncated TEL or chimeric TEL/PTPRR protein. Both molecules lost DNA binding to and trans-repression through EBS, but blocked the molecular function of wild-type TEL probably by heterodimerizing with it. TEL/PTPRR showed no tyrosine phosphatase activity. Notably, overexpression of TEL/PTPRR in factor-dependent human leukemia cell line UT7/GM led to factor-independent growth, suggesting the oncogenic potential of this chimeric molecule.

Materials and Methods

Case presentation. A 24-year-old woman presented with slight fever in April 2001. Her hemoglobin level was 9.1 g/dL, WBC count 4,200/µL with 29% blasts, and platelet count 191,000/µL. The bone marrow aspirate disclosed 79% blasts positive for myeloperoxidase, and a diagnosis of acute myelogenous leukemia (M2 according to the FAB criteria) was made. Flow cytometric assay revealed that the blasts were positive for CD13, CD15, CD33, and CD34. Cytogenetic analysis showed 46, XX, inv(12)(p13q13) (12) /46, XX (8). The patient achieved complete remission after one course of induction chemotherapy and underwent bone marrow transplantation in the first remission from her HLA-matched sibling in November 2001. She has thus far been free from relapse. All the following experiments were done under the written informed consent of the patient.

Fluorescence *in situ* hybridization analysis. The metaphase samples that were subjected to conventional cytogenetic studies were also applied to fluorescence *in situ* hybridization (FISH) analysis. LL12NCO1 cosmid probes (2G8, 163E7, and 184C4) which are located within the *TEL* gene were used in the assay (36). The probes were labeled with biotin-11-dUTP or digoxigenin-11-dUTP using PCR labeling after sequence-independent amplification, and were hybridized to metaphase samples as previously described (37, 38). The hybridization was detected with avium fluorescein or anti-digoxigenin rhodamine and the metaphase cells were subsequently counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. Images of the hybridized signals were captured under fluorescence microscopy.

Cloning of TEL/PTPRR. Total RNA was extracted from cryopreserved leukemic cells with inv(12)(p13q13) using RNeasy RNA miniprep system (Qiagen, Valencia, CA). We purified mRNA by oligo-dT column. The first-strand cDNA was synthesized from 2 µg of RNA using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) with random hexamers and Moloney murine leukemia virus reverse transcriptase. For 3′-rapid amplification of cDNA ends (RACE) procedure, we adopted Marathon cDNA amplification system (Clontech, Palo Alto, CA). Synthesis of the second-strand cDNA and adaptor ligation were carried out according to the instructions of the manufacturer. RACE-PCR was done for 40 cycles with primers TELf2 and activator protein (AP)-1, followed by nested PCR for 30 cycles with primers TELf4 and AP-2 (refer to the instructions of the manufacturer for the sequences of primers AP-1 and AP-2). PCR products were subcloned into the pCR2.1-TOPO cloning vector (Invitrogen) and nucleotide sequences were determined by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

PCR amplification. To amplify *TEL/PTPRR*, *PTPRR/TEL*, wild-type *TEL*, and wild-type *PTPRR* cDNAs, we used primer sets TELf2 and PTPRRr7a, PTPRRf6 and TELr5, TELf2 and TELr5, and PTPRRf6 and PTPRRr7a, respectively. PCR was done for 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Extension time was elongated to 2 minutes when we amplified full-length *TEL/PTPRR* cDNA with a set of primers TELf1 and PTPRRr14. Sequences of all PCR primers are given in the next section.

Oligonucleotides. TELf1 [nucleotide (nt) 25-47 of *TEL*]: 5′-ATGTC-TGAGACTCCTGCTCAGTG-3′; TELf2 (nt 88-111 of *TEL*): 5′-AGCC-CAGTGCCGAGTTACGCTTCC-3′; TELf3 (nt 328-347 of *TEL*): 5′-TTTCGCTATCGATCTCCTCA-3′; TELf4 (nt 376-405 of *TEL*): 5′-CAGCA-TATTCTGAAGCAGAGGAAACCTCGG-3′; TELr5 (complement, nt 637-660 of *TEL*): 5′-GAGGCGGCGGATCATGTTGTCCAG-3′; PTPRRf6 (nt 971-992 of *PTPRR*): 5′-ACCAGGAGATCCACCTATCGCC-3′; PTPRRr7a (complement, nt 1,313-1,334 of *PTPRR*): 5′-ACGACGTCCCTCAGCTGAGACC-3′; PTPRRr7b (complement, nt 1,193-1,216 of *PTPRR*): 5′-TCATGTCCAATGTAAGAGA-TACGT-3′; PTPRRf10 (nt 1,565-1,580 of *PTPRR*): 5′-CCACGCAGGGCCCCAT-3′; PTPRRr10 (complement, nt 1,565-1,584 of *PTPRR*): 5′-GATCATGGGGCC-CTGCGTGG-3′; PTPRRr14 (complement, nt 2,126-2,148 of *PTPRR*): 5′-TCA-CTGGACAGTCTCTGCTGAAA-3′.

Plasmid construction. Constructions of pME18S-HA-TEL, pME18S-FLAG-TEL, pcDNA3-HA-TEL, $(EBS)_3$tkLuc, and pSRαMSVtk*neo*-FLAG-wild-type-TEL were previously described (39, 40). Hemagglutinin or FLAG tag was inserted at both ends of *TEL/PTPRR* chimeric cDNAs by the PCR amplification method. The resultant cDNAs were subcloned into the *Eco*RI sites of pME18S, pcDNA3, and pCXN2 expression plasmids in the sense orientation. To obtain hemagglutinin-tagged wild-type *PTPRR* cDNA, a 5′ fragment spanning the initiation codon (nt 175 according to GenBank accession number U42361) and the *Apa*I site (nt 1,567) was amplified from a human brain total cDNA library (Clontech). Together with a 3′ *Apa*I (nt 1,567 in PTPRR cDNA)/*Xho*I (nt 355 in pCR2.1-TOPO vector) fragment derived from pCR2.1-TOPO-TEL/PTPRR-HA, it was subcloned into the *Xho*I site of pME18S. FLAG-tagged *human SUMO-1* cDNA was obtained by the PCR amplification method, and was subsequently cloned into the *Xba*I site of pME18S.

Cell culture. COS-7 cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% FCS and transfected with various expression plasmids by the DEAE-Dextran method. NIH3T3 fibroblasts were grown in DMEM with 10% bovine serum. HeLa cells were cultured in Eagle's MEM (Sigma) supplemented with 10% FCS and 1% nonessential amino acids. Human leukemia cells UT7/GM were maintained in Iscove's modified Dulbecco's medium (Invitrogen) containing 10% FCS and 1 ng/mL of human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF; Kirin, Gunma, Japan). KASUMI-1 cells were cultured in RPMI 1640 (Sigma) with 10% FCS.

Establishing bulk transfectants of NIH3T3 cells. NIH3T3 bulk population constitutively overexpressing FLAG-tagged wild-type TEL and its control were obtained by retroviral infection and G418 (Sigma) resistance as previously described (41).

Generating bulk and stable transfectants of UT7/GM cells. To obtain bulk transfectants for experiments, 1×10^7 of UT7/GM cells suspended in 500 µL PBS were electroporated at 380 V and 975 µF with pCXN2 plasmid carrying the aimed gene. Forty-eight hours after the electroporation, cells were selected with 0.8 mg/mL of G418. Stable UT7/GM clones overexpressing TEL/PTPRR were established by the limiting dilution method. For factor deprivation, UT7/GM cells were washed with PBS at least thrice and were finally suspended in factor-free media.

Metabolic labeling. Forty-eight hours after transfection, COS-7 cells were washed with phosphate-free DMEM and then cultured for 2 hours in DMEM supplemented with 400 µCi [^{32}P]orthophosphate/mL (Amersham Biosciences, Piscataway, NJ) and 10% dialyzed FCS.

Subcellular localization. NIH3T3 cells were transiently transfected with expression plasmids using TransFast (Promega). Forty-eight hours later, cells were suspended in hypotonic suspension buffer [10 mmol/L sodium phosphate (pH 7.0), 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride], and were separated into nucleic and cytoplasmic fractions using Dounce homogenizer (Wheaton, Millville, NJ). Equal volumes of aliquots were applied to Western blot analysis.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation were done as previously described (42). Anti-FLAG (M2) and anti-hemagglutinin (CH-7 or rabbit) antibodies were purchased from Sigma, and antibodies against TEL (N-19 and C-20), histone H1, actin, Bcl-X_L, signal transducers and activators of transcription (STAT)-3, and phospho-STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoretic mobility shift assay. The procedures for electrophoretic mobility shift assays (EMSA) were previously described (43). Lysates were *in vitro* prepared by TNT-Coupled Wheat Germ Extract System (Promega). The EBS oligonucleotide used as a probe or a specific competitor and its mutant used as a nonspecific competitor were previously described (2). End labeling of the double-stranded oligonucleotide was carried out with [α-^{32}P]dCTP (Amersham Biosciences) and Klenow enzyme (Takara, Shiga, Japan) at room temperature for 30 minutes. Unincorporated nucleotides were removed by G-50 Sephadex columns (Amersham Biosciences). Protein-DNA complexes were separated on a 4% polyacrylamide gel and visualized by autoradiography. In competition studies, a 300-fold molar excess of unlabeled oligonucleotide was added to the reaction. In supershift assays, we employed anti-TEL (N-19) supershift antibody of which epitope is encoded by NH_2-terminal *TEL* sequence in *tTEL* and *TEL/PTPRR* cDNAs. The antibody was preincubated with the lysates at 4°C for 1 hour.

Luciferase assay. HeLa cells in 24-well tissue culture plates were transfected with 1 μg of $(EBS)_3$tkLuc or $(mEBS)_3$tkLuc along with 1 μg of expression plasmids by using Tfx-20 (Promega). To equalize transfection efficiencies, total amounts of expression plasmids were kept constant in terms of weight by adding empty pME18S vector. Luciferase assays were done with Dual-Luciferase reporter assay system (Promega) as previously described (39, 42).

***In vitro* phosphatase assay.** Lysates of COS-7 cells overexpressing hemagglutinin-tagged wild-type PTPRR, tTEL, or TEL/PTPRR were immunoprecipitated with anti-hemagglutinin (CH-7) antibody conjugated with Sepharose A beads (Amersham Biosciences). The immunoprecipitates were washed and finally suspended in 150 μL of assay buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride]. One third of the mixture (50 μL) was added to 100 μL of assay buffer containing 15 mmol/L *p*-nitrophenyl phosphate (Sigma-Aldrich), followed by incubation at 37°C for 10 minutes. The reaction was stopped by adding 25 μL of 2.5 N NaOH. The absorption at 405 nm was read on a 96-well microtiter plate.

Results

Cloning of the *TEL/PTPRR* chimeric gene. Because the *TEL* gene on 12p13 is fused to various partners in a variety of hematologic malignancies, we suspected that inv(12)(p13q13) implicated it. To look whether the *TEL* gene is rearranged by the chromosomal abnormality, we first did FISH analysis using some cosmid probes spanning the *TEL* gene (Fig. 1*A*; ref. 36). Split signals were observed in bone marrow metaphases with inv(12)(p13q13) when we used cosmid 184C4 (Fig. 1*B*) or 163E7 (data not shown) as a probe. Because the procedure with cosmid 2G8 did not yield split signals (data not shown), it was likely that the inversion breakage occurred between introns 5 and 6 of the *TEL* gene. Subsequently, we adopted the 3′-RACE method to identify a fusion partner of the *TEL* gene. RACE-PCR products shown in Fig. 1*C* were subcloned into the pCR2.1-TOPO vector and resultant five clones were sequenced. Among them, four clones contained the wild-type *TEL* sequence only, but the last one included *TEL* exon 4–derived sequence followed by an unknown sequence. BLAST database searching revealed that the chimeric cDNA joined *TEL* exon 4 and *PTPRR* exon 7 with frameshift (human *TEL* was referred to GenBank accession number U11732 and human *PTPRR* to U42361; Fig. 2*A*).

Then, we did reverse transcription-PCR (RT-PCR) analysis to examine whether *TEL/PTPRR, PTPRR/TEL*, wild-type *TEL*, and wild-type *PTPRR* transcripts were expressed in the leukemic cells with inv(12)(p13q13). To detect each transcript, we used primer sets TELf2 (in *TEL* exon 2) and PTPRRr7a (in *PTPRR* exon 7) for *TEL/PTPRR*, PTPRRf6 (in *PTPRR* exon 6) and TELr5 (in *TEL* exon 5) for *PTPRR/TEL*, TELf2 and TELr5 for wild-type *TEL*, and PTPRRf6 and PTPRRr7a for wild-type *PTPRR*. As for *TEL/PTPRR*, two smaller bands (416 and 251 bp) besides a product of the expected size (551 bp) were observed (Fig. 1*D*). Sequencing revealed that these smaller cDNAs lacked exon 4 or exons 3 and 4 of the *TEL* gene, strongly suggesting the presence of alternative splicing mechanisms. On the other hand, reciprocal *PTPRR/TEL* mRNA was not

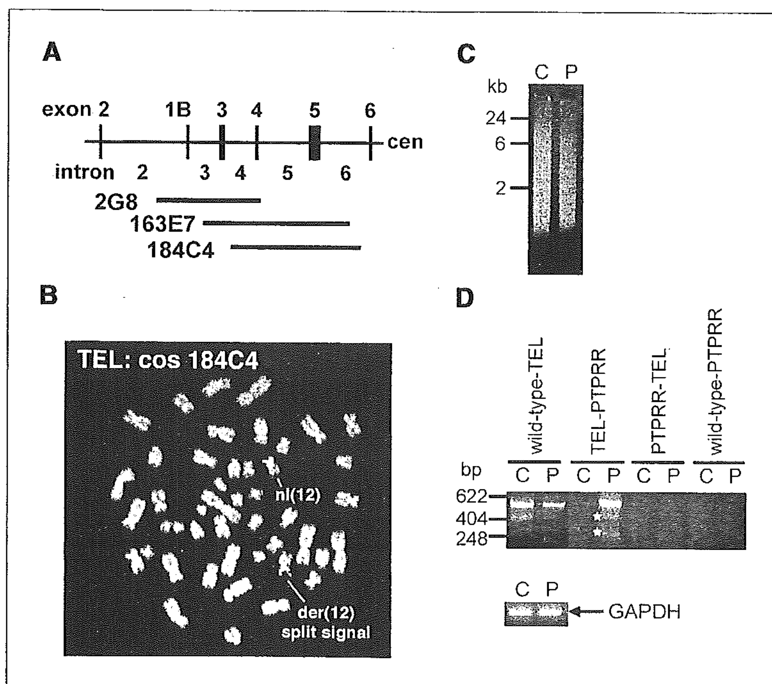


Figure 1. Detection of the fusion gene *TEL/PTPRR. A,* physical map of the *TEL* gene. The cosmid probes used in FISH analysis are shown. *B,* FISH analysis of metaphases with inv(12)(p13q13). Split signals of cosmid 184C4 were observed on the der(12) chromosome. *C,* 3′-RACE method adopted to identify the fusion partner for the *TEL* gene. A fraction of RACE-PCR products was electrophoresed on a 2% agarose gel. *C,* control cell line (HL60); *P,* patient's leukemic cells. *D,* RT-PCR analysis of inv(12)(p13q13)-carrying leukemic cells. Transcripts for wild-type *TEL, TEL/PTPRR, PTPRR/TEL,* wild-type *PTPRR,* and *GAPDH* were amplified. *Asterisks,* two minor *TEL/PTPRR* isoforms lacking exon 4 or exons 3 and 4 of the *TEL* gene. Primers used are described in Materials and Methods. *C,* control cell line (HL60); *P,* patient's leukemic cells.