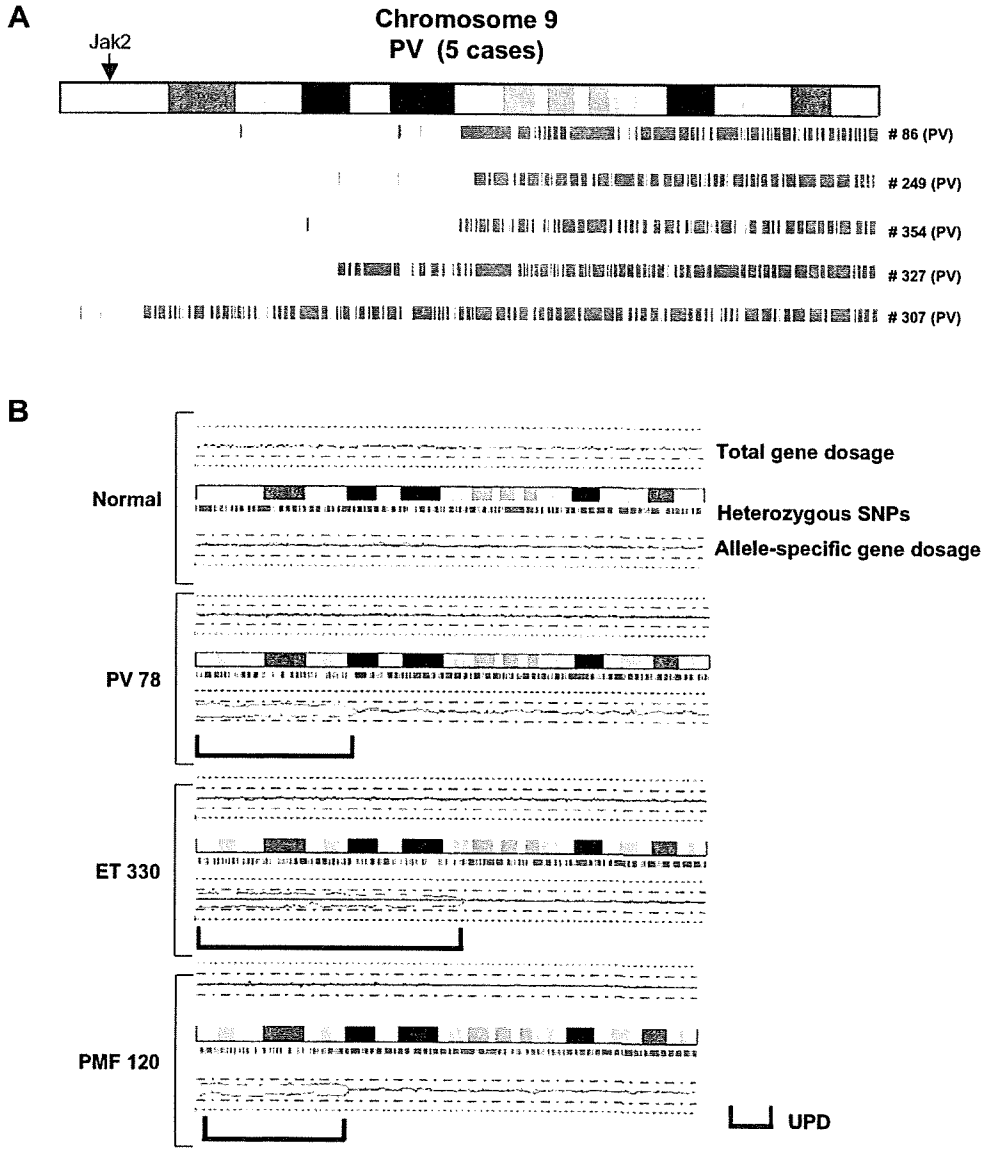


Figure 2. Uniparental disomy of 9 p in myeloproliferative disorder samples. (A) Uniparental disomy in dominant clones. Green bars indicate heterozygous single-nucleotide polymorphism (SNPs). Regions with pink lines show those regions with loss of heterozygosity but with normal DNA copy number (uniparental disomy). Position of *JAK2* gene is indicated by an arrow. Case numbers are shown to the right of the chromosomes. (B) Uniparental disomy in nondominant clone. Representative cases having nondominant clones with 9 p uniparental disomy are shown. In each panel, a blue line at the top indicates level of total gene dosage. Green bars under the chromosome indicate heterozygous SNP sites detected by SNP-chip analysis. Green and red lines in the bottom in each panel demonstrate levels of parental gene dosage. Uniparental disomy regions have decrease of one of the parental gene dosage (green line) and increase of gene dosage of the other parental allele (red line). ET = essential thrombocytosis; PMF = primary myelofibrosis; PV = polycythemia vera; UPD = uniparental disomy.

loci. For example, we identified deleted regions of PMF that include either the *RB1* (13q14) or *NF1* (17q11) locus.

Recently, uniparental disomy that is not detectable by conventional techniques has been reported in human cancers [14,25,26]. Most investigators have assayed for loss of heterozygosity using microsatellite markers, combined with fluorescence in situ hybridization to detect uniparental disomy [26]. A region was defined as having uniparental disomy when fluorescence in situ hybridization displayed an intact chromosome pattern and the loss of heterozygosity analysis demonstrated allelic imbalance at the locus [26]. However, this approach is both time- and labor-intensive compared with use of SNP-chip for detection of uniparental disomy. For example, we detected five PV cases with 9 p uniparental disomy. Each 9 p uniparental disomy case had two *JAK2* mutated

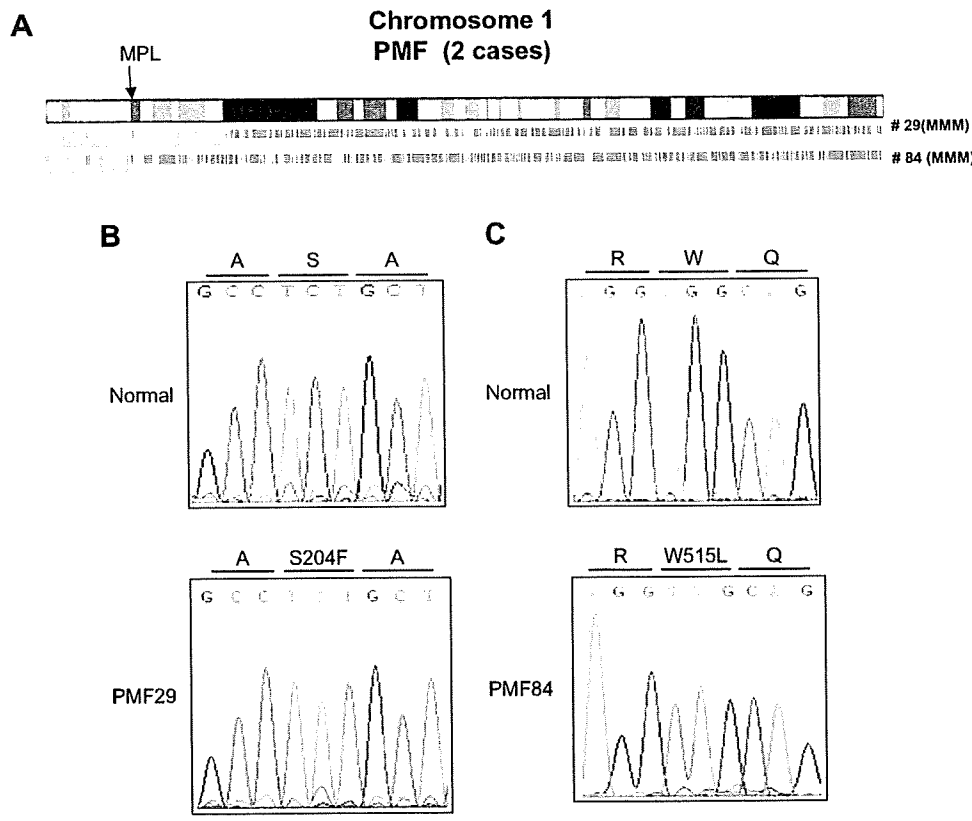


Figure 3. Uniparental disomy of 1 p and point mutations of *MPL* in primary myelofibrosis (PMF). (**A**) Two cases of PMF with clear uniparental disomy in the 1 p region are shown. Green bars indicate heterozygous single-nucleotide polymorphism (SNPs). Regions with pink lines show those regions with loss of heterozygosity but with normal DNA copy number (uniparental disomy). Position of *MPL* gene is indicated by an arrow. Point mutations of *MPL* found in two PMF cases are shown: S204F (**B**) and W515L (**C**). The sequences were determined by direct nucleotide sequencing. Sequences of normal alleles are shown in the upper panels. UPD = uniparental disomy.

alleles as confirmed by allele-specific PCR. As previously reported, uniparental disomy is a common mechanism for duplication of the mutant *JAK2*V617F allele in PV [6], presumably through mitotic recombination and subsequent positive selection for clones harboring two mutant alleles. We previously developed a new algorithm allowing us to evaluate allele-specific gene dosage (gene dosages of paternal and maternal alleles) without matched control samples [17]. This new algorithm can very sensitively and accurately detect uniparental disomy regions even though the clones with uniparental disomy are as small as 20% of whole tissues [17]. We previously found that 9 p uniparental disomy in nondominant clones were frequent events in MPD [17].

Using this method, we could detect nondominant clones with 9 p and/or 1 p uniparental disomy, which may be overlooked by standard algorithm used for SNP-chip analysis. Uniparental disomy was a very frequent event in PMF; three cases had 1 p uniparental disomy and seven cases had 9 p uniparental disomy. In contrast, only one of the ET cases in this study had uniparental disomy. In ET, we only found one sample with deletion of 5q23.1 and another with trisomy 9, as well as, 7 cases with a *JAK2* mutation. This observation could be explained by either lack of involvement of the granulocytic lineage (our source of DNA) in these ET patients, or by point mutations or other genetic abnormalities in these samples that were not detected by SNP-chip analysis. Clearly, the pathogenesis leading to ET, especially ET without a *JAK2* mutation, remains to be elucidated.

We detected a novel 1 p uniparental disomy in samples from three PMF and one PV individuals. Because chromosomal regions of uniparental disomy are hotspots of genes mutated in cancers, we screened all coding exons of several candidate genes, including *MPL*, in the 1 p region by direct nucleotide sequencing. In each case, we found a point mutation of the *MPL* gene; two samples involved the well-know mutation, W515L [10,11], and the other two were novel mutations (S204F and Y591D). Unfortunately, because the matched control samples of these cases were not available, we could not examined if these changes were found in their germline. Nevertheless, we did screen 100 DNA samples from normal individuals to determine if the nucleotide substitutions (S204F and Y591D) were polymorphic; we did not

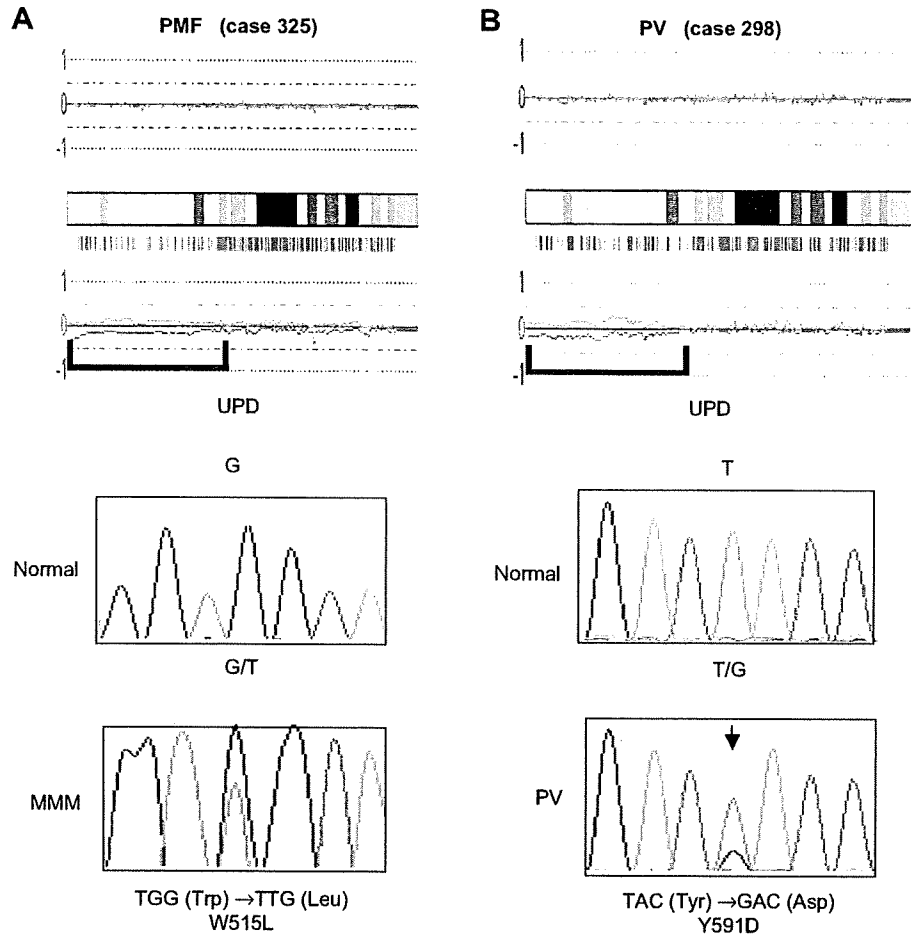


Figure 4. Nondominant clones with 1 p uniparental disomy in myeloproliferative disorder were detected by single-nucleotide polymorphism DNA microarray (SNP-chip). Top panels: Allele-specific gene dosage analysis. Blue lines above the chromosomal panels indicate levels of total gene dosage (0 indicates 2 N, normal diploid). Green and red lines below the chromosomal panels indicate respective parental allele gene dosage. Higher level of the red line and lower level of the green line indicates the regions of uniparental disomy. Middle and bottom panels: normal (middle) and mutated (bottom) nucleotide sequences of the *MPL* gene encoding amino-acid positions at 515 and 591; primary myelofibrosis (PMF) case 325 (**A**) had W515L mutation and polycythemia vera (PV) case 298 (**B**) had Y591D mutation. UPD = uniparental disomy.

detect these nucleotide substitutions in normal DNA (data not shown). We screened all of our remaining MPD cases for these mutations (S204F, W515L, and Y591D) and found none (data not shown). Amino-acid substitution of MPL was found in all cases with 1 p uniparental disomy, suggesting that these substitutions are mutations, not rare polymorphisms, and MPL gene is a target gene of 1 p uniparental disomy. 1 p uniparental disomy may be a hallmark for a *MPL* mutation in MPD. The precise function of novel MPL mutants (S204F and Y591) remains to be explored.

To elucidated functional significance of these novel mutations of the *MPL* gene, we expressed them in the IL3-dependent murine B-cell line, BaF/3 cells. Mutant S204F or Y591D MPL did not allow the cells to grow independently of IL-3, whereas W515L MPL did (data not shown). Unfortunately, because the peripheral blood and/or bone marrow cells from these patients were not available, we were not able to examine bone marrow hematopoietic stem cells for these abnormalities. How these novel MPL mutants contribute to development of PMF is unclear. Interestingly, one PV (case 298) had a point mutation of both a *JAK2* and *MPL* (Y591D), suggesting that mutations

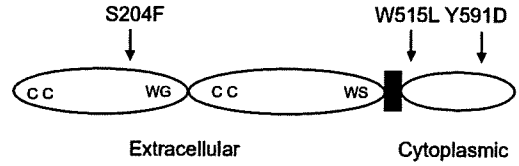


Figure 5. Position of point mutations of the *MPL* gene found in myeloproliferative disorder. Schema of the MPL protein. Position of the point mutations are indicated by arrows. CC = conserved cysteine residues; WG = WGXWS motif; WS = WSXWS motif; extracellular: extracellular domain; cytoplasmic: cytoplasmic domain; black box depicts the transmembrane domain.

of these two molecules may independently contribute to development of MPD, even though they are involved in the same Mpl/Jak/Stat signal transduction pathway.

Myelofibrosis, not thrombocytosis, is a prominent feature in PMF [1]. How an activating point mutation of *MPL* by itself can induce myelofibrosis is unclear. Interestingly, two cases with *MPL* mutations had deletion of the *RB1* gene. Mutation of *MPL* and deletion of *RB1*, may cooperate in development of PMF; or loss of *RB1* may potentiate acquisition of mutations of *MPL*.

In summary, we found recurrent genetic abnormalities, including 9 p uniparental disomy/*JAK2* mutations, 1 p uniparental disomy/*MPL* mutations, as well as deletions of the *RB* and the *NF1* genes in MPD. We found two novel point mutations of the *MPL* gene (S204F and Y591D) in PMF and PV samples with 1 p uniparental disomy. Genomic abnormalities, including point mutations, deletions, and uniparental disomy may cooperate in development of MPD. In regions of deletions, duplications/amplifications, and uniparental disomy, critical target genes may be altered. Accumulation of data of SNP-chip analysis will narrow the common abnormal genomic regions and help to clone novel mutated genes in MPD. SNP-chip analysis is a robust tool to detect genetic abnormalities, especially small deletions and uniparental disomy in MPD.

References

1. Tefferi A. Myelofibrosis with myeloid metaplasia. N Engl J Med. 2000;342:1255–1265.
2. Spivak JL. Diagnosis of the myeloproliferative disorders: resolving phenotypic mimicry. Semin Hematol. 2003;40(Suppl 1):1–5.
3. Thiele J, Kvasnicka HM, Orazi A. Bone marrow histopathology in myeloproliferative disorders—current diagnostic approach. Semin Hematol. 2005;42:184–195.
4. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365:1054–1061.
5. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434:1144–1148.
6. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352: 1779–1790.
7. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7:387–397.
8. Zhao R, Xing S, Li Z, et al. Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem.. 2005;280:22788–22792.
9. Scott LM, Tong W, Levine RL, et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med. 2007;356: 459–468.
10. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med. 2006;3:e270.
11. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006;108:3472–3476.
12. Levine RL, Belisle C, Wadleigh M, et al. X-inactivation-based clonality analysis and quantitative JAK2V617F assessment reveal a strong association between clonality and JAK2V617F in PV but not ET/MMM, and identifies a subset of JAK2V617F-negative ET and MMM patients with clonal hematopoiesis. Blood. 2006;107:4139–4141.
13. Garraway LA, Widlund HR, Rubin MA, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature. 2005;436:117–122.
14. Kawamata N, Ogawa S, Zimmermann M, et al. Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high resolution single nucleotide polymorphism oligonucleotide genomic microarray. Blood. 2008;111:776–784.
15. Lehmann S, Ogawa S, Raynaud SD, et al. Molecular allelokaryotyping of early stage untreated chronic lymphocytic leukemia. Cancer. 2008; 112:1296–1305.
16. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res. 2005;65:6071–6079.
17. Yamamoto G, Nannya Y, Kato M, et al. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. Am J Hum Genet. 2007;81:114–126.
18. Batard P, Sansilvestri P, Scheinecker C, et al. The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. Blood. 1996;87:2212–2220.
19. Lannutti BJ, Shim MH, Blake N, Reems JA, Drachman JG. Identification and activation of Src family kinases in primary megakaryocytes. Exp Hematol. 2003;31:1268–1274.
20. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005;115:3339–3347.
21. Carter NP. Methods and strategies for analyzing copy number variation using DNA microarrays. Nat Genet. 2007;39(Suppl):S16–S21.
22. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006;444:444–454.
23. Jakobsson M, Scholz SW, Scheet P, et al. Genotype, haplotype and copy-number variation in worldwide human populations. Nature. 2008;451:998–1003.
24. Sebat J, Lakshmi B, Troge J, et al. Large-scale copy number polymorphism in the human genome. Science. 2004;305:525–528.
25. Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Res. 2005;65:375–378.
26. Kralovics R, Guan Y, Prchal JT. Acquired uniparental disomy of chromosome 9p is a frequent stem cell defect in polycythemia vera. Exp Hematol. 2002;30:229–236.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exphem.2008.06.006.

Leukemia-related transcription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis

Minenori Eguchi-Ishimae,[1,2] Mariko Eguchi,[1,2] Kazuhiro Maki,[1] Catherine Porcher,[3] Ritsuko Shimizu,[4] Masayuki Yamamoto[5] and Kinuko Mitani[1,6]

[1]Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi 321-0293; [2]Department of Pediatrics, Ehime University, Ehime 791-0295, Japan; [3]MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, United Kingdom; [4]Institute of Basic Medical Sciences and Center for TARA, University of Tsukuba, Ibaraki 305-8577; [5]Medical Biochemistry, Tohoku University School of Medicine, Miyagi 980-8575, Japan

(Received 24 July, 2008/Revised November 7, 2008; December 18, 2008/Accepted December 24, 2008/Online publication March 11, 2009)

***TEL/ETV6* located at chromosome 12p13 encodes a member of the E26 transformation-specific family of transcription factors. *TEL* is known to be rearranged in a variety of leukemias and solid tumors resulting in the formation of oncogenic chimeric protein. Tel is essential for maintaining hematopoietic stem cells in the bone marrow. To understand the role of TEL in erythropoiesis, we generated transgenic mice expressing human *TEL* under the control of *Gata1* promoter that is activated during the course of the erythroid-lineage differentiation (*GATA1-TEL* transgenic mice). Although *GATA1-TEL* transgenic mice appeared healthy up to 18 months of age, the level of hemoglobin was higher in transgenic mice compared to non-transgenic littermates. In addition, CD71$^+$/TER119$^+$ and c-kit$^+$/CD41$^+$ populations proliferated with a higher frequency in transgenic mice when bone marrow cells were cultured in the presence of erythropoietin and thrombopoietin, respectively. In transgenic mice, enhanced expression of *Alas-e* and *β-major globin* genes was observed in erythroid-committed cells. When embryonic stem cells expressing human *TEL* under the same *Gata1* promoter were differentiated into hematopoietic cells, immature erythroid precursor increased better compared to controls as judged from the numbers of burst-forming unit of erythrocytes. Our findings suggest some roles of TEL in expanding erythroid precursors and accumulating hemoglobin. (*Cancer Sci* 2009; 100: 689–697)**

TEL (also known as *ETV6*) gene is frequently involved in recurring chromosomal translocations as well as deletions in various hematopoietic malignancies, suggesting its role as a tumor suppressor gene.[1,2] *TEL* encodes a member of the ETS family of transcription factors and has the ETS DNA binding domain in its C-terminal side[3] and the Pointed domain with oligomerization capacity in its N-terminal side.[4] Between the Pointed and ETS domains is the central domain that ascribes TEL with transcriptional repression activity by recruiting repressor complexes including histone deacetylase and nuclear corepressors,[5–9] one of the characteristic properties of TEL among ETS transcription factors.

Tel plays some important roles in development and hematopoiesis. Complete ablation of the *Tel* gene in mice results in embryonic lethal phenotype at day 10.5–11.5 post-coitus with impaired yolk sac angiogenesis.[10] At that time, primary erythropoiesis in the yolk sac is intact. Detailed analysis of hematopoietic differentiation and proliferation capacity of *Tel–/–* cells was carried out by means of generation of chimeric mice which consist of *Tel–/–* cells as well as wild-type cells to avoid embryonic lethality.[11] The observation that cells lacking both *Tel* alleles fail to contribute to hematopoiesis in the neonatal bone marrow, but do not in the yolk sac and fetal liver, indicating an active role of Tel on hematopoietic stem cells to recruit to the bone marrow microenvironment or to be maintained in the bone marrow niche to construct bone marrow hematopoiesis by producing their progeny. Conditional inactivation of *Tel* in adult mice results in complete loss of hematopoietic stem cells in the bone marrow[12] which is consistent with the finding obtained from the chimera analysis. These findings indicate the function of Tel is as a selective and essential regulator of stem cells. However, detailed functions of TEL in hematopoietic cell differentiation are still unknown. One approach to scrutinize the function of TEL is to see the outcome after enforced expression of *TEL* gene in a specific lineage and at a specific stage of hematopoietic differentiation, as expression of *TEL* gene is suggested to be ubiquitous and continuous during differentiation.[3,10,11] We have previously reported that upon induction of erythroid differentiation by chemical compounds, a murine erythroid leukemic cell line MEL differentiates to mature erythroid cells more effectively by overexpression of the *TEL* gene.[13] In addition, overexpressed *TEL* in an erythroid/megakaryocytic-committed human leukemic cell line UT7/GM promotes erythroid differentiation and inhibits megakaryocytic maturation.[14] All these data suggest that TEL might have some impact on terminal hematopoietic differentiation along the erythroid and megakaryocytic lineages.

GATA1, encoding a zinc-finger transcription factor, plays a central role in erythropoiesis by regulating transcription of genes such as *δ-aminolevulinic acid synthase-erythroid* (*ALAS-E*) and *α/β-globin* genes.[15–20] Gata1 is essential for primary erythropoiesis[21–23] and regulates maturation and apoptotic induction of definitive erythropoiesis.[24,25] Although *GATA1* is expressed in multipotential progenitor cells, albeit at a low level,[26,27] a drastic increase of *GATA1* expression is observed upon erythroid-lineage commitment, resulting in further progression of the erythroid differentiation pathway.[28,29] Regulation of *GATA1* expression at an appropriate stage is quite crucial for proper erythroid lineage development and its expression is controlled precisely through the erythroid-specific regulatory region of the gene.[30,31]

To understand the effects of *TEL* on erythropoiesis, we have established transgenic mice and embryonic stem (ES) cells that express human *TEL* specifically in the erythroid-committed cells under the control of the erythroid-specific *Gata1* promoter. Forced expression of *TEL* in the erythroid-committed cells resulted in higher hemoglobin (Hb) levels in the mice and promoted

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

doi: 10.1111/j.1349-7006.2009.01097.x

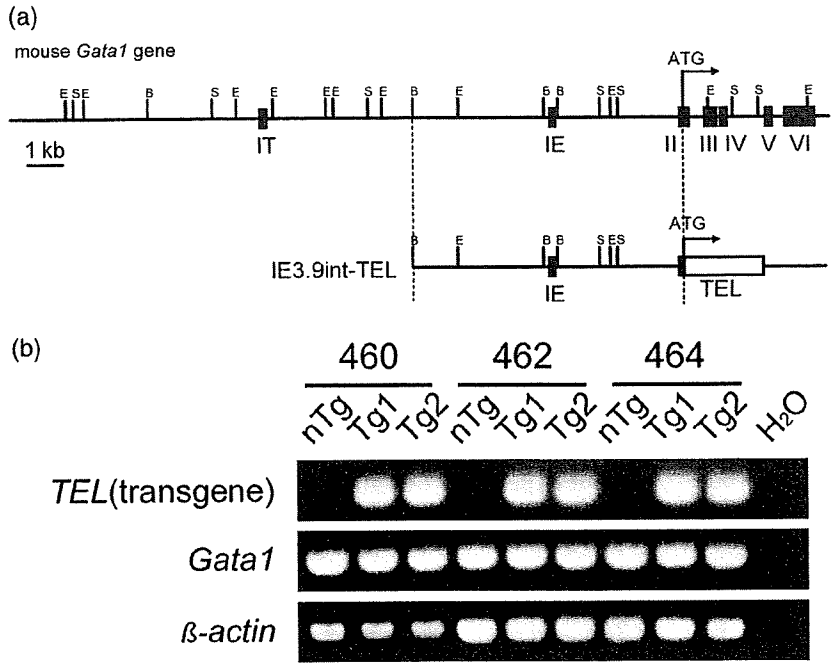


Fig. 1. Expression of TEL transgene in *GATA1-TEL* transgenic mice. (a) Schematic representation of the *Gata1* promotor region and the pIE3.9int-TEL construct used for the generation of *GATA1-TEL* transgenic mice. The pIE3.9int vector contains 3.9 kb *Gata1* promoter region upstream of exon IE.[30] The coding sequence of wild-type human *TEL* cDNA was connected to the first ATG codon in exon II of mouse *Gata1* gene. Abbreviations for the restriction enzyme sites are E, *Eco*RI; B, *Bam*HI; S, *Sac*I. (b) Expression of *Gata1*-driven *TEL* transgene was confirmed by reverse transcription polymerase chain reaction (RT-PCR) using bone marrow cells extracted from *GATA1-TEL* transgenic mice of lines 460, 462 and 464. Forward primers for the first and second PCR were both located on exon IE of mouse *Gata1* gene and reverse primers on exon II of human *TEL* gene. Tg, *Gata1-TEL* transgenic mouse; nTg, non-transgenic littermate.

expansion of erythroid progenitor following erythropoietin (EPO) stimulation *in vitro*. The erythroid-specific genes *Alas-e* and *β-major globin* were more highly expressed in CD71^{high}/TER119^{+} erythroid precursor in the bone marrow of *GATA1-TEL* transgenic mice than non-transgenic mice. In ES cell culture experiments, when day 7 embryoid body (EB) was subjected to hematopoietic colony assay, higher numbers of BFU-E were formed in *GATA1-TEL* transgenic cells. These data indicate that TEL could regulate both proliferation and differentiation of erythroid cells.

Materials and methods

Transgenic vector and generation of GATA1-TEL transgenic mice. pIE3.9intLacZ vector which contains mouse *Gata1* promoter region 3.9 kb upstream of exon IE was described previously.[30] LacZ-coding region was removed from pIE3.9intLacZ and replaced by a coding sequence of wild-type human *TEL* gene downstream of native ATG codon of *Gata1* (pIE3.9int-TEL, Fig. 1a). *GATA1-TEL* transgenic mice were generated by microinjection of pIE3.9int-TEL vector to fertilized mouse oocytes isolated from superovulated BDF1 mice (Clea Japan Inc., Tokyo, Japan). Genomic DNA was prepared from tails of liveborn mice and genotyping was performed by polymerase chain reaction (PCR) using a combination of primers located on an intron sequence upstream of exon II of mouse *Gata1* (G1 HD-8369f) and human *TEL* cDNA (TEL-91r, TEL-117r). Sequences of primers are listed in Supporting Table S1. Peripheral blood counts were performed using particle counter PCE-170 (ERMA Inc, Tokyo, Japan). Serum EPO levels were evaluated using Quantikine Mouse/Rat Epo Immunoassay (R & D Systems, Minneapolis, MN, US).

Reverse transcriptase-mediated PCR (RT-PCR). Total RNA was prepared using RNeasy kit (Qiagen, Valencia, CA, US) with DNaseI treatment and then reverse transcribed with random hexamers using MMLV reverse transcriptase (Stratagene, La Jolla, CA, US). Reverse transcription products were amplified by PCR with specific primers using standard procedures. To examine the expression of *GATA1-TEL* transgene, forward primers for PCR amplification were designed on exon IE of *Gata1* (mGATA1-3f, mGATA1-28f) and reverse primers on human *TEL* sequence (TEL-91r, TEL-117r). Upon transcription from integrated pIE3.9int-TEL sequences, exon IE of mouse *Gata1* is connected to human *TEL* sequence replacing *Gata1*-coding sequence, which could be assessed specifically by RT-PCR with this combination of primers. Expression of endogenous *Gata1* was examined with mGATA1-3f and mGATA1-336r (located on exon III of *Gata1*). The details of primer sequences are shown in Table S1. The products were electrophoresed on 2% agarose gels and stained by ethidium bromide.

Real-time quantitative PCR. Quantitative PCR was performed with a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, US) as indicated in the manufacturer's protocol using 10 ng cDNA template and 200 μM each primer per reaction. Reactions were run and analyzed on ABI7700 (Applied Biosystems). All reactions were performed in duplicate, and were analyzed using SDS software (Applied Biosystems). Primer sets to analyze expression levels of endogenous *Gata1*, transgenic *GATA1-TEL* and total (endogenous + exogenous) *TEL* transcripts were mGATA1–50f and mGATA1-303r, mGATA1-28f and GIHRD-TEL-r (*hTEL* in Supporting Table S2), TEL-1005f and TEL-1082r (for bone marrow cells, *hmTEL(3)* in Supporting Table S2), and TEL-829f and TEL-921r (for ES cells, *hmTEL(2)* in Supporting Table S2), respectively. Forward and reverse primers of both *hmTEL(3)* and *(2)* are located on exons V and VI of mouse and human *TEL* genes and can simultaneously amplify both mouse and human *TEL* transcripts because the sequences of this region are almost identical between these two species. *Hypoxanthine phosphoribosyl-transferase* (*Hprt*) was used as a control gene for normalization to account for variations in template input, as described previously.[32] The details of primer sequences are shown in Supporting Table S2.

Differentiation in liquid cultures. Bone marrow cells harvested from femurs of mice were dispersed into single cell suspensions and were cultured in the presence of recombinant murine EPO (3 U/mL) and stem cell factor (SCF) (50 ng/mL), or thrombopoietin (TPO, 20 ng/mL), interleukin (IL)-3 (10 ng/mL) and IL-6 (10 ng/mL). Cells were examined after 8 days by fluorescence activated cell sorting (FACS). Murine recombinant SCF, IL-3 and IL-6 were purchased from Peprotec (London, UK), and EPO from R & D Systems (Minneapolis, MN, US).

doi: 10.1111/j.1349-7006.2009.01097.x

Table 1. Peripheral blood count of GATA1-TEL transgenic mice and littermate controls

	TEL460		TEL462		TEL464	
	nTg	Tg	nTg	Tg	nTg	Tg
No. of mice	11	20	14	30	7	50
WBC ($\times 10^3/\mu L$)	9.8 ± 4.8	10.5 ± 3.5	11.1 ± 4.3	9.8 ± 3.4	8.6 ± 4.0	7.7 ± 3.0
RBC ($\times 10^6/\mu L$)	8.6 ± 0.5	9.1 ± 0.4*	8.7 ± 0.4	8.7 ± 0.4	8.7 ± 0.5	8.8 ± 0.4
Hb (g/dL)	16.5 ± 1.0	17.4 ± 1.0*	17.1 ± 0.8	17.8 ± 1.3*	16.4 ± 0.8	17.1 ± 3.9
Ht (%)	37.7 ± 3.5	38.9 ± 1.8	40.0 ± 3.1	41.0 ± 4.2	36.8 ± 1.9	37.7 ± 2.1
Plt ($\times 10^4/\mu L$)	164.4 ± 50.1	148.5 ± 32.6	128.1 ± 38.1	123.3 ± 56.0	122.0 ± 14.9	110.1 ± 25.9
MCV (fl)	43.5 ± 2.3	42.9 ± 1.2	46.1 ± 3.4	46.9 ± 3.9	42.5 ± 1.4	43.0 ± 1.4
MCH (pg)	19.1 ± 0.7	19.2 ± 0.8	19.8 ± 0.8	20.4 ± 0.9*	19.0 ± 0.7	19.5 ± 4.6*
MCHC (%)	44.1 ± 3.1	44.9 ± 1.7	42.9 ± 3.3	43.8 ± 4.4	44.7 ± 2.0	45.5 ± 10.5

*Significantly higher compared to littermate controls ($P < 0.05$).
Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate; Hb, hemoglobin; Ht, Hematocrit; Plt, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Flow cytometry and cell sorting. Single cell suspensions were prepared from bone marrow or cultured cells and were then analyzed by flow cytometry using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against c-kit, TER119 (BD Biosciences Pharmingen, San Diego, CA, US) or CD41, CD71 (eBioscience, San Diego, CA, US). The stained cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA, US) or sorted on FACSAria (Becton Dickinson).

ES cell growth and differentiation. Mouse ES cells (J1) were electroporated with pIE3.9int-TEL transgenic vector connected to *neomycin resistance* gene or mock pIE3.9int-neo vector, and selected with G418 (Sigma, St Louis, MO, US). ES cells were maintained on gelatinized plates in TX-WES cell culture medium (Thromb-X, Leuven, Belgium) with supplement of recombinant murine leukemia inhibitory factor (LIF, AMRAD, Melbourne, Australia). For the generation of EBs, ES cells were trypsinized and plated at various densities in differentiation cultures. Differentiation of EBs was carried out in 82-mm Petri-grade dishes in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine (Gibco/BRL, Gaithersburg, MD, US), 200 μg/mL transferrin, 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M 1-thioglycerol (Sigma). Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37°C.

Colony assays of EBs. To differentiate hematopoietic precursors, EBs were dissociated at day 7 and cells were plated in 1% methylcellulose containing 10% FCS, 5% protein-free hybridoma medium (PFHM-II; Gibco/BRL), 2 mM L-glutamine, 200 μg/mL transferrin and following cytokines for colony forming unit of granulocyte/erythrocyte/macrophage/megakaryocytic (CFU-GEMM) assay: SCF (100 ng/mL), TPO (5 ng/mL), EPO (2 U/mL), IL-11 (5 ng/mL), IL-3 (1 ng/mL), granulocyte/macrophage-colony stimulating-factor (GMCSF) (30 ng/mL), granulocyte-colony stimulating factor (G-CSF) (30 ng/mL), macrophage-colony stimulating factor (M-CSF) (5 ng/mL) and IL-6 (5 ng/mL), and for BFU-E assay: SCF (100 ng/mL), TPO (5 ng/mL) and EPO (2 U/mL). Murine recombinant GM-CSF, M-CSF, G-CSF and IL-11 were purchased from Peprotec. Cultures were maintained at 37°C with 5% CO_2. The numbers of colonies comprising more than 40 cells were scored after 7 days, and myeloid, erythroid and mixed colonies were defined based on their morphology.

***In vitro* differentiation of EB-derived c-kit$^+$/CD71$^+$ cells on OP9 layer.** EBs were dissociated at day 6 of differentiation and c-kit$^+$/CD71$^+$ cells were separated by FACSAria. Sorted c-kit$^+$/CD71$^+$ cells were plated onto OP9 stromal cell[33,34] layer supplemented with EPO (3 U/mL) and SCF (50 ng/mL) to promote erythroid differentiation and cultured for 8 days before FACS analysis. OP9 cells were maintained in α-modified minimum essential media (α-MEM, Gibco-BRL) supplemented with 20% FCS.

Statistical analysis. A two-tailed Student's *t*-test was used to determine the difference between non-transgenic and *GATA1-TEL* transgenic samples.

Results

Generation of *GATA1-TEL* transgenic mice. Three transgenic lines (460, 462 and 464) were established with pIE3.9int-TEL transgenic construct. Expression of transgene (i.e. human *TEL*) from integrated *Gata1-TEL* sequences was confirmed by RT-PCR with bone marrow cells of transgenic mice and their littermates. As expected, expression of the transgene was seen in the bone marrow of all of the transgenic mice examined, and the representative data are shown in Fig. 1(b). Expression of endogenous *Gata1* was also confirmed with the same bone marrow RNA samples. *GATA1-TEL* transgenic mice of all the lines appeared healthy up to 18 months of age without any symptoms.

Higher Hb concentration in *GATA1-TEL* transgenic mice. Blood counts were examined using peripheral bloods obtained from *GATA1-TEL* transgenic mice and their littermates (Table 1). As a result, Hb concentration was significantly higher in two transgenic lines (460 and 462), and red blood cell (RBC) count was also higher in one of the lines (460). Although not statistically significant, Hb concentration was also higher in the other transgenic line (464). There were no significant differences in white blood cell and platelet counts between *GATA1-TEL* transgenic mice and their littermate controls of any lines. Then, we evaluated serum EPO levels of *GATA1-TEL* transgenic mice and their litters of the three lines 460, 462 and 464. Mean EPO levels of transgenic and non-transgenic mice were 108 ± 21 pg/mL ($n = 10$) and 123 ± 30 pg/mL ($n = 10$) in line 460, 221 ± 65 pg/mL ($n = 8$) and 250 ± 105 pg/mL ($n = 11$) in line 462, and 134 ± 54 pg/mL ($n = 4$) and 142 ± 85 pg/mL ($n = 4$) in line 464. Although the differences were not statistically significant, there was a tendency that serum EPO levels were lower in the transgenic mice of all the lines, suggesting that serum EPO levels were negatively regulated by increased Hb in the transgenic mice.

CD71^{high}/TER119$^+$ cells expanded better from the bone marrow cells of transgenic mice than littermate controls. When populations of granulo-monocytic, erythroid, megakaryocytic and immature hematopoietic cells in the bone marrow were assessed by FACS analysis using antibodies against Gr-1/Mac-1, CD71/TER119, c-kit/CD41 and c-kit/CD34, no apparent difference between *GATA1-TEL* transgenic mice and their littermate controls was observed (data not shown). Colony forming cell (CFC) assay also revealed no significant difference between them (data not shown). However, when bone marrow cells were cultured in the

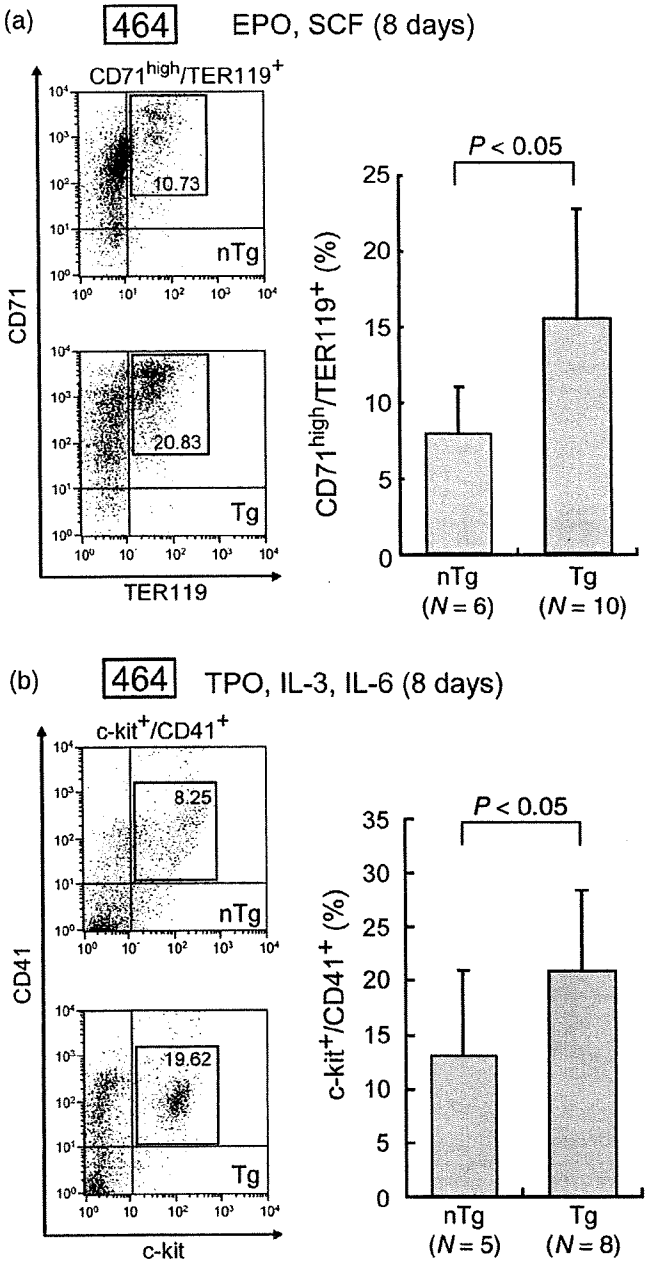


Fig. 2. Differentiation of bone marrow cells into erythroid and megakaryocytic precursors. Bone marrow cells were extracted and cultured in the presence of recombinant murine (a) erythropoietin (EPO) (3 U/mL) and Stem cell factor (SCF) (50 ng/mL), or (b) thrombopoietin (TPO) (20 ng/mL), interleukin (IL)-3 (10 ng/mL) and IL-6 (10 ng/mL). Cells were examined after 8 days of culture by fluorescence-activated cell sorter, which revealed that bone marrow cells obtained from *GATA1-TEL* transgenic mice showed higher populations of (a) CD71^{high}/TER119^{+} cells, or (b) c-kit^{+}/CD41^{+} cells compared to those from littermate controls. In the left panels, the representative data from non-transgenic (nTg) and transgenic (Tg) mice of line 464 are shown. In the right panel, indicated are average and standard deviation of five (a) or four (b) independent experiments using lines 460, 462 and 464. Numbers in parenthesis indicate numbers of mice analyzed in each group. Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.

presence of EPO and SCF for 7 days, CD71^{high}/TER119^{+} population, corresponding to proerythroblast to basophilic erythroblast[35] expanded more efficiently from the bone marrow cells of *GATA1-TEL* transgenic mice compared to those of littermate controls (Fig. 2a). In addition, c-kit^{+}/CD41^{+} population was also obtained more abundantly in the transgenic mice following 7 days of culture with TPO, IL-3 and IL-6 (Fig. 2b).

The expression levels of *Alas-e* and *β-major globin* genes are higher in CD71high/TER119^{+} erythroblast of transgenic mice than littermate controls. Given that *GATA1-TEL* transgenic bone marrow cells gave rise to more CD71^{high}/TER119^{+} erythroblast upon stimulation with EPO, *Gata1*-driven *TEL* expression might alter proliferation and/or differentiation abilities of immature erythroid progenitors. To find out the molecular basis, bone marrow cells were separated into three populations according to the expression levels of CD71 and TER119 (Fig. 3A-a,b,c), and expression of genes related to erythroid proliferation/differentiation was examined by quantitative PCR. The most differentiated erythroid population in the panel is represented as CD71^{-}/TER119^{+} (Fig. 3A-c), whereas the CD71^{high}/TER119^{+} population (Fig. 3A-b) contains more immature but erythroid-committed progenitors, which are derived from the CD71^{int}/TER119^{-} population (Fig. 3A-a) consisting of not only erythroid-committed progenitors but also other lineages-committed progenitors such as myeloid cell and megakaryocyte. CD71^{int}/TER119^{-} population was positive for c-kit, and gave rise to both myeloid and erythroid colonies (data not shown). The proportions of these three populations were comparable between *GATA1-TEL* transgenic mice and their littermates (data not shown).

Expression of endogenous *Gata1* existed in the CD71^{int}/TER119^{-} population at a low level, and was then highly induced to a maximum level at the CD71^{high}/TER119^{+} stage in both transgenic and non-transgenic mice (Table 2 and Fig. 3B). Corresponding to this *Gata1* expression, total expression of endogenous + exogenous *TEL* gene was maintained at a relatively high level at the CD71^{high}/TER119^{+} stage in the *GATA1-TEL* transgenic bone marrow cells, showing a striking contrast to the control cells in which endogenous *Tel* gene was markedly down-regulated to the lowest level at this stage (Table 2). This suggested that exogenous *TEL* expression overlaid endogenous *Tel* expression at this stage in the transgenic mice. Then, expression levels of the genes that are involved in erythropoiesis were examined in these three populations (Table 2). As a result, higher expression of *Alas-e* and *β-major globin* genes was constantly observed in the CD71^{high}/TER119^{+} cells of *GATA1-TEL* transgenic mice than control mice (the former with a statistical significance but the latter without; Table 2 and Fig. 3C). In addition, expression of *erythroid Kruppel-like factor* (*Eklf*) was higher in *GATA1-TEL* transgenic mice at the stage of CD71^{int}/TER119^{-} population, but without a statistical significance. There was no difference in expression levels of *Fli1*, *stem cell leukemia* (*Scl*) and other hematopoietic transcription factor-encoding genes as well as *EPO receptor* (*Epor*) gene between transgenic mice and littermate controls.

Because *GATA1-TEL* transgenic bone marrow cells produced a more abundant population of megakaryocytic progenitors (c-kit^{+}/CD41^{+}), we also separated c-kit^{+}/CD41^{+} populations from bone marrow cells of *GATA1-TEL* transgenic mice and their littermates, and the expressions of endogenous *Gata1* and *endogenous + exogenous TEL* genes in this population were examined by quantitative PCR. *Gata1* mRNAs were abundantly expressed at comparable levels in both types of mice, and the expression of *endogenous + exogenous TEL* gene was higher in *GATA1-TEL* transgenic mice with a statistical significance, as expected (Supporting Fig. S1). Thus, exogenous *TEL* expression might support expansion of c-kit^{+}/CD41^{+} megakaryocytic progenitors *in vitro*.

Generation of *Gata1-TEL*-expressing ES cells. To analyze the effects of TEL in early erythropoiesis, ES cells in which human *TEL*

doi: 10.1111/j.1349-7006.2009.01097.x

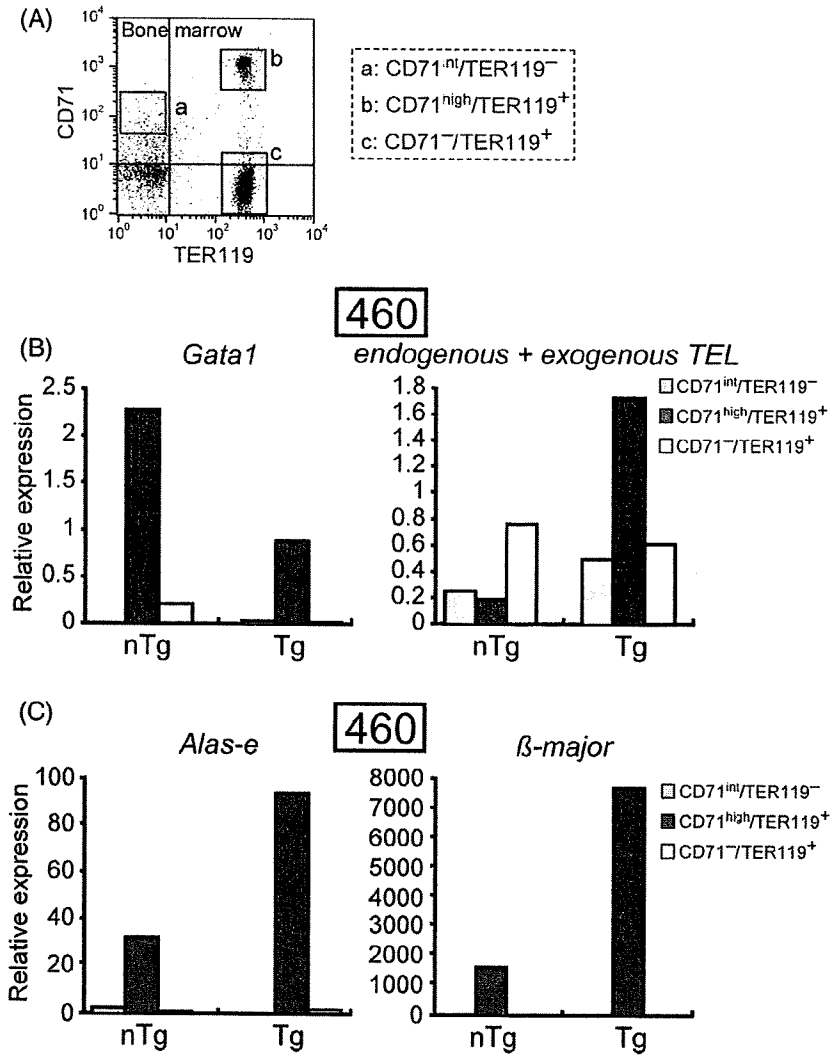
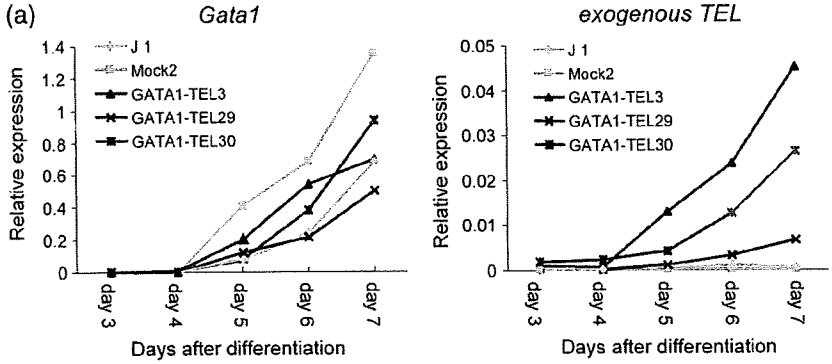


Fig. 3. Quantitative PCR of the genes involved in erythropoiesis. (A) To compare the expression of erythroid-related genes between *GATA1-TEL* transgenic mice and control littermates, bone marrow cells were sorted for $CD71^{int}/TER119^{-}$ (a), $CD71^{high}/TER119^{+}$ (b) and $CD71^{-}/TER119^{+}$ (c), representing different stages of erythroid differentiation, and then subjected to quantitative PCR analysis. The result of FACS analysis shown in Fig. 3 A came from a non-transgenic litter mouse. There was no difference in the expression pattern of each population between non-transgenic and transgenic mice. (B) Representative results of quantitative PCR for endogenous *Gata1* and endogenous + exogenous *TEL* in each stage of erythroid differentiation from animals of line 460. The highest expression of *TEL* gene was obtained in $CD71^{high}/TER119^{+}$ population in the *GATA1-TEL* transgenic mice, in concordance with the highest expression of endogenous *Gata1* among the three populations. (C) Representative results of quantitative PCR for *Alas-e* and *β-major globin* genes from animals of line 460. Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.

Table 2. Quantitative analysis of transcripts expressed in different stages of erythropoiesis

	$CD71^{int}/TER119^{-}$		$CD71^{high}/TER119^{+}$		$CD71^{-}/TER119^{+}$	
	nTg	Tg	nTg	Tg	nTg	Tg
Gata1	0.05 ± 0.05	0.63 ± 0.62	3.40 ± 2.41	1.46 ± 0.78	0.54 ± 0.29	0.57 ± 0.71
hmTEL	0.76 ± 0.67	1.73 ± 1.48	0.18 ± 0.05	1.20 ± 0.65*	1.19 ± 0.73	0.93 ± 0.48
Gata2	0.13 ± 0.08	0.90 ± 1.10	0.04 ± 0.01	0.22 ± 0.32	0.44 ± 0.36	0.41 ± 0.45
Runx1	0.38 ± 0.29	0.93 ± 0.71	0.47 ± 0.15	0.57 ± 0.34	0.79 ± 0.26	0.91 ± 0.20
Scl	0.06 ± 0.02	0.12 ± 0.09	1.79 ± 0.60	1.56 ± 0.75	0.12 ± 0.18	0.19 ± 0.31
Fli1	1.13 ± 1.01	4.32 ± 7.39	0.19 ± 0.01	5.77 ± 10.16	2.20 ± 0.98	3.31 ± 3.51
Eklf	0.11 ± 0.07	0.64 ± 0.52	10.6 ± 3.26	6.52 ± 4.53	1.39 ± 1.80	0.56 ± 0.12
Epor	0.19 ± 0.11	0.11 ± 0.11	1.89 ± 0.73	2.14 ± 1.05	0.73 ± 1.06	0.05 ± 0.06
β-major globin	2.10 ± 2.38	18.0 ± 5.80	2804 ± 1970	6730 ± 4775†	298 ± 288	83.3 ± 78.9
Alas-e	1.92 ± 1.36	1.01 ± 0.67	46.3 ± 12.2	143 ± 36**	10.7 ± 11.6	3.62 ± 1.97

Every numerical value indicates fold difference relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT) calculated by $2^{-(\Delta CT)}$.
(Δ cycle of threshold (CT), mean CT of indicated gene − mean CT of HPRT)
Average and standard deviation from two mice of line 460 and one mouse from 462 are shown.
*Significantly higher compared to control ($P < 0.01$).
**Significantly higher compared to control ($P < 0.003$).
†There is no significant difference though higher expression levels were observed in transgenic mice compared to controls in each experiment.
Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.



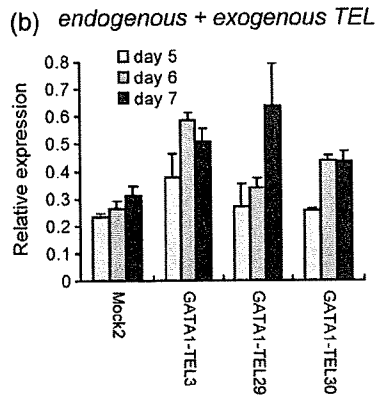


Fig. 4. Expression of *Gata1* and *TEL* during differentiation of embryonic stem (ES) cells. (a) Undifferentiated original J1, mock-transfected (Mock2) and *Gata1-TEL*-overexpressing (GATA1-TEL3, 29 and 30) ES cells were deprived of leukemia inhibitory factor to initiate differentiation and analyzed for the expression of endogenous *Gata1* gene and *TEL* transgene under the control of *Gata1* IE3.9int promoter. Expression of *TEL* transgene was observed from day 5 of differentiation in concordance with the expression of endogenous *Gata1* gene. (b) Total amount of *TEL* transcript (endogenous + exogenous) in differentiating embryoid body (EB) cells. Mouse and human *TEL* transcripts were simultaneously amplified as described in Materials and methods using primers TEL-829f and TEL-921r located on exons V and VI of mouse and human *TEL* gene. Average and standard deviation of two independent experiments are shown. After day 6 of differentiation, total amount of *TEL* was higher in *GATA1-TEL* EBs than in mock EBs.

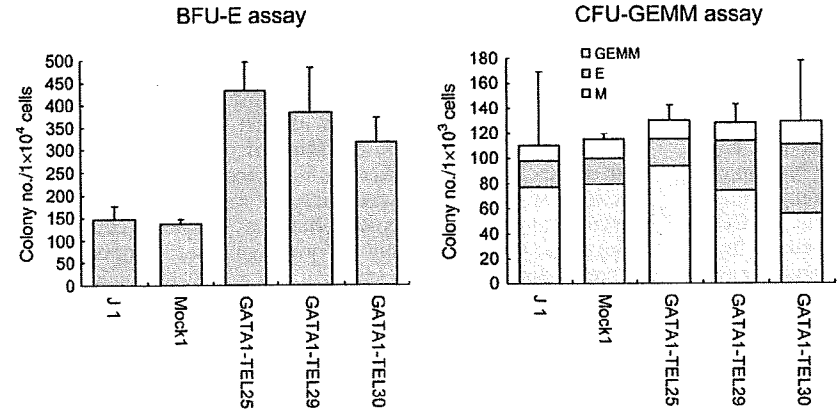


Fig. 5. Enhanced erythroid colony formation in *GATA1-TEL* embryoid body (EB) cells. Undifferentiated J1, mock-transfected (Mock1) and *Gata1-TEL*-overexpressing (GATA1-TEL25, 29 and 30) embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. EBs at day 7 of differentiation were collected and subjected to BFU-E (supplemented with SCF, thrombopoietin [TPO] and erythropoietin [EPO]) and CFU-GEMM (supplemented with SCF, TPO, EPO, interleukin [IL]-11, IL-3, GM-CSF, G-CSF, M-CSF and IL-6) assays. Average and standard deviation of at least two independent experiments are shown. *Gata1-TEL*-expressing EB cells showed higher BFU-E activity than controls, while no difference was observed in CFU-GEMM activity. GEMM, mixed colony; E, erythroid colony; M, myeloid colony.

gene is induced under the control of IE3.9int *Gata1* promoter (*GATA1-TEL* ES) were established. The ES cells were maintained and differentiated into hematopoietic cells as described previously.[36] When expression of *Gata1* and *TEL* was examined during differentiation of ES cells by quantitative PCR analysis, endogenous *Gata1* transcript began to increase from day 5 of removal of LIF, which gradually increased afterwards (Fig. 4a), possibly due to an increment of erythroid-committed cells in the whole cell population. There was no statistical difference in the amount of endogenous *Gata1* mRNA between *GATA1-TEL* ES cells and control cells during days 5–7 of EB culture. Exogenous *TEL* gene expression from the integrated *GATA1-TEL* vector showed precisely a similar pattern to endogenous *Gata1* expression, starting to express around day 5 of differentiation and gradually increasing afterwards. In *GATA1-TEL* ES cells, total amount of endogenous + exogenous *TEL* transcript was higher compared to control ES cells after day 6 of differentiation (Fig. 4b).

Higher erythroid activity of *Gata1-TEL*-expressing ES cells. Day 7 EBs following removal of LIF were subjected to CFC assay. Erythroid colony-forming activity (BFU-E) of *GATA1-TEL* EBs was significantly higher than that of control EBs, while there was no difference observed in the activity of multipotential progenitors (CFU-GEMM) between them (Fig. 5). This result indicated that *GATA1-TEL* EB cells might have an increased number of erythroid-committed progenitors or be more prone to commit to the erythroid lineage at day 7 of differentiation.

Day 6 EB-derived c-kit$^+$/CD71$^+$ cells efficiently differentiated into CD71^{High}/TER119$^+$ erythroid precursors on OP9. Erythroid differentiation of *GATA1-TEL* and control ES cells was also assessed by coculture with OP9 as described previously with some modifications.[37] Day 7 EBs were replated onto OP9 stromal

doi: 10.1111/j.1349-7006.2009.01097.x

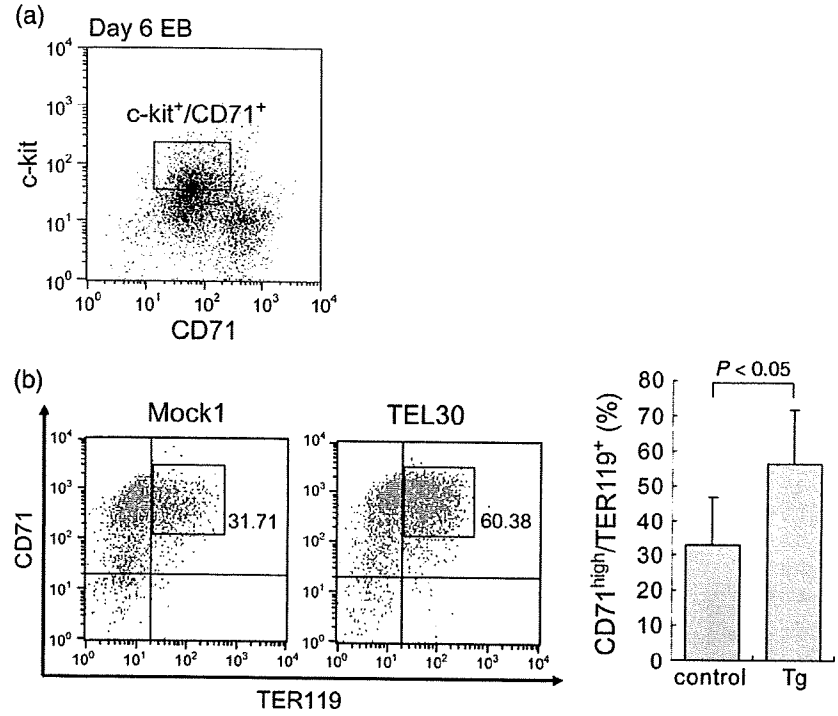


Fig. 6. *In vitro* erythroid differentiation of embryoid body (EB)-derived c-kit$^+$/CD71$^+$ cells. Undifferentiated embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. After 6 days of differentiation, c-kit$^+$/CD71$^+$ cells (shown in panel a) were separated by fluorescence-activated cell sorter (FACS) and subjected to erythroid differentiation assay on OP9 stromal cell layer. The result of FACS analysis shown in (a) came from non-transgenic cells. There was no difference in the population of EB-derived c-kit$^+$/CD71$^+$ cells between non-transgenic and transgenic cells. (b) The CD71^{high}/TER119$^+$ fraction after 8 days of culture with erythropoietin and SCF on OP9 layer. In the left panel, the representative data of mock-transfected (Mock1) and GATA1-TEL30 are shown. In the right panel, average and standard deviation of control and transgenic (Tg) cells are shown. The results of control and Tg are derived from the combined data in at least two independent experiments of J1, Mock1 and 2, and GATA1-TEL3, 25 and 30, respectively. Day 6 transgenic EB-derived c-kit$^+$/CD71$^+$ cells produced higher numbers of CD71^{high}/TER119$^+$ cells compared to the controls.

layer and cultured for another 8 days supplemented with EPO and SCF. Erythroid differentiation was then assessed by FACS analysis. *GATA1-TEL* and control EB cells produced comparable amounts of the CD71^{high}/TER119$^+$ erythroid progenitor population (data not shown). This result indicated the possibility that usage of whole EB cells avoided detecting increased abilities of transgenic erythroid progenitors to expand, and/or that timing could be earlier when transgenic erythroid progenitors in EBs showed increased abilities.

Erythroid differentiation of ES cells is considered to begin around day 5 corresponding to the initiation of expression of a key transcription factor, Gata1. Generally, when immature EBs differentiate into the erythroid lineage, increment of CD71 expression as well as loss of c-kit expression is observed. To figure out the effect of *TEL* transgene in immature hematopoietic progenitors, day 6 EB-derived c-kit$^+$/CD71$^+$ cells, which are considered to have multipotential in hematopoietic differentiation, were separated by FACS and subjected to short-term culture on OP9 stroma with EPO and SCF (Fig. 6a). There was no difference in the amount of day 6 EB-derived c-kit$^+$/CD71$^+$ populations between non-transgenic and transgenic EBs (data not shown). After 8 days of coculture with OP9, almost all EB-derived cells were differentiated to the erythroid lineage, showing a high level of CD71 expression (Fig. 6b). The population of CD71^{high}/TER119$^+$ cells, which are erythroid-committed and equivalent to proerythroblast, was significantly higher in the *GATA1-TEL* EB-derived c-kit$^+$/CD71$^+$ cells compared to the controls.

Discussion

For the purpose of investigating TEL's functions in erythropoiesis, we in this study generated transgenic mice and ES cells expressing human *TEL* under the control of erythroid-specific *Gata1* promoter. Each system could have highlighted different aspects of TEL's roles in *Gata1*-expressing cells. We have divulged two roles of the transcription factor in erythropoiesis; one is the expansion of immature erythroid precursor and the other is the augmentation of Hb accumulation. Thus, we conclude that TEL affects proliferation and differentiation of erythroid-committed cells by distinctive mechanisms.

We precisely studied the expression levels of endogenous *Gata1*, and endogenous and exogenous (*Gata1* promoter-driven) *TEL* transcripts during the progression of erythroid differentiation by fractionating CD71^{int}/TER119$^-$, CD71^{high}/TER119$^+$ and CD71$^-$/TER119$^+$ populations in the bone marrow of non-transgenic and transgenic mice. In both types of mice, endogenous *Gata1* transcripts were induced with the highest level in the CD71^{high}/TER119$^+$ cells belonging to the stage of proerythroblast[38] and then markedly declined afterwards, which represents essential functions of Gata1 to activate transcription of *globin* and heme biosynthetic genes. On the other hand, expressional changes of endogenous *Tel.* transcripts in a physiological setting of erythroid differentiation have not as yet been described. The endogenous TEL expression in the non-transgenic mice was found to be low in the CD71^{high}/TER119$^+$ population, while relatively high in both the CD71^{int}/TER119$^-$ and CD71$^-$/TER119$^+$ populations (Table 2), for which we could not uncover a biological meaning at this moment. Considering that the exogenous expression in the transgenic mice was up-regulated at the CD71^{high}/TER119$^+$ proerythroblast stage, consistent with the highest expression of endogenous *Gata1* at this stage, we can conclude that our transgenic system successfully led to overexpression of exogenous *TEL* transcript in the *Gata1*-expressing cells. In addition, c-kit$^+$/CD41$^+$ megakaryocytic progenitors also highly expressed endogenous *Gata1* gene and the transgenic c-kit$^+$/CD41$^+$ cells showed high expression of exogenous *TEL* gene as well.

This transgenic event caused several differences between the transgenic and control mice. One is further up-regulation of *Alas-e* and *β-globin* genes in the CD71^{high}/TER119$^+$ population of *GATA1-TEL* transgenic mice. These data indicate that TEL directly or indirectly exaggerates the transcription of genes involved in Hb synthesis. In *GATA1-TEL* transgenic mice, the level of Hb concentration in the peripheral blood was higher, and with a statistical significance. We have previously reported by evaluating Hb accumulation with benzidine staining that overexpressed *TEL* stimulates erythroid differentiation in UT7/GM and MEL

cells.[13,14] In both the cell lines, expressional levels of *β-globin* and *ALAS-E* mRNAs were higher in the *TEL*-overexpressing cells. These previous data are consistent with those observed here in the CD71^{high}/TER119^{+} population of bone marrow cells in the *GATA1-TEL* transgenic mice. The transgenic mice expressing deletion mutants of TEL that lack the Pointed or the ETS domain did not show any alterations in Hb concentration (data not shown). Because these deletions abolish major molecular functions of TEL as a transcription factor, TEL appears to reinforce Hb synthesis through transcriptional regulation at the CD71^{high}/TER119^{+} stage. Considering that endogenous *Gata1* expression in the erythroid fraction of transgenic bone marrow cells was not increased compared to that of non-transgenic cells, it could not be plausible that TEL up-regulates the transcription of *Gata1* gene itself. Although we do not have any evidence that TEL and GATA1 physically associate with each other, functions of each molecule may cross-talk in the transcriptional regulation of *β-globin* and *ALAS-E* genes. ETS-binding consensus sequences are not found in the promoter region of *Alas-e* gene, suggesting that TEL works indirectly to stimulate transactivation of the gene. On the other hand, because *β-globin* gene contains an ETS-binding consensus sequence (GGAA/T) in its promoter region, TEL might directly activate the expression of *β-globin* gene, although TEL is currently known only as a transcriptional repressor. Notably, the expression of *Eklf* that activates the promoter of *β-globin* gene[39] was higher at the immature CD71^{int}/TER119^{-} stage in the transgenic mice, which may also partly have contributed to the up-regulation of *β-globin* gene in the CD71^{high}/TER119^{+} stage.

Enforced *TEL* expression in transgenic mice not only caused accelerated Hb accumulation but also expanded the immature progenitor at the earlier stage where the expression of endogenous *Gata1 has* not been fully activated yet. When cultured in the presence of EPO and TPO, transgenic bone marrow cells produced a more abundant population of CD71^{high}/TER119^{+} (erythroid-committed) and c-kit^{+}/CD41^{+} (megakaryocyte-committed) cells than control cells, respectively. This observation suggests a stimulatory function of TEL in propagating immature erythroid progeny and possibly erythrocyte/megakaryocyte common progenitors that can make a commitment to either of the erythroid or megakaryocytic lineage. Although the levels of *Epor* transcript in *TEL*-expressing CD71^{high}/TER119^{+} cells were comparable to those in controls, we could not deny the possibility that TEL affects intracellular EPO signals. The molecular mechanisms underpinning TEL's functions in expansion of immature erythroid precursor remain unknown. On the other hand, we at this moment cannot discuss the exact reason that exogenous *TEL* expression led to *in vitro* expansion of megakaryocytic progenitor in the presence of TPO, but did not cause an increased production of platelets in mice. However, considering that overexpressed TEL accelerates erythroid differentiation but inhibits megakaryocytic maturation in UT7/GM cells,[14] TEL may preferably drive the erythroid commitment in erythrocyte/megakaryocyte common progenitors also in mice and its overexpression may not result in higher production of platelets.

We also took advantage of *in vitro* differentiation of ES cells to clarify TEL's role in early hematopoiesis. The expressions of endogenous *Gata1* and exogenous *TEL* concomitantly commenced at day 5 of EB culture in differentiation media without LIF, and gradually increased together subsequently. We found that total levels of endogenous + exogenous *TEL* transcripts were higher at day 6 or 7 in the *GATA1-TEL* transgenic EB cells than in control cells. Interestingly, when assayed day 7 EB-derived cells on methylcellulose, numbers of BFU-E colonies derived from *GATA1-TEL* transgenic EB cells revealed a significant increase compared to those from control cells. In the liquid culture on OP9 cells in the presence of EPO and SCF, c-kit^{+}/CD71^{+} cells sorted from day 6 transgenic EB produced a more abundant population of erythroid-committed CD71^{high}/TER119^{+} cells than non-transgenic control cells. These observations also argue the function of TEL in expanding erythroid progenitor or accelerating definitive erythroid commitment.

In summary, we verify two compelling functions of TEL exerted at the different stages of erythroid differentiation. At the earliest stage of erythroid differentiation, TEL could proliferate erythrocyte/megakaryocyte common progenitors and/or favor growth of the erythroid lineage-committed cells. At the late stage of differentiation, TEL can accelerate terminal erythroid differentiation through stimulating Hb synthesis. Although TEL is not essential for erythropoiesis in the fetus and adult mice, TEL could be activated under the condition of hematopoietic stresses such as anemia and hypo-oxygenemia. We currently have observed no difference between non-transgenic and transgenic mice in recovery of Hb levels after bleeding experiments. Further analyses with different strategies to induce hematopoietic stress are required to address this issue. Finally, to clarify precise mechanisms for TEL to promote the propagation of erythroid progenitor, unknown downstream target genes of TEL that could be critical in the erythroid commitment and proliferation, are under investigation in our laboratory using comprehensive microarray systems.

Acknowledgments

This work was financially supported by the Grants-in-Aid from the Ministries in Japan of Education, Culture, Sports, Science and Technology (17016068), and Health, Labour and Welfare, and Japanese Society for the Promotion of Science (17659295, 17390283, 18591085, 19591135).

The authors thank Professor T. Nakano (Department of Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University) for kindly providing us with OP9 cells. We also thank Kirin Brewery Co Ltd. for kindly supplying TPO for this project and Ms. Yuki Shinozaki for technical support.

References

1 Mavrothalassitis G, Ghysdael J. Proteins of the ETS family with transcriptional repressor activity. *Oncogene* 2000; **19**: 6524–32.
2 Bohlander SK. ETV6: a versatile player in leukemogenesis. *Semin Cancer Biol* 2005; **15**: 162–74.
3 Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, Tel., in chronic myelomonocytic leukemia with t (5;12) chromosomal translocation. *Cell* 1994; **77**: 307–16.
4 Kim CA, Phillips ML, Kim W *et al.* Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. *Embo J* 2001; **20**: 4173–82.
5 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.
6 Chakrabarti SR, Nucifora G. The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. *Biochem Biophys Res Commun* 1999; **264**: 871–7.
7 Lopez RG, Carron C, Oury C, Gardellin P, Bernard O, Ghysdael J. TEL is a sequence-specific transcriptional repressor. *J Biol Chem* 1999; **274**: 30132–8.
8 Guidez F, Petrie K, Ford AM *et al.* Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. *Blood* 2000; **96**: 2557–61.
9 Wang L, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. *Oncogene* 2001; **20**: 3716–25.
10 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.
11 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.
12 Hock H, Meade E, Medeiros S *et al.* Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev* 2004; **18**: 2336–41.
13 Waga K, Nakamura Y, Maki K *et al.* Leukemia-related transcription factor TEL accelerates differentiation of Friend erythroleukemia cells. *Oncogene* 2003; **22**: 59–68.

doi: 10.1111/j.1349-7006.2009.01097.x

14 Takahashi W, Sasaki K, Kvomatsu N, Mitani K. TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM. *Cancer Sci* 2005; **96**: 340–8.
15 Yamamoto M, Takahashi S, Onodera K, Muraosa Y, Engel JD. Upstream and downstream of erythroid transcription factor GATA-1. *Genes Cells* 1997; **2**: 107–15.
16 Ohneda K, Yamamoto M. Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. *Acta Haematol* 2002; **108**: 237–45.
17 Crispino JD. GATA1 in normal and malignant hematopoiesis. *Semin Cell Dev Biol* 2005; **16**: 137–47.
18 Migliaccio AR, Rana RA, Vannucchi AM, Manzoli FA. Role of GATA-1 in normal and neoplastic hemopoiesis. *Ann N Y Acad Sci* 2005; **1044**: 142–58.
19 Pan X, Ohneda O, Ohneda K *et al.* Graded levels of GATA-1 expression modulate survival, proliferation, and differentiation of erythroid progenitors. *J Biol Chem* 2005; **280**: 22385–94.
20 Shimizu R, Yamamoto M. Gene expression regulation and domain function of hematopoietic GATA factors. *Semin Cell Dev Biol* 2005; **16**: 129–36.
21 Pevny L, Simon MC, Robertson E *et al.* Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991; **349**: 257–60.
22 Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci USA* 1996; **93**: 12355–8.
23 Takahashi S, Onodera K, Motohashi H *et al.* Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J Biol Chem* 1997; **272**: 12611–15.
24 Weiss MJ, Keller G, Orkin SH. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev* 1994; **8**: 1184–97.
25 Suwabe N, Takahashi S, Nakano T, Yamamoto M. GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. *Blood* 1998; **92**: 4108–18.
26 Cheng T, Shen H, Giokas D, Gere J, Tenen DG, Scadden DT. Temporal mapping of gene expression levels during the differentiation of individual primary hematopoietic cells. *Proc Natl Acad Sci USA* 1996; **93**: 13158–63.
27 Shivdasani RA, Orkin SH. The transcriptional control of hematopoiesis. *Blood* 1996; **87**: 4025–39.
28 Nerlov C, Graf TPU. 1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev* 1998; **12**: 2403–12.
29 Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000; **404**: 193–7.
30 Onodera K, Takahashi S, Nishimura S *et al.* GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. *Proc Natl Acad Sci USA* 1997; **94**: 4487–92.
31 Kobayashi M, Yamamoto M. Regulation of GATA1 gene expression. *J Biochem* 2007; **142**: 1–10.
32 Vandesompele J, De Preter K, Pattyn F *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**: RESEARCH0034.
33 Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 1994; **265**: 1098–101.
34 Nakano T, Kodama H, Honjo T. *In vitro* development of primitive and definitive erythrocytes from different precursors. *Science* 1996; **272**: 722–4.
35 Zhang J, Socolovsky M, Gross AW, Lodish HF. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood* 2003; **102**: 3938–46.
36 Robertson SM, Kennedy M, Shannon JM, Keller G. A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development* 2000; **127**: 2447–59.
37 Kitajima K, Tanaka M, Zheng J, Sakai-Ogawa E, Nakano T. In vitro differentiation of mouse embryonic stem cells to hematopoietic cells on an OP9 stromal cell monolayer. *Meth Enzymol* 2003; **365**: 72–83.
38 Suzuki N, Suwabe N, Ohneda O *et al.* Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. *Blood* 2003; **102**: 3575–83.
39 Bieker JJ. Probing the onset and regulation of erythroid cell-specific gene expression. *Mt Sinai J Med* 2005; **72**: 333–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Quantitative PCR of the *Gata1* and *TEL* genes expressed in megakaryocytic progenitors.

Table S1. Sequences of primers used for polymerase chain reaction (PCR) and reverse transcription (RT-PCR).

Table S2. Sequences of primers used for quantitative polymerase chain reaction.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Int J Hematol (2009) 89:253–256
DOI 10.1007/s12185-009-0267-8

LETTER TO THE EDITOR

Enhanced expression of the *EVI1* gene in NUP98/HOXA-expressing leukemia cells

Minenori Eguchi-Ishimae · Mariko Eguchi · Kazuma Ohyashiki · Tetsuya Yamagata · Kinuko Mitani

Received: 7 August 2008 / Revised: 24 December 2008 / Accepted: 9 January 2009 / Published online: 26 February 2009

The chromosomal translocation t(7;11)(p15;p15) is mainly observed in Asian patients with acute myelogenous leukemia (AML), particularly in the M2 subtype according to the FAB classification, myelodysplastic syndrome, and blastic crisis of chronic myelogenous leukemia [1–3]. This is the first identified chromosomal translocation involving 11p15, and the presence of this chromosomal abnormality is associated with poor prognosis in AML. The t(7;11)(p15;p15) translocation causes the *NUP98* gene on 11p15 to fuse to three different members of the *HOXA* family gene on 7p15, leading to the production of three different fusion genes, *NUP98/HOXA9*, *NUP98/HOXA11* and *NUP98/HOXA13*. The *NUP98* gene encodes a nucleoporin protein, which comprises nuclear pore complexes that facilitate mRNA export from the nucleus. The *HOXA* family genes encode HOX family transcription factors, which play important roles in survival of hematopoietic stem cells and the development of body segmentation. The fusion of the *NUP98* gene and the *HOXA* family genes generate chimeric NUP98/HOXA proteins, which universally have the FG-repeat domain derived from the NUP98 molecule and the DNA-binding plus PBX-heterodimerizing domains derived from the HOXA molecules.

The generation of the NUP98/HOXA fusion proteins is believed to have critical roles in the development of leukemia with t(7;11)(p15;p15) translocation. To date, two mechanisms have been proposed for the leukemogenesis by the NUP98/HOXA fusion proteins; disruption of the formation of functional nuclear pore complexes, and dysregulated functions of the HOXA family transcription factors. For the latter mechanism, it has been shown that NUP98/HOXA fusion proteins up-regulate the HOXA family responsive genes such as *HOXA7*, *HOXA9* and *MEIS1* [4], of which the up-regulation of *MEIS1* is particularly critical for NUP98/HOXA to cause full-blown leukemia [5, 6]. To further investigate the molecular mechanism of the NUP98/HOXA chimeric proteins in leukemia development, we analyzed gene expressions in human leukemic samples that expressed the *NUP98/HOXA* fusion transcripts.

After having obtained written informed consent from patients, bone marrow cells were collected following protocols approved by institutional review board. Patient samples analyzed in this study included four cases that had the t(7;11)(p15;p15) translocation (Case 1, therapy-related AML; Cases 2 and 3, M2; Case 4, M4), and six control cases that did not have the t(7;11)(p15;p15) translocation (Case 5, M1; Case 6, M2; Case 7, M5a; Case 8, M0; Cases 9 and 10, M1). Total RNA was extracted from the bone marrow cells of these patients, and gene expressions were analyzed using reverse-transcript PCR (RT-PCR) method. Cases 1 and 2 expressed *NUP98/HOXA9* transcript, and Case 3 expressed *NUP98/HOXA11* transcript, as verified in

Electronic supplementary material The online version of this article (doi:10.1007/s12185-009-0267-8) contains supplementary material, which is available to authorized users.

M. Eguchi-Ishimae · M. Eguchi · T. Yamagata · K. Mitani (✉)
Department of Hematology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi-ken 321-0293, Japan
e-mail: kinukom-tky@umin.ac.jp

Present Address:
M. Eguchi-Ishimae · M. Eguchi
Department of Pediatrics, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan

K. Ohyashiki
First Department of Internal Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

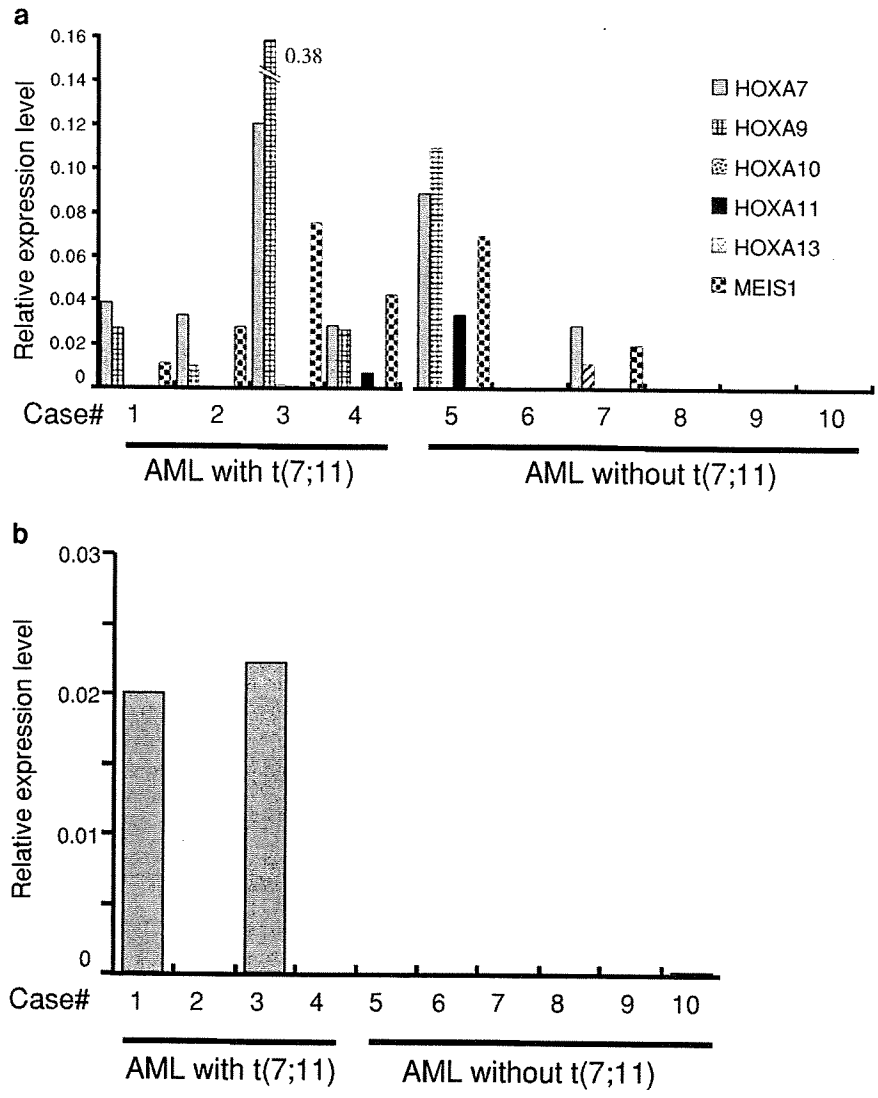


Fig. 1 **a** The expression levels of the *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11*, *HOXA13*, and *MEIS1* genes in bone marrow cells from the patients were analyzed in quantitative RT-PCR analysis. The expression levels of the genes were normalized with that of the *β2-microglobulin* gene. Cases 1–4 had the t(7;11)(p15;p15) abnormality, and Cases 1–3 expressed NUP98/HOXA-fusion transcripts. Cases 5–10 did not have the t(7;11)(p15;p15) abnormality. **b** The expression levels of the *EVI1* gene were analyzed in the same bone marrow samples. The expression levels were also normalized with that of the *β2-microglobulin* gene

RT-PCR (data not shown). Case 4 had the t(7;11)(p15;p15) translocation, but did not express NUP98/HOXA chimeric transcripts including *NUP98/HOXA9*, *NUP98/HOXA10*, *NUP98/HOXA11* or *NUP98/HOXA13*. Analysis of the fusion transcript in Cases 1, 2 and 3 showed the following fusion patterns; in *NUP98/HOXA9*-expressing samples (Cases 1 and 2), the exon 12 of the *NUP98* gene was fused to the exon 1B of the *HOXA9* gene, and in *NUP98/HOXA11*-expressing sample (Case 3), the exon 12 of the *NUP98* gene was fused to the exon 2 of the *HOXA11* gene.

Then we analyzed the expressions of five *HOXA* family genes, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11* and *HOXA13*, and that of the *MEIS1* gene with quantitative RT-PCR analysis using sets of primers shown in supplemental Table 1. Of the six genes, three genes, *HOXA7*, *HOXA9* and *MEIS1*, are reported to be up-regulated by the NUP98/HOXA chimeric proteins. Consistently, our analysis confirmed that these genes were significantly up-regulated in the samples expressing *NUP98/HOXA* chimeric transcripts (Cases 1–3) (Fig. 1a). Cases 4 and 5 also expressed *HOXA7*, *HOXA9* and *MEIS1*. FISH analysis for Case 5 showed no rearrangement in the *MLL* gene (data not shown), suggesting the absence of MLL-fusion proteins that are known to induce the *HOXA* family gene expression. Therefore, the expression of *HOXA7*, *HOXA9* and *MEIS1* in Cases 4 and 5 is due to mechanisms other than the generation of NUP98/HOXA chimeric proteins or the MLL fusion proteins.

We next evaluated the expression of the *EVI1* gene, since the expression of *EVI1* is highly associated with poor prognosis in myeloid malignancies [7]. Strikingly, the expression of *EVI1* was up-regulated in two of the three samples expressing *NUP98/HOXA* chimeric transcripts (Fig. 1b). No expression of *EVI1* was detected in Case 4,

which had t(7;11)(p15;p15) but did not express *NUP98/HOXA* chimeric transcripts. Also, the six control samples (Cases 5–10) did not express *EVI1*. These data suggest that there is a potential link between the expression of *NUP98/HOXA* chimeric mRNA and the over-expression of the *EVI1* gene.

We also measured the expression levels of a particular isoform of *EVI1* mRNA, *MDS1/EVI1*, since our PCR primers detect both EVI1 and MDS1/EVI1 isoforms [8]. It is important to distinguish these two transcripts because EVI1 and MDS1/EVI1 have opposing effects; EVI1 is highly oncogenic while MDS1/EVI1 has an anti-oncogenic effect over EVI1 [9]. Consistently, EVI1 is over-expressed in human leukemia with chromosome 3q abnormalities, while MDS1/EVI1 is mostly detected in normal hematopoietic cells [9]. We used PCR primers that distinguish *EVI1* and *MDS1/EVI1* transcripts [10]. Our analysis revealed that *MDS1/EVI1* transcript was not expressed in the three *NUP98/HOXA*-expressing samples (data not shown), indicating that only the oncogenic form of *EVI1* mRNA was expressed in the two *EVI1*-expressing samples.

To test if NUP98/HOXA chimeric proteins stimulate the expression of the *EVI1* gene through its 5′ regulatory region, we constructed a reporter plasmid that has the 6.5 kb upstream region of the human *EVI1* gene fused to the *luciferase* gene (pGL3-EVI1-Luc). We then transfected the EVI1 reporter plasmid along with expression plasmids of wild-type-HOXA9, NUP98/HOXA9 or NUP98/HOXA11 into 293T cells and evaluated luciferase activity. Cotransfection of NUP98/HOXA9 or NUP98/HOXA11 expression plasmid showed significant increase in the luciferase activity, while that of wild-type-HOXA9 plasmid caused almost no effect (Fig. 2). This result indicates that both NUP98/HOXA9 and NUP98/HOXA11 fusion proteins have capacity to enhance the promoter activity of the *EVI1* gene, while HOXA9 does not have such capacity.

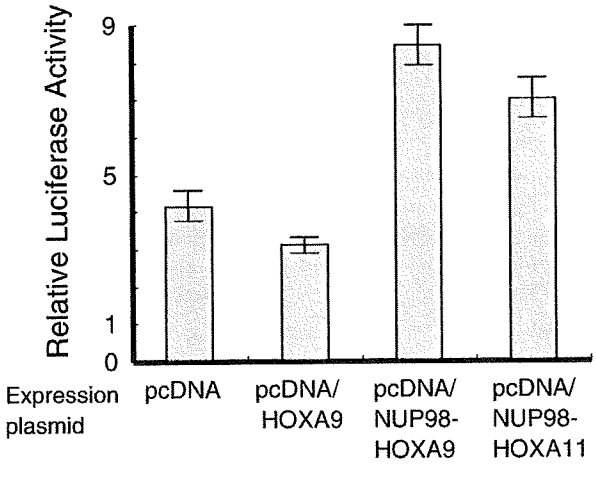


Fig. 2 The pGL3-EVI1-Luc (firefly) plasmid (320 ng) and pCMV-Luc (*Renilla*) (10 ng) were co-transfected into 293T cells with expression plasmids (80 ng) indicated. Cells were harvested 48 h post-transfection, and the firefly luciferase activity was normalized with control *Renilla* luciferase activity

We show that the *EVI1* gene is up-regulated in two out of three samples positive for *NUP98/HOXA* chimeric transcripts. This is the first study suggesting potential association between the presence of *NUP98/HOXA* chimeric transcripts and the over-expression of the *EVI1* gene. The over-expression of EVI1 is known to disrupt multiple signaling pathways, and is associated with various types of hematological malignancies [7]. Thus, this study suggests that the over-expression of *EVI1* causes the leukemic transformation of cells expressing *NUP98/HOXA* fusion proteins. Therefore, this study proposes another mechanism of leukemia development caused by t(7;11)(p15;p15) translocation, in addition to the two mechanisms introduced earlier.

Several studies have explored potential target genes down-stream of NUP98/HOX fusion proteins. Takeda et al. [11] have identified *EVI1* as one of the target genes induced by retrovirally transduced *NUP98/HOXA9* in human CD34-positive primary cells. Similarly, Palmqvist et al. [12] have identified *EVI1* among genes induced by two different NUP98-fusion proteins, NUP98/HOXA10 and NUP98/HOXD13, in murine bone marrow cells. In contrast, *EVI1* is not found in genes induced by NUP98/HOXA9 in a study by Jankovic et al. [13]. Despite the discrepancies, recurrent appearance of EVI1 in the two former studies suggests that EVI1 can potentially be induced by NUP98/HOX fusion proteins. The identification of *EVI1* transcript in our NUP98/HOXA-expressing samples provides clinical evidence that the induction of *EVI1* by NUP98/HOXA do occur in real leukemic cells.

Our reporter assay suggests that both NUP98/HOXA9 and NUP98/HOXA11 enhance the activity of the *EVI1* promoter. However, we could not find the specific consensus binding elements for HOXA9 or HOXA11 in the 6.5 kb promoter region. Thus, the activation of the *EVI1* promoter by NUP98/HOXA9 or NUP98/HOXA11 could be an indirect effect. Further study is needed to clarify the mechanism of the over-expression of the *EVI1* gene caused by the generation of NUP98/HOXA fusion proteins.

References

1. Nakamura T, Largaespada DA, Lee MP, et al. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nat Genet. 1996;12:154–8.
2. Nakamura T. NUP98 fusion in human leukemia: dysregulation of the nuclear pore and homeodomain proteins. Int J Hematol. 2005;82:21–7.

3. Argiropoulos B, Humphries RK. Hox genes in hematopoiesis and leukemogenesis. Oncogene. 2007;26:6766–76.
4. Calvo KR, Sykes DB, Pasillas MP, Kamps MP. Nup98-HoxA9 immortalizes myeloid progenitors, enforces expression of Hoxa9, Hoxa7 and Meis1, and alters cytokine-specific responses in a manner similar to that induced by retroviral co-expression of Hoxa9 and Meis1. Oncogene. 2002;21:4247–56.
5. Kroon E, Thorsteinsdottir U, Mayotte N, Nakamura T, Sauvageau G. NUP98-HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice. EMBO J. 2001;20:350–61.
6. Iwasaki M, Kuwata T, Yamazaki Y, et al. Identification of cooperative genes for NUP98-HOXA9 in myeloid leukemogenesis using a mouse model. Blood. 2005;105:784–93.
7. Maki K, Yamagata T, Mitani K. Role of the *RUNX1-EVI1* fusion gene in leukemogenesis. Cancer Sci. 2008;99:1878–83.
8. Vinatzer U, Mannhalter C, Mitterbauer M, et al. Quantitative comparison of the expression of EVI1 and its presumptive antagonist, MDS1/EVI1, in patients with myeloid leukemia. Genes Chromosomes Cancer. 2003;36:80–9.
9. Morishita K. Leukemogenesis of the EVI1/MEL1 gene family. Leukemogenesis of the EVI1/MEL1 gene family. Int J Hematol. 2007;85:279–86.
10. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003;101:837–45.
11. Takeda A, Goolsby C, Yaseen NR. NUP98-HOXA9 induces long-term proliferation and blocks differentiation of primary human CD34 + hematopoietic cells. Cancer Res. 2006;66:6628–37.
12. Palmqvist L, Argiropoulos B, Pineault N, et al. The Flt3 receptor tyrosine kinase collaborates with NUP98-HOX fusions in acute myeloid leukemia. Blood. 2006;108:1030–6.
13. Jankovic D, Gorello P, Liu T, et al. Leukemogenic mechanisms and targets of a NUP98/HHEX fusion in acute myeloid leukemia. Blood. 2008;111:5672–82.

Int J Hematol (2009) 90:91–93
DOI 10.1007/s12185-009-0336-z

CASE REPORT

Presentation of familial Mediterranean fever in a heterozygous *MEFV* mutation triggered by immunosuppressive therapy for myelodysplastic syndrome

Ko Sasaki · Toshiyuki Tahara · Kinuko Mitani

Received: 28 July 2008 / Revised: 18 February 2009 / Accepted: 22 April 2009 / Published online: 23 May 2009

Abstract Familial Mediterranean fever (FMF) is a recessively inherited disease characterized by recurrent episodes of systemic inflammation. The cause of this disease is the mutations affecting both the alleles of *MEFV* gene. We describe here a case in a heterozygous *MEFV* mutation complicated with myelodysplastic syndrome (MDS). Clinical symptoms and the effectiveness of colchicines in this patient are typical for FMF. The first attack of FMF in this patient was observed during immunosuppressive therapy for MDS. This case suggests the possibility that certain immunosuppressants may trigger FMF attack in asymptomatic cases carrying *MEFV* heterozygous mutation.

Keywords Myelodysplastic syndrome · Familial Mediterranean fever · Immunosuppressive therapy · *MEFV* gene

1 Introduction

Familial Mediterranean fever (FMF) is a recessively inherited disease characterized by recurrent episodes of systemic inflammation and is observed in Mediterranean and Middle Eastern populations. Ninety percent of FMF patients have the first attack by the age of 20. Typical events include abdominal, pleural and arthritic attacks that generally last 24–72 h. The cause of this disease is the mutations affecting both the alleles of *MEFV* gene. *MEFV* gene mapped at 16p13.3 was isolated independently by two groups [1, 2]. So far, various kinds of mutations in *MEFV* gene have been reported. Most of the disease-associated mutations are located in exon 10 of the gene with the smaller group in exon 2. Five founder mutations including this patient's type V726A, M694V, M694I, M680I and E148Q account for 74% of typical FMF cases [3]. More severe symptoms of systemic inflammation with a higher incidence of systemic amyloidosis are usually associated with M694V homozygotes.

2 Case report

In May 1998, a 19-year-old male was hospitalized in a practitioner's institute because of pneumonia. He was introduced to our hospital because pancytopenia was pointed out during the hospitalization. On admission, he had slight fever. Laboratory studies disclosed pancytopenia and increased levels of non-specific parameter for inflammation. Histological examination revealed no evidence of proliferation of immature blasts but with abundant ringed sideroblast in the bone marrow (Table 1). Chromosomal analysis of bone marrow cells showed 46,XY,del. Above all, he was diagnosed as myelodysplastic syndrome (MDS) associated with isolated del(5q) chromosome abnormality according to the WHO classification, although ringed sideroblasts were observed up to >15% among erythroid progenitors. After the introduction of oral cyclosporine A

K. Sasaki · K. Mitani (✉)
Department of Hematology,
Dokkyo Medical University School of Medicine,
880 Kitakobayashi, Mibu-machi, Shimotsuga-gun,
Tochigi 321-0293, Japan
e-mail: kinukom-tky@umin.ac.jp;
mitanik@dokkyomed.ac.jp

T. Tahara
Department of Gastroenterology,
Saiseikai Utsunomiya Hospital,
911-1 Takebayashi, Utsunomiya,
Tochigi 321-097, Japan
e-mail: toshiyuki_tahara@saimiya.com

(CsA) at a dose of 300 mg/day, pancytopenia was dissolved. However, administration of CsA at a dose of 50 mg/day was required to maintain complete hematological response. In 2007, he suddenly presented an abdominal pain with fever of 38°C or higher and diarrhea that lasted within 24 h. And this attack recurred afterward. His clinical course satisfied the diagnostic criteria for FMF [4]. His disease severity score was more than 4 (The presence of amyloidosis had not been checked.). His severity score was low in comparison with the mean score in both Iraqi Jews (6.25) and North Africans (9.24) [5]. A consulted physician of digestive tract internal medicine sought for mutation in the Mediterranean fever (*MEFV*) gene responsible for FMF. The sequence analysis was performed about whole coding regions of *MEFV* gene except exon 2. As a result, ATG of codon 694 in exon 10 was shown to be substituted to ATA in the single allele, indicating heterozygous mutation of *MEFV* gene (M694I). Even after the diagnosis of FMF, immunosuppressive therapy for MDS was not changed, and colchicines therapies were started in use to hang around. Colchicines therapies were very effective, and the symptoms of inflammation fever and abdominal pain were improved after the administration of colchicines.

Table 1 Laboratory findings on admission

Peripheral blood	
WBC	4300/μL
Stab.	0%
Seg.	74.0%
Eosino.	0%
Baso.	0%
Mono.	4.0%
Lymph.	19.0%
Atypical Ly	3.0%
RBC	220 × 10^4/μL
Hb	8.4 g/dl
Ht	23.4%
MCV	106.0 fl
HCH	38.0 pg
MCHC	35.9%
PLT	2.3 × 10^4/μL
Reti.	5‰
Biochemistry	
TP	6.6 g/dL
Alb	3.6 g/dL
T. Bil	1.1 mg/dL
D. Bil	0.4 mg/dL
I. Bil	0.7 mg/dL
AST	90 IU/L
ALS	165 IU/L
ALP	193 IU/L
γ-GTP	54 IU/L
LDH	769 IU/L
BUN	15 mg/dL
Cr	0.9 mg/dL
Na	137 mEq/L
K	4.1 mEq/L
Cl	103 mEq/L
UA	4.0 mg/dL
CRP	4.6 mg/dL
Bone marrow	
NCC	16.2 × 10^4/μl
Mgk	15/μl
Mbl	0.6%
Promyelo.	1.4%
Myelo.	8.4%
Meta.	11.2%
Stab.	20.4%
Seg.	20.0%
Eosino.	0.4%
Baso.	0%
Mono.	3.6%
Lymph.	9.2%
Plasma	0.6%
Ebl baso.	1.6%
poly.	20.2%
orth.	2.4%
Iron stain	
Ringed sideroblasts	27%/Ebl
Chromosome (Bone marrow)	
46,XY,del(9)	5/20

3 Discussion

All the patients with FMF do not necessarily conform to a recessive mode of inheritance. In the cases of heterozygous carriers, it is possible that various inflammatory stimuli predispose them to acute attacks of FMF [6]. One candidate inflammatory disease that may cause acute attacks in FMF heterozygotes is Behçet's disease. However, there was no symptom for Behçet's disease in this patient. The *MEFV* gene encoding pyrin (marenostine) is upregulated in response to inflammatory mediators [7] including TNF-α. On the other hand, CsA blocks the induction of TNF-α gene at a transcriptional level [8]. We thus speculated that this patient possibly had a reduced level of pyrin characterizing heterozygosity, and that administration of CsA further stressed the tendency through inhibiting TNF-α expression, which led to FMF attacks.

So far, direct evidence indicating that CsA suppresses the *MEFV* gene expression has not been reported. However, Khosroshahi [9] reported the FMF case that manifested the first attack during the immunosuppressive therapy after renal transplantation. The regimen of immunosuppressive therapy used for this case contained CsA. Therefore, this case indicates the possibility that CsA could trigger the first attack in FMF heterozygotes who are so far asymptomatic.

Here, we report a case of FMF patient carrying M694I heterozygous mutation of *MEFV* gene, whose first attack was presented during the course of immunosuppressive therapy for MDS. So far, no FMF cases complicated with MDS have been reported. This case suggests the possibility that certain immunosuppressants may cause attacks in cases carrying *MEFV* heterozygous mutation and also provides some warning for physicians to commence immunosuppressants for such patients.

References

1. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. Cell 1997;90(4):797–807. doi:10.1016/S0092-8674(00)80539-5.
2. The French FMF Consortium. A candidate gene for familial Mediterranean fever. Nat Genet. 1997;17(1):25–31. doi:10.1038/ng0997-25.
3. Touitou I. The spectrum of familial Mediterranean fever (FMF) mutations. Eur J Hum Genet. 2001;9(7):473–83. doi:10.1038/sj.ejhg.5200658.
4. Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, Lidar T, et al. Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum. 1997;40(10):1879–85. doi:10.1002/art.1780401023.
5. Pras E, Livneh A, Balow JE Jr, Kastner DL, Pras M, Langevitz P. Clinical differences between North African and Iraqi Jews with familial Mediterranean fever. Am J Med Genet. 1998;75(2):216–9. doi:10.1002/(SICI)1096-8628(19980113)75:2<216::AID-AJMG20>3.0.CO;2-R.
6. Livneh A, Aksentijevich I, Langevitz P, Torosyan Y, GS N, Shinar Y, et al. A single mutated MEFV allele in Israeli patients suffering from familial Mediterranean fever and Behcet's disease (FMF-BD). Eur J Hum Genet. 2001;9(3):191–6. doi:10.1038/sj.ejhg.5200608.
7. Centola M, Wood G, Frucht DM, Galon J, Aringer M, Farrell C, et al. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. Blood. 2000;95(10):3223–31.
8. Goldfeld AE, McCaffrey PG, Strominger JL, Rao A. Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor alpha gene promoter. J Exp Med. 1993;178(4):1365–79. doi:10.1084/jem.178.4.1365.
9. Khosroshahi HT, Tubbs RS, Shoja MM. Familial Mediterranean fever triggered by renal transplantation. Nephrol Dial Transplant. 2006;21(10):3000–1. doi:10.1093/ndt/gfl282.