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Multimodal treatment for resectable esophageal cancer

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Abstract Surgical resection has been traditionally the mainstay of treatment for localized esophageal cancers. However, survival after surgery alone for advanced esophageal cancer is not satisfactory. In Japan, the development of multimodal therapy for esophageal cancers has centered mainly on systemic chemotherapy plus surgery to control distant metastasis. Based on the results of the recent Japan Clinical Oncology Group (JCOG) 9907 study, preoperative chemotherapy (consisting of 5-FU and cisplatin) followed by surgery has emerged as the standard treatment. In Western countries, where chemoradiotherapy followed by surgery has been mainly explored for patients with resectable esophageal cancers, two large controlled trials that evaluated the effectiveness of preoperative chemotherapy reported conflicting results. However, a recent meta-analysis reported significant survival benefits for preoperative chemotherapy in patients with adenocarcinoma of the esophagus. We need to find new effective preoperative chemotherapeutic regimens, including molecular target agents, with response rates higher than that of the conventional chemotherapy of 5-FU and cisplatin. However, we also must compare the survival benefits of preoperative chemotherapy with preoperative chemoradiotherapy.

Key words Esophageal cancer · Chemotherapy · Neoadjuvant therapy Chemoradiation
Chemoradiotherapy

Introduction

Esophageal cancer remains an extremely difficult tumor to cure. Esophagectomy has been recognized as the standard treatment for resectable esophageal cancers. However, survival after surgery alone is still not satisfactory although new surgical techniques and perioperative care have been developed.^{1–3} Approximately half the patients who undergo curative resection for esophageal cancers develop local or distant recurrence postoperatively.^{4–8} To improve prognosis, multimodal therapy, including chemotherapy and radiotherapy, in addition to surgery has been developed. In Japan, three-field lymphadenectomy, which comprises cervical, mediastinal, and upper abdominal lymph node dissection, was introduced in the early 1980s, and there is a general agreement that this form of extended lymphadenectomy can achieve local tumor control.^{2,9–12} Therefore, in Japan, surgery combined with chemotherapy is mainly applied for the control of distant metastasis. On the other hand, in Western countries, efforts have been directed since the late 1980s toward analysis of the benefits of neoadjuvant chemoradiotherapy followed by surgery in improving local tumor control and eradicating distant micrometastases.^{13–20} In general, preoperative chemoradiation therapy, in particular, high-dose radiation therapy, is associated with increased perioperative morbidity although preoperative chemotherapy does not increase postoperative complications. By histopathological type, squamous cell carcinoma still accounts for the majority of esophageal cancers in Japan, the incidence of adenocarcinoma of the

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Table 1 Randomized trials of preoperative or postoperative chemotherapy for squamous cell carcinoma of the esophagus in Japan

Study	Arm	n	DFS	P value	5-year OS	P value
JCOG8806	Surgery alone	100	NA		45.1%	
	Surgery + CDDP/VDS	105	NA		48.3%	0.60
JCOG9204	Surgery alone	122	45%*		52%	
	Surgery + 5-FU/CDDP	120	55%*	0.037	61%	0.13
JCOG9907	Surgery + 5-FU/CDDP	166	2.0 years**		38.4%	
	5-FU/CDDP + surgery	164	3.0 years**	0.044	60.1%	0.013

CDDP, cisplatin; 5-FU, fluorouracil; VDS, vindesine; DFS, disease-free survival rate; OS, overall survival rate; NA, not available

*Five-year disease-free survival; **median progression-free survival.

esophagus has increased dramatically during the past three decades in Western countries, and adenocarcinoma has become the dominant histopathological type of esophageal cancer rather than squamous cell carcinoma.^{21,22} In operative procedures, the transhiatal approach has been widely performed in Western countries whereas it is rarely performed in Japan. Thus, this article reviews the accumulated evidence of systemic chemotherapy plus surgery in consideration of the differences in background between Japan and Western countries.

Systemic chemotherapy and surgery in Japan

In Japan, surgical resection has been the mainstay of treatment for clinical stage II and III esophageal cancers. The introduction of three-field lymphadenectomy improved the prognosis of patients with resectable esophageal cancers, but the 5-year survival rate remains relatively modest at less than 40%.²³ To compensate for this limitation of surgery, the role of adjuvant systemic chemotherapy was first explored in the late 1980s. The Japan Clinical Oncology Group (JCOG) conducted a randomized controlled trial (JCOG 8806) to compare the outcome of surgery alone and surgery followed by chemotherapy, consisting of fluorouracil (5-FU) and vindesine. The trial results indicated no significant benefits for postoperative chemotherapy²⁴ (Table 1).

Subsequently, the JCOG 9204 study compared postoperative adjuvant chemotherapy with surgery alone in patients who underwent curative resection (R0) for pathological stage II–IV esophageal cancers.²⁵ Patients in the postoperative chemotherapy group received two courses of chemotherapy comprising cisplatin at 80 mg/m² and 5-FU at 800 mg/m² for 5 days, separated by a 3-week interval. In that study, the overall survival was not significantly different between the two groups. However, the disease-free survival was significantly better in the postoperative chemotherapy group compared with surgery alone (5 year disease-free survival, 55% vs. 45%, $P=0.037$). These results suggest that postoperative chemotherapy seems to reduce recurrence after curative resection. In subgroup analysis,

postoperative chemotherapy showed survival benefit in patients with lymph node metastasis (pN1), but not those without lymph node metastasis (pN0). Based on these results, two cycles of postoperative chemotherapy consisting of 5-FU and cisplatin were recommended for patients with pathologically confirmed lymph node metastasis who underwent surgery for esophageal cancers.

Following the results of JCOG 9204, another study, the JCOG 9907 study, compared preoperative chemotherapy, consisting of 5-FU and cisplatin, with postoperative chemotherapy, applying a regimen similar to that used in patients with clinical stage II–III esophageal cancers.²⁶ In the study, all patients of the preoperative chemotherapy group received 5-FU and cisplatin regardless of the clinical diagnosis of lymph node metastasis; in the postoperative chemotherapy group, only patients who were confirmed pathologically to have lymph node metastasis received the treatment. The results showed that survival was significantly better for the preoperative chemotherapy group than the postoperative chemotherapy group (5-year overall survival, 60.1% vs. 38.4%). Furthermore, the preoperative group of the JCOG9907 study was superior with regard to the overall survival compared with the definitive chemoradiotherapy group of the JCOG 9906 study,²⁷ which was conducted to assess the effectiveness of definitive chemoradiotherapy in the same population of the JCOG 9907 (resectable clinical stage II/III) (5-year survival rate, 60.1% vs. 37%).^{26,27} Based on these results, preoperative chemotherapy consisting of 5-FU and cisplatin became the standard treatment for clinical stage II/III esophageal cancers in Japan. However, the JCOG 9907 study had several weak points that may be subject to criticism. Only 57% of patients received postoperative chemotherapy in the postoperative group because node-negative patients (pN0) were excluded from postoperative chemotherapy although most of the preoperative group received preoperative chemotherapy. The superiority of preoperative to postoperative chemotherapy was also evident in stage II patients, including node-negative patients, compared to those in stage III.

Table 2 Randomized trials of preoperative chemotherapy for esophageal cancers in Western countries.

Author	Year	Arm	Histology	<i>n</i>	MST (months)	OS	<i>P</i> value
Roth ²⁸	1988	CDDP/VDS/BLM + surgery	SCC	19	9	25% (3-year)	
		Surgery alone		20	9	5% (3-year)	
Schlag ²⁹	1992	5-FU/CDDP + surgery	SCC	21	10	20% (1-year)	
		Surgery alone		24	10	32% (1-year)	
Nygaard ³⁰	1992	CDDP/BLM + surgery	SCC	56	7.5	6% (2-year)	
		Surgery alone		50	7.5	13% (2-year)	
Maipang ³¹	1994	CDDP/VDS/BLM + surgery	SCC	24	17	31% (3-year)	
		Surgery alone		22	17	36% (3-year)	
Law ³²	1997	5-FU/CDDP + surgery	SCC	74	17	44% (2-year)	0.17
		Surgery alone		73	13	31% (2-year)	
Kelsen ³³	1998	5-FU/CDDP + surgery	SCC/Adeno	213	15	23% (3-year)	0.53
		Surgery alone		227	16	26% (3-year)	
Ancona ³⁴	2001	5-FU/CDDP + surgery	SCC	48	25	41% (3-year)	0.55
		Surgery alone		48	24	44% (3-year)	
MRC ³⁵	2002	5-FU/CDDP + surgery	SCC/Adeno	400	17	43% (2-year)	0.004
		Surgery alone		402	13	34% (2-year)	

VDS, vindesine; BLM, bleomycin; SCC, squamous cell carcinoma; Adeno, adenocarcinoma; MST, median survival time; OS, overall survival rate; NA, not available

Systemic chemotherapy and surgery in Western countries

To date, two large randomized controlled studies have compared preoperative chemotherapy followed by surgery with surgery alone (Table 2).^{28–35} In the U.S. intergroup trial (INT 113), 452 patients with adenocarcinomas or squamous cell carcinomas of the esophagus were randomly assigned to three cycles of 5-FU and cisplatin, followed by surgery, and additional postoperative chemotherapy, or surgery alone.³³ In this trial, there was no significant difference in overall survival or disease-free survival between perioperative chemotherapy and surgery alone. However, 19% of patients had an objective response to chemotherapy and those patients had a significantly better survival than those who had no objective response to chemotherapy or underwent surgery alone.

On the other hand, the UK Medical Research Council (MRC) trial showed survival benefits in preoperative chemotherapy.³⁵ In that trial, 802 patients with resectable esophageal cancers were randomized to two groups: patients who received two cycles of 5-FU and cisplatin followed by surgery or who underwent surgery alone. The curative resection (R0) rate was higher in the preoperative chemotherapy than the surgery alone group, although preoperative chemotherapy did not increase postoperative complications (R0 rate, 60% vs. 54%, $P < 0.0001$). Overall survival was significantly better in the preoperative chemotherapy group than surgery alone (2-year survival rate, 43% vs. 34%, $P = 0.004$). The

median survival time (MST) was also improved in the preoperative chemotherapy group compared with surgery alone (MST, 16.8 vs. 13.3 months).

Several factors could have caused the conflicting results of the US intergroup trial and UK MRC trial. First, there were slight differences in dosage and duration of preoperative chemotherapy (three cycles in the US intergroup vs. two cycles in the MRC trial). The larger total dose of chemotherapy provided in the US intergroup may explain the difference in the number of patients proceeding to resection (80% in US intergroup vs. 92% in MRC trial). Second, the proportion of cases with adenocarcinoma in the MRC trial was 66%, which was slightly higher than the rate in the U.S. intergroup, at 54%. Third, the larger sample size of the MRC trial compared with the U.S. intergroup trial may readily allow detection of slight but clinically important differences in survival.

In contrast to the conflicting results of the aforementioned large randomized controlled trials, a recent meta-analysis confirmed modest survival benefits of preoperative chemotherapy.³⁶ However, preoperative chemotherapy had no survival benefits in squamous cell carcinomas although the survival benefit was significant in adenocarcinomas. On the other hand, the meta-analysis showed a significant survival benefit for preoperative chemoradiation, irrespective of whether the histopathological type was squamous cell carcinoma or adenocarcinoma. This finding prompted the question of whether preoperative chemotherapy is superior to chemoradiotherapy. Only one study has compared

preoperative chemotherapy with preoperative chemoradiotherapy.³⁷ In that trial, 126 patients with locally advanced (T3-4NXM0) adenocarcinoma of the lower esophagus or gastric cardia were randomly allocated to one of two treatment groups: the chemotherapy group [2.5 courses of preoperative chemotherapy (cisplatin, 5-FU, and leucovorin) followed by surgery] and the chemoradiotherapy group [2 courses of the same chemotherapy followed by chemoradiotherapy (30 Gy), then surgery]. The rate of pathological complete response (pCR) was significantly higher in the preoperative chemoradiotherapy group than in the preoperative chemotherapy group (15.6% vs. 2%, $P = 0.03$), although there was no significant difference in the rate of curative resection between the two groups. Preoperative chemoradiotherapy tended to be associated with a better prognosis than preoperative chemotherapy, although this difference did not reach statistical significance because of early closure and the small sample size of this trial (3-year survival rate, 47.4% vs. 27.7%, $P = 0.07$). Based on the results of these trials, preoperative chemoradiotherapy followed by surgery has become the standard treatment strategy in Western countries for potentially resectable esophageal cancers.

Future directions

At present, chemotherapy consisting of 5-FU and cisplatin is accepted as the standard chemotherapy for esophageal cancers. However, the reported response rate to this chemotherapy for esophageal cancers remains at a 30–40% level.^{38,39} Previous studies that assessed the effectiveness of preoperative chemotherapy demonstrated a better survival of clinical responders.^{33,34,40} Thus, to improve the overall prognosis of patients who undergo preoperative chemotherapy followed by surgery, we need to explore new preoperative chemotherapeutic regimens that yield response rates higher than that of the conventional chemotherapy of 5-FU and cisplatin. One of the most promising chemotherapeutic regimens is the combination of 5-FU, cisplatin, and docetaxel. A gastric cancer TAX325 study, in which 445 patients with advanced gastric cancers were randomly assigned and treated with either docetaxel plus cisplatin and 5-FU (DCF) or cisplatin and 5-FU (CF), demonstrated that DCF is superior to CF with regard to progression-free survival and overall survival as well as the overall response rate.⁴¹ Including patients with advanced head and neck cancers, two recent large randomized trials (TAX323, TAX324) compared induction chemotherapy using docetaxel plus cisplatin and 5-FU or cisplatin and 5-FU, followed by chemoradiotherapy or radiotherapy. Both studies demonstrated that the addi-

tion of docetaxel significantly improved progression-free and overall survival, compared with the standard regimen of cisplatin and 5-FU.^{42,43} Thus, in head and neck cancer and gastric cancer, the superiority of chemotherapy of docetaxel/cisplatin/5-FU to the standard 5-FU/cisplatin has been established. In Japan, a phase I/II study (JCOG0807), designed to evaluate the effectiveness of chemotherapy of docetaxel/cisplatin/5-FU, is currently being conducted in patients with inoperable and/or recurrent esophageal cancers.

In addition to this, TS-1 is a potentially promising chemotherapeutic agent for esophageal cancers. TS-1 has been widely used in various cancers, such as head and neck cancer, lung cancer, and pancreatic cancer, and the combination chemotherapy of TS-1 plus cisplatin has been established as the standard chemotherapeutic regimen in gastric cancer. In esophageal cancers, the JCOG0604 study is currently being conducted to evaluate the effectiveness of definitive chemoradiotherapy consisting TS-1 plus cisplatin and 50.4-Gy radiotherapy in patients with clinical stage II/III esophageal cancers. In addition to TS-1, molecular target therapy agents for various cancers have been attracting much attention recently. A recent study showed that cetuximab, an epidermal growth factor receptor (EGFR) antibody, improved prognosis of patients with advanced head and neck cancer who received concurrent radiotherapy.⁴⁴ In esophageal cancers, the addition of cetuximab to cisplatin and 5-FU with or without radiotherapy has been examined in recent trials, and the results are encouraging for cetuximab.^{45,46} Studies of molecular target agents for esophageal cancers have just begun, and the clinical application of molecular target agents including cetuximab to systemic chemotherapy for esophageal cancers will be expected in the future.

Finally, in Japan, preoperative chemotherapy followed by surgery has been recognized as the standard treatment strategy for patients with resectable esophageal cancers. On the other hand, in Western countries, preoperative chemoradiotherapy followed by surgery is widely applied in patients with advanced esophageal cancers. Even after allowing for the difference in the dominant histopathological type and operative procedures between the two patient populations, there is a need to examine in the near future which of the treatment strategies is more effective in patients with resectable esophageal cancers.

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ORIGINAL ARTICLE

Eμ/miR-125b transgenic mice develop lethal B-cell malignancies

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MicroRNA-125b-1 (miR-125b-1) is a target of a chromosomal translocation t(11;14)(q24;q32) recurrently found in human B-cell precursor acute lymphoblastic leukemia (BCP-ALL). This translocation results in overexpression of miR-125b controlled by immunoglobulin heavy chain gene (*IGH*) regulatory elements. In addition, we found that six out of twenty-one BCP-ALL patients without t(11;14)(q24;q32) showed overexpression of miR-125b. Interestingly, four out of nine patients with *BCR/ABL*-positive BCP-ALL and one patient with B-cell lymphoid crisis that had progressed from chronic myelogenous leukemia overexpressed miR-125b. To examine the role of the deregulated expression of miR-125b in the development of B-cell tumor *in vivo*, we generated transgenic mice mimicking the t(11;14)(q24;q32) (Eμ/miR-125b-TG mice). Eμ/miR-125b-TG mice overexpressed miR-125b driven by *IGH* enhancer and promoter and developed IgM-negative or IgM-positive lethal B-cell malignancies with clonal proliferation. B cells obtained from the Eμ/miR-125b-TG mice were resistant to apoptosis induced by serum starvation. We identified *Trp53inp1*, a pro-apoptotic gene induced by cell stress, as a novel target gene of miR-125b in hematopoietic cells *in vitro* and *in vivo*. Our results provide direct evidence that miR-125b has important roles in the tumorigenesis of precursor B cells.

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Introduction

Chromosomal translocations involving the immunoglobulin heavy chain gene (*IGH*) locus have a pivotal role in the pathogenesis of human B-cell malignancies.^{1,2} *IGH* translocation brings target genes positioned on different chromosome loci into close apposition with transcription elements within the *IGH* locus, resulting in deregulated expression of the target genes. The vast majority of the target genes physiologically act as key regulators in cell growth, differentiation, signal transduction or apoptosis of B cells.¹ Thus, deregulated expression of the

translocated genes would alter B-cell fate leading to malignant transformation.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate protein synthesis through degradation of messenger RNAs or inhibition of translation.^{3,4} Many miRNAs are involved in a variety of important biological processes. Therefore, altered expression of miRNA affects cellular physiology and is responsible for some human disease.^{5,6}

An evolutionally conserved miRNA, miR-125b, has been implicated in human cancers.^{7–12} *MiR-125b-1*, mapped on 11q24, has been found to be a target of recurrent chromosomal abnormalities seen in both lymphoid and myeloid malignancies.^{7–10} We previously reported an insertion of *miR-125b-1* into the *IGH* locus in a patient with B-cell precursor acute lymphoblastic leukemia (BCP-ALL).⁷ Subsequently, other groups identified fusion sequences of *IGH* and *miR-125b-1* from t(11;14)(q24;q32).^{9,10} Bousquet *et al.*⁸ identified *miR-125b-1* as a gene involved in t(2;11)(p21;q23) observed in patients with myelodysplastic syndrome and acute myeloid leukemia (AML). This translocation is associated with upregulation of miR-125b from 6- to 90-fold. Recent studies show that ectopic expression of miR-125b in hematopoietic stem cells developed several hematological malignancies.^{13–15} However, *in vivo* oncogenic activities of miR-125b in B cells have not been fully understood.

In the present paper, we report molecular cloning of a novel breakpoint of t(11;14)(q24;q32) from a BCP-ALL patient, and show that transgenic mice mimicking this translocation develop lethal B-cell malignancies. Overexpression of miR-125b confers an anti-apoptotic property on cells along with inhibition of a pro-apoptotic gene, *Trp53inp1* (transformation-related protein 53 inducible nuclear protein 1), also known as *TP53INP1*, *TEAP*, *SIP* and *p53DINP1*,^{16–19} at the mRNA and protein levels. Of clinical relevance, we found overexpression of miR-125b in a variety of hematological malignancies. In particular, its overexpression in *BCR/ABL*-positive BCP-ALL supports the result showing the cooperative function of miR-125b in *BCR/ABL*-induced leukemia in an animal model.¹⁴

Materials and methods

Molecular analyses of patients showing fusion between *IGH* and *miR-125b-1*

A novel t(11;14)(q24;q32) breakpoint was cloned by long distant-inverse PCR as described.²⁰ An *IGH/miR-125b-1*

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chimera transcript was yielded by reverse transcriptase-PCR (RT-PCR) using cDNA from leukemic cells of the insertion case. Primer pairs and conditions are described in 'Supplementary Materials and methods'.

Establishment of $E\mu$ /miR-125b transgenic mice

A 422 bp of mouse genomic sequence including pre-miR-125b-1 was PCR amplified from C57BL/6 mouse genomic DNA. Primer pairs are described in 'Supplementary Materials and methods'. The fragment was inserted into the *EcoRI* site of p $E\mu$ /IGH plasmid (a kind gift from Dr Akagi, Saitama Cancer Institute, Japan). After digestion with *XbaI* and *Sall*, the recombinant DNA was injected into 300 eggs at Unitech Laboratory (Chiba, Japan). Two F0 mice were successfully generated as stable TG mice and designated as TG116 and TG005.

Southern blot analysis of the IGHJ region of the $E\mu$ /miR-125b-TG mice

Ten micrograms of genomic DNA was digested using *EcoRI* and subjected to Southern blot analysis using a digoxigenin-labeled 1.2 kb *EcoRI/HindIII* fragment of pMJH4 (JCRB, Osaka, Japan) recognizing the mouse IGHJ region.

Flow cytometric analysis

Red blood cell-depleted cells were stained with fluorescein isothiocyanate-conjugated anti-B220 Ab (Miltenyi Biotec, Gladbach, Germany), fluorescein isothiocyanate-conjugated anti-CD43 Ab (BD Biosciences, San Jose, CA, USA) or phycoerythrin-conjugated anti-CD19, IgM, CD3 and CD11b Abs (all from eBioscience, San Diego, CA, USA). For the apoptosis assay, cells were stained with fluorescein isothiocyanate AnnexinV and Propidium Iodide (BD Biosciences). Flow cytometric analysis was performed as described.^{21,22}

Morphological analysis

Cytospin preparations of bone marrow (BM) cells or peripheral blood were stained with Giemsa. Images were obtained with a BX51 microscope and DP21 camera (Olympus, Tokyo, Japan). Formalin-fixed and paraffin-embedded sections were stained with hematoxylin-eosin and examined using a microscope (Axio Imager A1, Carl Zeiss, Jena, Germany) with an AxioCam camera and AxioVision 4.6 software (Carl Zeiss).

Purification of B220-positive cells

B220-positive cells of BM cells or splenocytes were selected using an anti-B220 bearing magnetic beads (CD45R (B220) MicroBeads; Miltenyi Biotec) and columns attached by magnetic fields (MACS Separation Columns; Miltenyi Biotec) according to the manufacturer's protocol.

Vector construction

MicroRNA expression fragments were cloned into pMXsEF1-(GFP), which were pMXs-based self-inactivating retrovirus vectors expressing miRNA under an EF1a promoter along with a BGH polyA site and expressing GFP under a PGK promoter along with a 3' long terminal repeat. A miRNA expression cassette was constructed in the opposite direction from a GFP expression cassette. Expression fragments of miR-125b or IGH/miR-125b-1 were PCR amplified from C57BL/6 mouse genomic

DNA or cDNA of tumor derived from a BCP-ALL patient,⁷ respectively. Primer pairs are described in 'Supplementary Materials and methods'.

Real-time PCR

Total RNAs were treated with Deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA) and reverse transcribed by using miScript Reverse Transcription Kit (QIAGEN, Hilden, Germany). Real-time PCR was performed using a LightCycler Workflow System (Roche Diagnostics, Mannheim, Germany). Mature miR-125b expression was measured using a miScript SYBR Green PCR kit (QIAGEN). cDNA was amplified with miR-125b-specific primers (QIAGEN). Expression of U6 small nuclear RNA, as an internal control, was used for normalization of the results. For mRNA real-time PCR, a SYBR Premix EX Taq (Takara, Shiga, Japan) was used as described.²³ Primer pairs are described in 'Supplementary Materials and methods'. Patients' informed consents were obtained in accordance with the Declaration of Helsinki, and the studies were approved by the ethics committees of Wakayama Medical University (approval no. 72-2009) and the University of Tokyo (approval no. 21-44-1224). Human blood, peripheral leukocytes total RNA (Clontech, Palo Alto, CA, USA) was used as a normal control. Total RNAs from peripheral blood of the patients were isolated using Mouse RiboPure-Blood RNA Isolation Kit (Ambion, Austin, TX, USA).

Luciferase assay

The 3'untranslated region (UTR) of the mouse Trp53inp1 was PCR amplified from C57BL/6 mouse genomic DNA. Primer pairs are described in 'Supplementary Materials and methods'. The 3'UTR was cloned to downstream of the *Renilla* luciferase stop codon in pGL4.74 vector (Promega, Madison, WI, USA). To generate the mutant 3'UTR of the mouse Trp53inp1, two-step PCR mutagenesis was performed²⁴⁻²⁶ using the WT-3'UTR as a template. The Rluc-2 × 125b site vector contained two copies of a sequence designed to be perfectly complementary to the miR-125b downstream of the *Renilla* luciferase stop codon. 293T cells were co-transfected with the reporter vector (RLuc), miR-125b expression vector, and a transfection control vector expressing firefly luciferase (pGL3-control) using Fugene 6. Cells were harvested 30 h post-transfection and assayed with Dual Luciferase Assay (Promega). Three independent experiments were performed with a triplicate set.

Western blot analysis

Equal numbers of cells were lysed and western blotting was performed as described.^{22,27,28} Anti-Trp53inp1 Ab (ANASPEC, Fremont, CA, USA) and anti-ERK1/2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

Results

Overexpression of miR-125b in patients with BCP-ALL
 We found a new BCP-ALL patient with t(11;14)(q24;q32) and cloned the breakpoint. In this patient, the translocation resulted in breakage in JH6 of IGH on chromosome 14q32 and fusion to its 12.6 kb downstream of the miR-125b-1 locus on 11q24 (Figure 1a). The four breakpoints of the 11q24 so far identified by us and other groups^{9,10} are clustered within 18 kb downstream of the miR-125b-1 locus (Figure 1a). We previously reported a patient with BCP-ALL showing insertion of miR-125b-1

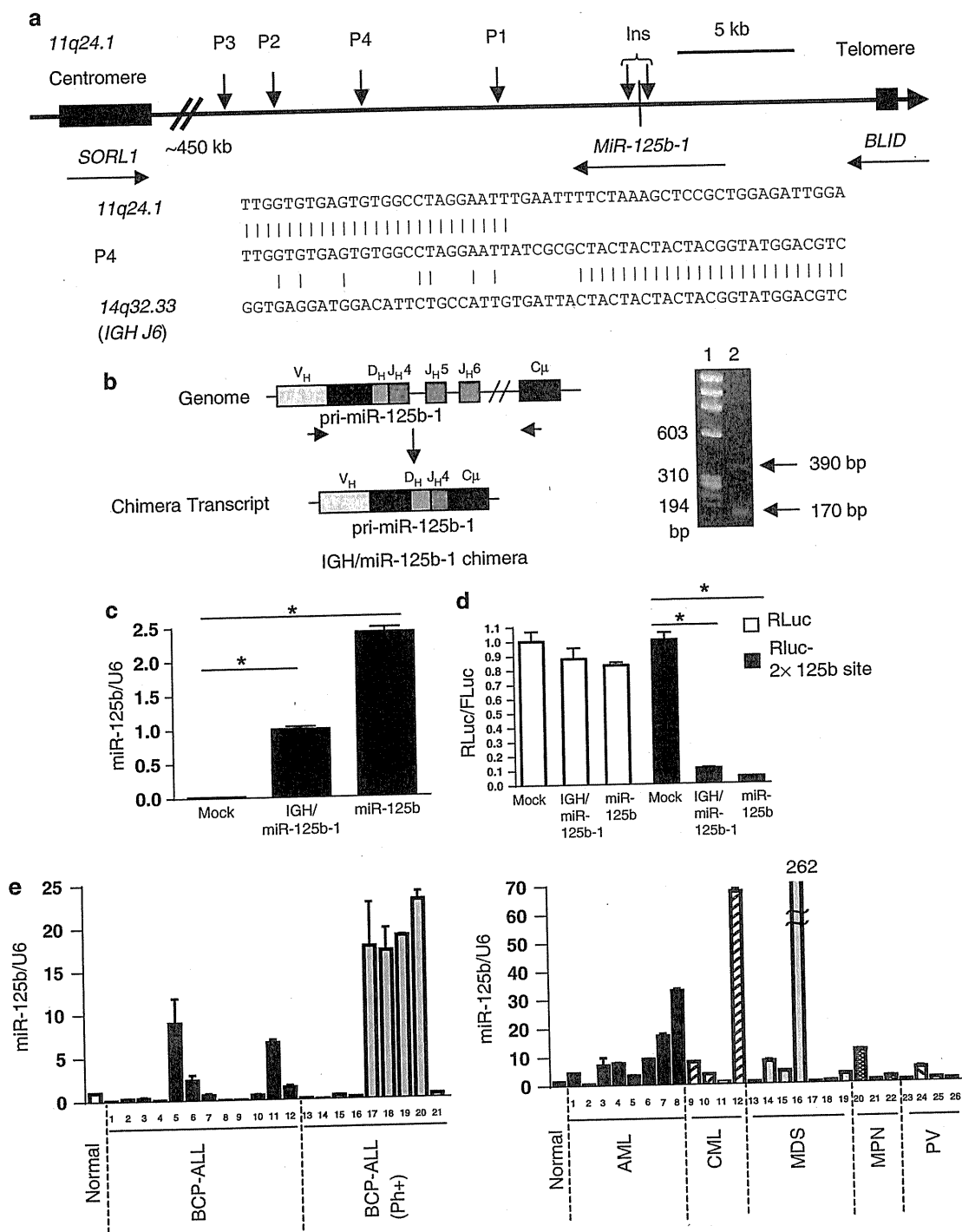


Figure 1 Molecular analyses of the expression of miR-125b in patients with BCP-ALL. (a) The 11q24 breakpoints cloned from BCP-ALL patients are clustered within 18 kb downstream of the *miR-125b-1* locus. Ins: Insertion points, P1, P2 and P3 were previously reported.^{9,10} P4 is a novel case showing t(11;14)(q24;q32). The nucleotide sequence cloned from P4 is shown. (b) The genome and chimera transcript structure observed in the insertion case.⁷ RT-PCR using primers set at V_H and C_μ produced two PCR products (390 and ~170 bp). (c) Relative expression levels of miR-125b were examined by real-time PCR in 293T cells transduced with pMXsEF1-IGH/miR-125b-1-(GFP), miR-125b expression vector (pMXsEF1-miR-125b-(GFP)) or mock (pMXsEF1-(GFP)). Data represent the average of two independent experiments. (d) *Renilla* luciferase (RLuc) vector or RLuc-2 × 125b site vector containing two copies of a sequence designed to be perfectly complementary to the miR-125b was co-transfected into 293T cells with a transfection control vector expressing firefly luciferase (pGL3 control) and pMXsEF1-IGH/miR-125b-1-(GFP), pMXsEF1-miR-125b-(GFP) or mock (pMXsEF1-(GFP)). Luciferase assays were performed with a triplicate set. (e) Relative expression levels of miR-125b were examined by real-time PCR in peripheral blood cells derived from patients with hematological diseases. Data represent the mean values ± s.e.m. of two independent experiments. *P*-values (*) of <0.05 were considered significant using a Student's two-tailed *t*-test. All data were representative of three independent experiments.

into a rearranged *IGH* allele.⁷ The genomic structure of the *IGH* allele inserted by pri-miR-125b-1 sequence is shown in Figure 1b. RT-PCR using primer settings at VH and C μ yielded 390 bp of chimera transcript consisting of VH, pri-miR-125b-1, DH, JH and C μ , and ~170-bp product (Figure 1b; Supplementary Figure 1). Sequence analysis of the ~170-bp product revealed diverse nucleotide alignments consisting of a VH/DH/JH/C μ complex sequence, suggesting that this PCR product might originate from polyclonal B cells existing in the tumor sample. To examine whether this chimera transcript could produce mature miR-125b, we cloned the chimera sequence from the cDNA derived from the tumor cells into pMXsEF1-(GFP) (hereafter pMXsEF1-*IGH*/miR-125b-1-(GFP)). Real-time PCR confirmed that the cells transduced with pMXsEF1-*IGH*/miR-125b-1-(GFP), as well as a positive control, expressed miR-125b (Figure 1c). Furthermore, luciferase assay showed that miR-125b produced from *IGH*/miR-125b-1 repressed the activity of the reporter construct (Rluc-2 \times 125b site) containing two copies of a sequence designed to be perfectly complementary to miR-125b (Figure 1d). These results demonstrated that the *IGH* allele inserted by the *miR-125b-1* sequence produces miR-125b when the promoter and enhancer are active.

For further analysis of the clinical relevance of miR-125b in hematological diseases, we examined expression of miR-125b in hematological tumors obtained from twenty-one BCP-ALL, eight AML, four chronic myelogenous leukemia, seven myelodysplastic syndrome, three myeloproliferative neoplasm and four polycythemia vera patients (Figure 1e; Supplementary Tables 1 and 2). We found that miR-125b was overexpressed in various patients; six BCP-ALL, five AML, two chronic myelogenous leukemia (accelerated phase and B-lymphoid blast crisis), one myeloproliferative neoplasm and two myelodysplastic syndrome patients showed more than fivefold higher expression of miR-125b compared with normal samples. Of note, miR-125b was highly expressed in four out of nine t(9;22)(q34;q11) positive BCP-ALL and one B-lymphoid blast crisis derived from chronic myelogenous leukemia.

We also investigated expression of miR-125a possessing the same seed sequence as that of miR-125b in these patients (Supplementary Figure 2). We found that one BCP-ALL patient showed more than fivefold higher expression of miR-125a compared with normal samples. These results indicate that overexpression of miR-125a in hematological malignancies is infrequent.

E μ /miR-125b-TG mice developed lethal lymphoid malignancies

We next generated *E μ /miR-125b-TG* mice to investigate whether deregulated expression of miR-125b could be responsible for the development of B-cell malignancy *in vivo*. The transgene consisted of human intronic enhancer of *IGH* (*E μ*), mouse VH promoter and rabbit β -globin-including mouse genomic sequence containing pre-miR-125b (Supplementary Figure 3a). Since the VH promoter and the *E μ* enhancer become active at the late pro-B cell stage,²⁹ miR-125b is thought to be expressed at this differentiation stage. We examined two TG lines, designated as *E μ /miR-125b-TG116* (TG116) and *E μ /miR-125b-TG005* (TG005). Southern blot analysis using a transgene-specific probe showed different patterns of integration, suggesting that a high copy number was introduced in TG116 compared with TG005 (Supplementary Figure 3). We confirmed expression of miR-125b in both TG mice (Figure 2a). All TG116 mice developed lethal lymphoid tumors within 35 weeks whereas five out of thirteen TG005 mice died within 50 weeks

with a disease similar to that of TG116 (Figure 2b). When the disease became overt, both TG116 and TG005 mice showed splenomegaly (Figure 2c; Supplementary Figure 4). Moreover, TG116 mice showed hepatomegaly and an increased number of BM cells, whereas the concentration of hemoglobin (Hb) of both TG116 and TG005 mice decreased compared with that of wild-type (WT) mice with statistical significance (Supplementary Figure 4).

Tumor cells with morphologically lymphoblastic features appeared in BM and peripheral blood (Figure 2d). Histological examination showed that atypical lymphoid cells filled the spleen and infiltrated the liver (Figure 2d). Using flow cytometric analysis, we found that nine morbid TG116 mice had developed IgM⁻ precursor B-cell tumors, one IgM⁺ B-cell tumor and one T-cell tumor, whereas four morbid TG005 mice had developed IgM⁺ B-cell tumors and one IgM⁺/B220⁻ tumor (Figure 2e and data not shown). Flow cytometric analysis delineated that most tumor cells of the morbid TG116 mice consisted of B220⁺/CD19⁺/IgM⁻/CD43⁺ B cells, whereas those of the morbid TG005 mice were B220⁺/CD19⁺/IgM⁺/CD43⁻ B cells. The number of B220⁺/CD19⁺/IgM⁻/CD43⁻ B cells increased in the TG116 mice as early as 4 weeks mice (Supplementary Figure 5a). To examine the tumor clonality, we performed Southern blot analysis; we detected rearranged bands in eight out of nine morbid TG116 mice and in all the five morbid TG005 mice, indicating clonal proliferation of B cells (Figure 2f and data not shown). One out of four young TG116 mice showed clonal rearranged bands before the onset of the disease, suggesting that clonal proliferation can occur even in 4 weeks after birth (Supplementary Figure 5b). Collectively, *E μ /miR-125b-TG* mice developed lethal IgM⁻ or IgM⁺ B-cell malignancies with clonal expansion.

MiR-125b inhibited apoptosis of hematopoietic cells

We next analyzed the mechanism by which miR-125b promoted leukemogenesis *in vivo*. To identify target genes of miR-125b, we performed a bioinformatics search using TargetScan (<http://www.targetscan.org>). There were some apoptosis-related genes, such as *Bak1*, *Bmf*, *KLF13*, *MCL-1* and *Trp53inp1*, among the candidate target genes of miR-125b. In addition, we performed microarray analysis of 32Dcl3 cells transduced with miR-125b or mock. In microarray analysis, we found that among the candidate target genes the expression levels of *Trp53inp1* mRNAs were reduced in 32Dcl3 cells transduced with miR-125b (data not shown). The 3'UTR of *Trp53inp1* contained one putative target site that matched the seed sequences of miR-125b. *Trp53inp1* is a pro-apoptotic gene induced by cell stress,¹⁶⁻¹⁹ and its repression is involved in tumorigenesis.^{30,31} To confirm that *Trp53inp1* is a direct target gene of miR-125b, the wild-type 3'UTR of *Trp53inp1* or the mutated 3'UTR was cloned to downstream of the *Renilla* luciferase open reading frame (Figure 3a). We performed luciferase assays and found that miR-125b directly represses the expression of the reporter gene with the *Trp53inp1*-3'UTR but not that with mutated 3'UTR of *Trp53inp1* (Figure 3a).

We then examined the expression levels of *Trp53inp1* mRNAs in 32Dcl3 cells at different time intervals after inducing apoptosis by IL-3 withdrawal (Supplementary Figure 6a). We found that the expression levels of *Trp53inp1* mRNAs increased after inducing apoptosis by IL-3 withdrawal but that those of *Trp53inp1* mRNAs were repressed in miR-125b-transduced 32Dcl3 cells compared with mock-transduced 32Dcl3 cells at each time point. In addition, the numbers of apoptotic cells decreased in miR-125b-transduced cells but not in miR-30a,

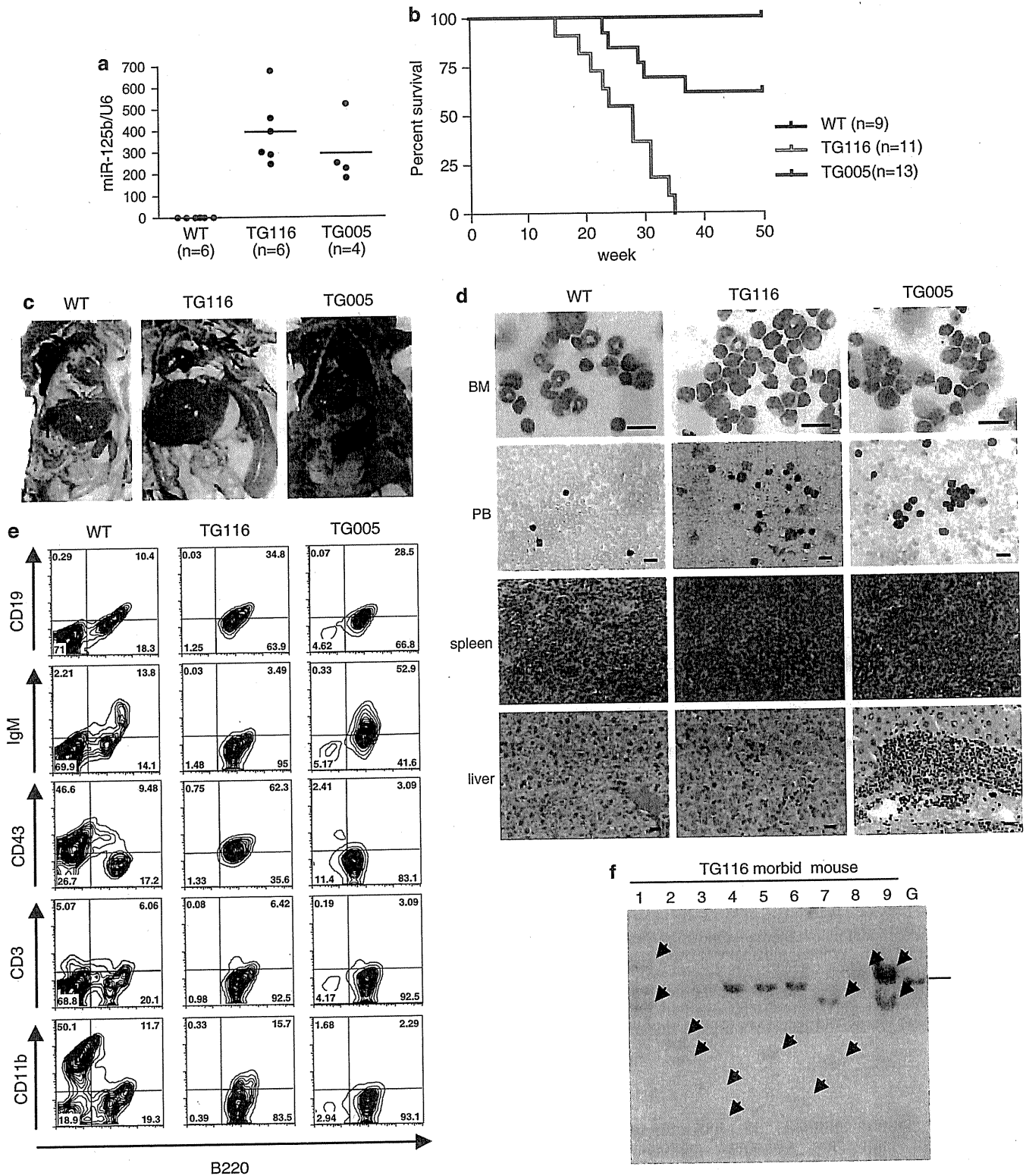


Figure 2 $\epsilon\mu$ /miR-125b-TG mice developed lethal B-cell tumors. (a) Relative expression levels of miR-125b were examined by real-time PCR in B220-positive BM cells derived from $\epsilon\mu$ /miR-125b-TG (TG116 and TG005) or WT mice. Data represent the average of two independent experiments. (b) Kaplan–Meier analysis for the survival of TG116, TG005 and WT mice. (c) Both TG116 and TG005 mice exhibit splenomegaly. (d) Hematoxylin and eosin staining of spleen and liver obtained from TG116, TG005 or WT mice. Bars, 20 μ m. (e) Flow cytometric analysis of BM cells derived from morbid TG116, morbid TG005 or WT mice. (f) Southern blot analysis of splenocytes derived from morbid TG116 mice (lanes 1–9). Clonal rearranged bands (arrows) were detected in lanes 1–3 and 5–9. G, germ line control.

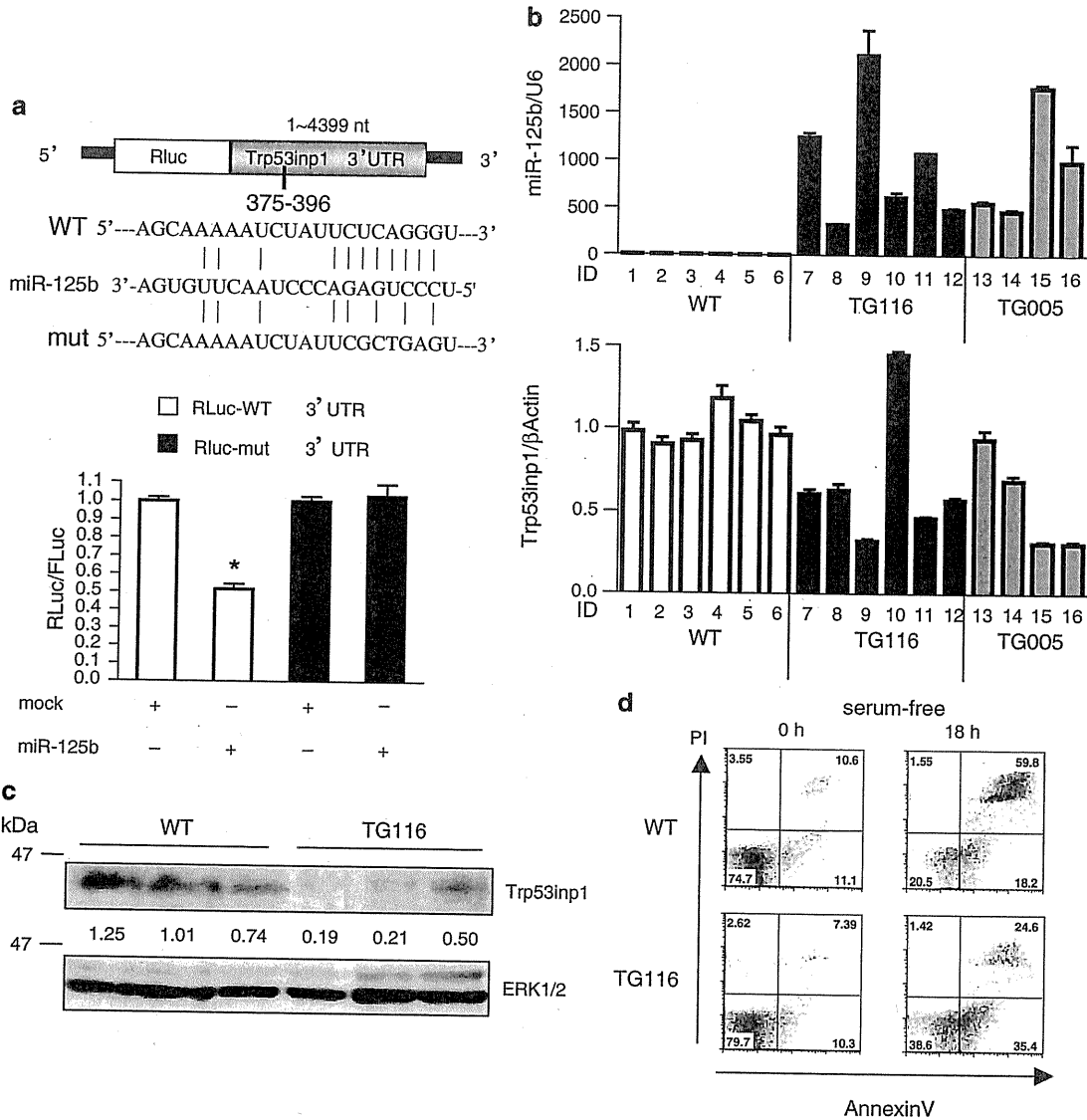


Figure 3 MiR-125b repressed expression of Trp53inp1 and inhibited apoptosis of B cells. (a) 293T cells were co-transfected with a transfection control vector (pGL3-control) and pMXsEF1-miR-125b-(GFP) or mock (pMXsEF1-(GFP)). Luciferase assays were performed with a triplicate set. (b) Relative expression levels of miR-125b were examined by real-time PCR in purified B220-positive splenocytes derived from TG116 mice (mouse IDs 7–12), TG005 mice (mouse IDs 13–16) or WT mice (mouse IDs 1–6) of two independent experiments. Relative expression levels of Trp53inp1 in these cells were examined by real-time PCR. Data points correspond to the mean values \pm s.e.m. of three independent experiments. (c) Expression of Trp53inp1 protein in purified B220-positive splenocytes of TG116 mice (lanes 4–6) or WT mice (lanes 1–3). Lysates were immunoblotted with anti-Trp53inp1 Ab or anti-ERK1/2 Ab as a control. Relative band intensities of Trp53inp1 were calculated by densitometric analysis and normalized to ERK1/2. (d) Purified B220-positive splenocytes of 20-week-old TG116 or WT mice were cultured without serum. Cells were stained with fluorescein isothiocyanate AnnexinV and Propidium Iodide. *P*-values (*) of <0.05 were considered significant using a Student's two-tailed *t*-test. All data were representative of three independent experiments.

used as a control miRNA, or mock-transduced cells (Supplementary Figure 6b). Moreover, expression levels of mature miR-125b and Trp53inp1 mRNA were inversely correlated. With one exception (Figure 3b, lane 10), TG116 mice tended to express lower levels of Trp53inp1 at the mRNA and protein levels compared with WT mice (Figures 3b and c). We also found that B220-positive splenocytes derived from TG116 mice became resistant to apoptosis induced by serum starvation when compared with WT mice (Figure 3d). Collectively, these results indicated that miR-125b inhibits apoptosis of hematopoietic cells at least partly through repressing Trp53inp1.

Discussion

A number of miRNAs are located on regions of the genome involved in chromosome alteration, such as deletion, amplification and translocation as observed in various malignant tumors.^{5,6} Although cloned chromosomal translocations involving *IGH* loci are common genomic alterations in B-cell malignancies, only *miR-125b-1* has so far been shown to be a target miRNA related to *IGH* translocations. In the present paper, we report a new patient with t(11;14)(q24;q32) and direct evidence for the oncogenic potential of miR-125b in B cells using transgenic mice mimicking the translocation.

Overexpression of miR-125b in BM cells was reported to induce myeloproliferative neoplasm in transplanted mice that progressed to AML,¹³ while BM-derived highly purified hematopoietic stem cells overexpressing miR-125b tended to differentiate into lymphoid lineages after transplantation.¹⁵ On the other hand, half of the mice transplanted with miR-125b-overexpressing fetal liver hematopoietic stem cells developed T-ALL, B-ALL or myeloproliferative neoplasm.¹⁴ These report indicated that miR-125b has important roles in proliferation and differentiation of hematopoietic stem cells. However, the functions of miR-125b in B cells have remained elusive.

In the present paper, we have shown that overexpression of miR-125b triggered by *IGH* enhancer and promoter induces two different types of lethal B-cell malignancies. TG116 mice mainly develop faster-onset IgM-negative tumors and TG005 mice develop slower-onset IgM-positive tumors. This difference could be explained by expression levels of miR-125b due to the different copy number of transgenes integrated into mouse chromosome (Supplementary Figure 3b). Alternatively, other factors such as epigenetic changes or inhibition of other miRNAs might have been involved in the development of the disease and were responsible for the difference in the disease phenotypes.

MiR-125 was suggested to confer anti-apoptotic characteristics on cells by reducing expression levels of apoptosis-inducing genes such as *Bak1*, *Bmf* and *KLF13* in mouse hematopoietic cells.^{15,32} In this study, we have identified *Trp53inp1* as a novel candidate of the miR-125b target gene in mice; its mRNA and protein levels decreased in B220-positive cells obtained from Eμ/miR-125b-TG mice. Indeed, *Trp53inp1* null mice were shown to be susceptible to cancer development.³¹

Of clinical relevance, we found that miR-125b was overexpressed in various hematopoietic diseases including BCP-ALL, AML and myelodysplastic syndrome without chromosomal translocations, t(11;14)(q24;q32) or t(2;11)(p21;q23). Mature miR-125b is also produced from *miR-125b-2* mapped on 21q21. However, the above-mentioned patients except for a BCP-ALL patient ID 7 showed no abnormalities of chromosome 21 (Supplementary Tables 1 and 2). These cases may express high amount of miR-125b by unknown mechanisms. Interestingly, we reported here that four out of nine *BCR/ABL*-positive ALL patients and one with B-cell lymphoid crisis of chronic myelogenous leukemia showed high expression levels of miR-125b. Another group also independently found overexpression of miR-125b in a *BCR/ABL*-positive ALL patient lacking t(11;14)(q24;q32).⁹ Recently, it was reported that miR-125b can cooperate in leukemogenic activity with *BCR/ABL*. Taking clinical and experimental results together, overexpression of miR-125b would be associated with hematological malignancies. As inactivation of oncomiR, such as miR-21, can decrease tumorigenesis,³³ inhibition of miR-125b could be a new strategy for treatment of hematopoietic malignancies. Our transgenic mice will be a useful tool in the development of the therapy for the patients with high miR-125b expression.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Two types of C/EBP α mutations play distinct but collaborative roles in leukemogenesis: lessons from clinical data and BMT models

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Two types of mutations of a transcription factor CCAAT-enhancer binding protein α (C/EBP α) are found in leukemic cells of 5%-14% of acute myeloid leukemia (AML) patients: N-terminal mutations expressing dominant negative p30 and C-terminal mutations in the basic leucine zipper domain. Our results showed that a mutation of C/EBP α in one allele was observed in AML after myelodysplastic syndrome, while the 2 alleles are mutated in de novo AML. Unlike an N-terminal frame-shift mutant (C/EBP α -N^m)-transduced cells,

a C-terminal mutant (C/EBP α -C^m)-transduced cells alone induced AML with leukopenia in mice 4-12 months after bone marrow transplantation. Coexpression of both mutants induced AML with marked leukocytosis with shorter latencies. Interestingly, C/EBP α -C^m collaborated with an Flt3-activating mutant Flt3-ITD in inducing AML. Moreover, C/EBP α -C^m strongly blocked myeloid differentiation of 32Dcl3 cells, suggesting its class II mutation-like role in leukemogenesis. Although C/EBP α -C^m failed to inhibit transcrip-

tical activity of wild-type C/EBP α , it suppressed the synergistic effect between C/EBP α and PU.1. On the other hand, C/EBP α -N^m inhibited C/EBP α activation in the absence of PU.1, despite low expression levels of p30 protein generated by C/EBP α -N^m. Thus, 2 types of C/EBP α mutations are implicated in leukemogenesis, involving different and cooperating molecular mechanisms. (*Blood*. 2011; 117(1):221-233)

Introduction

The CCAAT/enhancer binding protein α (C/EBP α) transcription factor is a critical regulator of proliferation and differentiation in myeloid cells.^{1,2} C/EBP α consists of an N-terminal transcriptional activation domain and a C-terminal basic leucine zipper (bZIP) domain.³⁻⁵ Two isoforms of C/EBP α proteins are generated from different translation start sites: a full-length 42-kDa protein (p42) and a truncated 30-kDa protein (p30) that lacks an N-terminal transcriptional activation domain. C/EBP α -p30 isoform inhibits C/EBP α -p42-mediated transcription.⁶⁻⁸ Importantly, C/EBP α promotes differentiation both by up-regulation of lineage-specific gene products⁹⁻¹¹ and by proliferation arrest.¹² Recent studies have indicated that C/EBP α -induced growth arrest is regulated by its interaction with other molecules involved in growth control: E2F,¹³⁻¹⁵ Max,¹⁶ and SWI/SNF chromatin remodeling complexes.¹⁷ For example, repression of E2F activity by E2F-C/EBP α interaction results in the down-regulation of c-Myc, leading to granulocytic differentiation.^{18,19}

According to several studies, *CEBPA* mutations are found in 5%-14% of acute myeloid leukemia (AML) patients belonging to the French-American-British subtypes M1, M2, or in some cases M4.^{8,20-22} The mutations of the *CEBPA* gene can be largely categorized into 2 types: one is an N-terminal frame-shift mutation disrupting p42 and producing p30 as a major product, and the other is a C-terminal in-frame mutation disrupting the bZIP region.

Interestingly, most AML patients with *CEBPA* mutations have both mutations simultaneously,²³⁻²⁵ and such patients displayed a favorable outcome.^{22,26} On the other hand, AML patients with single *CEBPA* mutations did not express a distinctive signature, presumably due to a variety of associating gene alterations, including Flt3 activating mutations. Related to this, involvement of single *CEBPA* mutations with myelodysplastic syndrome (MDS) remains to be clarified.^{27,28}

Analysis of mice with genetic alterations in the *CEBPA* locus has contributed to delineation of molecular mechanisms by which *CEBPA* mutations induce leukemia. Conditional deficiency of C/EBP α led to a differentiation block at the transition between common myeloid progenitors and granulocyte/monocyte progenitors but not to development of leukemia.¹ However, *Cebpa*^{LL} mice, expressing only p30 as C/EBP α protein, developed myeloid leukemia with complete penetration.²⁹ On the other hand, *Cebpa*^{BRM2/BRM2} mice, carrying a point mutation in the bZIP domain that dampened E2F interaction, showed only preleukemic features.¹⁴ Interestingly, the same group has recently reported combinatorial effects of the C-terminal and the N-terminal mutations on leukemogenesis by using *Cebpa*^{LL} mice, *Cebpa*^{KK} mice carrying the K313 duplication in the C-terminal domain, and *Cebpa*^{KL} mice.^{29,30} They proposed that efficient leukemogenesis is caused by the combination of both premalignant HSC expansion induced by

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C-terminal *CEBPA* mutation and residual myeloid lineage commitment maintained by the N-terminal *CEBPA* mutation.³⁰

Causative gene alterations in hematologic malignancies have been extensively studied, and it is now recognized that multiple mutations contribute to development of leukemia. These gene alterations are categorized into 2 groups, class I and class II mutations.^{31,32} Class I mutations include activating mutations of *FLT3*, *C-KIT*, *JAK2*, *SHP2*, and *RAS*, or inactivating mutations of *TP53* and *NF-1*, and induce proliferation or block apoptosis of hemopoietic cells. On the other hand, class II mutations disrupt normal functions of transcription factors and chromosome-modifying enzymes including *MLL*, *RUNX1*, *RARA*, and *PU.1* and hamper differentiation of hemopoietic cells. Combinations of class I and class II mutations are frequently observed in patients' leukemic cells.^{33,34} In addition, we and others presented evidence that class I and class II mutations collaborate in the development of leukemia in mouse models.^{35,36} Among a variety of gene alterations found in leukemia, *CEBPA* mutations are unique because different *CEBPA* mutations are frequently found on different alleles in leukemic cells of de novo AML.²²⁻²⁶

In the present study, we searched for mutations of the *CEBPA* gene in patients with myeloid malignancies, and found N- and C-terminal double mutations in patients with de novo AML. In patients with MDS/AML or therapy-related AML or MDS, only N- or C-terminal single mutation was identified. We chose a C-terminal mutation 304_323dup (hereafter called C/EBP α -C^m) and an N-terminal mutation (T60fsX159) (hereafter C/EBP α -N^m) for further analysis that had been isolated as double *CEBPA* mutations in a de novo AML patient. To evaluate the effects of these mutations on leukemogenesis, we used a mouse bone marrow transplantation (BMT) model. Interestingly, unlike the phenotype in Cebp^{K/K}, Cebp^{K/+}, Cebp^{BRM2/BRM2}, or Cebp^{BRM2/+} mice,^{14,29,30} C/EBP α -C^m alone induced AML with leukopenia in transplanted mice after BMT. We also confirmed the efficient induction of AML by coexpression of C/EBP α -C^m and C/EBP α -N^m. We will discuss the possible molecular mechanisms by which C/EBP α -N^m worked in concert with C/EBP α -C^m in accelerating leukemogenesis.

Methods

Patients and samples

We chose patients with hematologic diseases (224 MDS/AML patients, 71 therapy-related AML or MDS patients, and 89 de novo AML patients, who had been diagnosed at Hiroshima University Hospital between 1985 and 2007, are not a consecutive series of patients). All studies were approved by the Institutional Review Board at Hiroshima University and the ethics committee of the University of Tokyo (approval no. 20-10-0620). Patients' informed consents were obtained in accordance with the Declaration of Helsinki. *CEBPA* mutation screening by polymerase chain reaction (PCR)-single strand conformation polymorphism analysis and identification of *CEBPA*, *AML1*, *N-RAS*, *FLT3*, *PTPN11*, *C-KIT*, and *TP53* mutations was performed as described previously.³⁷

Retroviral vectors

We used 2 C/EBP α mutants, N^m or C^m, as well as C/EBP α wild-type (WT) and C/EBP α N-terminal truncated p30 (p30). C/EBP α -WT, p30, N^m, or C^m, which was tagged with a FLAG or Myc epitope at the C terminus, was inserted upstream of the internal ribosome entry site-enhanced green fluorescent protein (IRES-EGFP) cassette of pMYs-IG to generate pMYs-FLAG or Myc-tagged CEBP α -WT, p30, N^m, or C^m-IG, respectively. Similarly, these fragments were subcloned into pMXs-IRES-puro (pMXs-IP), pMXs-IRES-blasticidin (pMXs-IB), or pMYs-IRES-dsRED

(pMYs-IR). Flt3-ITD cDNA, which was derived from patient's leukemic cells harboring a 20-amino acid tandem duplication called M3^{38,39} was subcloned into pMYs-IG to generate pMYs-Flt3-ITD-IG. Human granulocyte colony-stimulating factor receptor (G-CSF-R) cDNA, a kind gift from Dr Shigekazu Nagata (Kyoto University, Kyoto, Japan), was subcloned into pMXs-IB to generate pMXs-G-CSF-R-IB.

Retroviral infection was done as described previously.⁴⁰ Briefly, retroviruses were generated by transient transfection of Plat-E packaging cells with FuGENE 6 (Roche Diagnostics).^{41,42} Growth of transduced 32Dcl3 cells, which were subject to the drug selection with 1 μ g/mL puromycin or 10 μ g/mL blasticidin, was estimated by quantitating luminescence as described previously.⁴³

Flow cytometric analysis

Briefly, cells were stained with phycoerythrin-conjugated antibodies (Abs) or biotinylated Abs and phycoerythrin/Cy5-streptavidin (eBioscience). Flow cytometric analysis of the stained cells was performed with FACSCalibur flow (BD Biosciences) equipped with FlowJo Version 7.2.4 software (TreeStar).

Real-time reverse-transcription PCR

Real-time reverse-transcription (RT) PCR was performed as described previously.⁴⁰ Reaction was subject to one cycle of 95°C for 30 seconds, 45 cycles of PCR at 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 10 seconds. The following primer pairs were used: 5'-AAGGCCAGTGTGTCTCTGT-3' (forward), and 5'-TACCAGCCCCAACTCAAAAC-3' (reverse) for G-CSF-R, 5'-AGAGGGAAATCTGTCGTGAC-3' (forward), and 5'-CAATAGTGATGACCTGGCCGT-3' (reverse) for β -actin, 5'-GCCCCCTAGTGCTGCATGAG-3' (forward) and 5'-CCACAGACACACATCAATTCTT-3' (reverse) for c-Myc.

Western blot analysis

Equal numbers of cells were lysed and Western blotting was performed as described previously.^{35,40} Anti-Flag (M2) Ab (Sigma-Aldrich), anti-c-Myc (9E10) Ab (Roche Diagnostics), anti-C/EBP α (14AA) or (N-19) Ab (Santa Cruz Biotechnology), ERK1/2, signal transducer and activator of transcription (STAT)3, STAT5, AKT1, or Flt3 Abs, and phospho-STAT3 Ab (Santa Cruz Biotechnology), anti-phospho mitogen-activated protein kinase and phospho-AKT Abs (Cell Signaling Technology), and phospho-STAT5 Ab (BD Biosciences) were used.

Luciferase assay

The 293T cells were transiently transfected with the luciferase reporter plasmid p(C/EBP)2TK (kindly provided by Atsushi Iwama, Chiba University, Japan), pMXs-C/EBP α -WT or mutants-IP, and pEF-BOS/PU.1 for C/EBP α transcriptional activity, or E2F α -TATA-LUC, pCMV-E2F1, and pCMV-DP1 (kindly provided by Claus Nerlov, EMBL Mouse Biology Unit, Italy) for E2F transcriptional activity.¹⁵ Luciferase assays were performed by Dual luciferase assay systems (Promega).

Immunostaining

Immunostaining of 293T cells transiently transfected with retrovirus constructs was performed as described previously.³⁵ After fixation with 1.5% paraformaldehyde, cells were immunostained with rabbit anti-Flag Ab or fluorescein isothiocyanate-conjugated mouse anti-c-Myc Ab (Sigma-Aldrich). The cells were then stained with Alexa Fluor 546-conjugated goat anti-rabbit immunoglobulin G secondary Ab (Molecular Probes). Nuclei were counterstained with Hoechst (H33342). Fluorescent images were analyzed on a confocal microscope (FLUOVIEW FV300 scanning laser biological microscope JX70 system; Olympus) equipped with SenSys/OL cold charge-coupled device (CCD) camera (Olympus). The objective lens (an LCPlanFI 60 \times /1.40 NA oil) was used.

Gel shift assay

Nuclear extracts from transfected 293T cells were incubated with 2 μ g of polydeoxyinosinic-deoxycytidylic acid and then with double-stranded

Table 1. Clinical features and genetic findings of the patients with *CEBPA* mutations

Patient no.	Age, y/sex	Diagnosis	N terminal C terminal			Karyotype	Other gene mutation*	Survival (years)
			p30 type	bZIP inframe	Frameshift			
MDS/AML								
142	79/F	AML following MDS	I62fsX160	-	-	46,XY[20/20]	-	1.0
829	70/M	AML following MDS	P51fsX160	-	-	45,XY,der(17;18)(q10;q10)[2/20] 46,XY[18/20]	-	0.4
769	69/M	AML following MDS	-	R297P	-	46,XY[20/20]	<i>AML1, PTPN11</i>	1.6
896	77/M	AML following MDS	-	K313del	-	46,XY[20/20]	-	0.6
22	71/M	AML following MDS	-	-	G176fsX317	47,XY,+1,der(1;15)(q10;q10)[20/20]	-	0.8
679	89/M	MDS(RAEB)	-	-	P235fsX318	46,XY[20/20]	-	1.9
806	77/M	AML following MDS	G38fsX107	-	R291fsX313	46,XY[20/20]	-	1.9
Therapy-related AML or MDS								
59	80/F	AML(M4)	L19fsX159	-	-	47,XX,+8,t(9;11)(p22;q23)[20/20]	<i>AML1</i>	1.7
158	72/F	AML following MDS	F106fsX154	-	-	46,XX[20/20]	-	1.0
811	76/M	MDS(RAEB)	E59X†	-	-	46,XY[20/20]	-	1.7
1068	66/M	MDS(RAEB)	-	Q305P	-	46,XY[20/20]	-	> 1.5
346	59/M	AML following MDS	-	-	S190fsX320	43,XX,del(5)(q31),-7,-15,-18,-21,+mar [20/20]	<i>N-RAS</i>	0.8
577	56/F	AML following MDS	-	-	C213X	46,XX[20/20]	-	2.4
629	89/F	AML following MDS	-	-	L350fsX360	46,XX[20/20]	-	1.2
920	69/F	AML(M5)	-	-	S348fsX422	46,XX,t(11;17)(p15;q21)[20/20]	-	0.2
De novo AML(M2)								
40	68/F	AML(M2)	A111fsX166	S299_L304dup	-	46,XX[20/20]	-	> 10.5
292	75/F	AML(M2)	F33fsX107	R297P	-	46,XX[20/20]	-	> 8.4
662	58/F	AML(M2)	S65fsX167	K313dup	-	46,XX[20/20]	-	> 5.0
888	70/F	AML(M2)	A111fsX166	N321D	-	47,XX,+8[20/20]	-	> 3.8
941	31/F	AML(M2Eo)	T60fsX159	304_323dup	-	46,XX,del(7)(q32)[20/20]	-	> 3.5

RAEB indicates refractory anemia with excess blasts.
 *Indicates that no mutation was detected in *AML1*, *N-RAS*, *FLT3*, *PTPN11*, *C-KIT*, and *TP53* genes.
 †Homozygous mutation.

CSF3R promoter oligonucleotide labeled with γ^{32} P-adenosine triphosphate. Cold competition and a super-shift reaction were carried out by adding a 40-fold excess of cold CSF3R oligo or 1.5 μ g of anti-C/EBP (14AA)X Ab (Santa Cruz Biotechnology), respectively. The resulting complexes were resolved on 4.5% polyacrylamide gel.⁴⁴

Colony assay

Infected mouse bone marrow (BM) mononuclear cells (1×10^4) were plated in methylcellulose medium (StemCell Technologies) supplemented with 50 ng/mL each of interleukin (IL)-3, IL-6, stem cell factor, and granulocyte/macrophage-colony-stimulating factor (GM-CSF; R&D Systems) in the presence of 1 μ g/mL puromycin. Colonies were counted after 1-week culture, and single-cell suspensions (10^4 cells) of drug-resistant colonies were subsequently replated.

Mouse BMT

Mouse BMT was performed as described previously.⁴⁰ Briefly, BM mononuclear cells were isolated from the femurs and tibias of C57BL/6 (Ly-5.1) donor mice 4 days after intraperitoneal administration of 150 mg/kg 5-fluorouracil. The cells were stimulated with 50 ng/mL of mouse stem cell factor, mouse FLT3 ligand, mouse IL-6, and human thrombopoietin (all cytokines were from R&D Systems). The prestimulated cells were infected for 60 hours with the retroviruses harboring pMYs-C/EBP α -C^m-IG, pMYs-C/EBP α -N^m-IG, pMYs-Myc-tagged C/EBP α -C^m-IG, pMYs-Flag-tagged C/EBP α -N^m-IR, pMYs-Flt3-ITD-IG, pMYs-IG, or pMYs-IR, using 6-well dishes coated with RetroNectin (Takara Bio). Then, $3-5 \times 10^5$ of the infected BM cells (that had not been sorted after either single or

double infection) were injected into sublethally γ -irradiated C57BL/6 (Ly-5.2) recipient mice. Overall survival of transplanted mice were estimated using the Kaplan-Meier method. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, The University of Tokyo.

Statistical analysis

Statistical significance was calculated using the Student *t* test for independent variables. *P* values < .05 were considered statistically significant.

Results

***CEBPA* mutations in patients with myeloid malignancies**

After we performed single-strand conformation polymorphism analysis to screen for *CEBPA* mutations in patients with hematologic disorders, *CEBPA* mutations were identified in 7 of 224 MDS/AML patients, 8 of 71 therapy-related AML or MDS patients, and 5 of 89 de novo AML patients (Table 1). Although the number of the de novo AML patients with *CEBPA* mutations were small, they all carried both an N-terminal mutation and a C-terminal bZIP in-frame mutation on the different alleles as reported previously.²⁰⁻²⁶ On the other hand, most MDS/AML or therapy-related AML or MDS patients had single *CEBPA* mutations. As exceptions, we found both an N-terminal mutation and a

C-terminal frame-shift mutation in 1 case of AML after MDS (patient [Pt] #806) and homozygous N-terminal frame-shift mutations in one case of therapy-related MDS/RAEB (Pt #811). Examination of other genes (*RUNX1*, *N-RAS*, *FLT3*, *PTPN11*, *C-KIT*, and *TP53* gene) in these patients demonstrated that one case of MDS/AML (Pt #769) had both *RUNX1* and *PTPN11* mutations and that patients with therapy-related AML or MDS (Pt #59 or #346) had a mutation of *RUNX1* or of *N-RAS*, respectively. Consistent with recent reports,^{22,26} our clinical data showed that overall survival was better in de novo AML patients with double *CEBPA* mutations compared with others with single *CEBPA* mutations (Table 1). These results suggested that double *CEBPA* mutations were able to induce AML, whereas single *CEBPA* mutation would lead to more aggressive AML, in concert with other gene alterations that have not been fully characterized.

The C-terminal but not N-terminal mutations of C/EBP α inhibited G-CSF–induced differentiation of 32Dcl3 cells into mature neutrophils

For further analysis, we chose an N-terminal mutant producing p30 designated as C/EBP α -N^m and a C-terminal b-ZIP in-frame mutant designated as C/EBP α -C^m (Figure 1A). C/EBP α -WT, C/EBP α -N^m, C/EBP α -C^m, C/EBP α -p30, or mock (pMXs-IP) was expressed in Plat-E cells. Expression of p42 protein or p30 protein generated by Myc-tagged C/EBP α -WT and mutants was verified by using anti-Myc Ab as bands corresponding to expected molecular weights (Figure 1B). In addition, we confirmed that N-terminal polypeptide produced by C/EBP α -N^m was detected by anti-C/EBP α Ab recognizing the N-terminal portion of C/EBP α (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Notably, the expression levels of p30 generated by C/EBP α -N^m were lower than those generated by C/EBP α -p30 (Figure 1B), indicating that deletion of the N-terminal part including the first start codon might increase the expression levels of p30 protein. We then infected 32Dcl3 cells with retroviruses harboring C/EBP α -WT or mutants, and the infected cells were subjected to drug selection. The 32Dcl3 cells expressing detectable levels of C/EBP α -WT were not obtained, presumably due to its strong inhibitory effect on proliferation. Western blot analysis showed that 32Dcl3 cells transduced with C/EBP α -C^m expressed the full length of C/EBP α -C^m at high levels (Figure 1B). C/EBP α -N^m or C/EBP α -p30 transduced into 32Dcl3 cells was detected as a band (30 kDa), but the expression level of the former was much lower than that of the latter (Figure 1B). Growth speed was comparable among these transfectants in the presence of IL-3 (Figure 1C). However, the potential of these transfectants to differentiate in response to G-CSF varied; G-CSF treatment induced terminal differentiation of 32Dcl3 cells transduced with mock, as indicated by the appearance of polymorphonucleated neutrophils and up-regulation of CD11b on the surface (Figure 1D-E). G-CSF–induced granulocytic differentiation of 32Dcl3 cells was profoundly inhibited by C/EBP α -C^m, while it was only weakly inhibited by C/EBP α -N^m (Figure 1D-E). The differentiation was also attenuated by C/EBP α -p30, as reported previously.⁴⁵ We reasoned that the difference of 32Dcl3 cells expressing C/EBP α -N^m or C/EBP α -p30 in the granulocytic differentiation levels was due to the dissimilar expression levels of a short form of C/EBP α (30 kDa). As for the expression levels of G-CSF-R transcripts, a target of C/EBP α , they were extremely or moderately decreased in 32Dcl3 cells expressing C/EBP α -C^m or C/EBP α -p30, respectively, compared with other transfectants (Figure 1F), implicating G-CSF-R in induction of granulocytic differen-

tiation. However, when human G-CSF-R was transduced into C/EBP α -C^m-expressing 32Dcl3 cells, G-CSF–induced granulocytic differentiation was not completely recovered (supplemental Figure 2). These results indicated that the differentiation block in 32Dcl3 cells expressing C/EBP α -C^m was presumably due to the suppression of C/EBP α activation, but not simply due to the decreased expression of G-CSF-R downstream of C/EBP α .⁴⁶

C/EBP α -C^m or C/EBP α -N^m suppressed the transcriptional activity of C/EBP α -WT by different mechanisms

We next analyzed the transcriptional activation of C/EBP α -WT and mutants in 293T cells using a luciferase construct harboring 2 C/EBP α binding sites. As expected, C/EBP α -WT strongly activated this promoter, while neither C/EBP α -C^m, C/EBP α -N^m, nor C/EBP α -p30 showed any transcriptional activation (Figure 2A). We next examined whether these C/EBP α mutants affected the transcriptional activation of C/EBP α -WT. Although C/EBP α -C^m reduced G-CSF-R expression and inhibited G-CSF–induced myeloid differentiation of 32Dcl3 cells even more efficiently than C/EBP α -N^m (Figure 1D-F), C/EBP α -N^m as well as C/EBP α -p30 but not C/EBP α -C^m decreased promoter activity in the luciferase assay in 293T cells (top panel in Figure 2A), which was in accordance with the data shown by Gombart et al.²⁰ Expression of C/EBP α WT and mutants in the transfected 293T cells was verified by Western blot analysis (bottom panel in Figure 2A). These results indicated that C/EBP α -C^m and C/EBP α -p30 suppressed expression of G-CSF-R by different mechanisms. Interestingly, transcriptional activation of C/EBP α -WT drastically increased in 293T cells when coexpressed with PU.1, although PU.1 itself did not stimulate the same promoter (top panel in Figure 2B). Notably, this synergistic effect was suppressed by C/EBP α -C^m and weakly by C/EBP α -N^m with low expression levels of p30 protein (top panel in Figure 2B). Expression of C/EBP α and PU.1 was also confirmed by Western blot analysis (bottom panel in Figure 2B). Therefore, we assumed that C/EBP α -C^m was interacting with other transcription factors such as PU.1, thereby suppressing the activation of C/EBP α -WT in hematopoietic cells. We also tested whether the C/EBP α mutants inhibit E2F activity. However, neither C/EBP α -C^m nor C/EBP α -N^m repressed E2F1/DP1-mediated transcription (supplemental Figure 3). In this regard, there was no difference between C/EBP α -C^m and C/EBP α -N^m. We then compared the DNA-binding ability of C/EBP α -WT and mutants by electrophoresis mobility shift assay. As shown in Figure 2C, C/EBP α -WT protein bound to the CSF3R (G-CSF-R) probe, and C/EBP α -p30 protein generated by C/EBP α -N^m less efficiently bound to the same probe. Binding was verified by super-shift of the DNA-protein complex by the anti-C/EBP α Ab. Remarkably, C/EBP α -C^m failed to bind the CSF3R probe (Figure 2C). Next, we examined subcellular localization of C/EBP α -WT and mutants. In line with previous reports, C/EBP α -WT and mutants localized in the nucleus of the interphase cells (Figure 2D). However, it was noteworthy that unlike C/EBP α -WT and C/EBP α -p30, C/EBP α -C^m was not localized on chromosome during the mitotic phase (Figure 2D). It is possible that C/EBP α -C^m without a DNA binding ability is stealing some interacting protein from chromosome. Taken together, these results indicated that C/EBP α -N^m suppressed the transcriptional activation of C/EBP α -WT by its direct binding to the promoter or by its heterodimerization with C/EBP α -WT, while C/EBP α -C^m showed the suppressive effect indirectly, probably through interaction with other transcription factors such as PU.1.

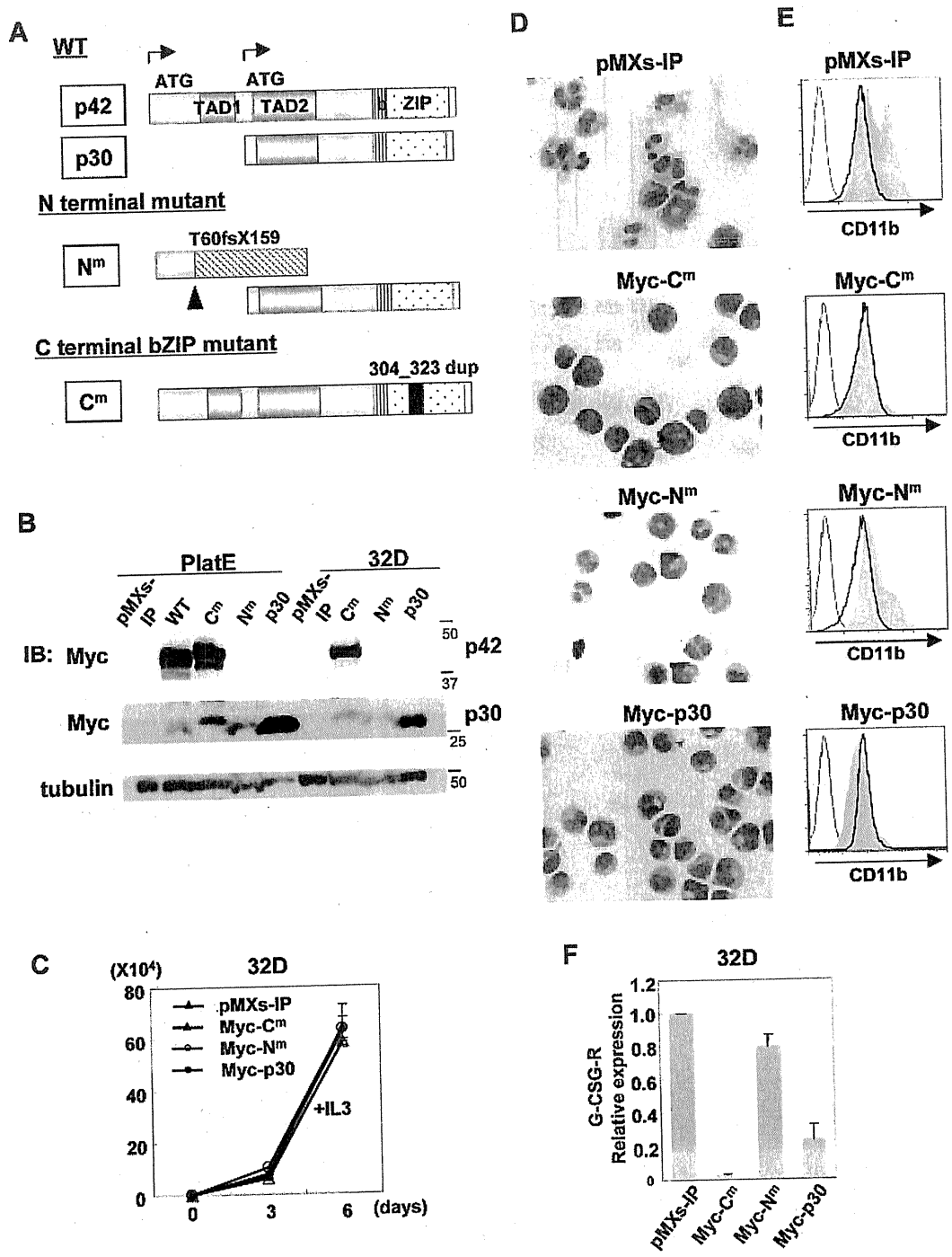


Figure 1. C/EBP α -C^m had the strong ability to block myeloid differentiation. (A) Schematic diagram of C/EBP α -WT (p42 and p30) and mutants, T60fsX159 (C/EBP α -N^m) and 304_323 dup (C/EBP α -C^m). TAD indicates the transcriptional activation domain; bZIP, basic region leucine zipper domain. (B) Expression of C/EBP α -WT and its mutants in Plat-E cells transiently transfected with a Myc-tagged C/EBP α -WT, C/EBP α -C^m, C/EBP α -N^m, or C/EBP α -p30 or an empty vector (pMXs-IP) and expression of C/EBP α mutants in 32Dcl3 cells transfected with Myc-tagged C/EBP α -C^m, C/EBP α -N^m, C/EBP α -p30, or mock (pMXs-IP). Cell lysates were subject to immunoblotting with anti-Myc Ab or anti-tubulin Ab as control. The results shown are representative of 3 independent experiments. (C) The growth of 32Dcl3 cells transfected with Myc-tagged C/EBP α -C^m, C/EBP α -N^m, C/EBP α -p30, or mock (pMXs-IP) in the presence of 1 ng/mL IL-3. All data points correspond to the mean and the standard deviation (SD) of 3 independent experiments. (D-E) 32Dcl3 cells transfected with Myc-tagged C/EBP α -C^m, C/EBP α -N^m, C/EBP α -p30, or mock (pMXs-IP) were cultured in the presence of 50 ng/mL G-CSF for 6 days. (D) Morphology of these cells was assessed by Giemsa staining. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); objective lens, $\times 40$. (E) Surface expression of CD11b in these transfectants after incubation with 1 ng/mL IL-3 (bold histograms) or 50 ng/mL G-CSF UplaniFI (Olympus); original magnification $\times 40$. (F) Relative expression levels of G-CSF-R in 32Dcl3 cells transfected with Myc-tagged C/EBP α -C^m, C/EBP α -N^m, C/EBP α -p30, or mock (pMXs-IP) were estimated by using real-time PCR. Data are representative of 3 independent experiments.

Retroviral transduction of C/EBP α -C^m, but not C/EBP α -N^m, immortalized BM hematopoietic cells
 To determine the effect of C/EBP α -C^m and C/EBP α -N^m on differentiation and proliferation of hematopoietic cells, we per-

formed serial colony-forming assays as described previously.⁴⁵ Mouse BM mononuclear cells were transduced with C/EBP α -C^m, C/EBP α -N^m, or C/EBP α -p30. Expression of C/EBP α mutants in the transduced BM cells was verified by Western blot analysis