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No *nucleophosmin* mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group

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Acute myeloid leukemia (AML) with normal karyotype had a heterogenous prognosis. In this subgroup, *FLT3*-internal tandem duplication (ITD) was strongly associated with a poor prognosis.[1–3] Recently, it was reported that mutations of *nucleophosmin* (*NPM*) gene occur in 50–60% of adult AML with normal karyotype and were frequently associated with *FLT3*-ITD. In the AML patients with normal karyotype and *FLT3*-ITD, patients with *NPM* gene mutations showed a better prognosis than those without *NPM* gene mutations.[4–6] However, the frequency and clinical impact of *NPM* gene mutations in pediatric AML patients with normal karyotype remained uncertain because there were a few number of reports.[7,8]

We searched for *NPM* gene mutations in 33 (20.9%) of 158 patients with normal karyotype who were treated on Japanese Childhood AML Cooperative protocol, AML 99 (0–15 years old, median 8 years old).[9] We amplified exon 12 of *NPM* gene using the primers; *NPM* cDNA Fow, 5′-AAAGGTGGTTCTCTTCCC AAA-3′ and *NPM* cDNA Rev, 5′-GCATTATAAAAAGGACAGCC AGA-3′ and directly sequenced on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).[9] We could not find any *NPM* gene mutations in this study.

It was reported that the frequency of *NPM* gene mutations in children (<18 years old) was very low (children 1 out of 47 (2.1%) versus adults 32 out of 126 (25.4%), $P<0.001$).[8] Furthermore, they suggested that *NPM* gene mutations were also rarely detected in patients younger than 40 years old (3 (3.5%) out of 85). On the other hand, it was reported that *NPM* mutations were found in seven (27.1%) of 26 pediatric AML patients with normal karyotype from Italy.[7] These seven patients ranged from 5.0–17.9 years old, 10 years old ($n=2$), 11 years old ($n=2$) and 5, 8, 17 years old ($n=1$). Notably, two AML patients with *NPM* gene mutations have been reported in adult Japanese AML study (15 and 16 years old).[5]

We also analyzed *FLT3*-ITD and *RAS* gene alterations in these patients and found *FLT3*-ITD in nine (27.3%), *NRAS* mutation in two (6.1%) and *KRAS* mutation in three (9.1%). The frequencies of these gene alterations were compatible with those of previous reports.[2,3]

We considered that *NPM* gene mutations may be infrequent in Asian pediatric AML patients with normal karyotype, especially less than 15 years old. Frequency of *NPM* gene mutations depends on age and may depend on races. Further larger studies of *NPM* gene analysis are needed to clarify this item.

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A Shimada[1], T Taki[2], C Kubota[1], A Tawa[3], K Horibe[4], M Tsuchida[5], R Hanada[6], I Tsukimoto[7] and Y Hayashi[1]
[1]*Department of Hematology/Oncology, Gunma Children's Medical Center, Hokkitsu, Shibukawa, Gunma, Japan;*
[2]*Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kamigyo-ku, Kyoto, Japan;*
[3]*Department of Pediatrics, National Hospital Organization Osaka National Hospital, Chuo-ku, Osaka, Japan;*
[4]*Clinical Research Center, National Hospital Organization Nagoya Medical Center, Naka-ku, Nagoya, Japan;*
[5]*Department of Pediatrics, Ibaraki Children's Hospital, Mito, Ibaraki, Japan;*
[6]*Division of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Saitama, Japan and*
[7]*Department of the First Pediatrics, Toho University School of Medicine, Oota-ku, Tokyo, Japan*
E-mail: hayashiy-tky@umin.ac.jp

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Identification of a Novel Fusion Gene *MLL-MAML2* in Secondary Acute Myelogenous Leukemia and Myelodysplastic Syndrome with inv(11)(q21q23)

Noriko Nemoto,[1] Kazumi Suzukawa,[1,2*] Seiichi Shimizu,[3] Atsushi Shinagawa,[4] Naoko Takei,[1] Tomohiko Taki,[5] Yasuhide Hayashi,[6] Hiroshi Kojima,[1] Yasushi Kawakami,[2] and Toshiro Nagasawa[1]

[1]Department of Hematology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan
[2]Department of Clinical Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan
[3]Department of Hematology, Tsuchiura Kyodo General Hospital, Ibaraki, Japan
[4]Department of Hematology, Hitachi General Hospital, Ibaraki, Japan
[5]Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, Japan
[6]Gunma Children's Medical Center, Gunma, Japan

We have identified a novel fusion partner of *MLL*, namely the mastermind like 2 (*MAML2* gene), in secondary acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) with inv(11)(q21q23). RT-PCR and sequencing revealed that exon 7 of *MLL* was fused to exon 2 of *MAML2* in the AML and MDS cells. The inv(11)(q21q23) results in the creation of a chimeric RNA encoding a putative fusion protein containing 1,408 amino acids from the NH2-terminal part of MLL and 952 amino acids from the COOH-terminal part of MAML2. The NH2-terminal part of MAML2, a basic domain including a binding site of the intracellular domain of NOTCH, was deleted in MLL-MAML2. MLL-MAML2 in secondary AML/MDS and MECT1-MAML2 in mucoepithelioid carcinoma, benign Wartin's tumor, and clear cell hidradenoma consist of the same COOH-terminal part of MAML2. A luciferase assay revealed that MLL-MAML2 suppressed HES1 promoter activation by the NOTCH1 intracellular domain. MAML2 involving a chimeric gene might contribute to carcinogenesis in multiple neoplasms by the disruption of NOTCH signaling. © 2007 Wiley-Liss, Inc.

INTRODUCTION

11q23 translocations are frequent in hematologic malignancies, occurring in 5–6% of acute myeloid leukemia (AML), 7–10% of acute lymphoblastic leukemia, 60–70% of acute leukemias in infants, and in most patients with therapy-related leukemias induced by inhibitors of topoisomerase II (Rowley, 1998). The *MLL* gene is rearranged as a consequence of 11q23 translocations, and at least 50 partner genes for *MLL* have so far been identified (Meyer et al., 2006).

Although the leukemogenic effect of MLL fusion proteins has been well established in a number of instances lack of functional information about the MLL partners has made it difficult to address their contribution to the oncogenic potential of MLL fusion proteins. A few observations indicated that fusion partners play essential roles in determining the oncogenic capacity of the MLL fusion proteins (So and Cleary, 2003; Liu et al., 2004). In the present study, we have identified the mastermind like 2 (*MAML2*) gene as a novel fusion partner of *MLL* in therapy-related AML and myelodysplastic syndrome (MDS) with inv(11)(q21q23), and provide evidence that the MLL-MAML2 fusion suppresses a promoter activation of the NOTCH target gene, *HES1*.

MATERIALS AND METHODS

Case Reports

Case 1. Details of the patient, a 48-year-old female, have been previously published (Takei et al., 2006). She initially had AML (M2 in the FAB classification) with t(8;21)(q22;q22). She achieved complete remission by chemotherapy. Consolidation and maintenance chemotherapies containing etoposide (VP-16; total dose 1,150 mg) were administered. Seven months after chemotherapy, a chromosomal abnormality, inv(11)(q21q23), appeared and was constantly detected in bone

*Correspondence to: Kazumi Suzukawa, Department of Clinical and Experimental Hematology, Major of Advanced Medical Applications, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan. E-mail: suzukazu@md.tsukuba.ac.jp

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TABLE I. Karyotype Analysis of Bone Marrow Cells (Patient 1)

Date	Number of 46,XX,inv(11) in 20 metaphases	Other abnormality	Status of the bone marrow
Jan 21, 1993	0	+*	AML
Jun 14, 1994	20	–	CR
Apr 3, 1995	19	–	CR
Dec 11, 1995	14	–	CR
Oct 6, 1997	4	–	NHL
Nov 14, 1997	1	–	CR
Feb 23, 1998	19	–	CR
Apr 21, 1998	17	–	CR
Apr 21, 1999	6	+	CR
Mar 13, 2000	19	+	t-AML
Apr 10, 2000	19	+	t-AML
Apr 24, 2000	12	+	t-AML
Jun 27, 2000	19	–	t-AML

*indicates t(8;21), 9q-. AML, acute myeloid leukemia; CR, complete remission; t-AML, therapy-related AML; NHL, non-Hodgkin's lymphoma.

marrow cells during hematologic remission (Table 1). After 6 years, she developed secondary AML with inv(11)(q21q23), and died in 2001.

Case 2. A 69-year-old male was admitted to a regional hospital due to phlegmon of the left thigh in March 2006. He had been diagnosed with rheumatoid arthritis and treated with bucillamine, prednisolone, salazosulfapyridine, and methotrexate (total dose 50 mg) for 8 years. He had no history of malignancy. After recovery of the phlegmon, mild anemia, leukocytosis (peak 136.4 × 10^9/l) and thrombocytopenia (30–40 × 10^9/l) persisted. He was referred to Hitachi General Hospital for further examination. His bone marrow was hypercellular with micromegakaryocytes and hypersegmentation of granulocytes. The karyotype was 46,XY,inv(11)(q21q23)[20]. Fluorescence in situ hybridization (FISH) analysis for *BCR-ABL1* was negative, whereas FISH analysis for *MLL* on peripheral blood revealed that 71% of the white blood cells had deletion of the 3′ part of the *MLL* gene. MDS was diagnosed, and he died of pneumonia in January 2007.

cDNA Panhandle Polymerase Chain Reaction (PCR)

Total RNA was extracted from bone marrow or peripheral blood cells using the acid guanidine thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987) and analyzed using a modified cDNA panhandle PCR method (Megonigal et al., 2000; Suzukawa et al., 2005). In brief, first-strand cDNAs were synthesized with *MLL*-random hexamer oligonucleotides, MLL-N. After primer 1 extension with MLL-1, and extension in stem-loop templates, the sample was amplified by first PCR with MLL-1 and MLL-2. Then, 1/25 of the products were used for nested PCR with MLL-3 and MLL-4. The *MLL*-random hexamer oligonucleotides and primers used were as follows: MLL-N, 5′-TCG AGG AAA AGA GTG AAG AAG GGA ATG TCT CNN NNN N-3′; MLL-1, 5′-TGA AGA ACG TGG TGG ACT CT-3′; MLL-2, 5′-GTC CAG AGC AGA GCA A AC AGA-3′; MLL-3, 5′-GTC AGA AAC CTA CCC CAT CA-3′; and MLL-4, 5′-TGT GAA GCA GAA AAT GTG TGG-3′.

Reverse Transcriptase PCR

Five μg of total RNA was reverse transcribed to cDNA in a total volume of 33 μl with random hexamers using the Ready-To-Go You-Prime First-Strand beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), after which 1/30 of the cDNA was amplified using PCR in a total volume of 50 μl with 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TAPS Buffer (pH 9.3 at room temperature), 0.4 μM of each primer, 0.2 mM of each dNTP, and 1 unit of Ex *Taq* polymerase (Takara-Bio, Siga, Japan). After 35 rounds of PCR (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C), 5 μl of the PCR product was electrophoresed in a 3% agarose gel. The primers used were as follows: MLL7s: 5′-TCC TCA GCA CTC TCT CCA AT-3′; MAML2R: 5′-GTC ATT TGG CCA TCC ATG TG-3′.

Plasmid Construction

The KIAA1816 cDNA clone in pBluescript II SK(+) vector was obtained from the KAZUSA DNA Research Institute in Japan. The GenBank accession number for KIAA1816 is AB058719. Full-length MAML2 and the MAML2 part of the MLL-MAML2 fusion gene (C-MAML2) cDNA were constructed by replacing the 5′ and 3′ untranslated region with PCR-amplified fragments. MLL exon 1–7 cDNA (N-MLL) was provided by Dr. H. Hirai (University of Tokyo, Tokyo, Japan). Both ends of N-MLL were replaced with PCR fragments for subsequent cloning into the mammalian expression vector, pcDNA3.1. FLAG(M2)-tagged MKK6 expression vector in pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was provided by Dr. T. Sudo (RIKEN, Saitama, Japan). MKK6 cDNA was replaced with MAML2, C-MAML2, and N-MLL for the construction of FLAG tagged genes. The 3′ end of FLAG-tagged N-MLL was replaced with an RT-PCR product containing an

MLL-MAML2 fusion sequence from the leukemia cells and C-MAML2 to construct full-length MLL-MAML2 fusion. All PCR-amplified fragments were confirmed by nucleotide sequence analysis. Further information regarding the primer sequences and cloning is available upon request. Notch1 intracellular domain tagged V5 (N1ICD) expression vector was provided by Dr. F. Ito (Tsukuba University, Japan) (Itoh et al., 2004). The luciferase reporter containing the promoter of the HES1 gene (pHES1-luc) was provided by Dr. R. Kageyama (Kyoto University, Japan) (Takebayashi et al., 1994).

Cell Lines and Antibodies

HEK293, a human embryonic kidney cell line, and KG-1, a leukemic cell line established from an AML patient, were purchased from RIKEN Bio-Resource Center (Tsukuba, Ibaraki, Japan). The HEK293 cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (SIGMA-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptmycin sulfate. The KG-1 cells were grown at 37°C in RPMI 1640 Medium (SIGMA-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptmycin sulfate.

The anti-FLAG polyclonal antibody (F7425) and the anti-MLL1 (BL1289) polyclonal antibody were purchased from Sigma and Bethyl Laboratory Inc. (Montgomery, TX), respectively.

Transfection and Immunoblotting

1×10^6 cells /well of HEK293 cells were seeded in six well plates. The next day, the cells were transfected with FLAG-tagged MLL-MAML2, MAML2, C-MAML2, or N-MLL expression vector using Lipofectamine 2000 (Invitrogen). The next day, the cells were washed with PBS twice and then lysed with RIPA buffer (1% TritonX-100, 0.1% SDS, 1% Soduim deoxycholate, 158 mM NaCl, 1 mM Na_3VO_4, 5 mM EGTA, 10 mM Tris-HCl, and pH 7.4). The cell lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were boiled and denatured in Sample buffer containing SDS and DTT (Invitrogen) followed by electrophoresis using SDS-PAGE gradient gel (2–15%) in Tris-Glycin buffer. The proteins were electro-transferred to nitrocellulose membrane (GE Healthcare Bioscience, Tokyo, Japan) using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad Japan, Tokyo, Japan). The resulting protein-bound membrane was blotted with Anti-FLAG (×1,000) or anti-MLL (×1,000) antibody and visualized using ECL reagents (GE Healthcare Bioscience). The cells for all samples were cultured and the protein extracts were also prepared at the same time.

Transfection and Luciferase Assay

The HEK293 cells were seeded (1×10^5 cells per well) in 24-well plates and transfected 48 hr later using Lipofectamine 2000 (Invitrogen) with reporter construct (HES1-luciferase, 100 ng), expression vector (300 ng), ICD expression vector (10 ng) and pRL-TK internal control vector (5 ng) (Promega, Madison, WI, USA). When increasing amounts of the expression vector were transfected, the amount of total DNA was kept constant by adding empty pcDNA3.1 vector to the transfection mixture. Forty-eight hours after transfection, the cells were washed with PBS twice, 100 μl of PLB lysis buffer of the Dual Luciferase Reporter Assay kit (Promega) was added. The samples were gently rocked for 15 min at room temperature. Luciferase activity was determined with 10 μl aliquots of the sample with Dual-Luciferase Reporter assay system (Promega) and Lumat LB9501 luminometer (Berthold Japan, Tokyo, Japan) by following the manufacture's protocol.

2×10^6 of the KG-1 cells were suspended in 100 μl of Nucleofector solution R (Amaxa biosystems, Cologne, Germany). Reporter construct (HES1-luciferase, 500 ng), expression vector (1.5 μg), ICD expression vector (50 ng), and the pRL-TK internal control vector (50 ng) were added to the suspension in the cuvette followed by electrotransfection using Nucleofector II Device (Amaxa biosystems). Transfected cells were incubated in culture medium for 48 hr, after which the cells were washed with PBS and centrifuged at 1,200 rpm for 5 min. PLB lysis buffer (100 μl) was added to the cells; then the samples were rocked for 15 min at room temperature. About 20 μl aliquots of cell lysate were used for quantification of luciferase activity. All experiments were performed in triplicate. Firefly luciferase activity was normalized by reference to the Renilla luciferase activity expressed by the pRL-TK vector.

Exon Nomenclature

The exon nomenclature for *MLL* was taken from a report (Rasio et al., 1996).

RESULTS

Isolation of *MLL-MAML2* Fusion Transcript from AML cells with inv(11)(q21q23)

FISH and Southern blot analyses revealed rearrangement of the *MLL* gene in case 1 (Takei et al.,

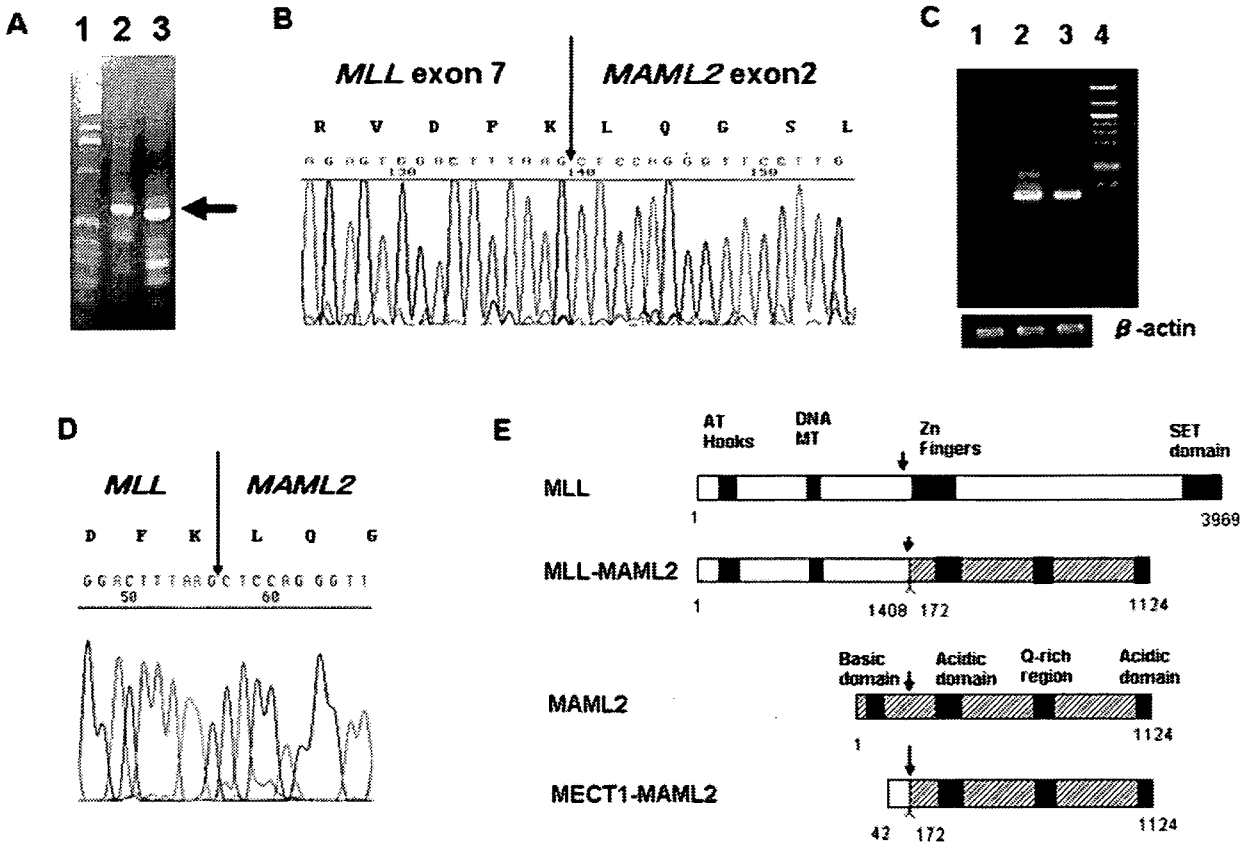

Figure 1. Identification of *MLL-MAML2* fusion transcript. (A) Panhandle PCR for cDNA product. Lane 1: λ *HindIII* digest (marker); lane 2: first PCR product; lane 3: second (nested) PCR product. An arrow indicates a DNA fragment whose sequence is shown in panel B. (B) Sequence analysis of the amplified panhandle PCR product from case 1 revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (arrow; GenBank accession no. 828759). (C) RT-PCR of two cases with inv(11)(q21q23). Lane 1: cDNA from normal peripheral blood; lane 2: cDNA from leukemic cells in case 1; lane 3: cDNA from peripheral blood in case 2; lane 4: 1 kb ladder marker. (D) Sequence analysis directly performed on the amplified RT-PCR product from case 2 revealed the same fusion between *MLL* and *MAML2*. (E) Schematic structures of wild-type *MLL*, *MLL-MAML2* fusion gene, wild-type *MAML2* and *MECT1-MAML2* fusion gene. *MT*, DNA methyltransferase homology domain; *Q-rich region*, glutamine rich region; Arrow, fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2006). To identify the *MLL* partner gene at 11q21, we performed cDNA panhandle PCR for total RNA from the patient's bone marrow cells. First and second PCR gave a specifically amplified DNA fragment (Fig. 1A). Sequence analysis of the fragment revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (Fig. 1B).

Detection of *MLL-MAML2* in Two Hematologic Malignancies with inv(11)(q21q23)

To confirm the presence of the *MLL-MAML2* fusion mRNA in the leukemic cells of the case 1 and peripheral blood of case 2, we performed RT-PCR analysis with MLL7s and MAML2R, which successfully yielded specific DNA fragment (Fig. 1C). We confirmed that the RT-PCR product from case 2 was an *MLL-MAML2* fusion transcript by sequencing (Fig. 1D). The putative MLL-MAML2 fusion protein of 2,389 amino acids (aa) contained 1,408 aa, from the NH2-terminal part of MLL and 981 aa from the COOH-terminal part of MAML2 (Fig. 1E).

MLL-MAML2 Suppresses HES1 Promoter Activation by N1ICD

To investigate the function of the MLL-MAML2 fusion gene, we constructed FLAG-tagged MLL-MAML2 (M-M2), MLL part of MLL-MAML2 (N-MLL), MAML part of the MLL-MAML2 (C-M2), and full-length MAML2 gene (M2) (Fig. 2A). The expression of recombinant proteins was confirmed by Western blot analysis (Fig. 2B). Immunohistchemical experiment using anti-FLAG antibody revealed that both M2 and M-M2 localized in the nucleus (data not shown). We evaluated the ability of MLL-MAML2 to participate in NOTCH signaling by examining the activation of a NOTCH target gene, *HES1*, the best characterized member of the HES gene family (Leong and Karsan, 2006). M-

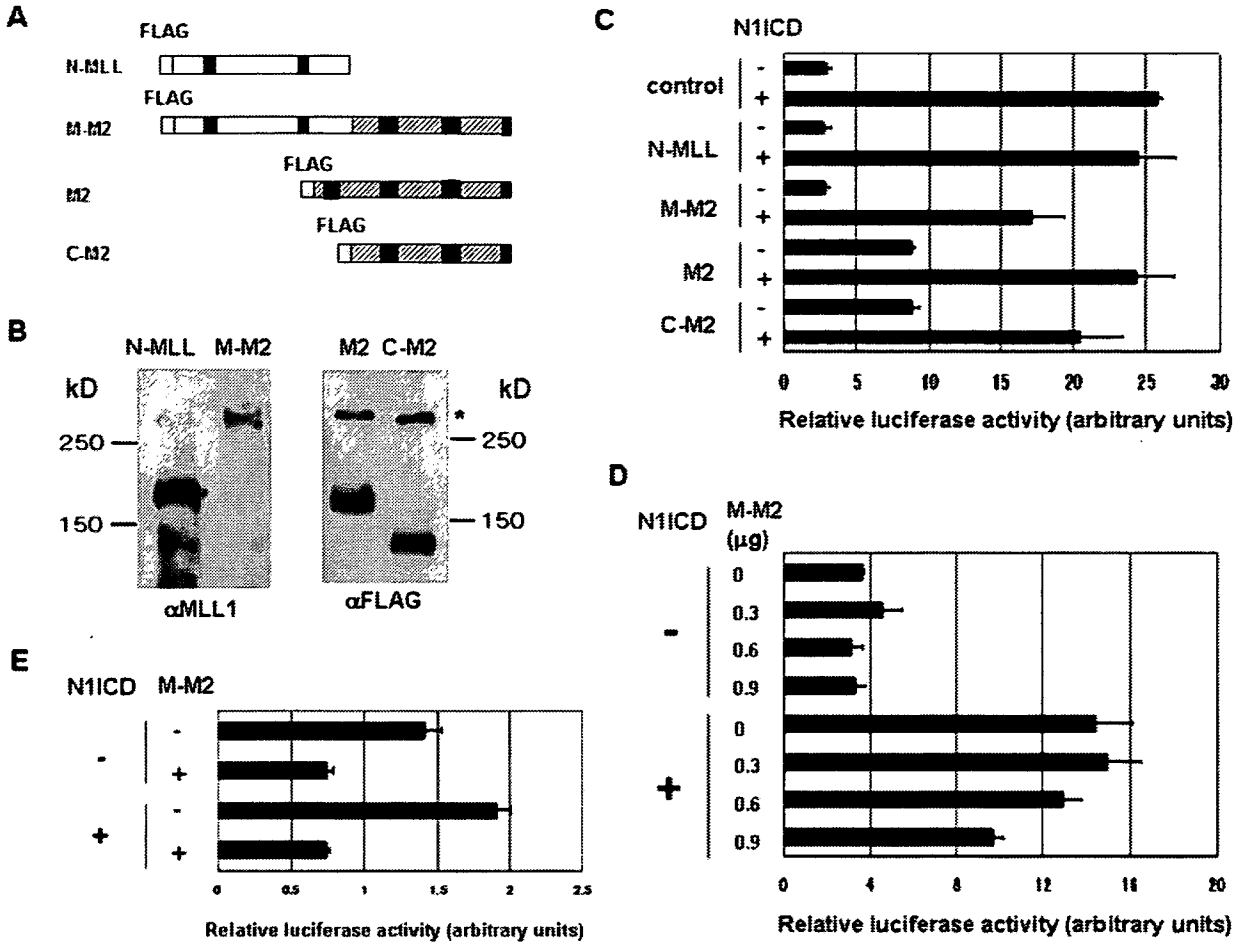

Figure 2. Functional analysis of the fusion gene. (A) a schematic presentation of recombinant genes. FLAG tag was fused to the NH2 terminal end of each recombinant construct. N-MLL: NH2 terminal part of MLL-MAML2: M-M2; MLL-MAML2 fusion gene: M2; full length MAML2: C-M2; COOH terminal part of MLL-MAML2. (B) Detection of recombinant proteins by Western blot analysis. Each protein was detected either anti MLL (left panel) or anti FLAG antibody (right panel). Asterisk in the right panel indicates a nonspecific band. (C) Comparison of basal (N1ICD-) and N1ICD-induced activation (N1ICD+) of HES1 promoter activity. Each recombinant gene was transfected as indicated. Empty pcDNA3.1 vector was used as control. (D) Dose escalation of MLL-MAML2 fusion gene. (E) Luciferase assay using KG-1 leukemia cell line. The error bars indicate the standard deviations ($n = 3$).

M2 did not increase the basal HES1 promoter activity, but suppressed N1ICD-induced HES1 promoter activation in a dose dependent manner (Figs. 2C and D). In contrast, MECT1-MAML2 was reported to activate the HES1 promoter, and this was independent of NOTCH stimulation (Tonon et al., 2003). Thus, the MAML2 containing fusion gene might disrupt NOTCH signaling by both activation and inhibition. This is not surprising because NOTCH activation can be both oncogenic and tumor suppressive in different tumors (Leong and Karsan, 2006), although there is a possibility that the difference is due to the different cell lines used for the experiments. N-MLL changed neither the basal nor N1ICD-induced HES1 promoter activity, suggesting that this part is not involved in the NOTCH signaling pathway. M2 and C-M2 enhanced the basal HES1 promoter activity. C-M2 suppressed the N1ICD-induced activation of the HES1 promoter, whereas M2 did not. Next, we used a leukemia cell line, KG-1, for luciferase assay. The N1ICD-induced activation of the HES1 promoter in KG-1 was less compared to the one in HEK293. M-M2 suppressed both basal and N1ICD-induced HES1 promoter activity to the same level (Fig. 2E).

DISCUSSION

The mammalian *MAML1*, *MAML2*, and *MAML3* genes are widely expressed in adult tissues and localize to nuclear bodies. They share a conserved basic domain in their N termini that binds to the ankyrin repeat domain of NOTCH, and contain a transcriptional activation domain in their C termini. They function as transcriptional co-activators for NOTCH, forming a complex in the nucleus

with the intracellular domain of an activated NOTCH receptor (ICN) and the CSL family of transcription factors, resulting in the activation of NOTCH downstream targets, such as *HES1* and *HES5* (Lin et al., 2002; Wu et al., 2002).

The acquisition of novel properties by the combination of MLL with the fusion partners, rather than the loss of wild-type MLL function leads to the generation of an active oncoprotein. Recent studies suggest that different mechanisms might be involved in the leukemogenesis by MLL fusion proteins (Li et al., 2005). The MLL fusion partners can be divided into nuclear and cytoplasmic factors, according to their compartment of protein expression/function. There is increasing evidence that the nuclear factors have transcription activity themselves and belong to important chromatin remodeling and transcription modulating complexes. A subgroup of fusion partners have oligomerization/dimerization domains, such as leucine zippers and a-helical coiled-coil domains. Recent reports indicate that the dimerization of the N-terminal portion of MLL fusion protein is oncogenic (Martin et al., 2003; So et al., 2003). In the case of MLL-MAML2, the N-terminal portion of MLL may acquire oncogenic activity by transcriptional activation, since the transcriptional activation domain of MAML2 is retained (Wu et al., 2005) and no self-association domain has been identified.

MLL is the second fusion partner of *MAML2* in human neoplasm. It has been reported that the same part of MAML2 as in MLL-MAML2 is fused to the NH2-terminal part of MECT1 in mucoepidermoid carcinoma, Warthin's tumor, and clear cell hidradenoma (Tonon et al., 2003; Enlund et al., 2004; Behboudi et al., 2005). A recent report demonstrated that not only MECT1, but also the MAML2 component is required for the transformation of RK3E cells (Wu et al., 2005). A common structural alteration of MAML2 in solid tumors and leukemia suggests that common functional aberration(s) of MAML2 contribute to carcinogenesis in multiple tissues. There are a few genes that are found as part of fusion genes in both leukemia and solid tumors. For example, the *ETS* family gene *ERG* is found as part of a fusion gene with different partner genes in myeloid leukemia, Ewing sarcoma, and prostate cancer (Ichikawa et al., 1994; Tomlins et al., 2005). The common alteration of *ERG* in these tumors is the overexpression of the COOH-terminal part, including the ETS domain. Especially, the same part of ERG is fused to either TLS/FUS or EWSR1 in myeloid leukemia or Ewing sarcoma, respectively. Thus, overexpression of the COOH-terminal portion of MAML2 may be required for common carcinogenesis. Functional alteration of partner gene might be required in tissue-specific carcinogenesis. In accordance with this, MLL-MAML2 did not transform RK3E cells (unpublished data), while transformation by MECT1-MAML2 was been reported (Wu et al., 2005). Intriguingly, both M-M2 and C-M2 lack the ICN binding site in the basic domain (Wu and Griffin, 2004).

Detection of chromosomal translocations involving MAML2 in not only benign tumors, Warthin's tumor (Enlund et al., 2004) and clear cell hidradenoma (Behboudi et al., 2005), but also in bone marrow cells before overt leukemia (Takei et al., 2006) and MDS suggests that the disruption of NOTCH signaling by the emergence of a MAML2 containing fusion gene is an early event of carcinogenesis. The inv(11)(q21q23) is rare in hematologic malignancies. There is another case report of secondary AML with inv(11)(q21q23) and rearrangement of the *MLL* (Obama et al., 1998). It is likely that the leukemia cells of the patient have MLL-MAML2 fusion gene. Although the clinical effect of this infrequent fusion hence may be limited, their characterization will certainly provide additional mechanistic insights into both MLL- and MAML2-mediated carcinogenesis.

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ORIGINAL ARTICLE

Diagnosis of acute myeloid leukemia according to the WHO classification in the Japan Adult Leukemia Study Group AML-97 protocol

Moe Wakui · Kazutaka Kuriyama · Yasushi Miyazaki · Tomoko Hata · Masafumi Taniwaki · Shigeki Ohtake · Hisashi Sakamaki · Shuichi Miyawaki · Tomoki Naoe · Ryuzo Ohno · Masao Tomonaga

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Abstract We reviewed and categorized 638 of 809 patients who were registered in the Japan Adult Leukemia Study Group acute myeloid leukemia (AML)-97 protocol using morphological means. Patients with the M3 subtype were excluded from the study group. According to the WHO classification, 171 patients (26.8%) had AML with recurrent genetic abnormalities, 133 (20.8%) had AML with multilineage dysplasia (MLD), 331 (51.9%) had AML not otherwise categorized, and 3 (0.5%) had acute leukemia of ambiguous lineage. The platelet count was higher and the rate of myeloperoxidase (MPO)-positive blasts was lower in AML with MLD than in the other WHO categories. The outcome was significantly better in patients with high (≥50%) than with low (<50%) ratios of MPO-positive blasts ($P < 0.01$). The 5-year survival rates for patients with favorable, intermediate, and adverse karyotypes were 63.4, 39.1, and 0.0%, respectively, and 35.5% for those with 11q23 abnormalities ($P < 0.0001$). Overall survival (OS) did not significantly differ between nine patients with $t(9;11)$ and 23 with other 11q23 abnormalities ($P = 0.22$). Our results confirmed that the cytogenetic profile, MLD phenotype, and MPO-positivity of blasts are associated with survival in patients with AML, and showed that each category had the characteristics of the WHO classification such as incidence, clinical features, and OS.

Keywords AML · WHO classification · Myeloperoxidase · Multilineage dyplasia · 11q23 abnormalities

M. Wakui · K. Kuriyama (✉)
Department of Clinical Laboratory Sciences, Hematoimmunology, School of Health Science, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan
e-mail: kuriyama@med.u-ryukyu.ac.jp

Y. Miyazaki · T. Hata · M. Tomonaga
Department of Hematology, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan

M. Taniwaki
Department of Hematology and Oncology, Kyoto Prefectural University of Medicine, Kyoto, Japan

S. Ohtake
Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

H. Sakamaki
Department of Hematology, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

S. Miyawaki
Department of Hematology, Saiseikai Maebashi Hospital, Maebashi, Japan

T. Naoe
Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan

R. Ohno
Aichi Cancer Center, Nagoya, Aichi, Japan

1 Introduction

The French-American-British (FAB) classification of acute myeloid leukemia (AML), based on morphological and cytochemical findings, was established in 1976 and has since become the standard classification [1, 2]. However, specific chromosomal and genetic abnormalities that have been extracted from analyses of prognostic factors for AML are recognized as important in selecting treatment strategies and are reflected in the AML classification as

factors that are required to establish the disease entity [3]. The 1999 World Health Organization (WHO) classification includes morphological, immunological, cytogenetic, genetic, and clinical features [4–6]. The WHO and FAB classifications differ in several aspects. The blast threshold required for a diagnosis of AML was reduced from 30 to 20%, and new AML categories have been added for cytogenetic abnormalities, the presence of multilineage dysplasia (MLD), as well as a history of chemotherapy and subtypes for acute basophilic leukemia, acute panmyelosis with myelofibrosis, and myeloid sarcoma. The WHO classification comprises more subtypes and is more comprehensive than the FAB classification.

Cytogenetic features are important prognostic factors in AML [3, 7–12]. However, 11q23 abnormalities have not yet been established as a cytogenetic risk classification. Over 30 partner genes with 11q23 abnormalities have been described, and some reports indicate that patients with $t(9;11)$ have a relatively more favorable prognosis than those with other partner chromosomes/partner genes [13–16].

In the present study, we reviewed stained smears of blood and bone marrow from patients who were registered in the Japan Adult Leukemia Study Group (JALSG) AML-97 trial, and classified them into FAB subtypes and WHO categories. We also evaluated their survival on the basis of the WHO classification, the myeloperoxidase (MPO)-positivity of blasts, and cytogenetic findings including 11q23 abnormalities.

2 Patients and methods

2.1 Patients

Between December 1997 and July 2001, 809 patients aged from 15 to 66 years with untreated AML (excluding M3) were registered from 103 institutions in the AML-97 trial of the JALSG. The patients were diagnosed with AML according to the FAB criteria at each institution. Patients with a history of MDS, hematological abnormalities before the diagnosis of AML, or a history of chemotherapy were not eligible for the AML-97 trial.

2.2 Treatment strategies

Details of the JALSG AML-97 treatment protocol are described elsewhere [17]. In brief, all patients underwent induction therapy consisting of idarubicin (3 days) and Ara-C (7 days). Patients who achieved complete remission were randomized into one of two arms of consolidation chemotherapy alone or in combination with maintenance chemotherapy. Patients who were placed into intermediate/poor risk groups according to the JALSG scoring system [17] and who had an HLA-identical sibling ($\leq$50 years old) were simultaneously assigned to receive allogeneic hematopoietic stem cell transplantation during their first remission.

2.3 Morphologic and cytochemical analyses

Peripheral blood and bone marrow smears from registered patients were sent to Nagasaki University for staining with May-Giemsa, MPO, and esterase, and the diagnosis was then reevaluated by the Central Review Committee for Morphological Diagnosis. Patients were subsequently categorized according to the FAB and WHO classifications. Dyserythropoietic features were defined as >50% dysplastic features in at least 25 erythroblasts and dysgranulopoietic features including $\geq$3 neutrophils with hyposegmented nuclei (pseudo-Pelger–Heut anomaly), and hypogranular or agranular neutrophils (>50% of $\geq$10 neutrophils). Dysmegakaryopoietic features were defined as $\geq$3 megakaryocytes that were micronuclear, multiseparate nuclear, or large mononuclear [18].

We assessed the ratios (%) of MPO-positive blasts on MPO-stained bone marrow smears using the diaminobenzidine method [19].

2.4 Cytogenetic analysis

Cytogenetic analysis was performed at either laboratories in participating hospitals or authorized commercial laboratories. The karyotypes of leukemic cells were collected through the JALSG AML-97 case report forms and reviewed by the Central Review Committee for Karyotyping. The patients were classified into favorable, intermediate, or adverse risk groups based on karyotypes according to results of the Medical Research Council (MRC) AML 10 trial [3]. The favorable risk group included patients with $t(8;21)$ and inv(16), whether alone or in combination with other abnormalities. The intermediate risk group included those with a normal karyotype and other abnormalities that were not classified as either favorable or adverse. The adverse risk group included patients with a complex karyotype with four or more numerical or structural aberrations, -5, deletion (5q), and -7, whether alone or in combination with intermediate risk or other adverse risk abnormalities.

2.5 Statistical analysis

The overall survival (OS) for all patients was defined as the interval from the date of diagnosis to that of death. We applied the Kaplan–Meier method to estimate OS and

Table 1 Patient characteristics

Age (year)	45 (15–66)
Male/female	390/248
WBC count ($\times 10^9$/l)	13.7 (0.4–709)
Hemoglobin (g/dl)	8.3 (3.8–17.2)
Platelet count ($\times 10^9$/l)	52 (0–890)
Bone marrow blasts (%)	56 (6–99)

Values are presented as the median (range)

WBC white blood cell

5-year survival. We compared survival rates between groups using the log-rank test (Stat View J 5.0). Differences were examined by the Chi-square test using Excel software. All *P*-values are two-sided, and values <0.05 were considered significant.

3 Results

3.1 Patient characteristics

Of the 809 registered patients, 638 were consistent with the WHO classification. Data were incomplete for 10 of the 638 patients. Table 1 lists the characteristics of the patients. The median age of all 638 patients (390 males and 248 females) was 45 years (range 15–66 years). The median values of WBC, hemoglobin (Hb), platelets, and the ratio of blasts in the bone marrow were 13.7 × 10^9/l, 8.3 g/dl, 52.0 × 10^9/l, and 56.0%, respectively.

3.2 FAB classification

Table 2 shows the FAB classification of the 638 patients. Most were classified as M2 (n = 261; 40.9%), followed by M4 (n = 148; 23.2%), and M1 (n = 109; 17.1%) with M0, M4Eo, M5a, M5b, M6, M7, and acute leukemia of ambiguous lineage comprising the remainder in that order.

3.3 WHO classification and clinical characteristics

Table 3 shows the patients categorized according to the WHO classification. The first category of AML with recurrent genetic abnormalities accounted for 171 patients (26.8%), 133 (20.8%) were in the second category of AML with MLD, 331 (51.9%) were in the fourth category of AML not otherwise categorized, and 3 (0.5%) were categorized as having acute leukemia of ambiguous lineage. Most patients in the second category were identical to those with a de novo MLD phenotype. We found that 144 patients diagnosed with the MLD phenotype comprised 133 (92.4%) in the second category, 10 (7.0%) with 11q23 abnormalities, and 1 (0.7%) with acute leukemia of ambiguous lineage. Figure 1 shows the OS of each category. The 5-year survival rates of the first, second, and fourth categories were 58.2, 22.5, and 40.9% (P < 0.0001), respectively.

Table 2 Number of patients according to the FAB classification

Subtype	Description	No. of patients	%
M0	Minimally differentiated acute myeloid leukemia (AML)	30	4.7
M1	AML without maturation	109	17.1
M2	AML with maturation	261	40.9
M4	Acute myelomonocytic leukemia (AMMoL)	148	23.2
M4Eo	AMMoL with eosinophils	23	3.6
M5a	Acute monoblastic leukemia	19	3.0
M5b	Acute monocytic leukemia	24	3.8
M6	Acute erythroleukemia	16	2.5
M7	Acute megakaryoblastic leukemia	5	0.8
Acute leukemia of ambiguous lineage		3	0.5
Total		638	100

Table 3 Number of patients according to the WHO classification

Category and subtype	No. of patients	%
I. AML with recurrent genetic abnormalities	171	26.8
t(8;21)(q22;q22);(AML1/ETO)	113	17.7
inv(16)(p13;q22) or t(16;16)(p13;q22);(CBFβ/MYH11)	26	4.1
t(15;17)(q22;q12)(PML/RARα)	–	–
11q23(MLL)abnormalities	32	5.0
II. AML with multilineage dysplasia	133	20.8
Following MDS	–	–
Without antecedent MDS	133	20.8
III. AML and MDS, therapy-related	–	–
Alkylating agent-related	–	–
Topoisomerase type II inhibitor-related	–	–
Other types	–	–
IV. AML not otherwise categorized	331	51.9
AML, minimally differentiated	25	3.9
AML without maturation	99	15.5
AML with maturation	108	16.9
Acute myelomonocytic leukemia (AMMoL)	63	9.9
AMMoL with eosinophilia	5	0.8
Acute monoblastic leukemia	8	1.3
Acute monocytic leukemia	16	2.5
Acute erythroid leukemia	6	0.9
Acute megakaryoblastic leukemia	1	0.2
Acute leukemia of ambiguous lineage	3	0.5
Total	638	100

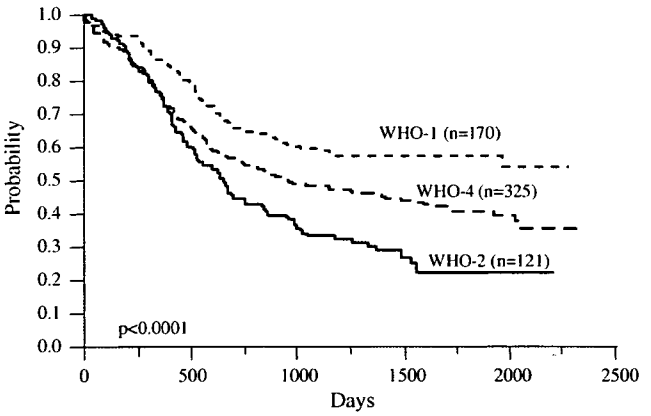

Fig. 1 Overall survival of patients categorized according to the WHO classification

Table 4 compares the clinical features among the WHO categories. The mean values of platelets, WBC, Hb, and the ratio (%) of blasts in bone marrow and of MPO-positive blasts significantly differed, whereas age did not significantly differ. Patients in the second category had a higher platelet count (111.0 × 10^9/l), whereas those with 11q23 abnormalities had a lower count (38.3 × 10^9/l) compared with those of other subtypes.

The WBC count of patients with *t*(8;21) was 1.4 × 10^9/l and lower than in other subtypes. The MPO-positive rate of blasts among patients with *t*(8;21) was higher (93.3%) and that of patients in the second category was lower (34.0%), than in other subtypes. All patients were grouped as high- or low-MPO according to ≥50% or <50% of MPO-positive blasts, respectively. A total of 339 patients (53.1%) were classified as high-MPO, 268 (42.0%) as low-MPO, and the MPO status of blasts could not be assessed in 31 (4.9%). Figure 2 shows the OS of patients with high- or low-MPO. The 5-year survival rate for patients with high or low-MPO was 50.7 and 29.6%, respectively ($P < 0.0001$).

3.4 Cytogenetics

All 638 patients were classified into favorable ($n = 139$; 21.8%), intermediate ($n = 413$; 64.7%), and adverse ($n = 54$; 8.5%) cytogenetic risk groups (Table 5). Figure 3 shows the OS according to this stratification. The 5-year survival rates were 63.4, 39.3, and 0.0% in the favorable, intermediate (except for those with 11q23 abnormalities), and adverse risk groups, respectively, and 35.5% in the group with 11q23 abnormalities ($P < 0.0001$).

The numbers of patients with or without MLD and high- or low-MPO in each cytogenetic risk group are listed in Table 6. None of those with the MLD phenotype were classified into the favorable risk group, while 129 (89.6%) and 15 (10.4%) of 144 patients with MLD were classified into intermediate or adverse risk groups, respectively. Only 15 patients (4.4%) in the high-MPO group were classified as having an adverse risk, while 11 (4.1%) in the low-MPO group were included in the favorable risk group.

The 32 patients with 11q23 abnormalities comprised 11 (34.4%) with *t*(11;19), 9 (28.1%) with *t*(9;11), 5 (15.6%) with del(11)(q23), 4 (12.5%) with *t*(6;11), and 3 (9.4%) with *t*(11;17). Figure 4 shows the OS of the intermediate risk group. The 5-year survival rate was 44.0% in patients with a normal karyotype, 35.5% in those with 11q23 abnormalities, and 30.6% in other patients including those with *t*(7;11), *t*(6;9), and Ph(+) abnormalities, respectively ($P = 0.033$).

Table 7 shows the relationship between *t*(9;11) ($n = 9$) and other 11q23 abnormalities ($n = 23$). More patients with low-MPO, without MLD, or with the FAB M5 subtype were found in the group with *t*(9;11) than with other 11q23 abnormalities. The survival rates between the two groups did not significantly differ ($P = 0.22$, data not shown).

4 Discussion

We attempted to classify selected patients who were reviewed morphologically and had available chromosomal data according to the WHO system. However, our series had some limitations in terms of analysis and patient selection. Although we obtained chromosomal data, genetic data were not available. Patients who were diagnosed with AML M3 or who had *t*(15;17), a history of MDS, or preceding hematological abnormalities, or who had previously undergone chemotherapy, were not eligible for the present study. However, multicenter trials might have some advantages in diagnosing AML according to the WHO classification, because morphological diagnoses and karyotypes are reviewed by the corresponding institutional committees.

The incidence of each category of the WHO classification was similar to those in several reports when patients with *t*(15;17) and therapy-related AML were excluded [20–22]. We and several others have shown that approximately 30% of patients have recurrent genetic abnormalities. Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) assays have recently been applied to analyze cytogenetic abnormalities [21, 23, 24]. This method might cause the frequency of the first WHO category to increase. Thus, the multiplex RT-PCR assay might have to be incorporated into the WHO system. The JALSG has started a cohort study in which all AML patients in participating hospitals are registered and analyzed according to the WHO classification. That study should clarify the real ratios of the AML subtypes in the WHO classification.

Table 4 Comparison of clinical findings of patients diagnosed according to the WHO classification

Category		Platelets ($\times 10^9$/l ± SE)	WBC ($\times 10^9$/l ± SE)	Hb (g/dl ± SE)	Age (year ± SE)	Blasts in bone marrow (%±SE)	MPO positivity of blasts (%±SE)
I	*t*(8;21)	76.7 ± 56.43 (113)[a]	1.4 ± 0.6 (113)	7.8 ± 0.2 (113)	41.6 ± 1.3 (113)	49.9 ± 2.0 (113)	93.3 ± 3.3 (108)
	inv(16)	57.8 ± 52.03 (26)	6.6 ± 1.2 (26)	9.2 ± 0.5 (26)	44.5 ± 2.6 (26)	50.5 ± 4.1 (26)	66.9 ± 6.7 (26)
	11q23	38.3 ± 30.8 (32)	4.3 ± 1.1 (32)	8.9 ± 0.4 (32)	41.6 ± 2.4 (32)	56.3 ± 3.7 (32)	43.6 ± 6.1 (32)
II		111.0 ± 121.5 (133)	3.0 ± 0.5 (133)	8.3 ± 0.2 (133)	44.2 ± 1.2 (133)	48.0 ± 1.8 (133)	34.0 ± 3.1 (126)
IV		72.8 ± 91.7 (330)	5.1 ± 0.3 (331)	8.8 ± 0.1 (330)	43.8 ± 0.7 (331)	65.7 ± 1.2 (328)	53.7 ± 1.9 (312)
		$P < 0.0001$	$P < 0.0001$	$P = 0.0004$	$P = 0.4077$	$P < 0.0001$	$P < 0.0001$

SE standard error, *WBC* white blood cell, *MPO* myeloperoxidase, *Hb* hemoglobin

[a] Number of patients

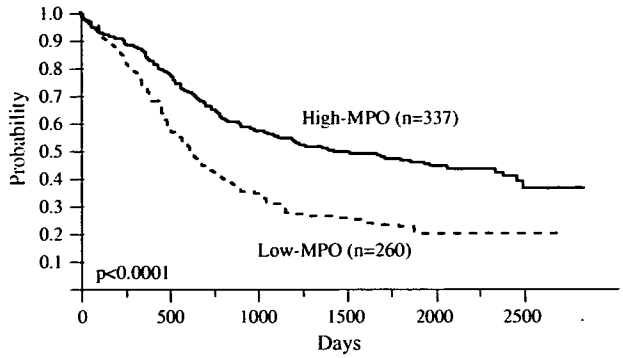

Fig. 2 Overall survival of patients with high or low MPO-positive blasts

Table 5 Distribution of patients classified by cytogenetic risk

Cytogenetic risk group	No. of patients	%
Favorable	139	21.8
t(8;21)	113	17.7
inv(16)	26	4.1
Intermediate	413	64.7
Normal karyotype	267	41.8
11q23	32	5.0
Ph(+)	7	1.1
t(7;11)(p15;p15)	4	0.6
t(6;9)	4	0.6
Other	131	20.5
Adverse	54	8.5
Complex	41	6.4
−7	2	0.3
abn3	5	0.8
del5q	2	0.3
−5	1	0.2
Other	3	0.5
Total	638	100.0

Few reports have included clinical data with the WHO classification. We found that the platelet count was higher among patients in the second category than in other categories. This supports our previous finding that the platelet count is higher in patients with AML accompanied by the MLD phenotype [25]. Among patients with MLD, none were in the favorable risk group, whereas the intermediate or adverse risk ratios among these patients were 89.6 and 10.4%, respectively. These differences might influence the finding that OS was better among patients without than with MLD ($P = 0.0002$, data not shown). Previous studies have also associated the MLD phenotype with a poorer outcome, although MLD is not significantly prognostic on multivariate analysis [18, 26], and a German group showed that dysplastic features correlate with adverse karyotypes

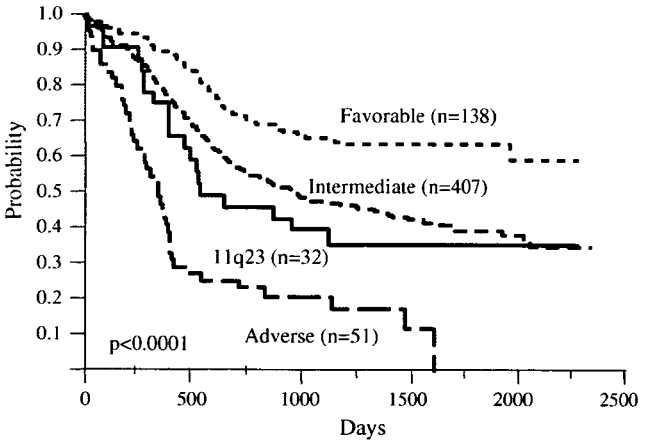

Fig. 3 Overall survival of patients stratified according to cytogenetic risk groups. Significant differences were observed between patients with a favorable, intermediate (except 11q23), and adverse karyotype ($P < 0.0001$)

Table 6 Relationship between cytogenetic risk groups and MLD phenotype or MPO-positive rates of blasts

	Favorable $n = 139$	Intermediate $n = 445$	Adverse $n = 54$	Total
MLD				
+	0	129 (89.5%)	15 (10.4%)	144
−	138 (28.2%)	292 (59.6%)	38 (7.8%)	490
Unknown	1	2	1	4
MPO				
High	123 (36.3%)	201 (59.3%)	15 (4.4%)	339
Low	11 (4.1%)	221 (82.5%)	36 (13.4%)	268
Unknown	5	23	3	31

High- and low-MPO indicates a percentage of myeloperoxidase positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

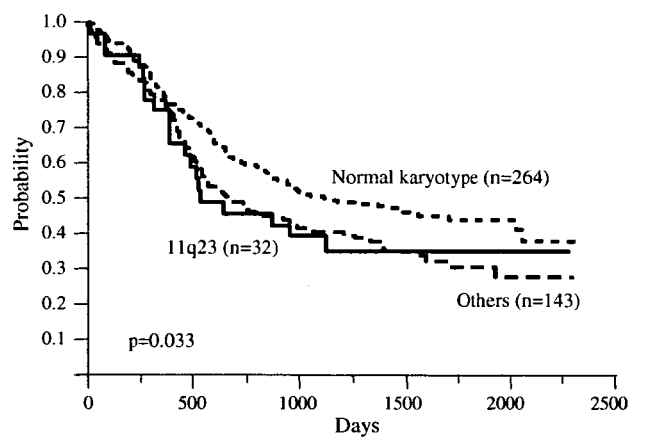

Fig. 4 Overall survival of patients with subtypes of intermediate cytogenetic risk. Significant differences were observed between patients with a normal karyotype and those with 11q23 abnormalities ($P = 0.033$)

[26]. Furthermore, patients in the second category had a lower MPO-positive rate of blasts, whereas those with $t(8;21)$ had a higher rate. Patients with high- and low-MPO were more frequently observed in the favorable and adverse risk groups, respectively. Multivariate analysis has shown that MPO is a significant factor affecting OS [19]. We did not assess prognostic factors by multivariate analysis here because the main theme of this study was to categorize patients according to the WHO classification, and we have already examined these in a previous series [18, 19].

Several studies have demonstrated the impact of specific cytogenetic abnormalities on survival in AML [3, 7–12, 20–22]. The cytogenetic risk groups stratified the AML patients in the present study according to the MRC system, as in these reports [3]. Therefore, we confirmed the clinical usefulness of cytogenetics as the first category of the WHO classification. We found that 32 patients had 11q23 abnormalities. The MRC system revealed that de novo and secondary AML patients with 11q23 abnormalities had an intermediate outcome with an OS rate of 45% at 5 years ($n = 60$; median age, 17 years) in a younger cohort [3] and an OS rate of 0% at 5 years ($n = 11$; median age 64 years) in an elderly cohort [7]. In contrast, SWOG/ECOG trials including adult de novo AML patients (age, 16–55 years) assigned those with 11q abnormalities to the unfavorable cytogenetic subgroup [8]. Our data showed that patients with 11q23 abnormalities have an intermediate rather than adverse outcome. The prognostic effect of 11q23 abnormalities might depend on the partner gene. Several studies have shown that 11q23 abnormalities with $t(6;11)$ and $t(10;11)$ are associated with a poor prognosis, whereas $t(9;11)$ is associated with a superior OS and such patients might respond well to intensive treatment, especially when the chemotherapy regimen includes high-dose cytarabine [15, 27–30]. The CALGB study has shown that the median OS of 13.2 months among 23 patients with $t(9;11)$ was significantly longer than the 7.7 months among 24 patients with other 11q23 rearrangements ($P = 0.009$) [30]. In a recent CALGB series of 54 patients with 11q23 abnormalities, 27 patients with $t(9;11)$ had an intermediate outcome and a median OS of 13.2 months, whereas those with $t(6;11)$ or $t(11;19)$ had a poor outcome of 7.2 or 8.4 months [15]. Conversely, Schoch et al. showed that 14 patients with $t(9;11)$ had a median OS of 10.0 months compared with the 12.8 months of 26 patients with other MLL rearrangements, and that the two cytogenetic groups did not significantly differ [13]. Our data showed that nine patients with $t(9;11)$ were more frequently involved in M5. The MPO and MLD features significantly differed between patients with $t(9;11)$ and those with other 11q23 abnormalities. However, the CALGB study found no significant differences in myelodysplastic features between the two

Table 7 Comparison of *t*(9;11) and other 11q23 abnormalities

	No. of patients	Auer		MPO*		MLD*		FAB						Median age (year)	Median survival (day)
		+	−	High	Low	+	−	M1	M2	M4	M4Eo	M5a**	M5b		
t(9;11)	9	0	9	1	8	0	9	0	0	3	0	6	0	39	1031.00
Other 11q23	23	5	18	13	10	10	13	1	3	13	1	2	3	48	520.00
Total	32	5	27	14	18	10	22	1	3	16	1	8	3	44.5	531.5

High- and low-MPO indicates a percentage of myeloperoxidase-positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

* $P < 0.05$, ** $P < 0.01$

cytogenetic groups [30]. In terms of OS, our results showed no significant differences between patients with *t*(9;11) and those with other 11q23 abnormalities ($P = 0.22$). Some problems are associated with the analyses of 11q23 abnormalities. We had few patients with these abnormalities, particularly individual translocations, and genetic analysis was not performed. Thus, the prognostic risk of 11q23 abnormalities cannot be concluded from the present study. Nonetheless, these abnormalities were never associated with a favorable risk. To classify 11q23 abnormalities into each prognostic risk group, further investigations and genetic analyses of a large number of patients with 11q23 abnormalities are required.

The fourth WHO category, which is not otherwise categorized, accounted for 52% of patients in the present study. Most of them were classified into the intermediate risk group, and no prognostic subdivisions were valuable. Using cytogenetic features as a prognostic factor in groups with a normal karyotype has limitations, and such patients accounted for 64.6% of the intermediate risk group (data not shown). Additional factors are required to stratify these patients. We and several others suggested that differences could be based on molecular genetic analysis [22, 31–35]. For example, FLT3 mutations are important biomarkers of a normal karyotype and might be valuable for stratifying the intermediate risk group. Further follow-up studies might also shed light on the roles of FLT3 ITD mutations in the development of AML and aid their use as novel molecular targeting agents against AML [22, 32]. Bienz et al. identified CEBPA mutations, FLT3-ITD, and differing levels of BAALC expression as having independent prognostic significance in patients with a normal karyotype [33]. If these genetic markers can be confirmed as being of clinical significance, genetic analyses will probably be incorporated into the WHO classification.

In summary, our results confirmed those of previous studies showing the prognostic significance of cytogenetics, MLD, and MPO-positivity of blasts in AML. Furthermore, we categorized patients with de novo AML according to the WHO classification and showed the clinical characteristics and OS of each category.

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Karyotype at diagnosis is the major prognostic factor predicting relapse-free survival for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy

Masamitsu Yanada,[1] Jin Takeuchi,[2] Isamu Sugiura,[3] Hideki Akiyama,[4] Noriko Usui,[5] Fumiharu Yagasaki,[6] Kazuhiro Nishii,[7] Yasunori Ueda,[8] Makoto Takeuchi,[9] Shuichi Miyawaki,[10] Atsuo Maruta,[11] Hiroto Narimatsu,[1] Yasushi Miyazaki,[12] Shigeki Ohtake,[13] Itsuro Jinnai,[5] Keitaro Matsuo,[14] Tomoki Naoe,[1] and Ryuzo Ohno[14] for the Japan Adult Leukemia Study Group

[1]From the Nagoya University Graduate School of Medicine, Nagoya; [2]Nihon University School of Medicine, Tokyo; [3]Toyohashi Municipal Hospital, Toyohashi; [4]Tokyo Metropolitan Komagome Hospital, Tokyo; [5]Jikei University School of Medicine, Tokyo; [6]Saitama Medical University, Saitama; [7]Mie University School of Medicine, Tsu; [8]Kurashiki Central Hospital, Kurashiki; [9]National Hospital Organization Minami-Okayama Medical Center, Okayama; [10]Saiseikai Maebashi Hospital, Maebashi; [11]Kanagawa Cancer Center, Yokohama; [12]Nagasaki University Graduate School of Biomedical Sciences, Nagasaki; [13]Kanazawa University Graduate School of Medical Science, Kanazawa; [14]Aichi Cancer Center, Nagoya, Japan

ABSTRACT

To identify factors associated with relapse-free survival (RFS), 80 patients with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia, enrolled in a phase II study of imatinib-combined chemotherapy, were analyzed. The median follow-up of surviving patients was 26.7 months (maximum, 52.5 months). Twenty-eight out of 77 patients who had achieved CR relapsed. The probability of RFS was 50.5% at 2 years. Multivariate analysis revealed that the presence of secondary chromosome aberrations in addition to t(9;22) at diagnosis constitute an independent predictive value for RFS (p=0.027), and increase the risk of treatment failure by 2.8-fold.

Key words: acute lymphoblastic leukemia, Philadelphia chromosome, BCR-ABL, imatinib, karyotype.

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Introduction

The treatment for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph$^+$ ALL) has changed dramatically since imatinib, a selective inhibitor of the ABL tyrosine kinase, was introduced.[1,2] Combined with chemotherapy, or even as a single agent, it can produce complete remission (CR) rates of 90% or higher in newly diagnosed patients.[3-9] We previously reported the results of a phase II study by the Japan Adult Leukemia Study Group (JALSG) to test the efficacy and feasibility of imatinib-combined chemotherapy for newly diagnosed Ph$^+$ ALL.[6] The rate of CR reached 96%, and that of BCR-ABL negativity in bone marrow 71%. However, despite a relatively short follow-up period, relapse occurred in a subset of the patients who had achieved CR.

On the other hand, remarkable progress is being made with the development of novel tyrosine kinase inhibitors with more potent *in vitro* and *in vivo* activities than imatinib.[10,11] Given this, we investigated factors associated with relapse-free survival (RFS).

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Correspondence: Masamitsu Yanada, MD, Department of Leukemia, Unit 428, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, Texas 77030, USA. E-mail: myanada@mdanderson.org

Design and Methods

Patients and treatments

Eligibility criteria included newly diagnosed Ph+ ALL, age between 15 and 64 years, an Eastern Co-operative Oncology Group performance status between 0 and 3, and adequate liver, kidney and heart function. Written informed consent was obtained from all patients prior to registration.

For remission induction therapy, imatinib was administered from day 8 to day 63 in combination with daunorubicin, cyclophosphamide, vincristine (VCR) and prednisolone (PSL). Consolidation therapy consisted of an odd course (C1) comprising high-dose methotrexate, high-dose cytarabine and methylprednisolone, and an even course (C2) with single-agent imatinib for 28 days. C1 and C2 were alternated for 4 cycles each. After completion of the consolidation therapy, patients received maintenance therapy consisting of VCR, PSL and imatinib for up to 2 years from the date CR had been achieved.[6] The daily dose of imatinib used in this study was 600 mg. Allogeneic hematopoietic stem cell transplantation (HSCT) was recommended if a matched sibling donor was available, and was allowed from an alternative donor.

The protocol was reviewed and approved by the institutional review board of each of the participating centers and was conducted in accordance with the Declaration of Helsinki.

Cytogenetic and molecular analysis

At diagnosis, bone marrow samples were examined for cytogenetic abnormalities with standard banding techniques. Karyotypes were classified according to the International System for Human Cytogenetic Nomenclature.[12] The number of BCR-ABL copies in bone marrow was determined at a central laboratory with the real-time quantitative RT-PCR test according to the previously described method.[13]

Statistical analysis

Kaplan-Meier survival analysis was performed to estimate the probabilities of RFS, event-free survival (EFS), and overall survival (OS), with differences between the groups compared by the log-rank test. Cumulative incidences of relapse were calculated with non-relapse mortality considered as a competing risk, and differences between the groups were compared with the Gray's test. For risk factor analysis, a Cox proportional hazards model was constructed. In multivariate analysis, variables with p values of <0.10 determined by univariate analysis were included in the final model. A hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI).

Results and Discussion

A total of 80 patients were recruited between September 2002 and January 2005. The median age was 48 years (range 15-63), with 49 males and 31 females. CR was achieved by 77 (96.2%) patients. During a median follow-up of 26.7 months (maximum 52.5 months), 28 patients relapsed. Of the 17 relapses observed during the consolidation therapy, 13 occurred during the imatinib course. The probabilities of EFS and OS were 48.5±5.7% and 58.1±5.7% at 2 years (Figure 1). For patients who had achieved CR, the probability of RFS was 50.5±5.9% at 2 years. Allogeneic HSCT was performed for 60 patients, including 24 from a sibling donor, 1 from a related donor other than a sibling, 25 from an unrelated donor, and 10 from unrelated cord blood. Disease status at the time of transplantation was first CR for 44 patients, second CR for 4 and non-CR for 12. The 2-year RFS for those who had undergone allogeneic HSCT during first CR was 62.6±7.5% and 62.1±12.3% for those who had not undergone allogeneic HSCT. When allogeneic HSCT was considered as a time-dependent covariate, it was shown to have no significant effect on RFS (HR, 1.03; 95% CI, 0.51-2.09; p=0.934). Major and minor BCR-ABLs were detected in 23 and 56 patients respectively. The transcript type of the remaining patient could not be determined because fluorescent *in situ* hybridization analysis was used instead of the PCR test. Neither transcript types nor copy numbers at diagnosis were associated with RFS (p=0.763 and 0.912). Pre-treatment cytogenetic results were not available for 4 patients because analysis was not performed (n=2) or was not successful (n=2). Of the remaining 76 patients, 22 showed only t(9;22) or variant translocations, 51 showed additional chromosome aberrations, and 3 showed normal karyotype. Additional aberrations exceeding a frequency of 10% comprised +der(22)t(9;22) in 17 patients, abnormalities involving the short arm of chromosome 9 [abn(9p)] in 17, monosomy 7 in 10, and trisomy 8 in 10. Figure 2 compares RFS for patients with and without additional chromosome aberrations. The presence of additional aberrations was significantly associated with shorter RFS (p=0.003). The relapse rate was also higher in patients with additional aberrations (41% *vs.* 20% at 2 years, p=0.0414). Analyses of the 4 recurrent abnormalities mentioned above demonstrated a statistically significant negative impact on RFS for +der(22)t(9;22) and abn(9p) (p<0.001 and p=0.005). Even after allogeneic HSCT, patients with additional aberrations appeared to have a trend for shorter RFS than those without (p=0.080), but this might reflect a larger proportion of transplantation beyond first CR in the former (31% *vs.* 17%). In patients allografted during first CR, there was no difference in cumulative incidences of relapse dated from the day of transplantation between the 2 groups