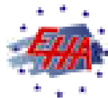

A new human acute monocytic leukemia cell line SHI-1 with t(6;11)(q27;q23), p53 gene alterations and high tumorigenicity in nude mice

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Background and Objectives. Human leukemia cell lines are of great value in leukemia research. Thus far 36 leukemia cell lines carrying the 11q23 translocation and *MLL* rearrangements,, including two cell lines with t(6;11)(q27;q23) and an *MLL-AF6* fusion gene have been described. We have established a new monocytic cell line with t(6;11), designated SHI-1, and herein describe its biological characteristics.

Design and Methods. Mononuclear cells isolated from the bone marrow of a patient with acute monocytic leukemia (AML-M5b) at relapse were inoculated and passaged by liquid culture. The biological features of the cell line were characterized by morphological assays, flow cytometry, cytogenetic analysis, reverse transcription polymerase chain reaction (RT-PCR), direct sequencing, fluorescence *in situ* hybridization (FISH), clonogenic culture, quantitative fluorescent PCR, zymography, short tandem repeating sequences- PCR (STR-PCR), multiplex-FISH (M-FISH), and tumorigenic capacity in nude mice.

Results. The SHI-1 cell line has been maintained in continuous culture without any external cytokines for three years. The morphology and immunoprofile of the cells show typical features of monocytic lineage. Karyotypic analysis demonstrated a t(6;11)(q27;q23) translocation accompanied by a deletion of 17p, which are the same abnormalities as were seen in the leukemia cells of this patient in relapse. The *MLL-AF6* fusion transcript and the loss of one p53 allele were proven by chromosome painting, FISH and RT-PCR analysis in both SHI-1 cells and the pimary leukemia cells. A point mutation of ATC→ACC at codon 195 of exon 6 in another p53 allele was found by direct sequencing of DNA in SHI-1 cells as well as in the primary leukemia cells. Neither Epstein-Barr virus nor mycoplasma was detected in SHI-1 cells. Tumor masses were found in all sixteen mice 9-19 days after subcutaneous injection of SHI-1 cells. DNA fingerprinting confirmed the authenticity of the cell line.

Interpretation and Conclusions. SHI-1 is a new monocytic leukemia cell line with the t(6;11) translocation, p53 gene alterations, and high tumorigenicity in nude mice. It could be a valuable tool in the study of leukemogenesis.

Keywords: acute monocytic leukemia, leukemia cell line, p53 gene, nude mice, tumorigenicity.

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Human leukemia-lymphoma (LL) cell lines are powerful tools for investigating basic and applied aspects of cell biology because, among other advantages, there is an unlimited supply, they offer worldwide availability of identical cell material and they can be cryopreserved.[1] These cell lines are characterized by their monoclonal origin, differentiation arrest, sustained proliferation *in vitro* with preservation of most cellular features and specific genetic alterations.[2] Since the first LL cell line, the Raji cell line, was established,[3] more than 1,000 human LL cell lines have been developed.[4] Among them, 36 cell lines have been described to carry 11q23 translocations and *MLL* fusion genes including 16 cell lines with t(4;11)(q21;q23), eight with t(11;19)(q23;p13), seven with t(9;11)(p22;q23), two with t(6;11)(q27;q23), and three cell lines with one of the following rare translocations, i.e., t(5;11)(q15;q23), t(11;16)(q23;p13), or t(X;11)(q13;q23).

We have recently established a novel human cell line, designated as SHI-1, from a patient with acute monocytic leukemia in relapse. It had t(6;11)(q27;q23) and the rearrangement of the *MLL* gene, accompanied by a point mutation of one p53 allele associated with loss of the other p53 allele and showed high tumorigenicity in nude mice. Here we present the phenotypic, genetic and functional properties of this cell line following the guidelines for characterization and publication of human malignant hematopoietic cell lines.[5]

Design and Methods

Case report

A 37-year old male was admitted to our hospital because of fever and fatigue in January 2002. Physical examination revealed bilateral inguinal lymph node swelling and hepatomegaly (3 cm below

the left costal margin). Peripheral blood (PB) examination showed Hb 110g/L, platelets 74×10^9/L and WBC 47.1×10^9/L, of which monoblasts and promonocytes accounted for 78%. The bone marrow (BM) was markedly hypercellular with 28.5% monoblasts and 60.5% promonocytes which had folded nuclei, prominent nucleoli, finely reticular chromatin, abundant blue-gray cytoplasm and finely azurophilic granules in the cytoplasm. These cells were negative for myeloperoxidase staining, while 24% of them were positive for periodic acid-Schiff staining. Flow cytometry immunophenotyping analysis showed positivity with CD13 (45.5%), CD33 (98.7%), CD14 (34.0%) and CD34 (24.4%), but negativity for lymphoid cell antigens (CD7, CD10 and CD19). The patient was diagnosed as having acute monocytic leukemia (M5b) according to the FAB criteria.[6] He was treated initially with two courses of a daunorubicin and cytosine arabinoside regimen, but no response was achieved. A complete remission was finally obtained after one course of mitoxantrone and cytosine-arabinoside regimen. However, his leukemia relapsed after a brief remission in April 2002. He then received an allogeneic PB stem cell transplantation from an HLA-matched sibling donor after a modified busulfan and cyclophosphamide conditioning regimen, but the leukemia cells reappeared in PB and BM, and invaded his skin 22 days after transplantation. Despite receiving intensive combination chemotherapy consisting of topotecan, intermediate dose of cytosine arabinoside and amsacrine, and donor lymphocyte infusion, the patient did not achieve another remission and died of intracranial hemorrhage in June 2002.

Cell culture

Mononuclear cells from a BM sample withdrawn on April 30, 2002 in the first relapse with informed consent were separated by Ficoll-Hypaque density gradient centrifugation and cultured in 25 cm^2 flasks at a density of 1×10^6cells/mL in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY, USA) supplemented with 15% heat-inactivated fetal calf serum (FCS), 20% supernatant (conditioned medium) of 5637 cells (5637 CM) which contains several cytokines including interleukin-3, interleukin-6, granulocyte-monocyte colony-stimulating factor, stem cell factor and others at 37° min a 5% CO_2 humidified atmosphere in an incubator. The medium was exchanged every 3-4 days depending on the rate of cell growth.

Morphological and cytochemical studies

Light microscopy examination was performed on Wright's stained cytospin preparations. Cytochemical staining for POX, α-naphthyl acetate esterase, and α-naphthyl butyrate esterase were also performed by standard methodology. Ultrastructural analysis was carried out as described previously.[7,8]

Cell surface marker analysis

Surface immunotyping of the primary leukemia cells and SHI-1 cells was performed by flow cytometry using a broad panel of monoclonal antibodies (MoAb). The cells were analyzed on a flow cytometer (Epicx XL-1, Beckman Coulter, France) for fluorescence intensity using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies. All the MoAb used were purchased from Immunotech (Marseille, France) and are listed in Table 1. For cytoplasmic CD3ε (cCD3ε) staining, the cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and stained with rabbit anti-CD3ε MoAb followed by FITC-conjugated goat anti rabbit antibody.

Cytogenetic analysis

Chromosomes of both the primary leukemia cells and the SHI-1 cells were analyzed. Chromosomes were prepared by a standard method and banded by the R-banding technique. The karyotypic abnormalities were identified according to the International System for Human Cytogenetic Nomenclature (ISCN 1995).

Fluorescence in situ hybridization (FISH) assay

FISH was performed according to the manufacturer's instructions using whole chromosome painting (WCP) probes for chromosomes 6 and 11 or chromosomes 7 and 13 labeled with digoxigenin and biotin, respectively, (Cambio, Cambrige, UK), two locus-specific probes for the *MLL* and p53 genes, labeled with digoxigenin (Qbiogene, USA), and a centromeric probe for chromosome 17 (a kind gift from Dr. Hervé Avet-Loiseau, Laboratoire de Cytogenetique Hematologique, Centre Hospitalier Universitaire de Nantes, France) labeled with biotin-16-dUTP (Boehringer, Mannheim, Germany). Images were captured using a Leica DMRXA fluorescence microscope equipped with a Sensys cooled CCD camera (KAF 1401 chip; Photometrics) and analyzed using multiple-FISH software (Leica, Germany).

Multiple- FISH (M-FISH)

M-FISH was performed using a commercially available set of combinatorially labeled WCP (SpectraVysion, Vysis, USA), essentially according to the manufacturer's protocol. Separate fluorochrome images were obtained using a Sensys cooled CCD camera mounted on an Olympus BX-60 microscope (Olympus Optical, Tokyo, Japan) with an 8-position filter turret containing specific filter sets for DAPI, SpectrumGold, SpectrumFred, SpectrumAqua, SpectrumRed, SpectrumGreen, using a 100-watt mercury

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Table 1. Immunophenotypic characterization of the SHI-1 cell line and the primary leukemia cells.

Antigen (CD)	Primary leukemia cells (%) at presentation	at the first relapse	SHI-1 % cell line
T/NK cell markers			
CD2	ND	4	1
CD3	ND	ND	1
cCD3	ND	ND	0
CD4	ND	ND	37
CD5	ND	ND	78
CD7	10	75	17
CD8	ND	ND	3
CD16/56	ND	ND	1
B cell markers			
CD10	1	2	4
CD19	1	1	0
CD20	ND	ND	0
CD22	ND	1	ND
Myelomonocytic markers			
CD13	46	30	95
CD14	34	11	37
CD15	ND	ND	95
CD33	99	91	80
MPO	ND	ND	26
Progenitor/activation markers			
CD34	24	1	0
CD38	96	43	88
HLA-DR	ND	ND	10
Adhesion markers			
CD11b	ND	ND	93
Megakaryocytic markers			
CD41	ND	ND	86
CD42b	ND	ND	2
CD61	ND	ND	36
Cytokine receptors			
CD25	ND	ND	92
CD116	ND	ND	59

lamp. The resultant images were analyzed using Powergene M-FISH software (Applied Imaging, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated as previously described.[9] Ten pmol of primers MLL5Sext (5'-GAGGATCCTGCCCCAAAGAAAAG-3') and AF6Sext (5'-CTCCGCTGAC TGCACTTCATAG-3') were used in the reaction. After an initial denaturation of 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C, followed by a final extension at 72°C for 10 min were performed in a Geneamp PCR systems 2400 (Perking Elmer Cetus, Norwalk, CT, USA). The PCR products were electrophoresed in 2.0% agarose gels. An aliquot of cDNA reagents from a normal individual was amplified by PCR as a negative control. β2 microglobulin was amplified as the internal control. Multiplex RT-PCR was performed according to the method described by Pallisgaard *et al.*[10] to detect the *MLL* partial tandem duplication (PTD) in SHI-1 cells. It used 29 sorts of primers for translocations and chromosomal aberrations in acute leukemia including the primers for *MLL* PTD.

Direct sequencing

Genomic DNA was amplified by PCR using p53-specific primer pairs which were as follows: exons 5 and 6: p53-e5-p5: GTTGCAGGAGGTGCTTACAC, p53-e6-p3: ATGGGGTTATAGGGAGGTCA_exon 7: p53-e7-p5: GAGCGAGATTCCATCTCAAA_p53-e7-p3:CTGAGTGGGAGCAGTAAGGA_exon 8: p53-e8-p5: AGCTTAGGCTCCA AAAGGA, p53-e8-p3: CCCAATTGCAGGTAAAACAG. After denaturing at 94°C for 5 min, 35 cycles of PCR amplification were performed as follows: denaturing of 94°C for 30 s, annealing at 62°C for 40 s for exons 5, 6, and 7, and at 62°C for 30 s for exon 8, extension at 72°C for 1 min for exons 5, 6, and 7, and at 72°C for 45 s for exon 8, followed by final extension at 72°C for 10 min. DNA fragments amplified by PCR were purified and concentrated and sequenced with a Taq DyeDeoxy™ Terminator Cycle Sequencing kit and an automated sequencer. The primers used for sequencing were the same as those listed above.

Detection of Epstein-Barr virus (EBV) and mycoplasma

Real-time PCR was performed to detect EBV genomic DNA according to the manufacturer's instructions (Daan Gene Company, Guangzhou, China). In brief, the genomic DNA was extracted from the cell line and amplified using EBV-specific primer pairs in an ABI PRISM 7700 Real-time thermal cycler (Applied Biosystems, USA). The following oligonucleotide primer sequences were used: sense primer, 5'-TCGCAGCTCTAACTTGGCATCTCTT-3'; antisense primer, 5'-TTGCTATCCGAATGGGG-3'; specific probe sequence, 5'-TCCTTCTGGCACGACT-GTTCC-3'. After an initial denaturation of 2 min at 93°C, 10 cycles of 45 s at 93°C and 1 min at 55°C, followed by 30 cycles of 30 s at 93°C and 45 s at 55°C, were performed.

The two classical detection methods, DNA fluorochrome staining with Hoechst 33258 and microbiological colony assay, were used to determine contamination by mycoplasma.

Authentication of the cell line

The identity of the SHI-1 cell line was checked using DNA fingerprint against a BM cell sample taken from the patient during progression of his disease. DNA was prepared from whole blood or whole BM using the QIAamp Blood Kit (Qiagen Company, Germany) according to the instructions provided by the manufacturer. The following 10 highly polymorphic short

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tandem repeat (STR) loci were tested by a multiplex PCR reaction: D3S1358 (on chromosome 3), VWA (on chromosome 12p12), FGA (on chromosome 4q28), X/Y (on chromosome X/Y), D8S1179 (on chromosome 8), D21S11 (on chromosome 21), D18S51 (on chromosome 18q21), D5S818 (on chromosome 5), D13S317 (on chromosome 13), and D7S820 (on chromosome 7). The multiplex PCR reaction conditions and primers were chosen according to the previous description, and the size of amplified products was determined by a DNA capillary electrophoresis apparatus, ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) at a single base definition.[11]

Clonogenic assay

A clonogenic assay was employed to evaluate the proliferation and renewal of the leukemia cell line. Briefly, the leukemia cells were mixed with 1 mL of IMDM containing 1.4% methylcellulose and 20% FCS. Cells were then plated in a 35 mm plastic dish (Falcon). After 7 days' incubation, colonies of more than 40 cells were counted under an inverted microscope. This assay was performed in quadruplicate.

Drug sensitivity and expression of multiple multidrug resistant related proteins

The sensitivity of SHI-1 cells to various chemotherapeutic drugs including daunorubicin (Pharmacia, USA), etoposide (Lizhu Company, Zhuhai, China), vincristine (Hualian Company, Shanghai, China), pacilitaxel (Sigma, USA), homoharringtonine (Minsheng Co., China), and cytosine arabinoside (Pharmacia, USA) was evaluated by the thizolyl blue method (MTT). Flow cytometry was used to study the expression of several multiple multidrug resistant related proteins including the P-glycoprotein, multidrug resistant related protein, lung resistance protein, GST-π and breast cancer related protein. HL60 and its drug-resistance cell line, HL60/VCR, were used as controls.

Tumorigenicity in nude mice

Four- week old NC or Balb/c nude mice were injected subcutaneously with the SHI-1 cell line (1×10^7 cells /injection). Sixteen mice were injected. If animals developed solid tumors they were killed. Tumors were fixed in 10% buffered formalin for histology. Tumors were also used to prepare viable cell suspensions for karyotypic analysis and RT-PCR assay to detect the *MLL-AF6* fusion transcript.

Zymography

Levels of secreted MMP-2 and MMP-9 (also referred to as gelatinase A and gelatinase B, respectively) were analyzed by zymography following the method described by Janowska- Wieczorek.[12] Several leukemic cell lines including K562, HL60, and NB4 were used as controls. Since the conversion of the latent proenzymes of MMP-2 and MMP-9 to the active enzymes results in the removal of a 10 kDa amino-terminal domain, each protease can be determined by zymography as indicated by clear bands of gelatinolytic activity. The levels of proteolytic activities were evaluated by densitometric analysis.

Results

Establishment of the SHI-1 cell line

One month after the beginning of the culture (April 2002), the cells showed slow but stable proliferation. The cells started to proliferate rapidly after 3 months. Since then, the cells have been maintained in continuous culture without 5637 CM for 3 years. Thus, it has been judged as a continuous cell line and designated as SHI-1. The SHI-1 cell line could be frozen in defined medium (70% medium, 20% FCS, 10% DMSO), stored in liquid nitrogen, thawed again (with viabilities of 85% or higher) and successfully expanded in culture. It grew as single cells in suspension with a few cells adhering to the flask. The SHI-1 cell line has a doubling time of about 36 h at a cell density of 1×10^6/mL. Cell cycle analysis by flow cytometry showed 56.3% of cells in G0/G1 phase, 30.3% in S phase, and 13.5% in G2/M phase. The main characteristics of the SHI-1 cell line remained stable during about 3 years of continuous culture as well as after freezing and thawing.

Morphology of SHI-1 cells

SHI-1 cells show a regular cell shape, folded nucleus with prominent nucleoli, finely reticular chromatin, and blue-gray cytoplasm with fine azurophilic granules in a part of the cells, which were similar to those of the primary leukemia cells (Figure 1A). Electron microscopy showed that most cells had numerous mitochondria, endoplasmic reticulum, lysosomes, vacuoles and folded nuclei with prominent nucleoli (Figure 1B). The cell line was positive for α-naphthyl butyrate esterase, α-naphthyl acetate esterase and the NaF inhibition test, while 95% of cells were negative for POX staining.

Immunophenotypic profiles

The immunoprofiles of the primary leukemia cells and the SHI-1 cell line are summarized in Table 1. Both of them have similar immunoprofiles. The SHI-1 cell line expresses typical antigens of myeloid and monocytic lineages such as CD11b, CD13, CD14, CD15, CD33, CD38, CD116, and myeloperoxidase. A few markers of megakaryocytic (CD41 and CD61) and T lymphocytic lineage (CD4, CD5 and CD25) are also present.

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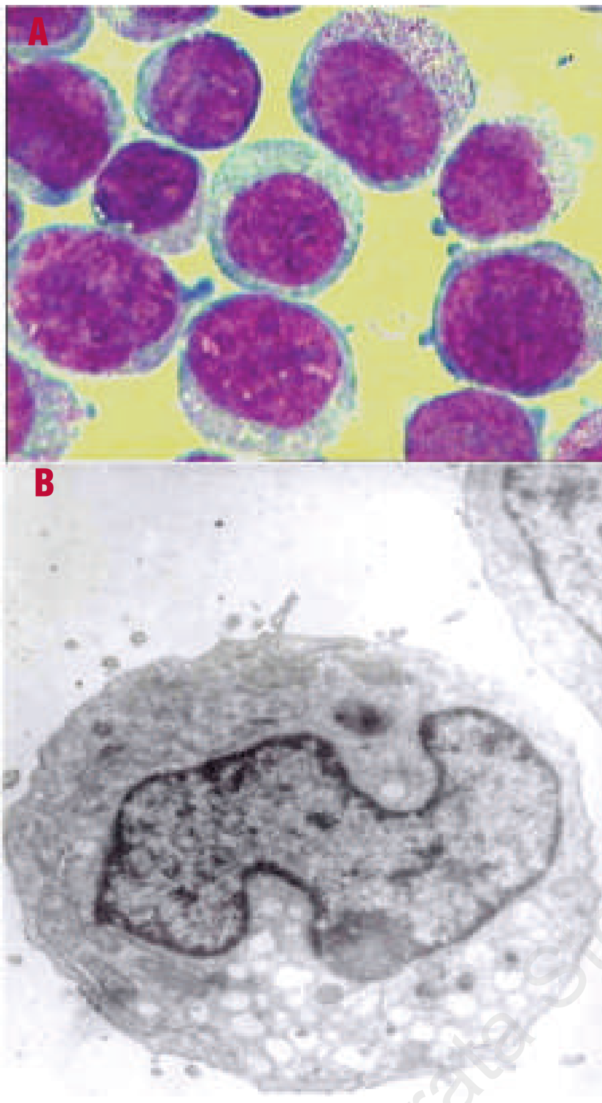

Figure 1. A. Morphology of SHI-1 cells on Wright's staining under a light microscopy (original magnification ×1,000). B. Electron microscopic photograph of SHI-1 cells showing numerous mitochondria, endoplasmic reticulum, lysosomes, vacuoles, an indented nucleus with prominent nucleoli (original magnification ×5,000).

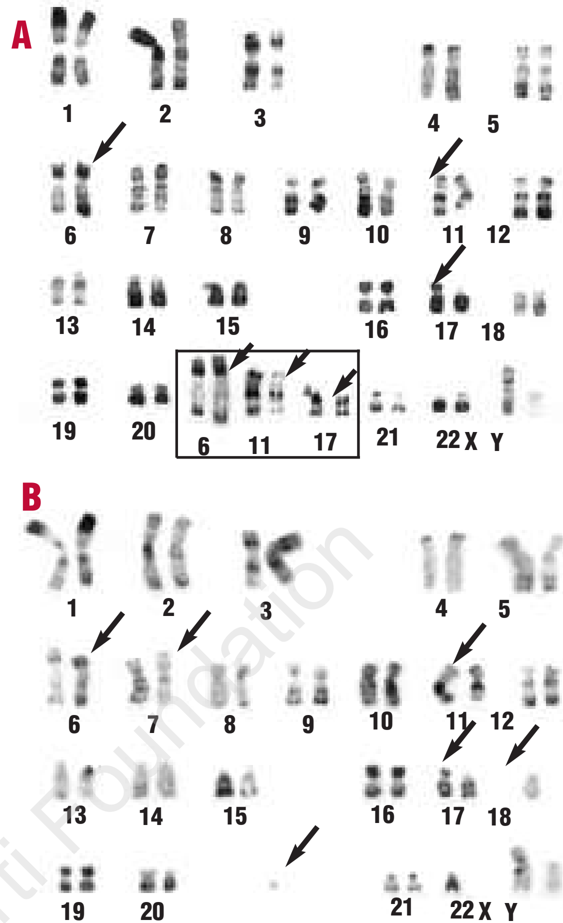

Figure 2. A. A representative R-banded karyotype of the SHI-1 cell line: 46,XY,t(6;11)(q27;q23), del(17)(p11). Arrows indicate a translocation t(6;11)(q27;q23) and a deletion of chromosome 17p. B. A representative R-banded karyotype of the SHI-1 cells passaged one year after beginning the cultures: 46,XY, t(6;11)(q27;q23),der(7)t(7;?),del(17)(p11),-18,minute.

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Cytogenetics

This patient received cytogenetic studies on three occasions. His BM cells showed a karyotype of 46,XY,t(6;11)(q27;q23) at presentation. The karyotype evolved from 46,XY, t(6;11)(q27;q23) to 46,XY,t(6;11)(q27;q23), del(17)(p11) seen at the first and the second relapses. Sequential cytogenetic studies were performed on SHI-1 cells in the logarithmic growth phase. SHI-1 cells showed a karyotype of 46,XY,t(6;11)(q27;q23),del(17)(p11) (Figure 2A), which was identical to that of the patient's BM cells at the first relapse, before March 2003. Since then some SHI-1 cells have shown extra abnormalities such as der(7)t(7;?),-18,minute in addition to t(6;11)(q27;q23) and del(17)(p11) (Figure 2B). Table 2 shows the results of sequential karyotypic analyses on the primary leukemia cells and SHI-1 cells.

FISH studies

Chromosome painting analysis using WCP 6 and 11 on both the primary leukemia cells and the SHI-1 cell line detected a red fluorescent signal on the terminal portion of the long arm of one chromosome 6 with a green fluorescent signal, whereas no green fluorescent signal was detected on the terminal of one chromosome 11 with a shorter red fluorescent signal than its homolog because the translocation segment from chromosome 6q was too small to be detected by WCP 6. This finding indicated a translocation between chromosomes 6 and 11 (Figure 3A).

Furthermore, the *MLL* gene probe labeled with digoxigenin was used to identify the rearrangement of the *MLL* gene in the primary leukemia cells and the SHI-1 cell line. The results showed that three green flu-

Table 2. The results of sequential karyotypic analysis of the primary leukemia cells and SHI-1 cell line.

Date	Karyotype
Primary leukemia cells	
Jan 2002 (at presentation)	46,XY,t(6;11)(q27;q23)[7]/46,XY[2]
April 2002 at the first relapse	46,XY,t(6;11)(q27;q23)[8]/46,idem, del(17)(p11)[8]/46,XY[6]
June 2002 (at the second relapse)	46,XY,t(6;11)(q27;q23),del(17)(p11)[6]/46, XY[4]
SHI-1 cell line	
August 2002	46,XY,t(6;11)(q27;q23),del(17)(p11)[11]
October 2002	46,XY,t(6;11)(q27;q23),del(17)(p11)[18]
March 2003	45,XY,t(6;11)(q27;q23),del(17)(p11)[1]/ 45,idem, der(7)t(7;?),-18[25]/45, idem,der(7)t(7;?),-18,minute[6]
July 2003	45,XY,t(6;11)(q27;q23),der(7)t(7;?), del(17)(p11), -18,minute[19]/45,XY, t(6;11)(q27;q23),der(7)t(7;?), del(17)(p11), -18[6]

orescent signals could be detected in most of the metaphases and the interphases from the primary leukemia cells and all the metaphases and the interphases from the SHI-1 cell line indicating a *MLL* rearrangement in the primary leukemia cells and SHI-1 cells. (Figure 3B) In addition, a probe for p53 gene labeled with digoxigenin and a centromere probe for chromosome 17 labeled with biotin were used to confirm the deletion of the p53 gene. The results showed that all of the metaphases and the interphases from the SHI-1 cell line presented a normal chromosome 17 with a green signal and a red signal, whereas only one red signal could be detected in the other chromosome 17 (Figure 3C). This means that deletion of p53 gene occurred in every cell of the SHI-1 cell line. Meanwhile, the deletion of a p53 gene could be detected in 32% of the primary leukemia cells.

M-FISH

M-FISH analysis was used to detect the chromosomal abnormalities of the SHI-1 cell line collected on March 3, 2003 passaged ten months after beginning the cultures. M-FISH confirmed the chromosomal abnormalities detected by conventional R-banded karyotyping such as t(6;11)(q27;q23), del(17)(p11) and -18. It also revealed that the der(7) resulted from a translocation between chromosomes 7 and 13. Moreover, 1 minute were found to derived from chromosome 8 in three out of ten metaphases analyzed. (Figure 4) The t(7;13) translocation was further confirmed by chromosome painting using WCP 7 and 13 in the SHI-1 cell line passaged 10 months later but not in the primary leukemic cells.

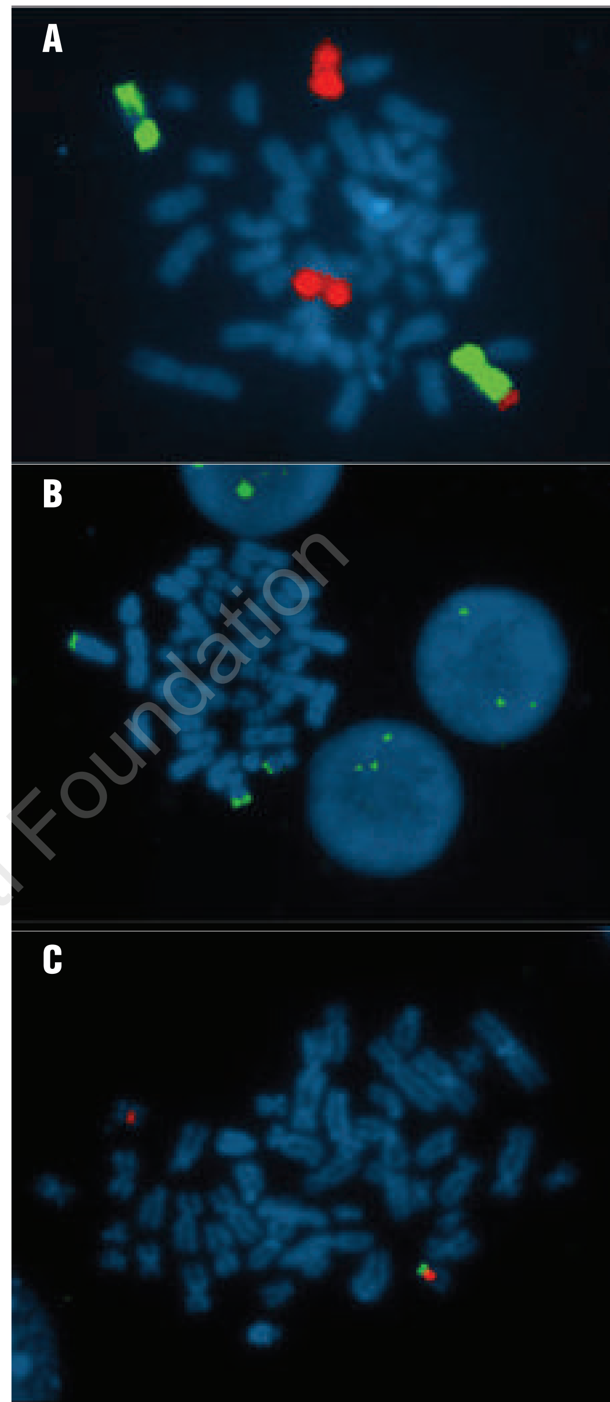

Figure 3. A. Chromosome painting using WCP 6 and 11 showing a red fluorescent signal on the terminal portion of the long arm of chromosome 6 with a green fluorescent signal, whereas no green signal was detected on the terminal of one chromosome 11 with a shorter red fluorescent signal than its homolog, indicating a translocation between chromosomes 6 and 11. **B.** A metaphase cell of SHI-1 cell line after FISH with a locus-specific probe for the *MLL* gene showing three green fluorescent signals on three chromosomes indicating rearrangement of the *MLL* gene. **C.** A metaphase cell of SHI-1 cells after FISH with a locus-specific probe for the p53 gene and a centromeric probe for chromosome 17 showing red and a green fluorescent signals on one chromosome 17 and a red fluorescent signal on another chromosome 17 with del(17)(p11), indicating the loss of one p53 allele.

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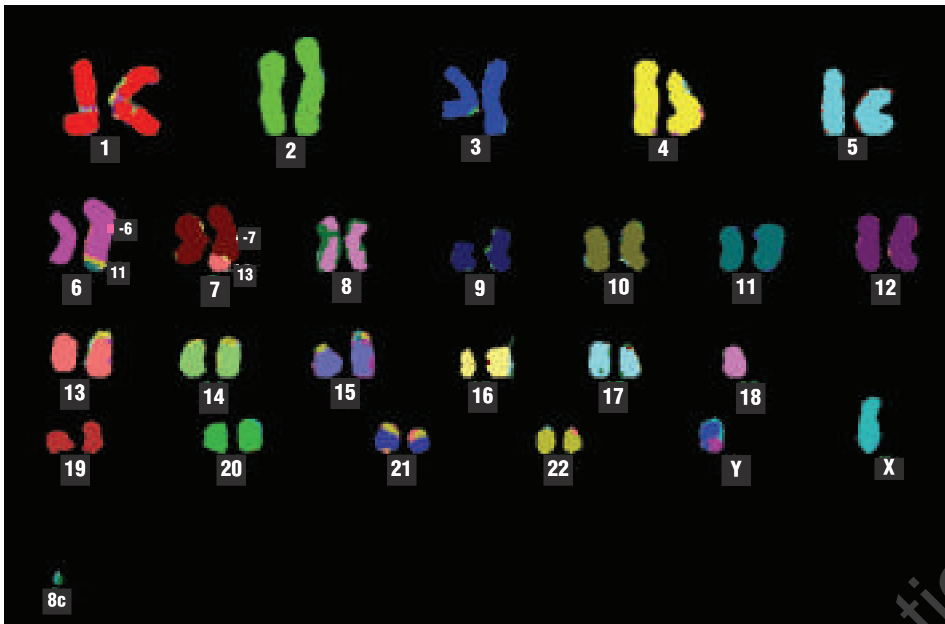

Figure 4. Karyotype analysis by M-FISH showing t(6;11)(q27;q23),der(7) t(7;13), del(17)(p11), monosomy 18, and a minute originating from chromosome 8.

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RT-PCR

RT-PCR using *MLL* and *AF6* sequence-specific primers was performed on total RNA from the primary leukemia cells and SHI-1 cells. Two alternatively spliced fusion mRNA could be detected in both of them (Figure 5). As previously reported, there was a product of 535-bp and another product of 461-bp corresponding to the two splicing types of the *MLL-AF6* fusion transcript (*MLL* exon 6/*AF6* exon 2 and *MLL* exon 5/AF6 exon 2).[9] Sequence analysis showed that the 535-bp PCR product was generated by fusion of *MLL* exon 6 to *AF6* exon 2 (*data not shown*). Multiplex RT-PCR revealed the *MLL-AF6* fusion transcript, but no *MLL* partial tandem duplication was detected.

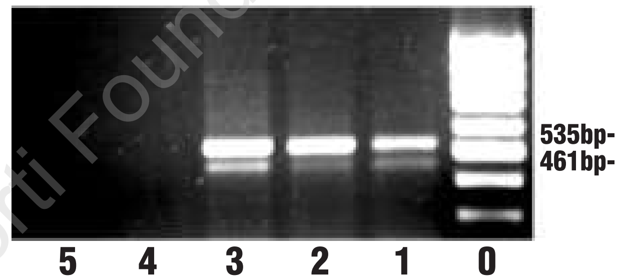

Figure 5. RT-PCR analysis of RNA from SHI-1 cells, showing two splicing types of *MLL-AF6* fusion transcript (535 bp and 461 bp, respectively). Lane 1, the primary leukemia cells; lane 2, SHI-1 cell line; lane 3, mononuclear cells of nude mice; lane 4, normal control from healthy individual; lane 5, ddH$_2$O.

Direct sequencing

As shown in Figure 6, sequence analysis of the DNA fragments amplified by PCR showed the conversion of T to C in exon 6 that changed codon 195 from ATC to ACC (Figure 6). This point mutation results in the substitution of isoleucine for threonine in the p53 protein.

Detection of EBV and mycoplasma

EBV genomic DNA was not detected by quantitative fluorescent PCR. The DNA fluorochrome staining and microbiological colony assay did not reveal mycoplasma contamination.

Authentication of the cell line

STR-PCR showed complete correspondence of the allele detected at the informative loci in the cell line with that in the primary leukemia cells, thus providing direct evidence for the derivation of the SHI-1 cell line from the leukemia cells of this patient.

Clonogenic assay

After incubation for 7 days, 467.5±30.4 colonies were counted under an inverted microscope. The colony formation rate was 33.4%. The appearance of the colonies was very loose.

Drug sensitivity and expression of multiple multidrug resistant related proteins

The results of MTT showed that the SHI-1 cell line was resistant to numerous drugs, especially etoposide. Flow cytomeetry revealed that the SHI-1 cell line was positive for lung resistance protein (92.86%, relative fluorescence intensity [RFI] 8.00) and GST-π (93.52%, RFI 6.91), but negative for P-glycoprotein (10.78%, RFI 1.02), multidrug resistant related protein (4.54%, RFI 1.38), and breast cancer related protein (3.84%, RFI 1.06).

Tumorigenicity in nude mice

Tumor masses were found in all sixteen mice injected with the SHI-1 cell line after 9-19 days. The sizes of the tumor masses ranged from 0.9×1.0 cm^2 to 1.5×1.8 cm^2. Conventional histopathology examination showed that the tumor masses were composed of leukemic cells in which blood vessels and areas of necrosis could be found. Chromosome analysis with R-bands was performed on mononuclear cells isolated from the tumor masses and revealed a karyotype of 46,XY,t(6;11)(q27;q23),del(17)(p11), with the same abnormalities as the SHI-1 cell line had (*data not shown*). The *MLL-AF6* fusion transcript was also detected. The *VEGF* gene was expressed in the SHI-1 cell line and the mononuclear cells isolated from the tumor masses (*data not shown*).

Zymography

Gelatin zymography analysis showed that the levels of expression of MMP-2 and MMP-9 in the supernatant of the SHI-1 cell line were significantly higher than those of the U937, K562, and NB4 cell lines (Figure 7).

Discussion

In the present study we describe a new cell line, SHI-1, established from a patient with AML M5b which was demonstrated by morphological, cytochemical and immunological characteristics such as monoblasts and promonocytes accounting for 89% of nucleated cells in the BM, negative myeloperoxidase staining for 95% of the blasts and positive expression of cell surface antigens for myeloid and monocytic lineages (CD13, CD14 and CD33). This patient was unique in that he only had a translocation t(6;11)(q27;q23) at presentation, while in the first relapse karyotypic evolution took place: 36.3% of metaphases had del(17)(p11) in addition to the t(6;11)(q27;q23) translocation. Chromosome painting and FISH demonstrated t(6;11), *MLL* rearrangement and loss of one p53 allele. RT-PCR revealed the *MLL-AF6* fusion transcript but no *MLL* partial tandem duplication was detected. Direct sequencing showed one point mutation of the other p53 allele in the sample taken at presentation and in the SHI-1 cell line.

Chromosomal translocations targeting the *MLL* gene at 11q23 have become a paradigm in acute leukemia.[1,13] These translocations result in an in-frame joining of the *MLL* gene with a partner gene to generate altered *HOX* gene expression, leading to leukemogenesis.[1,14] More than 30 partner genes have been reported to participate with *MLL* in the 60 known 11q23 translocations.[1,13] Among these, the t(6;11)(q27;q23), resulting in the *MLL-AF6* fusion gene represents 5% of 11q23 cases, which are mainly AML-M4 and AML-M5.[15] These forms of leukemia with t(6;11) translocation have a poor prognosis with an average survival of 12 months.[15] Besides the *MLL-AF6* fusion gene, our patient had a p53 gene mutation with 17p monosomy. This is a relatively unusual finding in *de novo* AML, although it is more frequent in solid tumors.[16,17] p53 is a nuclear phosphoprotein located at 17p13.1. It is related to important biological functions of cells such as regulation of the cell cycle, DNA repair, cell differentiation and cell apoptosis; p53 is also known as a tumor suppressor gene.[18] Thus mutation of

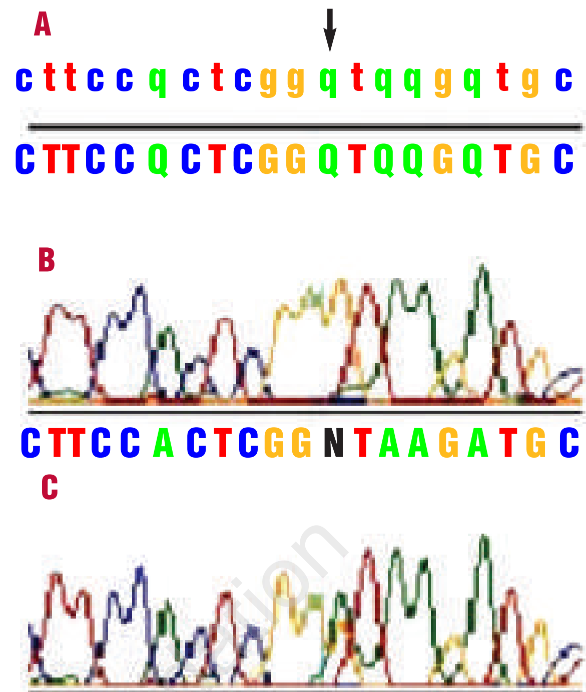

Figure 6. Direct sequence analysis of the PCR products showed the conversion of T to C in exon 6 that changed codon 195 from ATC to ACC. A. Normal p53 gene (antisense). The arrows indicate the position of base mutations; B. SHI-1 cell line (antisense); C: primary leukemia cells at presentation (antisense).

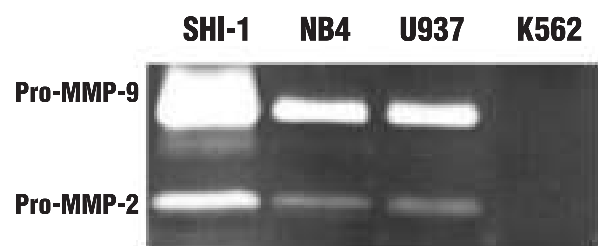

Figure 7. The result of gelatin zymography analysis showed that the SHI-1 cell line had significantly higher expression of MMP-2 and MMP-9 than did the U937, K562 and NB4 cell lines. 92KD corresponds to pro-MMP-9, 72 KD corresponds to pro-MMP-2.

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Table 3. Synopsis of data on the line SHI-1 cell line.

Parameter	*SHI-1*
Clinical data	
Patient	37-year old male
Diagnosis	AML M5b
Treatment status	At first relapse
Specimen	BM
Year of establishment	2002
Culture characterization	
Establishment	In 25 cm^2 flasks
Culture medium	IMDM+15% FCS+20% 5637 CM (the latter was withdrawn three months after beginning the cultures)
Growth pattern	Single cells in suspension
Doubling time	36h
Optimal cell density	1×10^6cells/mL
Optimal split	1:3 every 3-4 days
Cryopreservation	In 70% medium, 20% FCS, 10% DMSO
Morphology	Monoblasts and promonocytes
Viral status	Negative for EBV
Contamination	Negative for mycoplasma
Authentication	Yes (by DNA finger printing, cytogenetic characteristics, immunoprofile, and by p53 alterations)
Immunoprofiles	
Myelocytic	CD13+, CD15+, CD33+, MPO+
Monocytic	CD14+
Megakaryocytic	CD41+, CD42b-, CD61+
T/B cell	Negative except for CD4+
Adhesion	CD11b+
Progenitor/activation	CD25+, CD38+
Cytokine receptors	CD116+
Genetic characterization	
Cytogenetic profile (karyotypic analysis in conjunction with FISH and M-FISH)	46,XY,t(6;11)(q27;q23),del(17)(p11) before March 2003 45,XY,t(6;11)(q27;q23),der(7)t(7;13), del(17)(p11),-18,with or without minute after March 2003
Molecular profile	MLL/AF6 fusion transcript The point mutation (T?C in exon 5) of one p53 allele associated with loss of the other p53 allele
Functional characterization	
Clonality	The colony formation rate was 33.4%.
Expression of gelatin Zymes than other leukemia cell lines	Higher expression levels of MMP-2 and MMP-9 (NB4, U937 and K562)
Tumorigenicity in nude mice	Tumor masses in 16/16 nude mice
Scientific significance	(i) model for *MLL/AF6*-associated leukemogenesis (ii) model for p53-associated leukemogenesis (iii) model for inducing differentiation and apoptosis by As_2O_3 and TB (iv) model for tumorigenicity

Availability of cell line: on request to Dr. Yongquan Xue, Jiangsu Institute of Hematology, First Affiliated Hospital of Soochow University, 296 Shizi Street, Suzhou, 215006, PR China. Fax: 0086-512-65192662. E-mail: uujihsmc@publicl.sz.js.cn

one p53 gene coupled with loss of the other p53 allele, leading to inactivation of both p53 alleles is considered to play a role in oncogenesis.[19] Following development of del(17)(p11), this patient became resistant to combination chemotherapy and showed no response to a PB stem cell transplant. He then succumbed to a second leukemia relapse. These facts suggest advanced disease, probably resulting from a multistep process and the occurrence of several genetic events. p53 gene alterations could be one of the genetic events leading to tumor progression.[20] The SHI-1 cell line has been grown in culture for 3 years as single cells in suspension with a doubling time of 36 h. It proliferates autonomously and shows intensive colony formation ability. SHI-1 cells are negative for EBV virus and mycoplasma. The main features of this cell line have remained stable during the culture period and after freezing and thawing. Thus, SHI-1 represents a continuous cell line.[2] Essentially it has the same morphologic, immunologic, cytogenetic and molecular characteristics as the primary leukemia cells, with minor evolutions. The morphologic findings of SHI-1 were compatible with monoblasts and promonocytes. SHI-1 cells show positive expression of myeloid and monocytic antigens such as CD13, CD14, CD15 and CD33. They also show positive expression for a few markers of megakaryocytic (CD41 and CD61) and T lymphocytic lineages (CD4, CD5 and CD25) to a relatively small degree. SHI-1 cells have the identical karyotypic abnormalities, consisting of t(6;11)(q27;q23) and del(17)(p11), as those shown by the primary leukemia cells at the first relapse. Similarly, the *MLL-AF6* fusion transcript, mutation of one p53 allele associated with loss of the other p53 allele were also proven in both SHI-1 cells and the primary leukemia cells by RT-PCR and FISH.

After March 2003, SHI-1 cells showed karyotypic evolution from 46,XY,t(6;11)(q27;q23), del(17)(p11) to 46,XY,t(6;11)(q27;q23),der(7)t(7;13),del(17)(p11),-18,minute coming from chromosome 8, as proven by M-FISH and chromosome painting. In view of these facts we consider the SHI-1 cell line originated from the primary leukemia cells of our patient. More importantly, STR-PCR afforded powerful evidence for authenticating this cell line. Thus cell line cross-contamination could be definitively ruled out.

To our knowledge, only two cell lines with t(6;11)(q27;q23), the ML-2 cell line and the CTS cell line, have been established up to now.[21-23] The ML-2 cell line was established from a patient with AML M4 that developed after complete remission of T-cell lymphoma.[21] The ML-2 cells had no normal *MLL* gene on Southern blot analysis, indicating that an intact *MLL* gene is not necessary for the survival of leukemia cells.[22] The CTS cell line was established from a 13-year old girl suffering from AML-M1 in relapse.[23] CTS cells showed DNA rearrangement of the immunoglobulin heavy chain gene and the light κ chain gene, and deletions of the T-cell receptor δ 1 gene.[23] CTS cells showed no significant proliferation response to the cytokines, but were induced to differentiate to the T-cell, B-cell, erythroid or megakaryocytic lineage in the

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presence of particular cytokines.[23] Recently MLL partial tandem duplication has also been proven in the CTS cells.[24] Interestingly, this line had monosomy 17 in addition to t(6;11),[23] but, unfortunately direct sequencing was not done. Thus the possibility that there is co-existence of a mutation of the p53 gene cannot be ruled out. SHI-1 differs greatly from the ML-2 and CTS cell lines in the following aspects: (i) it has the p53 gene alterations mentioned above, which may endow SHI-1 cells with a growth advantage and is probably a prerequisite for the establishment of the SHI-1 cell line; (ii) it shows multidrug resistance, which may be associated with upregulation of the expression of the genes for LRP and GST-π; (iii) it shows high tumorigenicity in nude mice, which may be associated with the higher expression levels of MMP-2 and MMP-9 in the supernatant of the SHI-1 cell line. The salient features of the SHI-1 cell line and their scientific significance are summarized in Table 3. In summary, SHI-1 is a new acute monocytic leukemia-derived cell line with t(6;11)(q27;q23) translocation and p53 gene alterations. It has a clear biological background and provides a useful tool for leukemia research.

SC: contributed to the conception and design of the study, performed most of the experiments and data analysis, drafted the manuscript and corrected and approved its final version. YX: contributed to the conception and design of the study, writing, correction and approval of the submitted manuscript. XZ: participated in the conception and design of the study and approved the final version of the manuscript. YWu and JP: performed the cytogenetic analysis and approved the final version of the manuscript. YWa: performed the tumorigenicity studies in nude mice and approved the final version of the manuscript. JC: provided the guides for cell culture and the clonogenic assay and approved the final version of the manuscript. The authors declare that they have no potential conflicts of interest.

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