(1) to clarify the overall miRNA expression profile in these embryos/tissues and (2) to identify any changes in miRNA repertoire under pathological conditions. Expression of most miRNAs is known to be tightly regulated in a tissue- and development stage-dependent manner [10], further increasing the necessity to profile miRNA repertoire for a limited number of cells. mRAP was developed to enable such analyses, and we could indeed isolate candidates for novel miRNAs from early stages of embryos [10] or from fresh clinical specimens (data in submission). In addition, modification of mRAP may allow the identification of Piwi-interacting RNAs and other small RNAs which are slightly larger than miRNAs. mRAP and other emerging techniques for highly sensitive miRNA profiling should contribute to the complete understanding of RNA-mediated regulation of protein-coding genes or *vice versa*.

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Profiling of microRNA expression by mRAP

Shuji Takada[1] & Hiroyuki Mano[1,2]

[1]Division of Functional Genomics, Jichi Medical University, 3311-1 Yakushiji, Shimotsukeshi, Tochigi 329-0498, Japan. [2]CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan. Correspondence should be addressed to H.M. (hmano@jichi.ac.jp).

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MicroRNA (miRNA) amplification profiling (mRAP) is a sensitive method for the determination of miRNA expression profiles. The method relies on a long, optimized 5′ adaptor and the SMART (switching mechanism at the 5′ end of RNA templates of reverse transcriptase) reaction to yield miRNA-derived cDNAs flanked by synthesized oligomers at each end. The cDNAs are PCR-amplified with primers corresponding to the oligomers, and the products are concatamerized for nucleotide sequencing. The expression level of each miRNA can be estimated from the frequency of the occurrence of its sequence in the data set, provided that sufficient clones of the cDNAs are sequenced. This method potentially yields millions of miRNA-derived clones from as few as 1 × 10^4 cells, thus allowing the characterization of miRNA expression profiles with small quantities of starting material such as those available for fresh clinical specimens or organs of developing embryos. This protocol can be completed in 10 d.

INTRODUCTION

MicroRNAs (miRNAs) are small noncoding RNAs (20–24 nt) that either trigger the degradation or suppress the translation of target mRNAs through incomplete base-paring to their 3′-untranslated regions[1–4]. The transcription of miRNA genes in the nucleus, which is likely mediated by RNA polymerase II, produces long primary transcripts (pri-miRNAs)[5] that are subsequently processed to ~70-nt precursors with hairpin structures (pre-miRNAs) by an RNase III enzyme termed Drosha[6]. The pre-miRNAs are then exported from the nucleus to the cytoplasm, where they are further processed into mature miRNAs by the RNase III enzyme Dicer[7,8].

Many miRNAs are evolutionarily conserved across widely diverse phyla of eukaryotes and are therefore thought to be functionally important for the development of such organisms or for the growth or differentiation of their constituent cells[9–12]. Indeed, null mutations in the Dicer gene alter developmental timing in *Caenorhabditis elegans*[13,14] and *Drosophila*[7], and Dicer-deficient mice die during embryonic development[15,16]. Biological roles have recently been identified for a few miRNAs. The miRNA lin-4 thus controls the timing of larval development in *C. elegans*[17,18], and mir-1 plays an important role in heart development and physiology in mice[19,20]. Furthermore, dysregulation of the expression of certain miRNAs has been associated with human diseases including cancer. The miRNA cluster miR-17-92, for example, is often overexpressed in B-cell lymphomas[21] and lung cancer[22]. This miRNA polycistron may function as a human oncogene and is referred to as oncomiR-1 (ref. 21). These observations indicate that the ability to monitor changes in miRNA expression levels will be important for deciphering the molecular pathogenesis of a wide range of human disorders. Such monitoring will need to be effective with the small quantities of fresh clinical specimens that are usually available.

Profiling of miRNA expression

The ideal miRNA profiling method should fulfill several requirements: It should be (i) sensitive enough to determine miRNA profiles even with small amounts of starting material; (ii) easy to perform and not require equipment or reagents not readily available in a conventional molecular biology laboratory; (iii) capable of processing multiple samples in parallel; (iv) able to reproducibly detect a 1-nt difference between miRNAs and (v) capable of identifying novel miRNA candidates.

Several methods are currently available for miRNA expression profiling. The most widely adopted method is based on an miRNA cloning approach described by Lagos-Quintana *et al.*[23]. In brief, size-selected small RNAs are ligated consecutively to 3′- and 5′-adaptor oligonucleotides, and the RNA sequences are then amplified by reverse transcription (RT) and the PCR with primers that target the adaptor sequences. In another method described by Lee and Ambros[11], 'Achimera' (3′p-rA_6A_{24}), where rA and A represent the A ribonucleotide and A deoxyribonucleotide, respectively, is ligated to the 3′-terminus of size-selected small RNAs, which are then amplified by RT-PCR coupled with the SMART (switching mechanism at the 5′-end of RNA templates of reverse transcriptase) reaction. Both of these miRNA expression profiling methods fulfill the requirements described above with the exception of the sensitivity requirement. Both methods thus require > 100 μg of total RNA as the starting material.

Cummins *et al.* have developed a high-throughput profiling method, miRNA serial analysis of gene expression (miRAGE)[24], which also begins with ligation of a 3′ adaptor oligonucleotide to size-selected small RNAs but which subsequently follows the procedures of Long-SAGE and Digital Karyotyping. The advantage of miRAGE is that more sequence tags are obtained in a single sequence reaction (as many as 35 tags) compared with the number of small-RNA sequences obtained by other widely used methods (~5 tags per reaction). However, miRAGE requires 1 mg total RNA as a starting material.

Other strategies adopted to profile miRNA expression rely on massively parallel signature sequencing (MPSS)[25] technology, microarrays[26–32], bead-based flow cytometry[33] or stem–loop RT-PCR analysis[34]. The latter three of these strategies are sensitive; the stem–loop RT-PCR analysis offers the highest sensitivity, and can even profile miRNA from a single cell[35], though this method does require prior knowledge of the miRNA sequences for analysis. The MPSS-based method offers the highest throughput capacity for profiling available to date; however, it requires 20 ng size-selected small RNAs as a starting material and an as yet uncommon sequencing instrument[25].

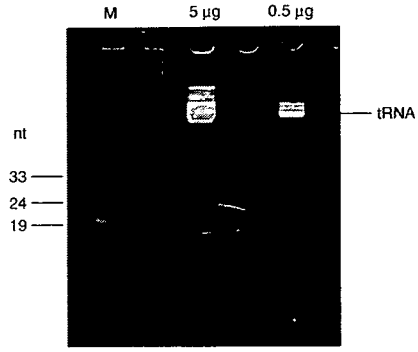

Figure 1 | Example of size selection of small RNAs. A fraction of Jurkat cell RNA consisting of molecules of <200 nt (5 or 0.5 µg) was subjected to denaturing PAGE on a 15% gel. The portion of the gel between the positions corresponding to 19- and 24-nt markers was excised, and the remaining gel was stained with SYBR Green II. The positions of RNA size markers (lane M) and tRNA are indicated on the left and right of the gel, respectively.

miRNA expression profiling by miRNA amplification profiling

To overcome the limitations of existing methods for miRNA expression profiling, we developed an approach, termed miRNA amplification profiling (mRAP)[36,37] that relies on the traditional miRNA cloning approach[23] but with substitution of the SMART reaction for the ligation of a synthetic primer to the 5′-end of small RNAs. Given that simple application of the SMART reaction to isolation of small RNA-derived cDNAs was not successful for small quantities of starting material, we designed and optimized a long, sophisticated 5′ adaptor for the SMART reaction to allow omission of steps (elution of nucleic acid from a polyacrylamide gel after 3′ and 5′ adaptor ligation) with a low recovery rate. In addition, the use of the optimized adaptor allows separation of the PCR-amplified fragments corresponding to miRNA sequences from the two major by-products of PCR by PAGE, thereby facilitating excision of the target fragments.

The mRAP method can be performed in a conventional molecular biology laboratory, readily allows the processing of multiple samples in parallel, is able to detect a 1-nt difference among miRNAs and is capable of identifying both new miRNAs and mutations in known miRNAs. The major advantage of mRAP over its widely adopted competitors, however, is its sensitivity. We have previously cloned miRNA from as few as 1×10^4 cells of the human Jurkat T cell line[36], corresponding to ~100 ng total RNA. This sensitivity also allowed us to obtain miRNA expression profiles from a pool of mouse embryos at embryonic day 6 ($n = 14$) or embryonic day 7 ($n = 5$)[36]. While mRAP is a complex, multistep procedure, we still believe that mRAP may currently be the best choice for obtaining expression profiles for both known and unknown miRNAs or for identification of sequence alterations in miRNAs with small amounts of starting material.

Analysis of mRAP products with recently developed high-throughput sequencing systems such as pyrosequencing[38] would be a robust approach to extensively profile miRNAs in a given tissue. Although the mRAP method was originally developed for miRNA profiling, it could also be applied to the profiling of Piwi-interacting RNA (piRNA), repeat-associated small interfering RNA, small interfering RNA or other small RNAs. Indeed, we have succeeded in cloning piRNAs with this method (data not shown).

mRAP step by step

The mRAP method starts with the isolation of <200-nt RNA molecules. Concentration of the miRNA fraction is achieved by subjecting these RNA molecules of <200 nt to denaturing PAGE on a 15% gel and then eluting RNAs of defined size from the gel (**Fig. 1**). The resolution of size fractionation is usually higher with small RNAs of <200 nt as the starting material than with total RNA. Although we routinely use RNA molecules of <200 nt for this reason, it may be possible to use total RNA as a starting material for mRAP. The volume of the solution of RNA molecules eluted from the gel is reduced by 2-butanol extraction, and the RNAs are then precipitated with ethanol. The extraction step with 2-butanol is necessary because small RNA molecules are not precipitated efficiently from a large volume of solution.

The size-selected RNAs are then dephosphorylated to prevent their ligation to each other during the next step (**Fig. 2**). The dephosphorylated RNA is ligated with the 3′ adaptor (self-ligation of the 3′ adaptor cannot occur because its 3′-end is blocked with inverted deoxythymidine) and subjected to RT with the RT primer (**Fig. 2**). In this reaction, RT often adds a few C nucleotides at the 3′-end of the generated cDNAs as a result of its terminal transferase activity. The poly(C) overhang hybridizes with the 3′-end of the 5′ adaptor, and the enzyme extends the cDNA product through to the end of the 5′ adaptor according to the SMART reaction. The cDNAs thus obtained are subjected to PCR for amplification of the sequences derived from the size-selected RNAs.

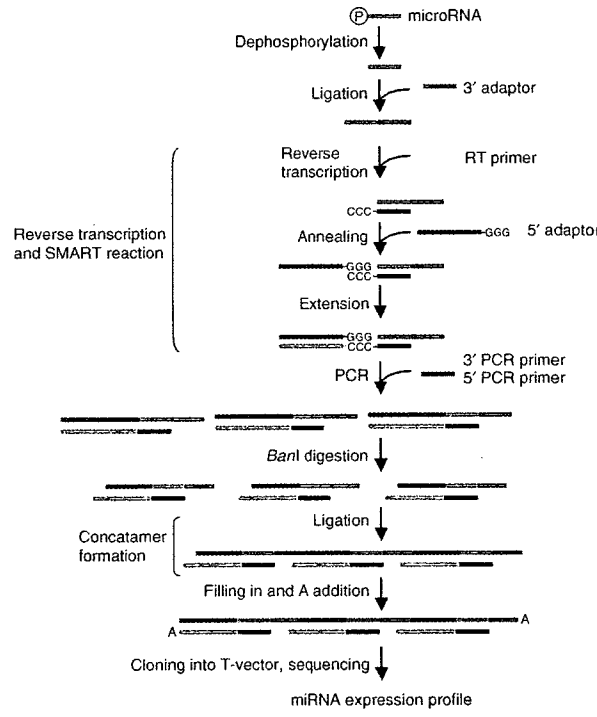

Figure 2 | Schematic representation of the reactions that constitute the microRNA (miRNA) amplification profiling (mRAP) method. MicroRNAs are dephosphorylated, ligated to the 3′ adaptor and subjected to reverse transcription (RT) and the SMART (switching mechanism at the 5′-end of RNA templates of reverse transcriptase) reaction. The resulting cDNAs are annealed with the 5′ adaptor and amplified by PCR. The amplification products are digested with *BanI* and concatamerized. The protruding ends of the products are filled in, and an A nucleotide is added to the 3′-ends of the concatamers for cloning into a T-vector.

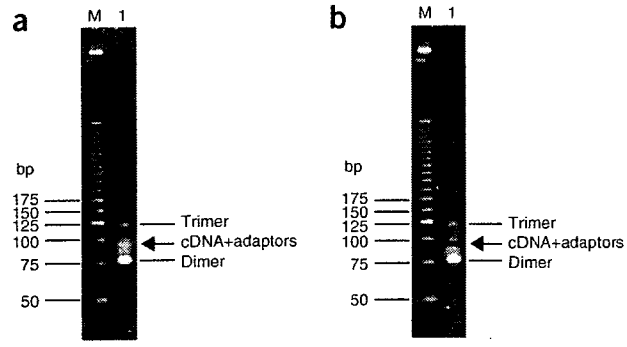

Figure 3 | Example of isolation of the cDNA fraction from the dimer and trimer by-products of PCR. (**a**) PCR products derived from small RNAs of Jurkat cells were subjected to PAGE on a 10% gel (lane 1). (**b**) The portion of the gel shown in (**a**) containing cDNAs (90–95 bp) was excised. The positions of size markers are shown at the left of the gel, and the identity of the bands is indicated at the right. Lane M contains a 25-bp DNA ladder.

Analysis of the amplification products by PAGE usually reveals three major bands of ~70, ~90 and ~120 bp (**Fig. 3**). The ~70-bp band corresponds to the 5′ adaptor–3′ adaptor dimer, a by-product of the SMART reaction. Given that the RT primer is complementary to the 3′ adaptor, they hybridize to each other and the SMART reaction may take place at the 3′-end of the RT primer without the involvement of an miRNA sequence (**Fig. 2**). The ~90-bp band corresponds to the miRNA-derived cDNAs (more precisely, if the miRNA is 22 nt, then the amplicon is 92 bp). Finally, the ~120-bp band corresponds to a 3′ adaptor–5′ adaptor–5′ adaptor trimer, another by-product of the SMART reaction that is synthesized from the first by-product and the 5′ adaptor. The SMART reaction may further take place with the ~120-bp product, yielding a by-product of ~170-bp. This latter product is usually not observed in the gel because its generation is less efficient than that of the trimer. The desired band of ~90 bp is thus observed between the two major by-products of ~120 and ~70 bp (**Fig. 3**).

The next step of mRAP is cloning of the ~90-bp DNA fragments (**Fig. 2**), which are first subjected to concatamer formation with the use of the restriction endonuclease *Ban*I. Given that nonpalindromic recognition sequences of *Ban*I are present in the 5′ and 3′ PCR primers, the concatamers are formed by directional ligation in a tandem manner. Cloning (TA) into commercially available T-vectors is facilitated by filling in of the termini of the concatamers and by the addition of an A nucleotide overhang at their 3′ ends by *Taq* DNA polymerase (**Fig. 2**). The concatamers thus generated are then size-fractionated by PAGE on a 10% gel to obtain products of >500 bp (**Fig. 4**). The size-selected concatamers are cloned into a T-vector, and the resulting plasmid library is the miRNA library.

We usually check the quality of the library by nucleotide sequencing of the cDNAs on a small scale. To this end, we transform *Escherichia coli* with a small volume of the library and purify plasmid DNA from 20 to 96 white colonies (color selection is based on the *lacZ*/*lacI*^{q} system of the T-vector) in order to determine insert size and the percentage of plasmids containing inserts by *Not*I digestion. If mRAP has been successful, 80–100% of plasmids should contain inserts of ≥500 bp. Nucleotide sequencing of the cDNAs and comparison of the resultant sequence information with public databases such as miRBase (http://microrna.sanger.ac.uk) reveal whether the library contains miRNAs (**Fig. 5**). Confirmation of the quality of the library can then be followed by large-scale sequencing of cDNAs and determination of the miRNA expression profile.

MATERIALS

REAGENTS

- Fresh or cryopreserved tissue or cells (or cell lines) derived from animals or possibly from plants ▲ **CRITICAL** RNA should be intact.
- mirVana miRNA isolation kit (Applied Biosystems, cat. no. AM1560) ! **CAUTION** Contains phenol, which is poisonous; contact with eyes and skin should be avoided. ▲ **CRITICAL** Other equivalent kits for preparation of <200-nt RNA can be used. Also total RNA can be used.
- Diethylpyrocarbonate-treated water (DEPC water; Sigma-Aldrich, cat. no. D5758) ! **CAUTION** Harmful if swallowed.
- SequaGel sequencing system (National Diagnostics, cat. no. EC-833), consisting of SequaGel concentrate, SequaGel diluent and SequaGel buffer ! **CAUTION** Contains acrylamide, which is poisonous and should be handled with care.
- SYBR Green II (Cambrex, cat. no. 50523)
- 2-Butanol (Wako, cat. no. 020-11215)
- Ethanol (Wako, cat. no. 057-00456)
- Chloroform (Wako, cat. no. 038-02606) ! **CAUTION** Toxic; should be handled with care.
- Phenol (Wako, cat. no. 160-12725) ! **CAUTION** Poisonous; contact with eyes and skin should be avoided.
- Glycogen (Roche Diagnostics, cat. no. 901393)
- Calf intestinal alkaline phosphatase (CIAP; New England Biolabs, cat. no. M0290S)
- T4 RNA ligase (New England Biolabs, cat. no. M0204S)
- Acetylated BSA (Invitrogen, cat. no. 15561020)
- ATP (Takara Bio, cat. no. 4041)
- DMSO (Sigma-Aldrich, cat. no. D5879)
- PowerScript reverse transcriptase (Clontech, cat. no. 639500)
- Deoxynucleoside triphosphates (dNTPs; GE Healthcare, cat. no. 28-4065-51)
- Ampli*Taq* Gold polymerase (Applied Biosystems, cat. no. N808-0244)
- Sodium acetate (Wako, cat. no. 192-01075)
- 10× Tris-borate-EDTA (TBE; Sigma-Aldrich, cat. no. T4415)
- Acrylamide (Bio-Rad, cat. no. 161-0101) ! **CAUTION** Poisonous; should be handled with care.
- *N,N*′-Methylene-bis-acrylamide (bis-acrylamide) ! **CAUTION** Poisonous; should be handled with care.
- Ammonium persulfate (APS; Wako, cat. no. 016-08021)
- Tetramethylethylenediamine (TEMED) ! **CAUTION** Poisonous; should be handled with care.
- 25-bp DNA ladder (Invitrogen, cat. no. 10597-011)

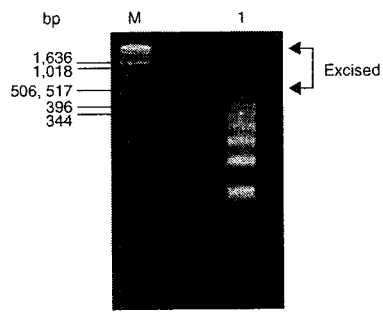

Figure 4 | Example of size selection of concatamers. The concatamers synthesized from Jurkat cDNA were subjected to PAGE on a 10% gel (lane 1). The portion of the gel containing products of >500 bp was excised on the basis of the positions of size markers, and the remaining gel was then stained with ethidium bromide (EtBr). The positions of size markers are shown at the left of the gel. Lane M contains a 1-kb DNA ladder.

- Ethidium bromide (EtBr; Sigma-Aldrich, cat. no. E8751) ! CAUTION Carcinogenic; should be handled with care.
- *Ban*I restriction endonuclease (New England Biolabs, cat. no. R0118S)
- *Not*I restriction endonuclease (Takara Bio, cat. no. 1166A)
- Ligation high (Toyobo, cat. no. LGK-101)
- BIOTAQ DNA polymerase (Bioline, cat. no. BIO-21040)
- Ammonium acetate (Wako, cat. no. 019-02835)
- 1-kb DNA ladder (Invitrogen, cat. no. 15615-016)
- pGEM-T Easy vector (Promega, cat. no. A1360)
- DH5alpha competent cells (Takara Bio, cat. no. 9057)
- 3′ adaptor: 5′-(Pu)uuAACCGCGAATTCCAG(idT)-3′ (lowercase letters indicate RNA, uppercase letters indicate DNA, Pu denotes 5′-phosphorylated Urd, and idT represents 3′-inverted deoxythymidine) (synthesized by Dharmacon)
- 5′ adaptor: 5′-GACCACGCGTATCGGGCACCACGTATGCTATCGAT CGTGAGATGGG-3′ (synthesized by Operon Biotechnologies)
- RT primer:5′-GACTAGCTGGAATTCGCGGTTAAA-3′ (synthesized by Operon Biotechnologies)
- 5′ PCR primer: 5′-GCGTATCGGGCACCACGTATGC-3′ (synthesized by Operon Biotechnologies)
- 3′ PCR primer: 5′-GACTAGCTTGGTGCCGAATTCGCGGTTAAA-3′ (synthesized by Operon Biotechnologies)
- 19-nt RNA oligomer: r(CGUACGCGGAAUACUUCGA)(synthesized by Dharmacon)
- 24-nt RNA oligomer: r(CGUACGCGGAAUACUUCGAAAUGU) (synthesized by Dharmacon)
- 33-nt RNA oligomer: r(CCAUCGAUAAAAAAUAUGGAGAGC UUCCCGAAG) (synthesized by Dharmacon)
- Small RNA markers (see REAGENT SETUP)

EQUIPMENT

- Vertical electrophoresis apparatus (Nihon Eido, cat. no. NA1113 or equivalent; System Instruments, cat. no. SE8010 or equivalent)
- Submarine electrophoresis apparatus (Nihon Eido, cat. no. NB1012 or equivalent)
- ProbeQuant G-50 micro columns (GE Healthcare, cat. no. 27-5335-01)
- D-Tube (Novagen, cat. no. 71504-3)

REAGENT SETUP

Small RNA markers Dissolve 10 μg each of 19-, 24- and 33-nt RNA oligomers in 100 μl DEPC water.

PROCEDURE

Casting the denaturing 15% polyacrylamide gel ○ TIMING ~1.5 h

1| Mix 15 ml SequaGel concentrate, 7.5 ml SequaGel diluent, 2.5 ml SequaGel buffer, 200 μl 10% APS and 10 μl TEMED for a gel with dimensions of 0.1 × 16 × 16 cm^3. Leave the gel at room temperature (18–24 °C) for >1 h to polymerize.

▲ CRITICAL STEP Freshly prepared 10% APS and TEMED should be added last, immediately before pouring the gel.

▲ CRITICAL STEP Steps 1–31 should be performed in a room or, at least, in a bench specialized for RNA handling to keep RNA samples away from contaminating RNase. In addition, reagents for Steps 1–31 should be maintained separate from those for subsequent steps.

Small-RNA preparation ○ TIMING ~1 h

2| Prepare RNA from fresh or cryopreserved tissue or cells (or cell lines) with the use of a mirVana miRNA isolation kit, following the manufacturer's instructions and using the small-RNA fraction option, which allows purification of small RNA molecules of <200 nt. A 100 μl sample is obtained.

3| Precipitate the RNA from the 100 μl sample obtained in Step 2 by adding 5 μl of 5 M NaCl and 250 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 80% ethanol before allowing it to air dry. Dissolve the RNA precipitate in 5 μl DEPC water. If the yield of RNA is thought to be >0.5 μg, determine the RNA concentration by measurement of absorbance at 260 nm. If the concentration of RNA is >1 μg μl^{-1}, then it should be adjusted to 1 μg μl^{-1} with DEPC water. When the concentration is <1 μg μl^{-1}, then proceed to the next steps.

Size selection of small RNAs ○ TIMING ~16 h

4| Subject the gel (Step 1) to a constant current of 40 mA for 10–30 min in 1× TBE.

5| Mix 5 μl RNA solution (<5 μg) and an equal volume of 2× gel loading buffer II (included in the mirVana microRNA isolation kit). Separately, mix 0.25 μl small RNA markers, 4.75 μl DEPC water and 5 μl of 2× gel loading buffer II.

6| Incubate sample and the markers separately at 90 °C for 20 s and then place them on ice.

7| After stopping the electrophoresis (initiated in Step 4), wash the wells of the gel with 1× TBE. Apply 10 μl of the sample and 5 μl of the markers to the gel separated by an empty lane and subject them to electrophoresis at a constant current of 40 mA for 1 h.

8| Separate the marker and sample lanes, then wrap the gel portion containing the sample in plastic film wrap.

GATTGCGTATCGGGCACCACGTATGCTATCGATCGTGAGATGGGTGTGCAAATCTATGCA
AAACTTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGTAGTGTTT
CCTACTTTATGGATTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGG
GTAGTGTTTCCTACTTTATGGATTTTAACCGCGAATTCGGCACCACGTATGCTATCGATC
GTGAGATGGGAGCTCAGCGGTTACTTCGACATTTAACCGCGAATTCGGCACCACGTATGC
TATCGATCGTGAGATGGGGTAGTGTTTCCTACTTTATGGATTTAACCGCGAATTCGGCAC
CACGTATGCTATCGATCGTGAGATGGGGGGAGCGGGCGGGCGGTCCGCCGCTTTAACCGC
GAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGGGTCGGGGCGGCGGCGGCGGC
GTTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGGAGTTAAAGAC
TTTTTCTCTGACCTTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGG
GTAGTGTTTCCTACTTTATGGATTTAACC

Figure 5 | Example of a nucleotide sequence of one insert obtained from Jurkat cells by the microRNA (miRNA) amplification profiling (mRAP) procedure. Red and green letters denote the cDNA sequences for miRNAs and other sequences such as ribosomal RNA, respectively. Black letters indicate the adaptor sequences, with *Ban*I sites underlined.

9| Stain the marker lane with SYBR Green II (1:10,000 dilution in water) for 5–10 min and wrap it in plastic film wrap. Photograph the stained gel portion aligned with a ruler.

10| Align the ruler with the gel portion containing the sample, and mark the plastic wrap at the positions corresponding to the 19- and 24-nt markers with a red pen.

11| Excise the piece of the gel containing RNA molecules of 19–24 nt.

12| Stain the remainder of the gel portion for confirmation of correct handling and to check the quality of the RNA preparation. Nothing should appear below the tRNA bands (**Fig. 1**).
? TROUBLESHOOTING

13| Chop the excised gel piece into small fragments and transfer them into a microcentrifuge tube.

14| Add 125 μl DEPC water to the tube and incubate at 4 °C overnight with gentle agitation.

15| Centrifuge the tube briefly, collect 100 μl of the water phase, and transfer it into another microcentrifuge tube.

16| Add 250 μl 2-butanol into the tube containing the 100 μl of the water phase (from Step 15), invert it several times and centrifuge it briefly.

17| Check the volume of the water phase; if it is >10 μl, then repeat Step 16 with addition of 25 μl 2-butanol.
▲ **CRITICAL STEP** Precipitation of a small amount of small RNAs requires that the volume of the solution be small.

18| Discard the 2-butanol (upper) phase.

19| Add 50 μl chloroform into the tube, invert it several times and centrifuge it at 18,000*g* for 1 min at room temperature. Transfer the supernatant into another microcentrifuge tube.

20| Precipitate RNA from the tube containing the supernatant from Step 19 by adding 0.3 μl glycogen (20 mg ml^{-1}), 0.5 μl of 5 M NaCl and 25 μl of ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 80% ethanol, before allowing it to air dry. Dissolve the RNA precipitate in 8.75 μl DEPC water.
□ **PAUSE POINT** The RNA solution can be stored at −80 °C for several weeks.

Dephosphorylation of size-selected RNA molecules ○ **TIMING 1 h**
21| Add 1 μl of 10× NEBuffer 3 (supplied with CIAP) and 0.25 μl CIAP to the sample and then incubate the mixture at 50 °C for 30 min.

22| Add 0.5 μl of 5 M NaCl and 10 μl of water-saturated phenol to the tube, invert it several times and centrifuge it at 18,000*g* for 5 min at room temperature. Transfer the supernatant to another microcentrifuge tube.

23| Perform chloroform extraction on the supernatant from Step 22, as described in Step 19, then precipitate RNA by adding 0.3 μl glycogen (20 mg ml^{-1}), 0.5 μl of 100 μM 3′ adaptor, 0.5 μl of 5 M NaCl and 25 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 80% ethanol, before allowing it to air dry. Dissolve the RNA precipitate in 3 μl DEPC water.
□ **PAUSE POINT** The RNA solution can be stored at −80 °C for several weeks.

Ligation of 3′ adaptor to RNA molecules ○ **TIMING 1.5 h**
24| Add 1 μl of 10× ligation buffer (supplied with T4 RNA ligase), 1 μl acetylated BSA (1 mg ml^{-1}), 1 μl of 1 mM ATP and 3 μl of 50% DMSO to the sample.

25| Incubate the mixture at 90 °C for 30 s and then on ice for 20 s.

26| Add 1 μl of T4 RNA ligase to the mixture and incubate it at 37 °C for 1 h.

27| Perform Steps 19, 20 and 22 consecutively but dissolve the final RNA precipitate in 4 μl DEPC water.
□ **PAUSE POINT** The sample can be stored at −80 °C for several weeks.

RT and the SMART reaction ○ **TIMING 1.5 h**
28| Add 0.5 μl of 100 μM RT primer and 0.5 μl of 100 μM 5′ adaptor to the sample. Incubate it at 70 °C for 2 min and then on ice.

29| Add 2 μl of 5× RT buffer (supplied with PowerScript reverse transcriptase), 1 μl of 0.1 M DTT (supplied with PowerScript reverse transcriptase), 1 μl of 10 mM dNTPs and 1 μl of PowerScript reverse transcriptase.

30| Incubate the mixture at 42 °C for 1 h.

31| Add 40 μl water, and then incubate the sample at 72 °C for 7 min to inactivate the enzyme. After that, add 160 μl water.
PAUSE POINT The sample can be stored at −20 °C for several months.

PCR amplification of small RNA-derived cDNAs ○ TIMING 4.5 h

32| Take 49 μl sample from Step 31 and add 1,510 μl water, 245 μl of 10× PCR buffer (supplied with AmpliTaq Gold), 245 μl of 2 mM dNTPs, 196 μl of 10 μM 5′ PCR primer, 196 μl of 10 μM 3′ PCR primer and 9.8 μl AmpliTaq Gold. Transfer 50 μl of the resulting mixture into a 0.5-ml tube.
▲ CRITICAL STEP The remaining sample can be stored at −20 °C.

33| Perform PCR with a condition of 95 °C for 4.5 min; 32 cycles of 95 °C for 30 s and 65 °C for 30 s; 72 °C for 5 min.
PAUSE POINT The PCR products can be stored at −20 °C for several months.

34| Subject the reaction mixture to ethanol precipitation by adding 240 μl of 3 M sodium acetate (pH 5.2) and 5 ml ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 70% ethanol, before allowing it to air dry.

35| Dissolve the precipitate in 60 μl of 2× loading buffer (supplied with *Not*I as 10× loading buffer).
PAUSE POINT The sample can be stored at −20 °C for several months.

Casting the 10% polyacrylamide gel ○ TIMING ~1.5 h

36| Mix 6.25 ml of 40% acrylamide mix (acrylamide:bis-acrylamide weight ratio of 29:1), 2.5 ml of 10× TBE, 16 ml water, 250 μl of 10% APS and 12.5 μl TEMED for a gel with dimensions of 0.1 × 16 × 16 cm^3. Leave the gel at room temperature for >1 h to polymerize.
▲ CRITICAL STEP The freshly prepared 10% APS and TEMED should be added last, immediately before pouring the gel.

Purification of PCR amplification products ○ TIMING 10 h

37| Subject the gel to a constant current of 40 mA for 10–30 min in 1× TBE.

38| Wash the wells of the gel with 1× TBE. Apply 10 μl sample (from Step 35) to each well (6 wells per sample to avoid over loading) as well as the 25-bp DNA ladder to separate wells. Perform electrophoresis at a constant current of 40 mA until the bromophenol blue dye reaches the bottom of the gel.

39| Stain the gel with EtBr, and cut out the portion of the gel from all six lanes containing 90- to 95-bp DNA molecules under long-wave ultraviolet illumination (365 nm is preferable, but 302 nm can also be used) (**Fig. 3**).
▲ CRITICAL STEP Do not include the upper and lower of the three major bands during excision of the gel portion.
? TROUBLESHOOTING

40| Chop the excised gel pieces collected from all six lanes into small fragments and transfer them into a microcentrifuge tube.

41| Add 200 μl of 0.3 M NaCl to the tube and incubate it at 37 °C for >8 h.

42| Centrifuge the tube briefly, collect the water phase and transfer it into another microcentrifuge tube.

43| Perform ethanol precipitation on the water phase from Step 42 by adding 0.3 μl glycogen (20 mg ml^{-1}) and 500 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 70% ethanol, before allowing it to air dry. Dissolve the DNA precipitate in 43 μl water.
PAUSE POINT The DNA solution can be stored at −20 °C for several months.

Concatamer formation ○ TIMING 8 h

44| Add 5 μl of 10× NEBuffer 4 (supplied with *Ban*I restriction endonuclease) and 2 μl *Ban*I (20 U μl^{-1}) to the sample.

45| Incubate the sample at 37 °C for 2 h.
PAUSE POINT The incubation can be extended up to 16 h.

46| Centrifuge a ProbeQuant G-50 micro column at 800*g* for 2 min and then transfer the column to another microcentrifuge tube. Apply the sample mixture to the column and centrifuge again at 800*g* for 2 min.

47| Add 7 μl of 3 M sodium acetate (pH 5.2) and 50 μl phenol (saturated with Tris-HCl (pH 8.0))–chloroform (1:1) to the eluate, invert the tube and centrifuge it at 18,000*g* for 5 min at room temperature. Transfer the supernatant to another microcentrifuge tube.

48| Perform chloroform extraction on the supernatant from Step 47, as described in Step 19, and ethanol precipitation by adding 0.3 μl glycogen (20 mg ml^{-1}) and 150 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 70% ethanol, before allowing it to air dry. Dissolve the DNA precipitate in 2 μl water.
PAUSE POINT The DNA solution can be stored at −20 °C for several months.

49| Add 2 μl ligation high to the sample.

50| Incubate the mixture at 16 °C for 4 h.
PAUSE POINT The incubation can be extended up to 16 h.

Filling in and adding A nucleotide to concatamers ○ TIMING 2.5 h
51| Prepare the following mixture (*Taq* mix) 20 min before the end of the ligation reaction: 76 μl water, 10 μl of 10× ammonium (NH_4) buffer (supplied with BIOTAQ DNA polymerase), 3 μl of 50 mM magnesium chloride ($MgCl_2$) (supplied with BIOTAQ DNA polymerase), 10 μl of 2 mM dNTPs and 1 μl BIOTAQ DNA polymerase.

52| Incubate the *Taq* mix at 95 °C for 10 min and then keep it at 72 °C.

53| Transfer the ligation mixture (from Step 50) to the *Taq* mix (from Step 52).

54| Incubate the resulting mixture at 72 °C for 30 min.

55| Perform ethanol precipitation by adding 15 μl of 10 M ammonium acetate and 250 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 70% ethanol, before allowing it to air dry. Dissolve the DNA precipitate in 5 μl of 2× loading buffer.
PAUSE POINT The DNA solution can be stored at −20 °C for several months.

Casting the 10% polyacrylamide gel ○ TIMING ~1.5 h
56| Mix 2.5 ml of 40% acrylamide mix (acrylamide:bis-acrylamide ratio of 29:1), 1 ml of 10× TBE, 6.4 ml water, 100 μl of 10% APS and 5 μl TEMED for a gel with dimensions of 0.1 × 8.5 × 7 cm^3. Leave the gel at room temperature for >1 h to polymerize.
▲ CRITICAL STEP The freshly prepared 10% APS and TEMED should be added last, immediately before pouring the gel.

Size selection of concatamers ○ TIMING 10 h
57| Subject the gel to a constant current of 20 mA for 10 min in 1× TBE.

58| Wash the wells of the gel with 1× TBE. Apply 5 μl sample prepared in Step 55 and a 1-kb DNA ladder to the gel. Perform electrophoresis at a constant current of 20 mA until the bromophenol blue reaches the middle of the gel.

59| Separate the marker and sample lanes and wrap the gel portion containing the sample in plastic film wrap.

60| Stain the marker lane with EtBr for 5–10 min, remove excess stain by incubation with 1× TBE, and wrap the gel portion in plastic film wrap. Photograph the stained gel portion aligned with a ruler.

61| Mark the plastic film containing the sample lane at the position corresponding to 500 bp with a red pen.

62| Excise the piece of the gel containing DNA molecules of >500 bp.

63| Stain the remaining piece of gel for confirmation of concatamer formation. If successful, a ladder should be observed in the region corresponding to <500 bp (**Fig. 4**).
? TROUBLESHOOTING

64| Chop the excised piece of gel and transfer the fragments to a D-Tube moistened with water. Fill The D-Tube with 1× TBE.

65| Transfer the assembly to a submarine electrophoresis apparatus filled with 1× TBE.

66| Perform electrophoresis at a constant voltage of 100 V for 4 h.

67| Harvest the solution and subject it to ethanol precipitation by adding 0.3 μl glycogen (20 mg ml^{-1}), 25 μl of 3 M sodium acetate (pH 5.2) and 500 μl ethanol, keep it on dry ice for 5 min, centrifuge it at 18,000*g* for 5 min at 4 °C, discard the supernatant and rinse the precipitate with 70% ethanol, before allowing it to air dry. Dissolve the DNA precipitate in 2.4 μl water.
PAUSE POINT The DNA solution can be stored at −20 °C for several months.

Cloning of the size-selected concatamers ○ **TIMING 4.5 h**

68| Add 0.6 μl pGEM-T Easy and 3 μl ligation high to 2.4 μl of the size-selected concatamers (from Step 67).

69| Incubate the mixture at 16 °C for 4 h. The resulting product is the miRNA library.

PAUSE POINT The incubation can be extended up to 16 h.

Examination of the quality of the library ○ **TIMING Depends on the number of plasmids to be analyzed, 4 d for 96 plasmids**

70| Transform DH5alpha with 0.5 μl library according to the manufacturer's instructions.

71| Prepare plasmid DNA from the transformants, and check the insert size by digesting the DNA with *Not*I restriction endonuclease. Determine the sequence of the inserts to verify whether miRNA sequences are included in the library (quality check).

? TROUBLESHOOTING

72| If the quality of the library is sufficient, proceed with large-scale sequencing.

73| From the sequence reads, determine the sequences of the 20–25 bp regions (in red/green on **Fig. 5**) between the sequences of the 5′- and 3′-PCR primers (adaptor sequences shown in black on **Fig. 5**), to obtain miRNA sequence and determine expression profiles.

○ **TIMING**

Steps 1–3, casting the denaturing 15% polyacrylamide gel and preparing RNA samples: ~2.5 h
Steps 4–20, size selection of small RNAs: ~16 h
Steps 21–23, dephosphorylation of size-selected RNA molecules: 1 h
Steps 24–27, ligation of 3′ adaptor to RNA molecules: 1.5 h
Steps 28–31, RT and the SMART reaction: 1.5 h
Steps 32–35, PCR amplification of small RNA-derived cDNAs: 4.5 h
Step 36, casting the 10% polyacrylamide gel: ~1.5 h
Steps 37–43, purification of PCR amplification products: 10 h
Steps 44–50, concatamer formation: 8 h
Steps 51–55, filling in and adding A nucleotide to concatamers: 2.5 h
Step 56, casting the 10% polyacrylamide gel: ~1.5 h
Steps 57–67, size selection of concatamers: 10 h
Steps 68 and 69, cloning of the size-selected concatamers: 4.5 h
Steps 70–73, examination of the quality of the library: depends on the number of plasmids to be analyzed, 4 d for 96 plasmids

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table

Step	Problem	Possible reasons	Solution
12	Smear is observed between tRNA and 24-nt position	RNA is damaged	Begin with fresh tissue or cells Use fresh reagents to ensure they are RNase free Treat electrophoresis apparatus with 0.4 N sodium hydroxide for 30 min to remove RNase
39	Only two bands of ~70 and ~120 bp and a smear between them are observed	The amount of starting material is small	This is a common problem when the amount of RNA used as a starting material is small or recovery rate of small RNA from a gel at Step 14 is low. Given that 90 to 95-bp products should exist within the smear, excise the corresponding region based on the positions of the size markers. This process may result in excision of a fraction of incorrect size, but it is safe also to cut out portions of the gel above and below this region. Treat the three pieces of gel in parallel in the subsequent steps. Sequencing will reveal which region contains molecules of the correct size
63	Ladder is not observed	Region of the gel cut out at Step 39 is too broad	Restart from Step 32 with care to excise the 90 to 95-bp region at Step 39

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solution
71	Only 5′ and 3′ adaptor sequences are obtained without any cDNA sequence	PCR by-products of ~70 and ~120 bp contaminate the region of the gel excised in Step 39	Given that the abundance of the adaptor dimer (~70-bp band) and trimer (~120-bp band) is much greater than that of the ~90-bp products, excise only the region of the gel corresponding to the 90 to 95-bp region, without inclusion of the dimer and trimer bands. Extending the electrophoresis time or use of a larger gel may increase the separation of the dimer and trimer from the ~90-bp products
		Ligation of small RNAs and 3′ adaptor does not work	To ensure that all reactions are working well, it is recommended to include 0.2 μg of 24-nt RNA oligomer at Step 5 in parallel with a sample. If the reaction is working, discrete 94-bp band can be observed at Step 39 and multiple sequences of 24-nt RNA oligomer are read by sequencing at Step 71
	Average length of the inserts or percentage of insert positive clones is less than desired	Small concatamer is recovered at Step 62. Recovery rate of PCR product at Step 41 or of the concatamer at Step 66 is poor	Restart from Step 32 using remaining sample of Step 31. To improve average insert length, extend the electrophoresis time at Step 58 or use of a larger gel may increase the separation of <500-bp concatamer. To improve the percentage of insert positive clones, two times scale of experiment can be performed from Step 32 to Step 43, then dissolve the precipitate at Step 43 with 43 μl of water in total. After that, proceed following steps as a single experiment scale

ANTICIPATED RESULTS

High-quality starting materials and successful handling result in recovery of 80–100% of plasmids containing inserts of >500 bp. Nucleotide sequencing typically reveals two to nine small-RNA sequences for each read. An example of such a sequence is shown in **Figure 5**. The authors recommend scientists to start with a control experiment where mRAP is conducted directly on a synthetic 24-nt RNA oligomer RNA. In such experiment, PCR amplification of miRNA-derived cDNAs should yield only the cDNA–adaptor complex at Step 39, but not other dimers or trimers in **Figure 3**. Another appropriate control experiment is to perform mRAP on a sequentially diluted small RNA samples isolated from the same cell line, as shown in our initial report for mRAP[36]. A small-scale sequencing of mRAP products from 1,000, 100 or 10 ng of starting RNAs will clarify whether mRAP has been acceptably performed.

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LETTER TO THE EDITOR

MicroRNA expression profiles of human leukemias

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MicroRNAs (miRNAs) are small noncoding RNAs of 20–24 nucleotides (nt) that negatively regulate the translation of target mRNAs through incomplete base-pairing with their 3′-untranslated regions.[1] Evidence indicates that miRNAs play an important role in the development of human cancers including leukemias, with one of the most well-characterized examples being association of miR-15a and miR-16a with chronic lymphocytic leukemia. Almost half of chronic lymphocytic leukemia patients harbor a chromosome deletion that encompasses 13q14, a region that includes the genes for miR-15a and miR-16a, and the abundance of these miRNAs is reduced in chronic lymphocytic leukemia cells with the chromosome deletion.[2] Several other miRNAs, such as miR-155 and miR-17-92, have also been implicated in the pathogenesis of lymphoma.[3] It is therefore important that the entire miRNA repertoire of clinical specimens be characterized and compared among various hematologic malignancies.

Reliable assessment of the global expression profiles of miRNAs, especially for the small amounts of clinical specimens available, is not straightforward, however. Microarray-based detection of miRNAs is prone to the generation of false-positive data that may result from mishybridization of probes, although improvements have recently been developed for this technology.[4] A large-scale cloning strategy would be an ideal approach to reliable estimation of the expression level of miRNAs, provided that a sufficient number of clones were to be analyzed. However, conventional methods for isolation of miRNAs require $>10\,\mu g$ of total RNA, which is not always obtainable from clinical specimens.

We recently developed a sensitive method, mRAP (micro RNA amplification profiling)[5] that readily allows the isolation of miRNA clones from $\leqslant 1 \times 10^4$ cells. To examine the miRNA expression profiles for leukemias with mRAP, we first purified $CD34^+$ cells from individuals ($n=12$) with *de novo* acute myeloid leukemia, acute myeloid leukemia secondary to myelodysplastic syndrome, acute lymphoid leukemia or biphenotypic acute leukemia (Table 1). Column affinity-chromatography to isolate $CD34^+$ cells yielded 10–50% of the input cells with a purity of $\geqslant 90\%$ as judged by flow cytometry (data not shown). As a normal control, we also purified a $CD34^+$ cell fraction from bone marrow mononuclear cells of a healthy volunteer. Then mRAP procedure was applied to 1.1×10^6–1.0×10^8 of the purified $CD34^+$ cells from each individual in order to obtain short RNA clones.

Sequencing and computer filtering[5] of the mRAP amplicons identified a total of 38 858 qualified reads for the 13 study subjects. BLAST analysis then isolated 32 867 reads that match the human genome sequence (ncbi 36 assembly), among which 2054 reads were mapped to transfer RNA genes, 2720 to ribosomal RNA genes and 9474 to repetitive sequences. From the remaining sequences, we identified 7191 reads corresponding to 143 independent known miRNAs (Supplementary Table 1). We further searched for candidate sequences corresponding to novel miRNAs whose surrounding genome sequences (of ~100 nt) potentially fold into a hairpin structure with a single notch. In this analysis, we did not exclude miRNA candidates that were not detected in the genomes of other species, given that some miRNAs are species-specific or have arisen recently during evolution.[6]

We isolated an unexpectedly large number ($n=170$) of independent candidates for novel miRNAs among 296 sequence reads (Supplementary Table 1, Supplementary Data). The proportion of reads for such novel candidate miRNAs among all miRNA reads ranged from 1.7 to 9.5% per sample (mean, 4.7%). Of the 170 candidates, 19 were identified in at least two samples, supporting the notion that they are *bona fide* miRNAs. The surrounding genome sequence for one such candidate (designated Hsj_376) is conserved among human, cow and hedgehog (Figure 1a). Hsj_376 was found in two acute myeloid leukemia samples (corresponding to a total of 52 reads) in our data set and folds into a single hairpin (Figure 1a). In contrast, we obtained only one read for a candidate miRNA (Hsj_41) whose surrounding genome sequence also folds into a single hairpin structure (Figure 1b). However, this read was independently identified in our experiments performed both in Japan and in the Netherlands. The nucleotide sequence of all the miRNA candidates and their flanking sequences are presented in Supplementary Data.

The genomic sequences for some of the candidate miRNAs mapped in the vicinity ($\leqslant 20$ kbp) of those for other miRNAs in the human genome. For example, the gene for one candidate (Hsj_360) and hsa-miR-560 are present on the long arm of chromosome 2 separated by a distance of ~1 kbp (Supplementary Figure 1). In this instance, the genome sequences for the two miRNAs are not conserved in other species, indicative of recent evolution.

Expression of some of the candidate miRNAs was confirmed by northern blot analysis with small RNA fractions isolated from

Table 1 Clinical characteristics of the study subjects

ID (no.)	*Age (years)*	*Sex*	*Sample origin*	*Disease*	*Karyotype*
3	64	M	PB	ALL	46,XY,t(9;22)
4	45	M	BM	AML (M4)	46,XY,inv(16)
7	78	F	BM	MDS-derived AML	46,XX
10	21	F	PB	AML (M0)	46,XX,t(9:15)
12	58	M	BM	AML (M2)	46,XY
32	43	M	BM	AML (M2)	46,XY,t(8;21)
33		M	PB	AML (M1)	46,XY
44	71	M	PB	MDS-derived AML	46,XY,t(8:21)
46	61	M	PB	AML (M2)	46,XY
47	61	M	BM	AML (M3)	46,XY,t(15;17)
48	29	M	PB	BAL	46,XY
49	58	M	PB	MDS-derived AML	46,XY

Abbreviations: ALL, acute myeloid leukemia; AML, acute lymphoid leukemia; BAL, biphenotypic acute leukemia; MDS, myelodysplastic syndrome; BM, bone marrow; F, female; M, male; PB, peripheral blood.

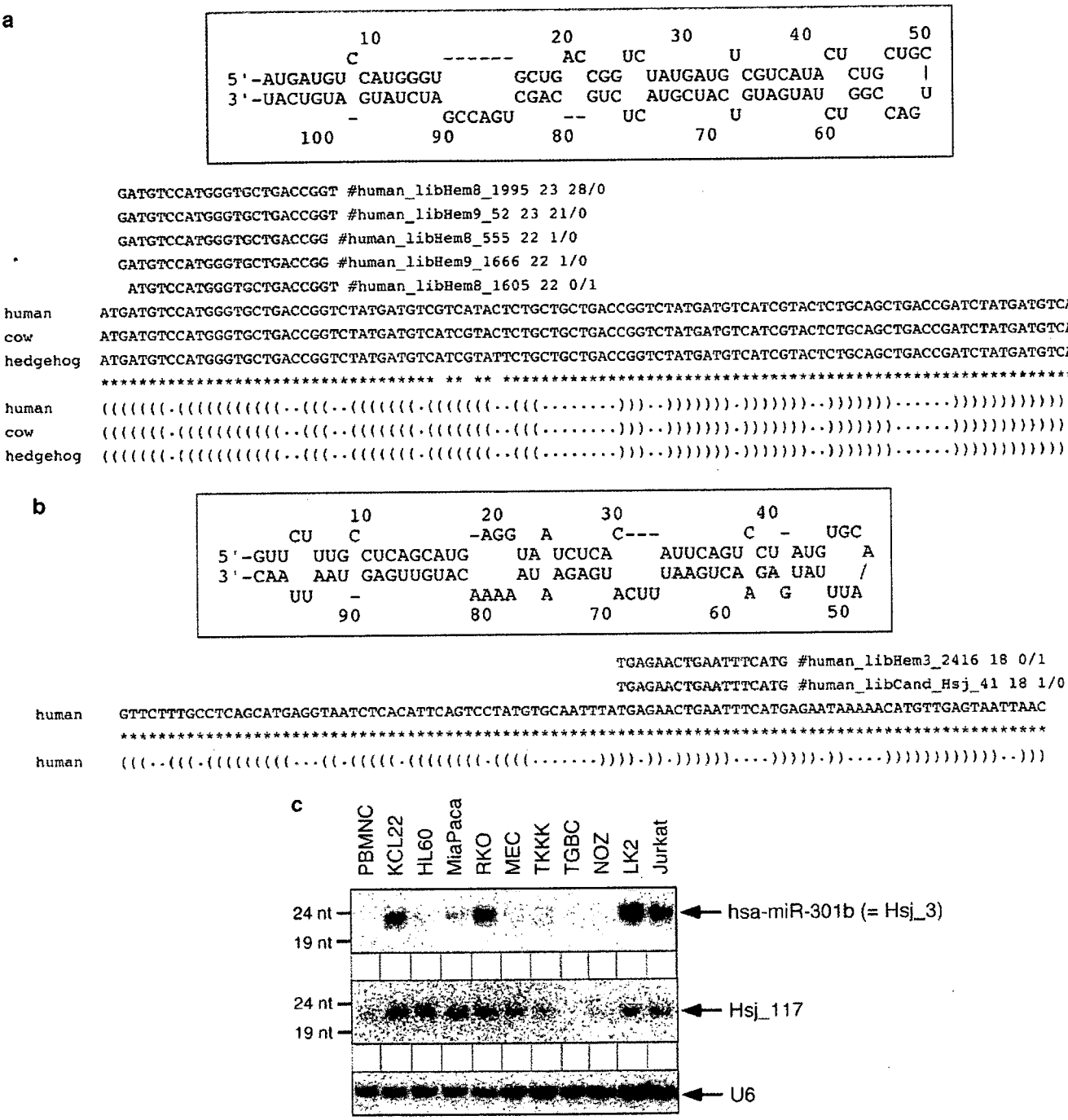

Figure 1 Nucleotide sequence and expression of novel miRNA candidates. Nucleotide sequences (red) of genes for the predicted novel miRNAs Hsj_376 (**a**) and Hsj_41 (**b**) are aligned with genomic sequences of human and other species. Asterisks indicate conserved nucleotides. Possible base-pairing schemes for the respective miRNA precursors are shown in the upper insets. (**c**) Small-RNA fractions (800 ng per lane) purified from the indicated cell lines with the use of a mirVana RNA isolation kit (Ambion, Austin, TX, USA) were subjected to northern blot analysis with 'locked' nucleic acid probes for the candidate miRNAs Hsj_3 or Hsj_117 or for U6 small nuclear RNA (internal control). Hjs_3 has been very recently deposited into the miRBase database as hsa-miR-301b. The positions of 24- and 19-nt size markers are indicated on the left. miRNA, microRNA.

a variety of human cancer cell lines, including KCL22 (chronic myeloid leukemia), HL60 (acute myeloid leukemia), MiaPaCa (pancreatic carcinoma), RKO (colorectal carcinoma), MEC (cholangiocarcinoma), TKKK (intrahepatic bile duct carcinoma), TGBC (gallbladder carcinoma), NOZ (gallbladder carcinoma), LK2 (lung squamous cell carcinoma) and Jurkat (T-cell leukemia) (Figure 1c).

The relative expression profile of miRNAs was then calculated for each sample as shown in Figure 2. Whereas some miRNAs, such as miR-124a, miR-142, miR-143 and miR-146a, were expressed in different types of leukemia, most miRNAs were expressed in a sample-specific manner. For instance, miR-29b was abundant in only two samples (ID nos. 46 and 47), with the reads for this miRNA accounting for <1% of all miRNA reads in

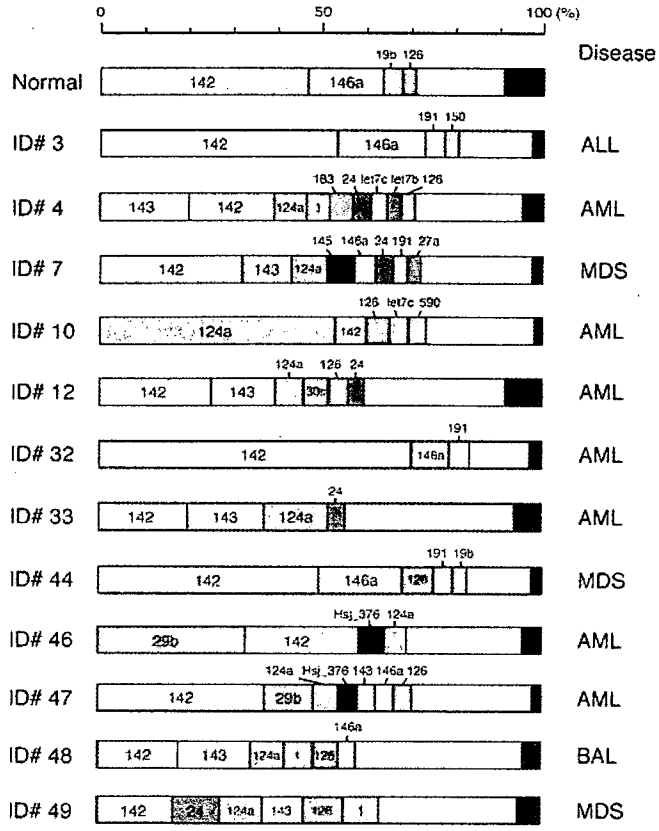

Figure 2 Expression profiles of miRNAs in CD34$^+$ specimens. The percentage contribution of each miRNA to the total miRNA population was calculated for each study subject. Abundant miRNAs are represented as color-coded, with candidates for novel miRNAs shown in red. The disease type of each individual is also indicated on the right. ALL, acute myeloid leukemia; AML, acute lymphoid leukemia; MDS, myelodysplastic syndrome; miRNA, microRNA.

each of the other specimens. Similarly, the novel miRNA candidate Hsj_376 was abundant in the same two samples but not in the others. Both hsa-miR-183 and hsa-miR-590 were detected in only single samples (ID nos. 4 10, respectively).

To examine further the similarities and differences in the miRNA profiles among the study subjects, we performed a hierarchical clustering analysis for the subjects based on the expression patterns of all known and novel miRNAs (Figure 3a). Leukemia specimens with a normal karyotype were clustered in the same branch, indicative of a relative homogeneity of these samples, at least with regard to miRNA expression. Nevertheless, the healthy volunteer was placed in a different branch, suggesting that leukemic blasts with a normal karyotype possess a miRNA profile distinct from that of nonleukemic CD34$^+$ cells with a normal karyotype.

We further attempted to identify miRNAs whose expression level was significantly linked to blast karyotype. Application of Student's *t*-test to the miRNA expression data with a Benjamini and Hochberg false discovery rate [7] of <0.05 resulted in the isolation of six miRNAs (hsa-miR-29c, hsa-miR-124a, hsa-miR-150, hsa-miR-183, hsa-miR-382 and hsa-miR-590). Hierarchical clustering of the study subjects based on the expression profiles of these 'karyotype-associated miRNAs' revealed that the healthy volunteer was again placed apart from the leukemic patients with a normal karyotype.

In conclusion, application of the mRAP procedure to CD34$^+$ leukemic blasts yielded 7487 reads for potential miRNA clones. We previously showed that mRAP readily allows the isolation of $>1\times10^6$ miRNA concatamers from $\leqslant1\times10^4$ cells and is thus suitable for miRNA profiling of clinical specimens.[5] Indeed, mRAP functioned well with the small number of purified specimens in the present study, with the result that sequencing capacity, rather than specimen quantity, is likely to be the limiting factor for the size of the final data set in most studies.

Although, in the present study, the total number of sequence reads per sample (average = 2989 reads) was not high, we were able to discover a relatively large number ($n=170$) of novel miRNA candidates from our sequence reads. Candidates for novel miRNAs continue to be identified, making it likely that the total number of human miRNAs has not yet reached saturation.[8] Our results show that CD34$^+$ leukemic blasts express a wider range of miRNAs than previously appreciated and that overall miRNA expression profiles generally reflect blast karyotype. Such karyotype-specific miRNAs may play a role in the malignant transformation of blasts of the corresponding karyotype, a possibility that needs to be confirmed by analysis of a large number of samples.

It is possible that some of the miRNA candidates identified in our study are not genuine miRNAs but rather degradation products of RNA or DNA. We believe, however, that a substantial proportion of the candidate miRNAs are indeed novel miRNAs because (i) many of them were identified in different samples in different laboratories (in Japan and in the Netherlands), (ii) many of them (together with the surrounding sequences in the genome) are conserved across various species and (iii) the expression of some of them was confirmed by northern blot analysis.

We have identified 170 novel miRNA candidates in, and demonstrated a high level of diversity in miRNA profiles among, leukemic blasts. Our data thus suggest that the miRNA repertoire of human leukemias has not yet been exhausted, and they should provide a framework for future studies in this regard.

Note added in proof

Hsj_117 and Hsj_360 have the miRBase accession numbers hsa-miR-590 and hsa-miR-663b, respectively.

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S Takada[1], Y Yamashita[1], E Berezikov[2], H Hatanaka[1], S-i Fujiwara[1], K Kurashina[1], H Watanabe[1], M Enomoto[1], M Soda[1], YL Choi[1] and H Mano[1,3]

[1]*Division of Functional Genomics, Jichi Medical University, Shimotsukeshi, Tochigi, Japan;*

[2]*Hubrecht Institute, Utrecht, The Netherlands and*

[3]*CREST, Japan Science and Technology Agency, Saitama, Japan*

E-mail: hmano@jichi.ac.jp

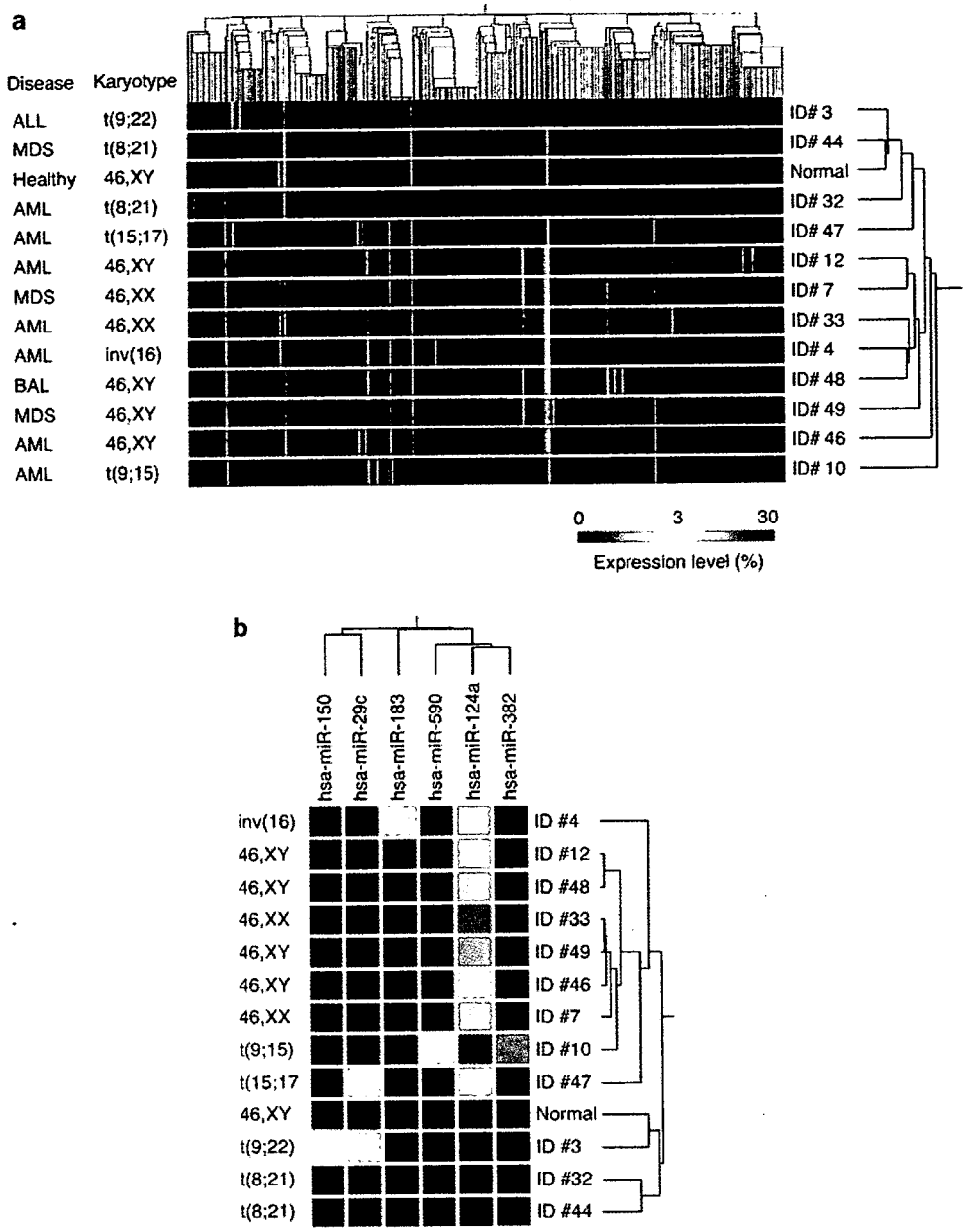

Figure 3 Hierarchical clustering of the study subjects based on miRNA expression profiles. (**a**) Subject tree generated by two-way clustering analysis with the expression profiles of all known and novel miRNAs. Each row corresponds to a separate sample, and each column to a miRNA whose expression is color-coded according to the indicated scale. The disease type and karyotype of each subject are shown at the left. (**b**) Six karyotype-associated miRNAs identified with Student's *t*-test and a false discovery rate of <0.05 were used for two-way clustering analysis as in (**a**). ALL, acute myeloid leukemia; AML, acute lymphoid leukemia; BAL, biphenotypic acute leukemia; MDS, myelodysplastic syndrome; miRNA, microRNA.

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ORIGINAL ARTICLE

Phase II study of weekly chemotherapy with paclitaxel and gemcitabine as second-line treatment for advanced non-small cell lung cancer after treatment with platinum-based chemotherapy

Kiyoshi Mori · Yukari Kamiyama · Tetsuro Kondo · Yasuhiko Kano · Tetsuro Kodama

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Abstract
Purpose We evaluated the tolerability and activity of the combination of weekly paclitaxel (PTX) and gemcitabine (GEM) in second-line treatment of advanced non-small cell lung cancer (NSCLC) after treatment with platinum-based chemotherapy.
Patients and methods PTX (100 mg/m^2) and GEM (1,000 mg/m^2) were administered to patients with previous treated NSCLC on days 1 and 8 every 3 weeks.
Results A total of 40 patients (performance status 0/1/2, 7/27/6 pts) were enrolled. The response rate was 32.5% (95% confidence interval: 18.0–47.0%). The median survival time was 41.7 weeks (95% confidence interval: 28.5–54.7 weeks). The median time to disease progression was 19 weeks. Hematological toxicities (grade 3 or 4) observed included neutropenia in 60%, anemia in 15%, and thrombocytopenia in 12.5% of patients. Non-hematological toxicities were mild, with the exception of grade 3 diarrhea, pneumonitis, and rash in one patient each. There were no deaths due to toxicity.
Conclusion The combination of weekly PTX and GEM is a feasible, well-tolerated, and active means of second-line treatment of advanced NSCLC.

Keywords Non-small cell lung cancer · Second-line chemotherapy · Weekly chemotherapy · Gemcitabine · Paclitaxel

K. Mori (✉) · Y. Kamiyama · T. Kondo · Y. Kano · T. Kodama
Department of Thoracic Diseases, Tochigi Cancer Center, 4-9-13, Yonan, Utsunomiya, Tochigi 320-0834, Japan
e-mail: kmori@tcc.pref.tochigi.jp

Y. Kamiyama
e-mail: ykamiyam@tcc.pref.tochigi.jp

T. Kondo
e-mail: tkondo@tcc.pref.tochigi.jp

Y. Kano
e-mail: ykano@tcc.pref.tochigi.jp

T. Kodama
e-mail: tkodama@tcc.pref.tochigi.jp

Introduction

The clinical usefulness of second-line chemotherapy has been established for cases of advanced non-small cell lung cancer (NSCLC) in which tumor has recurred or exhibits resistance to treatment after first-line chemotherapy. The effectiveness of docetaxel, pemetrexed, and elrotinib for second-line chemotherapy for NSCLC has been demonstrated in phase III clinical studies [13, 23, 24]. Furthermore, paclitaxel (PTX) and gemcitabine (GEM) have been shown to be effective against NSCLC resistant to platinum preparations [5, 16, 20]. There appears to be partial non-cross-resistance between these drugs and platinum preparations.

In previous attempts at second-line chemotherapy for NSCLC, the response rate was 0–38% for patients treated with PTX alone at intervals of 3 weeks [12, 21, 25] and 8–37.5% for patients treated with low-dose weekly PTX therapy [5, 16, 26, 28]. On the other hand, the rate of response to uncombined GEM therapy was 6–21% [7, 11, 17, 20, 22].

In combined PTX and GEM therapy, the two drugs exhibit interactions with each other but no overlap or synergism of adverse reactions. When this combined

regimen was applied to previously untreated patients with NSCLC, the response rate was high, at 29–46% [1, 3, 4, 8, 15, 18]. When a combination of PTX (administered every 3 weeks) and GEM was used for second-line chemotherapy, the response rate was either 18 or 39% [2, 14].

Weekly chemotherapy for lung cancer has recently been attempted at several facilities [3, 9]. Favorable results of weekly chemotherapy have also been reported for recurrent NSCLC [5, 16, 26, 28]. Compared to standard regimens of chemotherapy, with administration of drugs at intervals of 3–4 weeks, weekly chemotherapy has certain advantages. For example, the single dose level of anti-cancer drugs can be reduced with weekly chemotherapy, and the dose level can be adjusted after the start of treatment depending on signs of hematological toxicity of the drugs or the general condition of individual patients. In comparison with treatment at intervals of 3–4 weeks, weekly chemotherapy was of equal efficacy but had fewer side effects [3]. Weekly chemotherapy is thus a promising means of treating cases of recurrent NSCLC in which bone marrow function has been compromised by first-line chemotherapy.

The present study was undertaken to evaluate the effectiveness and safety of weekly chemotherapy using a combination of PTX and GEM in cases of advanced NSCLC in which tumor had recurred or relapsed after platinum-based first-line chemotherapy or platinum-based first-line chemotherapy had failed to exert efficacy.

Patients and methods

Patient selection

Patients were required to have histologically or cytologically confirmed non-resectable or metastatic NSCLC that had progressed during or after one or more chemotherapy regimens. The trial was initiated after a rest period of at least 4 weeks following previous chemotherapy (2 weeks in the case of radiotherapy). Patients were required to have recovered completely from prior therapy, and to have no ongoing toxicity greater than grade 1. Other eligibility criteria were as follows: measurable lesions; life expectancy of at least 12 weeks; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; adequate bone marrow reserve (defined as absolute granulocyte count $\geq$ 2,000/ml and platelet count $\geq$ 100,000/ml); adequate hepatic and renal function (defined as serum creatinine level ≤ 2 mg/dl, AST and ALT ≤ 1.5 times the upper limit of normal, and bilirubin ≤ 1.5 mg/dl). Exclusion criteria included pre-existing motor or sensory neurological signs or symptoms $\geq$ grade 2 (Common Terminology Criteria for Adverse Events version 3.0) and active infections. Asymptomatic treated or untreated patients with brain metastases were not excluded from the study. The Ethics Committee of the Tochigi Cancer Center approved the study protocols. Written informed consent was obtained from every patient stating that the patient was aware of the investigational nature of this treatment regimen.

Treatment

Paclitaxel was administered at a dose of 100 mg/m^2 intravenously during a 1-h infusion on days 1 and 8 of the treatment cycle. Gemcitabine was administered at a dose of 1,000 mg/m^2 intravenously during a 30-min infusion on days 1 and 8 of the treatment cycle. Prior to each treatment, patients were given diphenhydramine 50 mg orally, and an H2 blocker intravenously along with dexamethasone 16 mg 30 min before PTX administration. Granisetron 3 mg was administered intravenously as an antiemetic. The length of each chemotherapy cycle was 21 days. Patients who experienced grade 4 leukopenia or neutropenia that lasted for 3 or more days, or who experienced grade 4 thrombocytopenia or reversible grade 2 neurotoxicity or liver dysfunction, received reduced doses of both PTX and GEM (PTX 80 mg/m^2, GEM 800 mg/m^2) for the next cycle. If non-hematological toxicities of grade 3 or higher occurred, treatment was stopped. Subsequent courses of chemotherapy were started after 3 weeks when the leukocyte count was 3,000/mm^3 or more, the neutrophil count was 1,500/mm^3 or more, the platelet count was 75,000/mm^3 or more, serum creatinine were less than 1.5 mg/dl, GOT and GPT were less than twice the upper limit of the normal range, and neurotoxicity was grade 1 or less. If these variables did not return to adequate levels by the first day of the next course of chemotherapy, treatment was withheld until full recovery. If more than 6 weeks passed from the time of the last treatment before these criteria were met or if change in treatment more significant than reduction of dose was indicated, the patient was removed from the study at that time, but still included in the analysis of its results.

Evaluation of responses and toxicity

Pretreatment evaluation included medical history, physical examination, complete blood count, bone marrow examination, serum biochemical analyses,

chest roentgenogram, electrocardiogram, and urinalysis. All patients underwent radionuclide bone scan, magnetic resonance or computerized tomography (CT) of the brain, and CT of the thorax and abdomen. Complete blood count, biochemical tests, serum electrolytes, urinalysis, and chest roentgenograms were obtained before patients received chemotherapy.

Responses and toxicity were evaluated on the basis of tumor images obtained by CT and other techniques, laboratory data, and subjective/objective symptoms and signs before, during, and after administration of the study drugs and during the period from completion of treatment to final analysis. Measurable disease parameters were determined every 4 weeks by various means such as computerized tomography. Evaluation was performed in compliance with the Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines for antitumor activity and with Common Terminology Criteria for Adverse Events version 3.0 for safety. Patients were withdrawn from the study if evidence of tumor progression was obtained. The Institutional Ethical Review Committee gave approval to the study.

The primary endpoint of the study was the response rate. Simon's two-stage optimum design was used to determine sample size and decision criteria. It was assumed that a response rate of 30% among eligible patients would indicate potential usefulness while a rate of 10% would be the lower limit of interest, with $\alpha = 0.05$ and $\beta = 0.10$. Using these design parameters, the fist stage of the study was initially to enroll 18 patients, and this regimen was to be rejected if fewer than two patients had an objective response. If two or more patients responded, accrual was to be continued to 36 patients. Considering the percentage of probable dropout cases, 40 patients were required. Secondary endpoints were toxicity and overall survival. Response and survival rates were both calculated on an intent-to-treat basis. Overall survival and time to progression were measured from the start of this treatment up to the time of death or up to the date of the last follow-up clinical assessment. Survival curves were constructed using the Kaplan–Meier method.

Results

Patient characteristics

Forty patients were enrolled in this study from October 2000 to July 2003. All patients were assessable for toxicity, response, and survival. Characteristics of the 40 patients are listed in Table 1. All 40 patients had received a prior platinum-based chemotherapy regimen (Table 1). Two of these patients had received more than one chemotherapy regimen. All 40 patients were eligible for toxicity assessment. Four patients had received prior chemotherapy in the neoadjuvant setting. Of the 40 patients, 15 had initially responded to platinum-based therapy, 24 patients had achieved stable disease (SD), and one had progressive disease (PD).

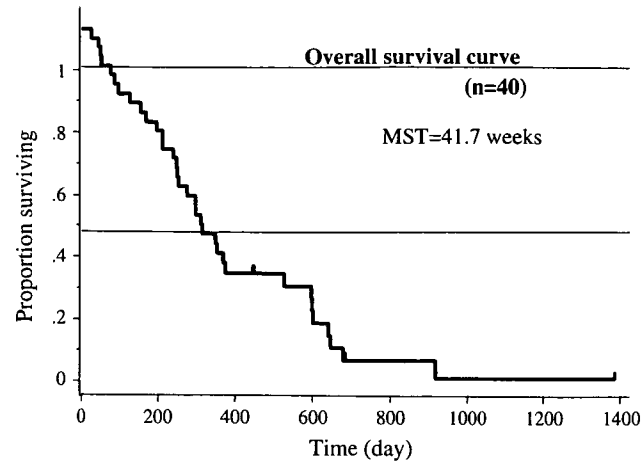

Fig. 1 Kaplan–Meier estimated overall survival curves. Median survival time, 41.7 weeks; 1-year survival rate, 38%

Efficacy of treatment

The mean number of cycles administered per patient was 4, and number of cycles ranged from one to twelve. Three patients required reduction of dose due to neutropenia and thrombocytopenia. Thirteen patients exhibited partial response (PR). Overall response rate was 32.5% (13/40) [95% confidence interval (CI): 18–47%]. SD was achieved in 26 patients (65%), and one (2%) achieved PD. All 40 patients were included in the survival analysis, with a median follow-up time of 82.9 weeks (range 56–263 weeks). The overall median survival time was 41.7 weeks (95% CI: 28.5–54.7 weeks). The 1-year survival rate was 37.5% (15/40) (Fig. 1). The median time to disease progression was 19 weeks.

Toxicities (Table 2)

Table 2 lists toxicities observed during this study. Hematological toxicities included high incidences of leukopenia and neutropenia, with leukopenia and neutropenia of grade 3 or higher occurring in 45 and 60% of patients, respectively. Anemia and thrombocytopenia of grade 3 or higher occurred in 15 and 12.5% of patients, respectively. Non-hematological toxicities

Table 1 Patient characteristics

Eligible patients	40
Gender	
Male	27
Female	13
Age (years)	
Median	59
Range	33–75
Performance status	
0	7
1	27
2	6
Histology	
Adenocarcinoma	30
Squamous cell	8
Large cell	2
Stage III	10
Stage IV	30
Number of metastatic sites	
Median	2
Range	0–3
Location of metastases	
Bone	13
Lung nodules	12
Brain	10
Lymph nodes	7
Liver	5
Adrenals	3
Subcutaneous	1
Prior surgery	4
Prior irradiation	15
Lung only	9
Brain only	4
Lung and bone	2
Prior chemotherapy	40
Cisplatin/vinorelbine	32
Cisplatin/docetaxel	5
Cisplatin/irinotecan	3
Response to prior chemotherapy	
Partial response	15
Stable disease	24
Progressive disease	1

observed included grade 3 pneumonitis in one patient, who exhibited rapid recovery following administration of steroids, grade 3 diarrhea in one, and grade 3 rash in one. Other non-hematological toxicities observed were of grade 2 or less and included nausea in 47.5%, vomiting in 20%, alopecia in 45%, sensory neuropathy in 35%, and fatigue in 32.5% of patients. All of these toxicities disappeared or were improved by symptomatic treatment. There were no deaths due to toxicity.

Discussion

Although a standard regimen of chemotherapy for recurrent NSCLC is being established, it is still important to determine how the outcome of treatment of this cancer can be improved [13, 23, 24]. At this point, the results of large-scale phase III clinical trials indicate single-agent chemotherapy with docetaxel, erlotinib, or pemetrexed as the standard chemotherapy regimen for recurrent NSCLC. In recent years, however, many reports have been published investigating two-drug combined therapy rather than single-agent therapy for recurrent NSCLC, with the objective of further improving therapeutic outcomes [2, 5, 7, 11–14, 20–26, 28].

A large number of reports have been published concerning salvage chemotherapy for recurrent NSCLC. Platinum-based chemotherapy is now used as the first-line chemotherapy at most medical facilities. Reports on second-line chemotherapy for NSCLC published to date have principally concerned uncombined drug therapy or two-drug combined therapy using non-platinum preparations [2, 5, 7, 11, 12, 14, 16, 17, 20–22, 25, 26, 28]. At several facilities, weekly administration chemotherapy has been adopted [5, 16, 26, 28]. Weekly-administration chemotherapy allows single dose levels to be reduced, thus making it possible to adjust the dose levels of anti-cancer agents after the start of treatment depending on adverse reactions or the general condition of individual patients.

Table 3 summarizes the results of two-drug combined therapy for recurrent NSCLC using non-platinum preparations [2, 6, 9, 10, 14, 19, 27]. The studies shown in this table were phase I-II in the case of that reported by Iaffaioli [14], phase III in that by Fossella [9], and phase II in the other studies. The overall response rate varied widely among studies, from 0.8 to 39%. The overall median survival time was 24–47 weeks and the one-year survival rate was 19–46%. Major adverse reactions observed in these studies were signs of hematological toxicity (particularly neutropenia), excluding the studies involving prophylactic G-CSF treatment reported by Androulakis [2] and Wachters [27]. Signs of non-hematological toxicity varied depending on the drugs used, and symptoms and signs unique to each drug were noted.

For combined PTX and GEM therapy for recurrent NSCLC, Androulakis [2] reported an overall response rate of 18%, an overall median survival time of 47 weeks, and a median time to disease progression of 34 weeks. Compared to the present study, the overall response rate reported by Androulakis was lower, while the overall median survival time and median time to disease progression were more favorable in the study by Androulakis. The dosing regimen used by Androulakis involved administration of PTX (175 mg/m^2; day 8), GEM (900 mg/m^2; days 1 and 8), and granulocyte colony-stimulating factor (G-CSF; days

Table 2 Maximum toxicity over 152 cycles (40 patients)

	CTCAE v 3.0 grade (number of patients)					Grade 3 ≤ (%)
	0	1	2	3	4	
Leukopenia	7	4	11	15	3	18 (45)
Neutropenia	6	5	5	17	7	24 (60)
Febrile neutropenia	–	–	–	2	–	2 (5)
Anemia	4	8	22	5	1	6 (15)
Thrombocytopenia	9	21	5	3	2	5 (12.5)
Pneumonitis	36	1	0	1	0	1 (2.5)
Diarrhea	27	9	3	1	0	1 (2.5)
Rash	22	15	2	1	0	1 (2.5)
Nausea	21	19	0	0	0	
Vomiting	32	3	5	0	0	
Fatigue	27	11	2	0	0	
Alopecia	22	17	1	0	0	
Neuropathy-sensory	26	14	0	0	0	
Edema	32	8	0	0	0	
Arthralgia	33	7	0	0	0	

CTCAE v 3.0 Common terminology criteria for adverse events version 3.0

Table 3 Non-platinum regimens used as second-line treatment of non-small cell lung cancer

First author (Ref.)	No. of patients	Regimen and schedule			Response rate (%)	Survival	
						Median (weeks)	1-year (%)
Androulakis [2]	49	P	175 mg/m^2	d 8 q 3w	18	47	37
		G	900 mg/m^2	d 1,8 q 3w			
		G-CSF	150 μg/m^2	d 9–15			
Iaffaioli [14]	37	P	90–240 mg/m^2	d 1 q 3w	39	40	46
		G	1,000 mg/m^2	d 1,8 q 3w			
Fossella [9]	123	FO	2 g/m^2/day	d 1–3 q 3w	0.8	24	19
		V	30 mg/m^2	d 1,8,15 q 3w			
Kosmas [19]	43	D	100 mg/m^2	d 8 q 3w	33	36	28
		G	1,000 mg/m^2	d 1,8 q 3w			
Cao [6]	33	CPT11	300 mg/m^2	d 1 q 4w	9	25	23
		V	30 mg/m^2	d 1,14 q 4w			
Georgoulias [10]	76	CPT11	300 mg/m^2	d 8 q 3w	18.4	38	24.5
		G	1,000 mg/m^2	d 1,8 q 3w			
Wachters [27]	52	CPT11	200 mg/m^2	d 1 q 3w	10	27	30
		D	60 mg/m^2	d 1 q 3w			
		G-CSF	150 μg/m^2	d 2–12			
Present study	40	P	100 mg/m^2	d 1,8 q 3w	32.5	42	38
		G	1,000 mg/m^2	d 1,8 q 3w			

P paclitaxel, *G* gem citabine, *F0* infostamide, *V* vinorebine, *D* docetaxel, *CPT-11* irinotecan, *G-CSF* granulocyte colony-stimurating factor, *d* day, *q* every

9–15), with each cycle of treatment lasting for 3 weeks. Because their regimen involved prophylactic administration of G-CSF, the incidence of grade 3 or worse neutropenia was lower than that in the present study (12 vs. 60%). However, the incidence of grade 2 or worse fatigue (a sign of non-hematological toxicity) was lower in the present study (4%) than in that reported by Androulakis (51%).

Belani [19] reported the results obtained with combined use of PTX and GEM as first-line chemotherapy for NSCLC. In their study, PTX was administered using two regimens and a comparison was made between treatment with PTX on day 1 (200 mg/m^2) and weekly treatment with PTX on days 1 and 8 (100 mg/m^2/dose; identical to the regimen used in the present study). According to their report, the response rate was 45% for the first regimen and 46% for the second regimen, the median survival time was 42 and 39 weeks and the 1-year survival rate 46 and 41% for the first and second regimens, respectively. Efficacy thus did not differ