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Expression of the *JAK2* V617F mutation is not found in *de novo* AML and MDS but is detected in MDS-derived leukemia of megakaryoblastic nature

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A somatic point mutation in the tyrosine kinase *JAK2* (V617F) is detected in most patients with polycythenia vera (PV) and in approximately half of those with essential thrombocythemia (ET), as well as in other myeloid disorders such as *de novo* acute myeloid leukemia (AML; ~6%), myelodysplastic syndrome (MDS; ~4%), chronic myelomonocytic leukemia (CMML; ~20%) and atypical myeloproliferative disease (MPD). In contrast, *JAK2* V617F was not detected in the patients with acute or chronic lymphoid malignancies.[1] In these studies, most of these mutations were heterozygous mutation and homozygous mutation was only detected in 30% of patients with *JAK2* mutation using DNA samples.[2] Thus, the prevalence of the expression of *JAK2* V617F in myeloid malignancies remains unclear.

In the present study, we examined the expression of *JAK2* V617F in 241 Japanese patients with myeloid malignancies. The morphologic subtypes of AML and MDS were initially classified according to the French–American–British (FAB) criteria. A diagnosis of transformation from MDS to AML (MDS-AML) was made when the bone marrow (BM) consisted of more than 30% blasts. Leukemic cells from BM were collected after informed consent was obtained. Mononuclear cells were isolated using the Ficoll–Hypaque density gradient centrifugation method, followed by total cellular RNA extraction. Expression of the *JAK2* gene was determined by reverse transcription-PCR, as described previously.[3] Mutation analysis of V617F was then performed by direct sequencing of these PCR products.

A homozygous *JAK2* V617F mutation was detected in the HEL cell line, which was established from a patient with erythroleukemia (AML M6 as defined by the FAB classification) and in which the JAK2/STAT5 signal transduction pathway is constitutively activated.[4] Among *de novo* AML, only heterozygous *JAK2* V617F mutation was detected in the patients with AML M6 (1 of 53 patients; 2%) and AML M7 (2 of 11 cases; 18%).[5,6] None of the 198 patients with *de novo* AML tested showed expression of *JAK2* V617F, including nine cases with M6 and eight cases with M7 (Table 1). These observations suggest that the prevalence of the expression of *JAK2* V617F in *de novo* AML appears to be less frequent than the reported detection rate of the genetic mutation using DNA samples.

In contrast to *de novo* AML, expression of the *JAK2* V617F mutation was found in 2 of 18 cases (11%) of MDS-AML but not

Table 1 Prevalence of the expression of *JAK2* V617F in myeloid malignancies

Disease		*Tested samples*	*JAK2 V617F*	*% Mutation*
De novo AML	M0	5	0	0
	M1	21	0	0
	M2	90	0	0
	M3	12	0	0
	M4	32	0	0
	M5	21	0	0
	M6	9	0	0
	M7	8	0	0
MDS	RA	8	0	0
	RAEB	17	0	0
MDS-AML		18	2	11

Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.

Table 2 Clinical and laboratory features of MDS-AML showing expression of *JAK2* V617F

Patient	*Age/sex*	*WBC* ($\times 10^9$/l)	*Hb* (g/dl)	*Platelets* ($\times 10^9$/l)	*% blasts in BM*	*Myelofibrosis*	*Surface marker*[a]	*Karyotype*	*Splenomegaly*
1396	51/M	1.6	5.6	8.0	85	—	CD34,CD33,CD13,CD41,CD7	46,XY	—
2704	52/M	12.6	7.9	115.0	75	—	CD34,CD41	46,XY	—

Abbreviations: BM, bone marrow; Hg, hemoglobin; M, male; WBC, white blood cells; —, not found.
[a]Positive antigens on the blasts.

in eight cases with refractory anemia (RA) or 17 cases with refractory anemia with excess blasts (RAEB) (Table 1). In both cases with the mutation, most of fluorescent intensity at codon 617 was V617F and the wild type was only faintly visible, suggesting that most of the leukemic cells expressed V617F. A previous study found a high occurrence of the *JAK2* V617F mutation in RA with ringed sideroblasts associated with marked thrombocytosis (71%),[7] and this mutation has also been detected in MDS with myelofibrosis (33%).[8] In our two cases with the mutation, neither thrombocytosis nor myelofibrosis was detected (Table 2). Interestingly, the blastic cells of these two cases were small with pale agranular cytoplasms and cytoplasmic blebs, and expressed CD41 antigen, suggesting that blastic cells have a megakaryoblastic nature. The progenitor cells of MDS are believed to undergo a multistep process during transformation into overt acute myelogenous leukemia. Several genetic abnormalities have been detected in both MDS and MSD-AML patients, which have suggested that genetic alterations are closely associated with disease progression of MDS.[3] Therefore, MDS serves as a useful model for studying the abnormal genetic events that occur in leukemogenesis. We detected expression of *JAK2* V617F in MDS-AML but not in RA or RAEB, suggesting that expression of *JAK2* V617F may be one of the genetic factors involved in the progression of MDS to AML with a megakaryoblastic nature.

In conclusion, detection of the expression of the *JAK2* gene mutation in MDS-AML patients may enhance both the management of these patients and the application of adequate therapeutic strategies such as tyrosine kinase inhibitors.

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Quantification of *ex vivo* generated dendritic cells (DC) and leukemia-derived DC contributes to estimate the quality of DC, to detect optimal DC-generating methods or to optimize DC-mediated T-cell-activation-procedures *ex vivo* or *in vivo*

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There is a need for less intensive (post-remission) immunotherapies to maintain stable remissions in AML and at least stable diseases in MDS before or after stem cell transplantation (SCT). The significance of T cells to mediate cellular anti leukemic reactions has been demonstrated by donor-lymphocyte-infusion (DLI) therapy of relapsed AML,[1] although not all of the patients treated respond to this therapy. Professional antigen-presenting cells like dendritic cells (DC) could be used to overcome this therapeutic resistance and (re)-activate anti-leukemia-directed allogeneic or autologous T cells.[2] Leukemic

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Retroviral expression screening of oncogenes in natural killer cell leukemia

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Abstract

Aggressive natural killer cell leukemia (ANKL) is an intractable malignancy that is characterized by the outgrowth of NK cells. To identify transforming genes in ANKL, we constructed a retroviral cDNA expression library from an ANKL cell line KHYG-1. Infection of 3T3 cells with recombinant retroviruses yielded 33 transformed foci. Nucleotide sequencing of the DNA inserts recovered from these foci revealed that 31 of them encoded KRAS2 with a glycine-to-alanine mutation at codon 12. Mutation-specific PCR analysis indicated that the *KRAS* mutation was present only in KHYG-1 cells, not in another ANKL cell line or in clinical specimens ($n = 8$).

Keywords: Aggressive NK cell leukemia; cDNA expression library; Retrovirus; KRAS2 oncogene

1. Introduction

Outgrowth of $CD3^{-}CD16/CD56^{+}$ natural killer (NK) cells in peripheral blood is diagnosed as either chronic NK lymphocytosis (CNKL) or aggressive NK cell leukemia (ANKL) [1,2]. Whereas the former condition has an indolent clinical course with few symptoms, the latter is characterized by chemoresistance and multiorgan failure and has a poor outcome.

The Epstein–Barr virus (EBV) genome is frequently present episomally in ANKL cells [3], suggesting a role for EBV in disease pathogenesis. However, little is known of how infection with EBV might trigger clonal growth of NK cells. Inactivation of tumor suppressor genes has been associated with NK cell neoplasia. For instance, a homozygous deletion of the genes for p16INK4A, p15INK4B, or p14ARF has been detected [4]. Additionally, inactivating mutations of the FAS gene have been found in nasal NK/T cell lymphoma [5].

A few studies have identified a potential contribution of oncogenes to NK cell malignancy. Mutations that affect codons 13 or 22 of *KRAS2* were found in NK/T cell lymphoma [6] but not in ANKL [7]. Furthermore, although mutations in *KIT* were associated with NK/T cell lymphoma, transforming activity of the mutant KIT proteins was not demonstrated [8]. A role for oncogenes in ANKL has not been identified to date.

Functional screening based on transforming ability is one potential approach to the efficient isolation of tumor-promoting genes in ANKL. Focus formation assays with

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mouse 3T3 fibroblasts have indeed proved successful for the identification of oncogenes in human cancer [9]. In such screening assays, genomic DNA isolated from cancer specimens is used to transfect 3T3 cells and the formation of transformed cell foci is then evaluated. Expression of the exogenous genes in such experiments is driven by their own promoters or enhancers, however, so that oncogenes can exert transforming effects in 3T3 cells only if their regulatory regions are active in fibroblasts. Given the distinct developmental origins of NK cells and fibroblasts, expression of oncogenes associated with ANKL in 3T3 cells under these conditions is not guaranteed.

This problem might be expected to be overcome by the expression of test cDNAs under the control of an ectopic promoter in 3T3 cells. We have therefore constructed a retroviral cDNA expression library from the ANKL cell line KHYG-1 [10] and used this library to infect 3T3 cells. In preparation of the cDNA library, we took advantage of a polymerase chain reaction (PCR)-based system that preferentially amplifies full-length cDNAs. The resulting library was found to have sufficient complexity and to contain a high percentage of full-length cDNAs. Focus formation assays with 3T3 cells resulted in the identification of *KRAS2* as a transforming gene in KHYG-1 cells.

2. Materials and methods

2.1. Cell culture and clinical samples

KHYG-1 and NKL cells [11] were kindly provided by M. Yagita and Y. Yokota, respectively, and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and human interleukin-2 (20 U/mL) (Roche, St. Louis, MO). The BOSC23 packaging cell line for ecotropic retroviruses [12] and mouse 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM)–F12 (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Mononulear cells were isolated by Ficoll–Hypaque density gradient centrifugation from peripheral blood of the subjects with informed consent. The cells were incubated with anti-CD3 MicroBeads (Miltenyi Biotec, Auburn, CA), and loaded onto MIDI-MACS magnetic cell separation columns (Miltenyi Biotec) to remove $CD3^+$ cells. The flow-through was then mixed with anti-CD56 MicroBeads (Miltenyi Biotec), and was subjected to a MINI-MACS column for the "positive selection" of $CD56^+$ cells. Cells bound specifically to the column was then eluted according to the manufacturer's instructions.

2.2. Construction of a retrovirus library

Total RNA was extracted from KHYG-1 cells with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA), and first-strand cDNA was synthesized from the RNA with PowerScript reverse transcriptase, a SMART IIA oligonucleotide, and CDS primer IIA (Clontech, Palo Alto, CA). The resulting cDNA molecules were then amplified for 12 cycles with 5′-PCR primer IIA and a SMART PCR cDNA synthesis kit (Clontech), with the exception that LA Taq polymerase (Takara Bio, Shiga, Japan) was substituted for the Advantage 2 DNA polymerase provided with the kit. The PCR products were treated with proteinase K, rendered blunt-ended with T4 DNA polymerase, and ligated to a *Bst*XI adapter (Invitrogen). Unbound adapters were removed with a cDNA size fractionation column (Invitrogen), and the modified cDNAs were ligated into the pMX retroviral plasmid (kindly provided by T. Kitamura) [13] that had been digested with *Bst*XI. The pMX-cDNA plasmids were introduced into ElecroMax DH10B cells (Invitrogen) by electroporation.

2.3. Focus formation assay

BOSC23 cells (1.8×10^6) were seeded onto 6-cm culture plates, cultured for 1 day, and then transfected with a mixture comprising 2 μg of retroviral plasmids, 0.5 μg of pGP plasmid (Takara Bio), 0.5 μg of pE-eco plasmid (Takara Bio), and 18 μL of Lipofectamine reagent (Invitrogen). Two days after transfection, polybrene (Sigma, St. Louis, MO) was added at a concentration of 4 μg/mL to the culture supernatant, which was then used to infect 3T3 cells for 48 h. For the focus formation assay, the culture medium of 3T3 cells was changed to DMEM–high glucose (Invitrogen) supplemented with 5% calf serum and 2 mM L-glutamine. Transformed foci were isolated after 3 weeks of culture.

2.4. Recovery of cDNAs from 3T3 cells

Each 3T3 cell clone was harvested with a cloning syringe and cultured independently in a 10-cm culture plate. Genomic DNA was subsequently extracted from the cells and subjected to PCR with 5′-PCR primer IIA and LA Taq polymerase for 50 cycles of 98 °C for 20 s and 68 °C for 6 min. Amplified genomic fragments were purified by gel electrophoresis and ligated into the pT7Blue-2 vector (EMD Biosciences, San Diego, CA) for nucleotide sequencing.

2.5. Mutation-specific PCR for KRAS2

Detection of $KRAS2^{G12A}$ cDNA was performed as described previously [14] but with minor modifications. In brief, a 5′-region of *KRAS2* cDNA was amplified from oligo(dT)–primed cDNA by PCR with 5′-RAS primer (5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′; the underlined cytosine was incorporated to generate a *Bst*NI site) and 3′-RAS primer A (5′-CTGTGTCGAGAATATCCAAGAGACA-3′). The PCR product was subjected to digestion with *Bst*NI (New England Biolabs, Beverly, MA) and then to a second PCR with 5′-RAS primer and 3′-RAS primer B (5′-CTGTGTCGAGAATCCAGGAGACA-3′; the under-

lined guanine was incorporated to generate a *Bst*NI site). The second PCR product was then also subjected to digestion with *Bst*NI, and the resulting DNA fragments were separated by agarose gel electrophoresis.

3. Results

3.1. Construction of a full-length cDNA expression library for KHYG-1 cells

Full-length cDNAs were selectively amplified from mRNA of KHYG-1 cells and ligated into the retroviral vector pMX. We obtained a total of 5.61×10^6 colony-forming units (cfu) of independent plasmid clones. To evaluate the quality of the library, we randomly selected 40 clones and examined the incorporated cDNAs. Thirty-nine of the 40 clones contained inserts with an average size of 2.03 kbp. The cDNA inserts from 20 out of these 39 clones were sequenced from both ends, and the determined sequences were used to screen, with the BLAT search program [15], the nucleotide sequence database assembled as of July 2003 by the Genome Bioinformatics Group of the University of California at Santa Cruz (http://genome.ucsc.edu/). Both ends of 14 of the 20 cDNAs could be matched to the mRNA sequences of known genes, and 13 of these cDNAs included complete open reading frames (data not shown). We therefore concluded that the retroviral cDNA expression library was of sufficient complexity and sufficiently enriched in full-length cDNAs for the present study.

3.2. Identification of $KRAS2^{G12A}$ in KHYG-1 cells

We generated a recombinant ecotropic retrovirus library by introducing 7.1×10^5 cfu of the generated plasmids into a packaging cell line. This library was then used to infect mouse 3T3 fibroblasts. After culture of the infected cells for 3 weeks, we detected 33 transformed foci (Fig. 1). Each focus was isolated, expanded independently, and subjected to extraction of genomic DNA for the recovery of retroviral inserts by PCR with the primer used originally to amplify the cDNAs during construction of the library. In most instances, a single major DNA fragment was recovered from each genome (Fig. 2A), suggestive of original infection of a single 3T3 cell with a single retrovirus.

The recovered cDNA fragments were sequenced from both ends for all 33 clones. Screening of the human genome sequence database with the insert sequences revealed that those from 31 of the 33 clones (#1–#29, #31, #33) matched, with >98% identity, the sequence of human *KRAS2* (GenBank accession number, NM_004985). The genome of 3T3 clone ID #30 yielded two PCR fragments (Fig. 2A); the larger (~1.4 kbp) and the smaller (~0.9 kbp) fragments were revealed to be derived from β-actin (*ACTB*; GenBank accession number, NM_001101) and profilin 1 (*PFN1*; GenBank accession number, NM_005022) genes, respectively. The final 3T3 clone (#32) yielded a major PCR fragment corresponding to the gene for isocitrate dehydrogenase 3 (NAD^+) β (*IDH3B*; GenBank accession number, NM_006899).

KRAS2 belongs to the *RAS* gene family and is involved in a wide variety of human cancers [16]. Given that point

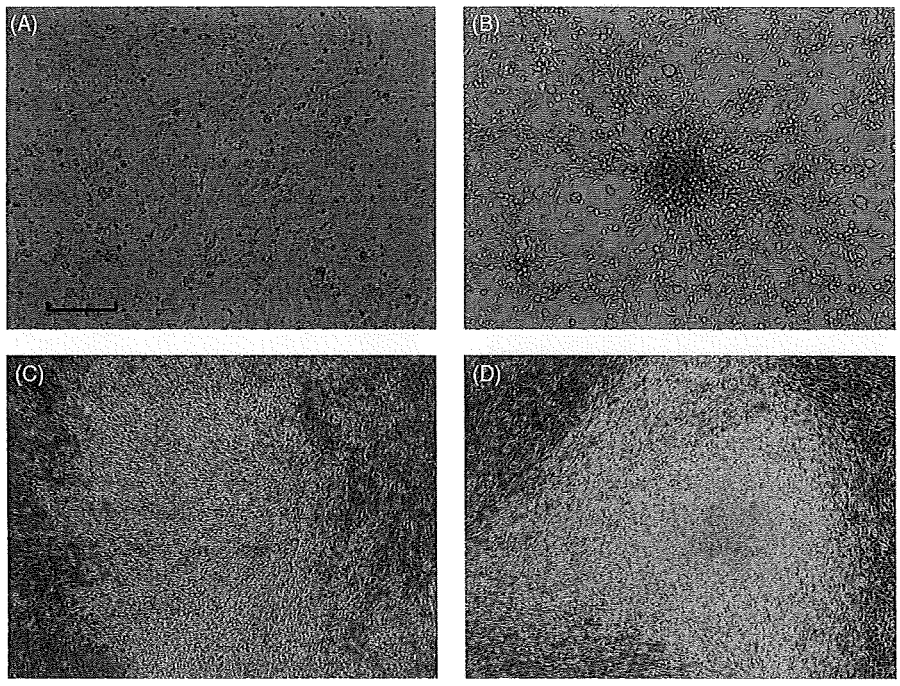

Fig. 1. Focus formation assay with a retroviral library derived from KHYG-1 cells. Mouse 3T3 cells were infected with the empty virus (A), a retrovirus expressing v-Ras as a positive control (B), or retroviruses from the KHYG-1 cell library (C and D). The cultures were photographed 3 weeks after infection. Scale bar, 100 μm.

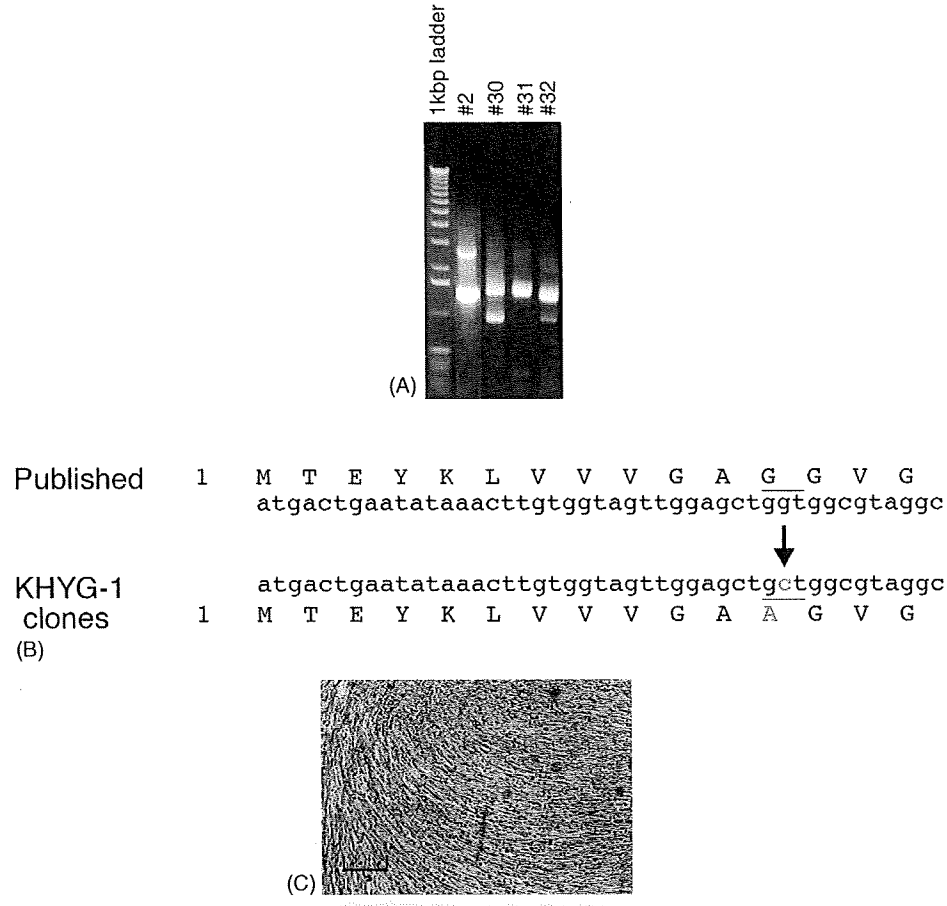

Fig. 2. Identification of a *KRAS* mutant gene with transforming activity: (A) Genomic DNA isolated from transformed 3T3 cell foci (clones #2, #30, #31, and #32) was subjected to PCR for amplification of the DNA inserts. The left lane contains DNA size markers (1-kbp DNA ladder; Invitrogen); (B) The nucleotide sequences of the DNA inserts derived from 31 of the 33 transformed foci matched that of *KRAS* with a single nucleotide substitution (G to C) in the second position of codon 12 that results in a change in the encoded amino acid from glycine to alanine; (C) A recombinant retrovirus encoding KRAS2^{G12A} was used to infect 3T3 cells. The cells were photographed after culture for 2 weeks. Scale bar, 50 μm.

mutations in *KRAS2* confer oncogenic activity, we compared the nucleotide sequences of the *KRAS2* cDNAs derived from KHYG-1 cells with the published sequence of the wild-type gene. Although oncogenic mutations have previously been shown to affect codons 12, 13, and 59 of *KRAS2*, all of the KHYG-1 cell cDNAs harbored the same mutation: the GGT sequence of codon 12 was changed to GCT, resulting in the substitution of an alanine residue for the glycine normally present at this position (Fig. 2B). To verify the transforming ability of *KRAS2*^{G12A}, we inserted the corresponding cDNA into the pMX retroviral vector and generated recombinant retroviruses for infection of 3T3 cells. The recipient 3T3 cells indeed underwent transformation (Fig. 2C), confirming that *KRAS2*^{G12A} possesses oncogenic activity.

3.3. Screening for *KRAS2*^{G12A} in NK cell leukemia

To determine whether *KRAS2*^{G12A} is frequently associated with NK cell leukemia, we applied a slightly modified version of a rapid screening method previously described by Kahn et al. [14]. *KRAS2* cDNA was first amplified by PCR with 5′-RAS primer and 3′-RAS primer A (Fig. 3A). The 3′ end of 5′-RAS primer corresponds to codon 11 of *KRAS2* but contains a cytosine substitution in the first position of codon 11, which generates a *Bst*NI recognition site [CC(T/A)GG] that includes the first and second positions of codon 12. The presence of a point mutation at the first or second position of codon 12 would therefore prevent digestion of the PCR product by *Bst*NI.

After *Bst*NI digestion, the PCR product was subjected to a second PCR with 5′-RAS primer and 3′-RAS primer B. Given that *Bst*NI digestion removes the binding site for 5′-RAS primer, only *KRAS2* cDNA with a mutation at the first or second position of codon 12 should yield a second PCR product. Even if *Bst*NI digestion of the initial PCR product was not complete and the second PCR amplified a trace amount of wild-type *KRAS2* cDNA, a second *Bst*NI digestion further discriminates between the wild-type and mutant genes. The 3′-RAS primer B thus contains a guanine substitution that generates a *Bst*NI site. The second PCR product of wild-type *KRAS2* cDNA would thus contain two *Bst*NI sites, whereas that of mutant *KRAS2* contains only one.

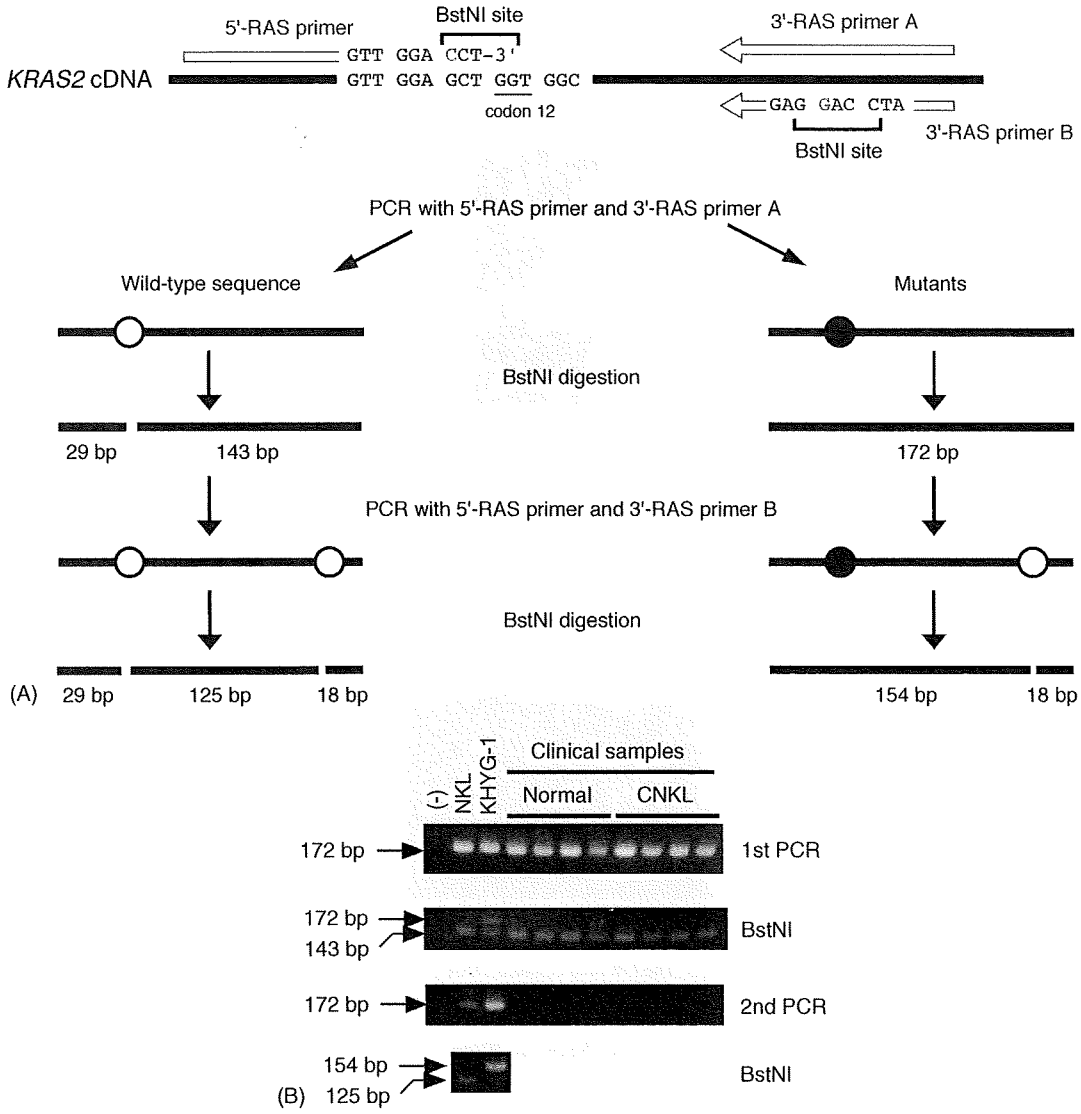

Fig. 3. Mutation-specific PCR analysis of NK cell leukemia cell lines and clinical specimens: (A) *KRAS2* cDNA was amplified with 5′-RAS primer and 3′-RAS primer A. The PCR product was subjected to digestion with *Bst*NI and then to a second PCR with 5′-RAS primer and 3′-RAS primer B. The second PCR product was also subjected to digestion with *Bst*NI. The nucleotides shown in red were incorporated into the primers to generate a *Bst*NI site. Open circles indicate *Bst*NI sites; closed circles indicate corresponding mutant sequences that are not susceptible to *Bst*NI; (B) cDNA isolated from the NKL and KHYG-1 cell lines as well as from $CD3^{-}CD56^{+}$ NK cell fractions derived from healthy volunteers (Normal) or individuals with CNKL was subjected to mutation-specific PCR analysis. A reaction without input DNA was also performed as a negative control (–). The size of DNA fragments is indicated on the left.

With this approach, we analyzed cDNA prepared from two ANKL cell lines (NKL and KHYG-1) and from $CD3^{-}CD56^{+}$ NK cell fractions purified from the peripheral blood of healthy individuals ($n=4$) and patients with CNKL ($n=4$). The first PCR step yielded a single DNA fragment of 172 bp from all samples. Furthermore, only the PCR product from KHYG-1 cells was refractory to *Bst*NI digestion, indicating that only KHYG-1 cells harbor a codon 12 mutation of *KRAS2*. The presence of the 143-bp band may indicate that KHYG-1 cells are heterozygous for the *KRAS2* mutation. The second PCR generated a 172-bp DNA fragment only with the NKL and KHYG-1 cell samples. Whereas this fragment derived from NKL cells was completely digested by *Bst*NI to generate a 125-bp band, *Bst*NI digestion of the fragment derived from KHYG-1 cells generated a band of 154 bp. Of all the samples analyzed, therefore, mutation of the first or second position of codon 12 of *KRAS2* was detected only in KHYG-1 cells.

4. Discussion

We have constructed a retroviral cDNA expression library for an ANKL cell line. Given that >97% (39/40) of the viral plasmids contained cDNA inserts and that the overall clone number was $>5 \times 10^6$, our library likely represents most of

the transcriptome of KHYG-1 cells. The high probability that the incorporated cDNAs are full length is also an important advantage for functional screening.

In our screening, most of the 3T3 transformants were found to have incorporated a single cDNA fragment corresponding to *KRAS2*^{G12A}, with only two transformants found to contain other cDNAs. One of these two cDNA inserts was derived from the gene for PFN1, a protein that binds to unpolymerized actin [17]. Homozygous deletion of *Pfn1* results in embryonic death in mice, with the encoded protein apparently being indispensable for cell growth or differentiation during embryonic development [18]. The other cDNA insert isolated from 3T3 transformants contained the entire open reading frame for IDH3B, which catalyzes the oxidative decarboxylation of isocitrate and is a key enzyme in the tricarboxylic acid cycle [19]. Neither *PFN1* nor *IDH3B* has previously been shown to possess oncogenic activity. It is currently under examination whether a long terminal repeat (LTR)-driven overexpression of *PFN1* or *IDH3B* leads focus formation in 3T3 cells.

Comparative genomic hybridization analysis identified a wide variety of genetic alterations at a high frequency in ANKL cells [20], suggesting that leukemogenesis in ANKL is associated with multiple steps of oncogene activation. An analysis of patients with NK cell neoplasia failed to detect any changes in the genes for members of the RAS and MYC families of proteins [7], however. This previous study did find a frequent increase in the abundance of the cell cycle regulator MDM2.

In contrast, we have detected a transforming *KRAS2* mutant gene in an ANKL cell line. Given that the mutation in codon 12 of this gene was detected by two different approaches (retroviral screening of PCR-amplified cDNAs and mutation-specific PCR), we conclude that it was not an artifact of PCR. KRAS2 is a GTP-binding protein with a relative molecular mass of ~21 kDa. Together with HRAS and NRAS, it plays an important role in cell growth and differentiation. Many mitogenic signals promote the loading of KRAS2 with GTP, which in turn triggers various downstream signaling events including activation of the mitogen-activated protein kinase (MAPK) pathway.

Activating mutations of *KRAS2* have been identified in a wide range of human cancers. Mutations of codon 12, for example, are associated with acute lymphoblastic leukemia [21], lung cancer [22], and pancreatic cancer [23]. No such mutations have previously been detected in association with ANKL, however. Although we have now identified a *KRAS2* mutation affecting codon 12 in the ANKL cell line KHYG-1, we did not detect this mutation in another ANKL cell line (NKL) or in $CD3^{-}CD56^{+}$ NK cell fractions isolated from healthy volunteers or from individuals with CNKL. Mutation of *KRAS2*, at least of codon 12 of this gene, might therefore be an infrequent event in NK cell neoplasia. Indeed, it remains possible that the detected *KRAS2* mutation is specific to the KHYG-1 cell line. Nevertheless, our identification of an activating *KRAS2* mutation in KHYG-1 cells might provide insight into the role of the RAS-MAPK signaling pathway in ANKL carcinogenesis. Furthermore, given the high quality of our retroviral expression library, the strategy adopted in the present study also might prove useful for the functional screening of genes associated with various clinical characteristics of ANKL, such as chemoresistance.

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mRAP, a sensitive method for determination of microRNA expression profiles

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Abstract

MicroRNAs (miRNAs) are noncoding RNA molecules of 21–24 nucleotides that regulate the expression of target genes in a posttranscriptional manner. Although evidence indicates that miRNAs play essential roles in embryogenesis, cell differentiation, and pathogenesis of human diseases, extensive miRNA profiling in cells or tissues has been hampered by the lack of sensitive cloning methods. Here we describe a highly efficient profiling strategy, termed miRNA amplification profiling (mRAP), that relies on the use of a long, optimized 5′ adaptor, the SMART (switching mechanism at the 5′ end of RNA templates of reverse transcriptase) method, the polymerase chain reaction, and cDNA concatamerization after BanI digestion. This approach is highly sensitive, readily allowing the isolation of $>1 \times 10^4$ independent miRNA-derived cDNAs from $\leqslant 1 \times 10^4$ cells. The mRAP method thus makes it possible to analyze miRNA expression profiles for small quantities of tissue or cells such as fresh clinical specimens.

Keywords: MicroRNA; SMART; Cloning; cDNA library

1. Introduction

MicroRNAs (miRNAs) are short noncoding RNAs that bind target mRNAs by incomplete base-pairing to their 3′ untranslated regions. Mainly through translational repression, miRNAs regulate the expression of target genes and thereby have a plethora of effects on cellular functions [1,2]. Given that changes in miRNA expression have been implicated in tumorigenesis [3,4], it is important to characterize miRNA expression profiles in fresh cancer specimens.

Isolation of miRNA clones has not been a simple task with limited amounts of tissue or small numbers of cells, however. Given that conventional miRNA isolation procedures require $\geqslant 100$ μg of total RNA as starting material [5], several strategies have been employed to overcome this obstacle with the use of stem-loop reverse transcription-polymerase chain reaction (RT-PCR) analysis [6], the SMART (switching mechanism at the 5′ end of RNA templates of reverse transcriptase) method [7], serial analysis of gene expression (SAGE) [8], and bead-based flow cytometry [9], for example.

We have attempted to isolate cDNAs for miRNAs from fresh clinical specimens. A simple application of the SMART method to cDNA cloning did not allow an efficiency sufficient for the isolation of miRNA-derived clones, especially from small numbers of cells. We therefore developed a highly sensitive miRNA cloning strategy, mRAP (miRNA amplification profiling), that relies on a long, optimized 5′ adaptor and the SMART method. This approach readily allows the isolation of $>1 \times 10^4$ miRNA-derived clones even from $\leqslant 1 \times 10^4$ cells, making it possible to determine miRNA expression profiles for small quantities of tissue or cells such as fresh clinical specimens.

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2. Isolation and fractionation of small RNA molecules

A small-RNA fraction is isolated from cells or tissue, the RNA molecules are separated by denaturing polyacrylamide gel electrophoresis (PAGE), and the region of the gel containing RNA molecules of 19–24 nucleotides (nt) is excised. The portion of the gel containing the sample RNA should not be stained with a dye or exposed to ultraviolet light.

1. Purify small RNA molecules with the use of a *mir*Vana miRNA isolation kit (Ambion, Austin, TX) and the small-RNA fraction option. Other protocols or kits suitable for the enrichment of small RNAs may be used.
2. Prepare the sample mixture: 5 μl of isolated RNA (≤5 μg) and 5 μl of 2× Dye (*mir*Vana).
3. Prepare the size-standard mixture: 0.25 μl of size standards (synthesized RNA oligonucleotides of 19, 24, and 33 nt at a concentration of 0.1 mg/ml each), 4.75 μl of water, and 5 μl of 2× Dye (*mir*Vana).
4. Incubate each mixture at 90 °C for 20 s and then place on ice.
5. Subject the sample and size-standard mixtures to PAGE on a 15% gel (SequaGel Sequencing System; National Diagnostics, Atlanta, GA).
6. Cut out and stain only the marker lane with SYBR Green II (Takara Bio, Shiga, Japan) or with ethidium bromide for 5–10 min and then photograph the gel aligned with a ruler.
7. Excise the portion of the sample lane containing RNA molecules of 19–24 nt.
8. Chop the isolated gel into small fragments and then transfer them to a microcentrifuge tube.
9. Add 125 μl of water to the tube and maintain it at 4 °C overnight with gentle agitation.
10. Separate the gel pieces by brief centrifugation, and transfer 100 μl of the supernatant containing the small RNA molecules to another microcentrifuge tube.
11. Add 250 μl of 2-butanol to the RNA, invert the tube several times, and centrifuge the mixture briefly.
12. Check the volume of the bottom, water phase (should be <10 μl) and discard the upper, 2-butanol phase.
13. Subject the water phase to chloroform extraction followed by ethanol precipitation with 1 μl of glycogen (Roche Diagnostics, Mannheim, Germany; 20 mg/ml stock) as a carrier.
14. Dissolve the purified RNA molecules in 8.75 μl of water.

3. Synthesis of cDNAs, PCR, and concatamer formation

The mRAP procedure is depicted schematically in Fig. 1. The miRNAs isolated by gel electrophoresis are ligated at their 3′ ends to a 3′ adaptor with the use of RNA ligase. Complementary DNAs corresponding to the miRNAs are then synthesized with the use of reverse transcriptase and an RT primer complementary to the 3′ adaptor. Given that some reverse transcriptases possess terminal deoxynucleotidyl transferase activity, the synthesized cDNA strands frequently have a small poly(C) overhang at their 3′ ends. After annealing of a long 5′ adaptor to such poly(C) overhangs, PCR is used to amplify the miRNA-derived cDNAs. As shown in Fig. 1, there are usually three main types of PCR product of different sizes (~70, ~90, and ~120 bp). The ~90-bp products are the ones that contain the cDNAs; the smaller and larger products comprise dimers and trimers of the 5′ adaptor and 3′ adaptor (without cDNA). The desired products are purified, digested with the restriction endonuclease BanI (the target sites of the enzyme are incorporated into the PCR primers), and ligated to generate cDNA concatamers.

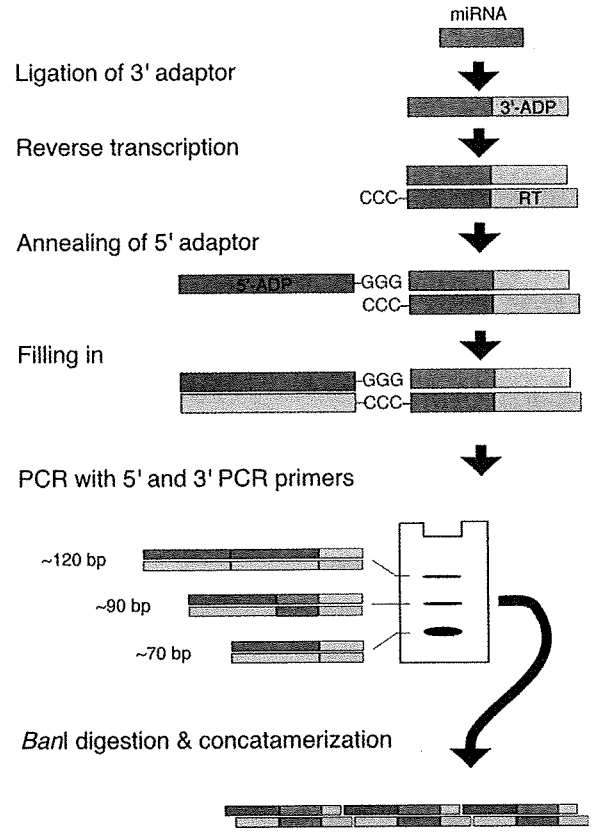

Fig. 1. The mRAP protocol. Isolated small RNA molecules are ligated to the 3′ adaptor (3′-ADP) and subjected to reverse transcription with the RT primer. After annealing of the 5′ adaptor (5′-ADP) to the poly(C) overhang at the 3′ end of the synthesized cDNAs, the latter are subjected to PCR with the 5′ and 3′ PCR primers. Of the three major sizes of amplicon generated, only the middle one includes cDNAs derived from miRNAs. These cDNAs are isolated, digested with BanI, and self-ligated to yield concatamers. Reproduced from [10] with permission of Oxford University Press.

3.1. Required oligonucleotides

3′ adaptor: 5′-(Pu)uuAACCGCGAATTCCAG(idT)-3′ (lowercase letters indicate RNA, uppercase letters

indicate DNA, Pu denotes 5′-phosphorylated uridine, and idT represents 3′-inverted deoxythymidine [Dharmacon, Chicago, IL])
5′ adaptor: 5′-GACCACGCGTATCGGGCACCACG TATGCTATCGATCGTGAGATGGG-3′
RT primer: 5′-GACTAGCTGGAATTCGCGGTTAA A-3′
5′ PCR primer: 5′-GCGTATCGGGCACCACGTAT GC-3′
3′ PCR primer: 5′-GACTAGCTTGGTGCCGAATTC GCGGTTAAA-3′

3.2. Synthesis of cDNAs from miRNAs

1. Mix the small RNAs (8.75 μl) with 1 μl of 10× NEB buffer 3 (New England Biolabs, Ipswich, MA) and 0.25 μl of calf intestinal alkaline phosphatase (New England Biolabs) in a microcentrifuge tube, and incubate the mixture at 50 °C for 30 min.
2. Subject the mixture to phenol–chloroform extraction and chloroform extraction followed by ethanol precipitation in the presence of 1 μl of glycogen (20 mg/ml stock) and 0.5 μl of 100 μM 3′ adaptor.
3. Isolate the RNA by centrifugation and allow it to dry before dissolving it in 3 μl of water.
4. To the dissolved RNA, add the following: 1 μl of 10× Ligation Buffer (New England Biolabs), 1 μl of acetylated bovine serum albumin (Invitrogen, Carlsbad, CA; 1 mg/ml stock), 1 μl of 1 mM ATP, and 3 μl of 50% dimethyl sulfoxide. Incubate the mixture at 90 °C for 30 s and then place it on ice for 20 s.
5. Add 1 μl of T4 RNA ligase (New England Biolabs), and incubate the mixture at 37 °C for 1 h. Perform phenol–chloroform extraction, chloroform extraction, and ethanol precipitation. Isolate the RNA by centrifugation and dissolve it in 4 μl of water.
6. Add 0.5 μl of 100 μM RT primer and 0.5 μl of 100 μM 5′ adaptor. Incubate the mixture at 70 °C for 2 min, and then place it on ice.
7. Add the following: 2 μl of 5× RT buffer (Clontech, Mountain View, CA), 1 μl of 0.1 M dithiothreitol, 1 μl of a mixture of the four deoxynucleoside triphosphates (dNTPs) each at 2.5 mM, and 1 μl of PowerScript reverse transcriptase (Clontech). Incubate the resulting mixture at 42 °C for 1 h.
8. Add 40 μl of water, and incubate the mixture at 72 °C for 7 min. Add 160 μl of water.

3.3. PCR amplification of miRNA-derived cDNAs

1. Mix the following: 49 μl of cDNA solution, 1706 μl of water , 245 μl of 10× AmpliTaq buffer (Applied Biosystems, Foster City, CA), 245 μl of a mixture of the four dNTPs each at 2 mM, 196 μl of a mixture of the 5′ PCR primer and 3′ PCR primer each at 10 μM, and 9.8 μl of AmpliTaq Gold DNA polymerase (Applied Biosystems).
2. Transfer 50 μl of the reaction mixture to each of 48 PCR tubes (0.5-ml scale).
3. Perform PCR with an initial incubation at 95 °C for 4.5 min; 32 cycles of 95 °C for 30 s and 65 °C for 30 s; and a final incubation at 72 °C for 5 min.
4. Subject each reaction mixture to sodium acetate–ethanol precipitation as follows: add 1/10th volume of 3 M NaOAc (pH 5.5), mix, add two volumes of 100% ethanol, mix, and chill at −70 °C for 15 min. Centrifuge the tubes, and dissolve the final precipitates in water.

3.4. Concatamer formation

1. Subject the PCR products and size standards to PAGE on a 10% gel under nondenaturing conditions.
2. Stain the gel with ethidium bromide, and excise the portion of the gel containing DNA molecules of 90–95 bp.
3. Chop the gel portion into small fragments, transfer the fragments to a microcentrifuge tube, and add 200 μl of 0.3 M NaCl. Incubate the mixture at 37 °C for $\geqslant$8 h.
4. Briefly centrifuge the tube and harvest the supernatant containing the cDNAs.
5. Subject the cDNA preparation to ethanol precipitation (in the presence of glycogen). Isolate the DNA by centrifugation and dissolve it in 43 μl of water.
6. Add 5 μl of 10× K buffer (New England Biolabs) and 2 μl of BanI (New England Biolabs; 20 U/μl stock). Incubate the mixture at 37 °C for 2 h or overnight.
7. Purify the DNA with ProbeQuant G50 (GE Healthcare Bio-Sciences, Uppsala, Sweden) and then subject it to phenol-chloroform extraction, chloroform extraction, and ethanol precipitation (in the presence of glycogen). Isolate the DNA by centrifugation and dissolve it in 2 μl of water.
8. Add 2 μl of Ligation High solution (Toyobo, Tokyo, Japan), and incubate the resulting mixture at 16 °C for 4 h or overnight.
9. Twenty minutes before the end of the ligation incubation, prepare the following mixture (Taq mix): 76 μl of water, 10 μl of 10 × NH_4 buffer (Bioline, London, UK), 3 μl of 50 mM $MgCl_2$, 10 μl of a mixture of the four dNTPs each at 2 mM, and 1 μl of BioTaq DNA polymerase (Bioline).
10. Incubate the Taq mix at 95 °C for 10 min and then maintain it at 72 °C.
11. Add the ligation mixture to the Taq mix and incubate at 72 °C for an additional 30 min.
12. Subject the mixture to ammonium acetate–ethanol precipitation as follows: add 1/4th volume of 10 M ammonium acetate (i.e., 2 M final), mix, add two volumes of 100% ethanol, mix, and chill at −70 °C for 15 min. Centrifuge the tubes, and dissolve the final precipitates in water.

13. Fractionate the DNA molecules by PAGE on a 10% gel under nondenaturing conditions.
14. Excise the portion of the gel containing DNA molecules of 500–2000 bp.
15. Chop the excised region of the gel into small fragments and transfer them to a D-tube (EMD Biosciences, San Diego, CA). Subject the fragments to electrophoretic elution at 100 V for 4 h in Tris–borate–EDTA buffer.
16. Harvest the solution and subject it to sodium acetate–ethanol precipitation in the presence of glycogen.
17. Isolate the cDNA concatamers by centrifugation and dissolve them in water. Ligate the concatamers into a TA cloning vector such as pGEM-Teasy (Promega, Madison, WI) for nucleotide sequencing.

4. Sequencing of mRAP products

We usually first assess the quality of the mRAP plasmid library by sequencing the inserts of ~100 randomly isolated plasmids. The plasmid inserts typically contain two to six cDNAs (Fig. 2). BLAST searching of the insert cDNAs against the genome sequence of interest has revealed that about one-third of the cDNAs correspond to miRNAs, one-third to ribosomal or transfer RNA, and the remaining one-third to other sequences [10]. In our experience, a high proportion of "other sequences" indicates poor quality of the initial RNA preparation. Abundant miRNA sequences can be obtained even from $\leqslant 1 \times 10^4$ cells, provided that the isolated RNA is in good condition.

Our initial trial with mRAP to characterize the entire miRNA repertoire of the mouse resulted in the identification of a total of 77,736 small RNA reads [10]. Even at this scale, many miRNAs (both known and predicted) were present in only one read per tissue, indicating that our trial did not exhaust the miRNA repertoire. It might therefore be of interest to couple mRAP to recently developed high-throughput sequencing technologies. Pyrosequencing or other recent methods are capable of simultaneously determining short nucleotide sequences for 1×10^5–1×10^7 clones [11]. Sequencing of mRAP products on such a scale should allow characterization of a complete body map of miRNA profiles for any organism.

5. Troubleshooting

5.1. Three bands are not observed in the gel after RT-PCR at step 3.4.2

If small amounts of starting material, such as fresh clinical specimens, are used, distinct bands around 90–95 bp may not be observed at step 3.4.2; instead a smear may appear. Since the cDNA/adaptor products should exist in this smear (at 90–95 bp), cut the corresponding region according to the size marker. Since this process may result in cutting out a region of an incorrect size, also individually cut out the fractions of the gel above and below this region. Treat these three fractions in parallel in succeeding steps described above. Sequencing reveals which fraction contains the targets.

5.2. Most of the sequences obtained are derived from the adaptors used in mRAP

The reason for this is that ~70 bp and/or ~120 bp bands are contaminated at step 3.4.2. Contamination of ~70 bp or ~120 bp bands leads to obtaining the sequences for "5′ adaptor-3′ adaptor" or "5′ adaptor-5′ adaptor-3′ adaptor", respectively. Since abundances of the adaptor-derived bands (~70 and ~120 bp) are much greater than that of the desired adaptor/cDNA band (90–95 bp), cut out only the gel at 90–95 bp without even touching the ~70 and ~120 bp bands. An extended time of electrophoresis facilitates separating adaptors and cDNA/adaptor bands. For this purpose electrophoresis can run until BPB dye is about to run out the bottom edge of the gel.

6. Concluding remarks

Given the emerging roles of miRNA in animal development or pathogenesis of human disorders, it is mandatory

GCACCACGTATGCTATCGATCCTGAGATGGGTGTTGCACTTGTCCCGGCCTGTTTTAACC
GCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGTAGTGTTTCCTACTTTATG
GATTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGCGGGCGGCGGC
GGTCGGCGGGCTTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGA
AGCGGGTTTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGATGGGGTAGTG
TTTCCTACTTTATGGATTTAACCGCGAATTCGGCACCACGTATGCTATCGATCGTGAGAT
GGGGTAGTGTTTCCTACTTTATGGAAATTTAACCGCGAATTCGGCACCACGTATGCTATC
GATCGTGAGATGGGGGGCTGGGGCGCGAAGCGGGGCTTTAACCGCGAATTCGGCACCACG
TATGCTATCGATCGTGAGATGGGGGGTCGGGGCGGCGGCGGCGGTTTAACCGCGAAT
TCGGCACCACGTATGCTATCGATCGTGAGATGGGTGTGCAAATCTATGCAAAACTG

Fig. 2. Example of a plasmid insert of an mRAP library. The nucleotide sequence of one insert in a library prepared from human Jurkat cells is shown. Black letters indicate the sequences of adaptors, whereas red or blue letters indicate the cDNA sequences for miRNAs or for other sequences (ribosomal RNA or genomic DNA sequences), respectively. BanI sites are underlined.

(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

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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].

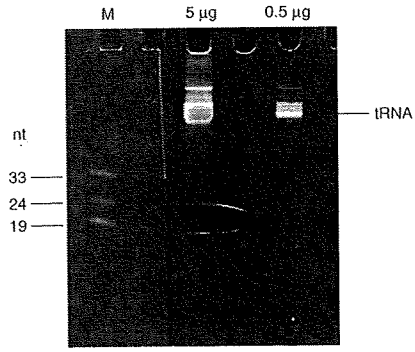

Figure 1 | Example of size selection of small RNAs. A fraction of Jurkat cell RNA consisting of molecules of <200 nt (5 or 0.5 μg) was subjected to denaturing PAGE on a 15% gel. The portion of the gel between the positions corresponding to 19- and 24-nt markers was excised, and the remaining gel was 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.

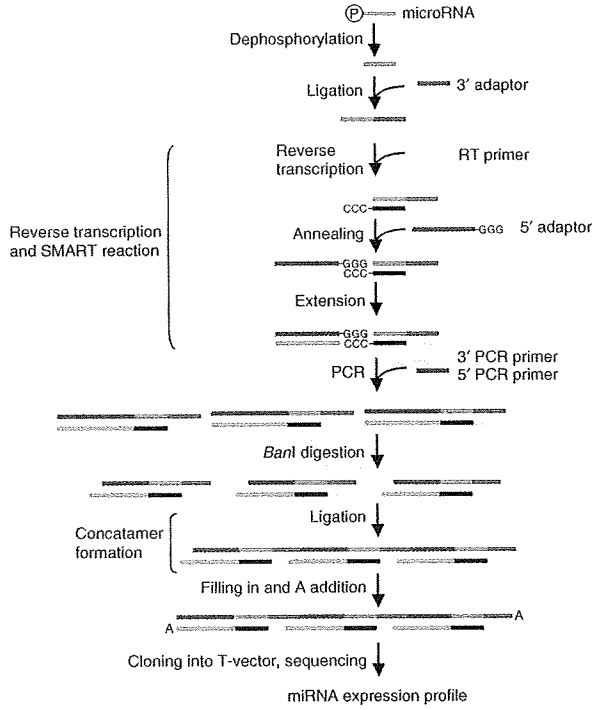

Figure 2 | Schematic representation of the reactions that constitute the microRNA (miRNA) amplification profiling (mRAP) method. MicroRNAs are dephosphorylated, ligated to the 3′ adaptor and subjected to reverse transcription (RT) and the SMART (switching mechanism at the 5′-end of RNA templates of reverse 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.

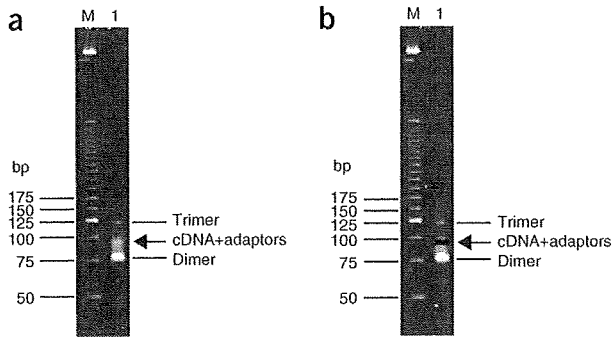

Figure 3 | Example of isolation of the cDNA fraction from the dimer and trimer by-products of PCR. (**a**) PCR products derived from small RNAs of Jurkat cells were subjected to PAGE on a 10% gel (lane 1). (**b**) The portion of the gel shown in (**a**) containing cDNAs (90–95 bp) was excised. The positions 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)

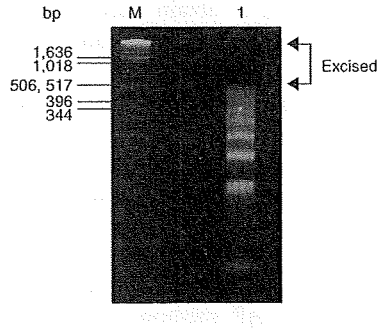

Figure 4 | Example of size selection of concatamers. The concatamers synthesized from Jurkat cDNA were subjected to PAGE on a 10% gel (lane 1). The portion of the gel containing products of >500 bp was excised on the basis of the positions of size markers, and the remaining gel was then stained with 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.