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Real-time expression profiling of microRNA precursors in human cancer cell lines

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ABSTRACT

Our previous study described a real-time PCR method to quantify microRNA (miRNA) precursors using SYBR green detection [T. D. Schmittgen, J. Jiang, Q. Liu and L. Yang (2004) *Nucleic Acids Res.*, 32, e43]. The present study adapted the assay to a 384-well format and expanded it to include primers to 222 human miRNA precursors. TaqMan minor groove binder probes were used to discriminate nearly identical members of the let-7 family of miRNA isoforms. The miRNA precursor expression was profiled in 32 human cell lines from lung, breast, colorectal, hematologic, prostate, pancreatic, and head and neck cancers. Some miRNA precursors were expressed at similar levels in many of the cell lines, while others were differentially expressed. Clustering analysis of the miRNA precursor expression data revealed that most of the cell lines clustered into their respective tissues from which each cell line was ostensibly derived. miRNA precursor expression by PCR paralleled the mature miRNA expression by northern blotting for most of the conditions studied. Our study provides PCR primer sequences to all of the known human miRNA precursors as of December 2004 and provides a database of the miRNA precursor expression in many commonly used human cancer cell lines.

INTRODUCTION

Mature microRNAs (miRNAs) are endogenous, ~21 nt noncoding RNAs whose primary function is believed to be translational repression of protein coding mRNAs [reviewed in (1,2)]. The mature miRNA is processed from longer precursor molecules by the enzymes Drosha (3) and Dicer (2). While >220 miRNAs have been discovered in humans alone (4), the miRNA's targets, expression levels and function in normal and diseased cells remain largely unknown.

Up until very recently, the most common method of quantifying miRNAs was Northern blotting. Northern blotting has the advantage of simultaneously detecting the precursor and mature miRNAs, however, they are insensitive methods with poor throughput. Over the past year, a number of different approaches have been described to quantify miRNAs including cDNA arrays (5–8), a modified Invader assay (9) and real-time PCR to measure miRNA precursors (10,11). Each of these methods has distinct advantages and disadvantages. Real-time PCR has unparalleled sensitivity and specificity; however, these assays quantify the miRNA precursor and not the active, mature miRNA. cDNA arrays have excellent throughput, however arrays are temperamental. Arrays and the Invader assay do not amplify the miRNA and thus the sensitivity is often compromised.

Our earlier study reported a real-time PCR method to quantify hairpin-containing miRNA precursors (11). This method used gene-specific primers and a thermostable RT to convert the hairpin of the primary precursor and precursor miRNA to cDNA. The cDNA was subsequently amplified and quantified using real-time PCR and SYBR green detection. Our prior study described assays to 23 miRNA precursors and studied the expression levels in six cancer cell lines (11). The expression levels of the miRNA precursors (PCR assay) were validated to the mature miRNA (northern blotting) for three miRNAs in three cancer cell lines (11). We expand the assay here to include PCR assays to 222 human miRNA precursors representing all of the known human miRNAs as of December 2004 (4).

Of the 222 human miRNA genes discovered thus far, 38 are grouped into families of nearly identical isoforms (4). The largest of the human families include let-7 (14 members) and miR-30 (6 members). miRNA isoforms may be one of two types. The first type is when the mature miRNAs are of nearly identical sequences, usually differing by 1–3 nt. These families are designated with a letter (e.g. let-7b and let-7c) (Figure 1). The second designation is for miRNA genes that produce the identical mature miRNA from a slightly different precursor gene (e.g. let-7a-1 and let-7a-2) (Figure 1). These are designated with a number implying that both genes, let-7a-1

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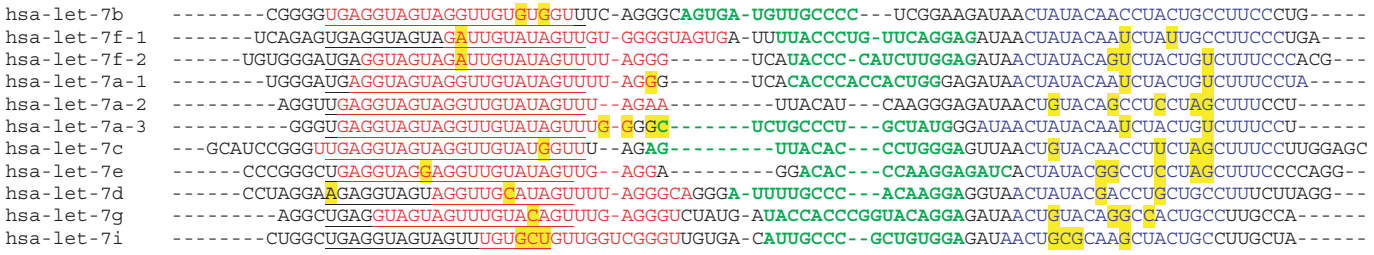


Figure 1. Primer and TaqMan probe sequences to let-7 miRNA isoforms. The sequences of the miRNA precursors for the members of the human let-7 family of miRNA isoforms are shown. Underlined, sequence of the mature miRNA; red, sequences of the forward PCR primers; blue, sequences of the reverse PCR primers; green sequences of the TaqMan MGB probe; and yellow, priming sequences that differ among isoforms. Sequences are in the 5′ to 3′ direction.

and let-7a-2, produce the identical mature miRNA (let-7a). Each isoform is usually located on different chromosomes. TaqMan minor groove binder (MGB) probes were designed to the loop portion of the miRNA precursor allowing discrimination of individual members of the let-7 miRNA isoforms.

MATERIALS AND METHODS

Cell lines, tissues and tissue culture

The following human tumour cell lines were used: K-562 (chronic myelogenous leukemia); HL-60 (promyeolocytic leukemia); Daudi and Ramos (Burkitt lymphoma); Jurkat (T-cell leukemia); LNCaP, PC3, PPC-1, DU145 and TSU-PR1 (prostate); SCC17A, SCC17B, SCCD12, SCC10B and SCC5 (head and neck squamous cell carcinoma); MDA231, T47D, SKBR3, MDA361 and MCF7 (breast cancer); SW620, HCT8, HCT116, HT29 and HCT15 (colorectal carcinoma); Panc1 and Hs 766T (pancreatic); H23, H522, HOP62, A549 and H719 (lung cancer); RH30, RH3, CW9019, SMS-CTR and RD2 (rhabdomyosarcoma) and SK-Hep1, PLC/PRF5, SNU387 and SNU449 (liver cancer). Cells were obtained from American Type Culture Collection (Manassas, VA) or were obtained from various laboratories. Cancer cell lines were cultured in a humidified atmosphere of 95% air, 5% CO_2 using RPMI 1640 or other suitable media and 10% fetal bovine serum. Total RNA from normal human liver and skeletal muscle tissue was purchased from Ambion (Austin, TX). Hepatocellular carcinoma tumours were received from Dr Lewis Roberts, Mayo Clinic, Rochester, MN. Collection and distribution of liver tissues were approved by the appropriate Institutional Review Boards at both institutions.

Cloning

The sequences of six miRNA isoforms (let-7a-1, let-7a-2, let-7a-3, let-7f-1, let-7f-2 and let-7d) were amplified from genomic DNA (Roche Applied Science, Indianapolis, IN) using primers listed in Supplementary Table 2. Primers were designed to span ~100–125 bp on each side of the hairpin. The amplicons were cloned into plasmids using TOPO TA cloning (Invitrogen, Carlsbad, CA). The sequences of the let-7 isoforms were verified by DNA sequencing.

Primers and TaqMan MGB probes

Primers were designed to all of the known human miRNAs as of December 2004 (4). These 222 miRNA genes include 38 families of isoforms. Many of the miRNA isoforms differed by only 1–3 bp in the primer binding sequence (Figure 1 and Supplementary Figure 1). If the difference in sequence occurred towards the 5′ end of the primer, then the same pair of primers was used to amplify both isoforms. If the sequence difference occurred towards the 3′ end of the primer or there were multiple differences, then a unique pair of primers was designed to each isoform. An example for the miR-30 isoforms is shown in Supplementary Figure 1. For this particular isoform family, four different primer pairs were used, one for miR-30a/miR-30e, one for miR-30c-1/miR-30c-2 and one for each of miR-30b and miR-30d (Supplementary Figure 1). Throughout the study we refer to the expression of 222 miRNA precursors. In actuality, primers were designed to and data are presented on 201 miRNA precursors since several isoforms were amplified by the same pair of primers.

Primers were designed to the primary precursor molecule for several miRNAs. These are designated by the letter 'P' in Supplementary Table 1. Primers were designed to the primary precursor if we were unable to successfully design primers to the hairpin-containing precursor. In addition, some primers were designed to the primary precursors of miRNA isoforms. Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA) using the criteria described previously (11). TaqMan MGB probes were designed using Primer Express software. Probes were designed to have a 5′ FAM and an MGB at the 3′ end. TaqMan MGB probes were synthesized by Applied Biosystems. Sequences of the TaqMan MGB probes are listed in Supplementary Table 3. Primers were validated on human genomic DNA (Roche), mouse genomic DNA, cDNA synthesized from Universal Human Reference RNA (Stratagene) and no template control reactions.

RNA extraction, DNA extraction and reverse transcription

cDNA was synthesized from total RNA using gene-specific primers as described (11). Briefly, total RNA was extracted from 3×10^6 cells using TRIZOL (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Total RNA was extracted from the liver tumors using TRIZOL, after pulverizing the tumours in a stainless steel mortar and pestle that was chilled on dry ice. Total RNA was briefly exposed to RNAase-free DNAase I and 1 μg was reverse transcribed to cDNA using gene-specific primers and Thermoscript RT (Invitrogen). The gene specific primers included a mixture of 10 μM

each of the antisense primers to all of the miRNAs and U6 RNA listed in the Supplementary Table 1. Following an 80°C denaturation step and 60°C annealing, the cDNA was reacted for 45 min at 60°C as described (11). Genomic DNA from NIH 3T3 mouse fibroblasts was isolated as described (12).

Real-time PCR

The expression of the miRNA precursors was determined using real-time quantitative PCR as described (11) with several modifications. Master mix (3 µl) containing all of the reaction components except the primers was dispensed into a 384-well real-time PCR plate (Applied Biosystems) using a 12-channel repeating pipette (Model EDP3-Plus, Rainin Instruments, Woburn, MA). The master mix contained 0.5 µl of 10× PCR buffer, 0.7 µl of 25 mM $MgCl_2$, 0.1 µl of 12.5 mM dNTPs, 0.01 µl UNG, 0.025 µl Amplitaq Gold DNA polymerase, 0.5 µl of dilute cDNA (1:50) and water to 3 µl. All of the PCR reagents were from the SYBR green core reagent kit (Applied Biosystems). A 2 µM solution of each pair of primers listed in Supplementary Table 1 was stored in 12-well PCR strip tubes. Each primer (2 µl) was dispensed into duplicate wells of the 384-well plate using the 12-channel repeating pipette. Everything was identical for the TaqMan assays except the TaqMan core reagent kit (Applied Biosystems) and 200 nM of the TaqMan MGB primers were used. Each miRNA listed in Supplementary Table 1 and U6 RNA was assayed in duplicate in the 384-well reaction plate. Real-time PCR was performed on an Applied Biosystems 7900HT real-time PCR instrument equipped with a 384-well reaction block. PCR was performed for 15 s at 95°C and 1 min at 60°C for 40 cycles followed by the thermal denaturation protocol. TaqMan and SYBR green assays may be run simultaneously on the 7900HT real-time instrument. The expression of each miRNA relative to U6 RNA was determined using the $2^{-\Delta CT}$ method (13). To simplify the presentation of the data, the relative expression values were multiplied by 10^5.

Northern blotting

Northern blotting was performed as described (11,14). DNA oligonucloetides of the reverse compliment to the mature miRNA were used as probes. Blots were successfully stripped and re-probed up to three times.

Data analysis

Hierarchical clustering was performed on PCR data (15). ΔCT values were used in the analysis (16). Values for each miRNA were median-centered before clustering. Additional statistical analysis was done using TIGR Mutiple Array Viewer software package (TMeV version 3.0) (17). Data were grouped in seven groups according to the tissue origin of the cell lines. Gene expression data were then filtered for significance using ANOVA multiple groups test (18,19) with *P*-values obtained from permutation tests (1000 permutations) using Pearson Correlation as a distance. This subset of 85 significant genes (Supplementary Table 6) was used for unsupervised hierarchical clustering based on Complete Linkage method with Pearson Correlation as a distance (15). Support trees (20) for samples were constructed using gene resampling by Jackknifing (resampling without replacement) with 1000 permutations.

RESULTS

TaqMan assay to discriminate miRNA isoforms

TaqMan MGB probes were designed to anneal to the loop portion of the miRNA precursors (Figure 1). TaqMan probes are typically designed with a melting temperature (T_m) that is 10°C higher than that of the primers. As shown in Figure 1, the space in between the primer annealing site is very short (~15–20 bp). The presence of the MGB allows the design of short TaqMan probes with T_ms ranging from 61–68°C (Supplementary Table 3).

An experiment was performed to determine if it is possible to distinguish one miRNA sequence among nearly identical isoforms using TaqMan MGB probes. The sequences of six miRNA isoforms (let-7a-1, let-7a-2, let-7a-3, let-7f-1, let-7f-2 and let-7d) were cloned into plasmids using PCR and TOPO TA cloning. The identity of the sequences was verified by DNA sequencing. Real-time PCR was attempted on seven different reactions. Each reaction contained the plasmid for each miRNA precursor and primers specific to each miRNA isoform. The TaqMan MGB probe for let-7d was added to each PCR. Only the reaction containing the let-7d plasmid gave a detectable signal (Figure 2A). Following the real-time PCR, a portion of each reaction was resolved on an agarose gel to verify that amplification had occurred in each PCR (Figure 2B).

Real-time PCR was attempted on plasmids containing each of the five miRNA precursors (let-7a-1, let-7a-3, let-7f-1, let-7f-2 and let-7d) using primers and TaqMan MGB probes specific to each miRNA. Only those reactions containing primers and probes to the intended target produced a detectable C_T (Figure 3). These experiments verified that it is possible to use TaqMan MBG probes to quantify miRNA precursors and that the detection is highly specific to the intended isoform.

Validation of miRNA precursor primers, SYBR green

Each pair of primers listed in Supplementary Table 1 was validated on human genomic DNA, cDNA synthesized from Universal Human Reference RNA, mouse genomic DNA and no template control reactions. All of the primers listed in Supplementary Table 1 worked successfully on human genomic DNA (not shown). Successful amplification was defined by the presence of a single dissociation peak on the thermal melting curve. For those reactions that produced multiple dissociation peaks, a new pair of primers were designed to the primary precursor miRNA. These primers are listed with the designation 'p', e.g. miR-9-1(p) (Supplementary Table 1). Many of the miRNA genes that required priming of the primary precursor were miRNA genes with known isoforms (e.g. miR-9-1, -19b-1 and -106a). About 70% of the human primers successfully amplified mouse genomic DNA (Supplementary Table 1). The ability of primers to amplify both human and mouse miRNA genes is probably owing to the similarity in sequence among these genes. Human miRNA primers were not tested on mouse cDNA.

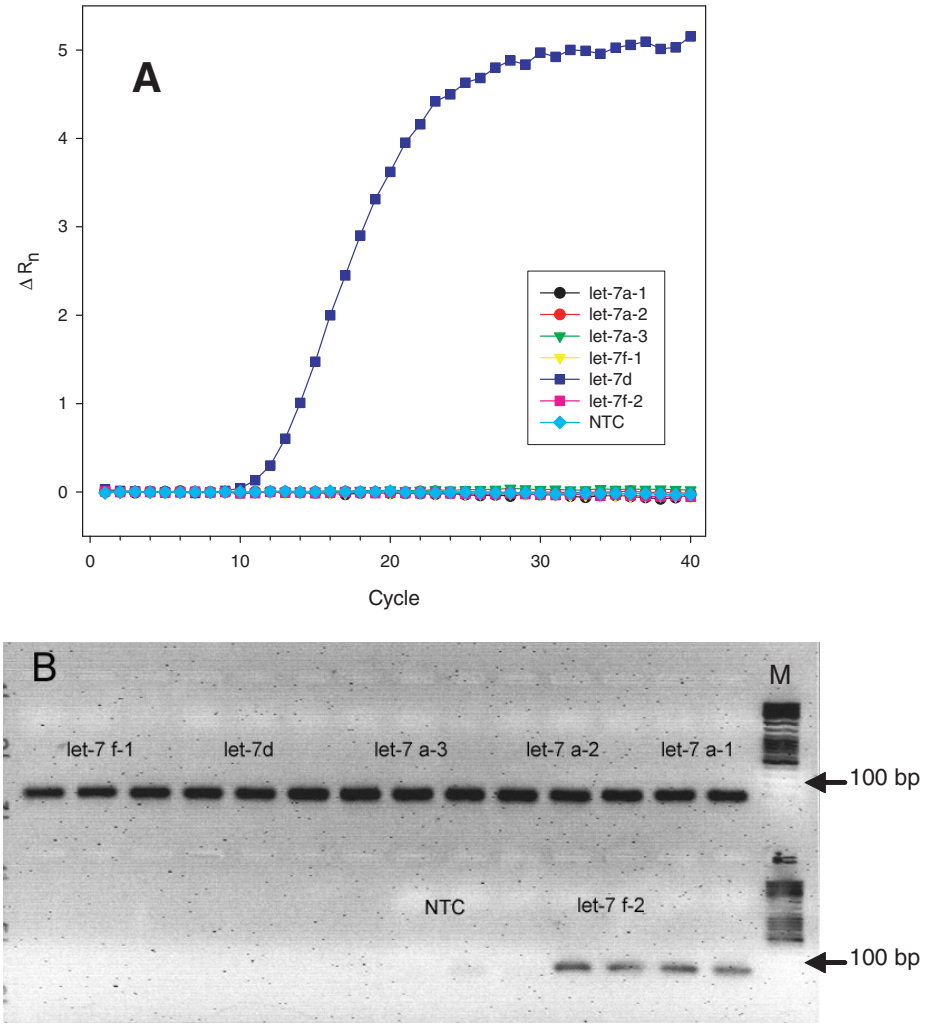


Figure 2. Real-time PCR of miRNA precursor isoforms. The sequences of six miRNA precursor isoforms (let-7a-1, let-7a-2, let-7a-3, let-7f-1, let-7f-2 and let-7d) were cloned into plasmids. Real-time PCR was attempted on seven different reactions (in triplicate) containing each plasmid and primers specific to each isoform. Each reaction contained the TaqMan MGB probe for let-7d. Only the reaction containing the let-7d plasmid gave a detectable signal (**A**). Following the real-time PCR, a portion of each reaction was run on an agarose gel to demonstrate that PCR had occurred in each reaction (**B**). NTC, no template control. M, 100 bp DNA ladder.

		let-7 miRNA target					
		let-7a-1	let-7a-3	let-7d	let-7f-1	let-7f-2	
miRNA	let-7a-1	100	0	<0.01	0	0	Relative detection (%)
	let-7a-3	<0.01	100	0	<0.01	0	
	let-7d	0	0	100	0	<0.01	
	let-7f-1	0	<0.01	<0.01	100	0	
	let-7f-2	0	0	0	<0.01	100	

Figure 3. Discrimination of let-7 miRNA precursor isoforms. Real-time PCR was attempted on miRNA precursor genes contained within plasmids using gene-specific primers and TaqMan MGB probes. The relative detection was calculated based upon the C_T difference between the perfectly matched and mismatched targets.

miRNA precursor expression profiling in cancer cell lines

The expression of 222 miRNA precursors was profiled in 32 commonly used cell lines of lung, breast, head and neck, colorectal, prostate, pancreatic, and hematopoietic cancers. Gene expression data was normalized to U6 RNA. U6 was validated as an internal control by comparing its expression levels in each of the cell lines. U6 RNA was consistently expressed in each of the 32 cell lines (Figure 4), thus U6

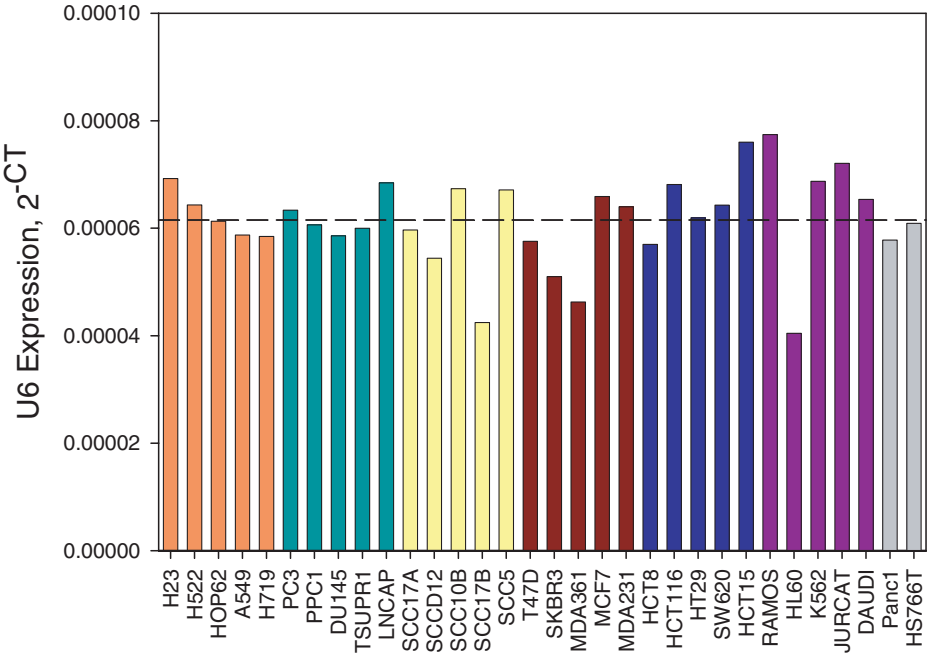


Figure 4. U6 RNA expression in cancer cell lines. The expression of U6 RNA internal control in lung (orange), prostate (green), head and neck (yellow), breast (brown), colorectal (blue), hematopoietic (violet) and pancreatic (grey) cancer cell lines. Dashed line, mean U6 value.

RNA is an acceptable internal control for quantitative PCR in these cell lines.

The relative expression was determined for each of the 222 miRNA precursors (Supplementary Table 4). The relative expression for all 222 miRNA precursors was clustered using unsupervised hierarchical clustering and presented as a heatmap (Figure 5). Unsupervised hierarchical clustering was performed on the data presented as ΔC_T (16), the raw data may be found in Supplementary Table 5. The heatmap and dendrogram demonstrate that most of the cell lines clustered into their respective tissues from which each cell line was ostensibly derived (Figure 5). Five of five hematopoietic and head and neck cell lines and two of two pancreatic cell lines produced unique clusters. Four of five lung and colorectal cell lines produced unique clusters as well. The breast cancer and prostate cancer cell lines tended to cluster together with four of five of the prostate cell lines forming a cluster (along with one breast cancer cell line) and three of five breast plus one prostate forming another cluster (Figure 5B).

Unsupervised hierarchical clustering was performed on a subset of 85 genes which were differentially expressed ($P = 0.01$) among groups of cell lines as determined by ANOVA multi-group comparison test. The subset of the 85 significant genes are listed in Supplementary Table 5. The resulting support tree (Supplementary Figure 2) shows high level of support (80–100%) and somewhat better separation of cell lines according to their tissue of origin. The hematopoietic and pancreatic lines group into unique clusters (Supplementary Figure 2). The remaining five cell lines group more closely than in the unfiltered analysis (Figure 5); two breast lines being the only exception (Supplementary Figure 2). However, each of the cell lines does not group into pure clusters, some trees of the dendrogram contain more than five cell lines (Supplementary Figure 2).

There were also individual or small groups of cell lines with miRNA expression that differed significantly from the rest of the group. All five head and neck cancer cell lines expressed miR-205 at levels that were 36-fold higher compared with the other cell lines (Table 1). Increased miR-205 expression in the head and neck cell lines was validated by northern blotting (Figure 6). Other examples include increased let-7c precursor expression in the prostate cancer cell line LNCaP (53-fold) and in the lung cancer cell line H522 (7-fold). miR-7-3 was increased by 122-fold in the colorectal cancer cell line SW620. Some miRNAs were significantly decreased compared to the entire group including miR-134 in HL-60 cells. The expression of miRNAs cloned from pancreas tissue (miR-375 and miR-376) was expressed at higher levels in the mouse pancreas and pancreatic islet cells compared with mouse brain, heart and liver tissue (21). While expression of miR-376 precursor in the pancreatic cancer cell line Panc-1 was among the highest of the cell lines studied, expression of miR-375 in the two pancreatic cancer cell lines did not differ from the other cell lines (Supplementary Table 4).

Validation of miRNA precursor expression data

The PCR assay quantifies the miRNA precursors (primary precursor and precursor) and not the active, mature miRNA. In order to validate the miRNA precursor data, northern blotting was performed on total RNA from eight of the cell lines. RNA from MDA-231, SW-620, TSU, PPC-1, DU-145, SCC-5, HCT-8 and SCC-17A cells was probed for miR-21, -205, -100 and U6 RNA. The expression of the miRNA precursors from the PCR assay paralleled that of the mature miRNA by northern blotting, although the correlation was somewhat better for miR-100 and miR-205 compared with miR-21 (Figure 6).

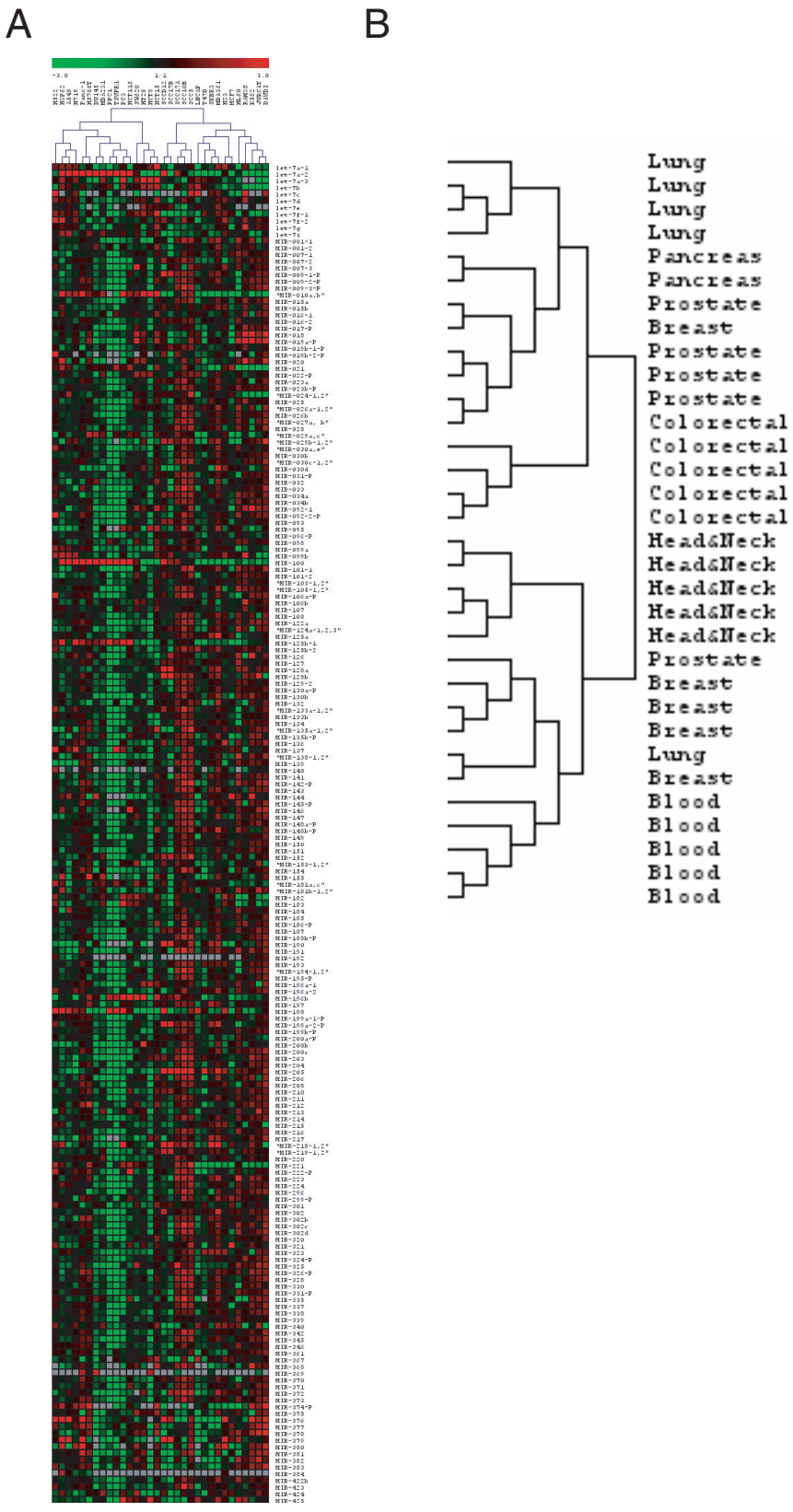


Figure 5. Heatmap of miRNA precursor expression in 32 human cancer cell lines. (**A**) The names of the 32 cancer cell lines are listed on the top of the figure. The names of the miRNAs that were profiled in the cancer cell lines are listed to the right of the figure. The relative expression of each gene was determined by real-time PCR; data are presented as ΔC_T. Unsupervised hierarchical clustering was performed using PCR primers to 201 miRNA precursors. Data were unfiltered prior to clustering. A median expression value equal to one was designated black; red increased expression; green, reduced expression; grey, undetectable expression. (**B**) Dendrogram of clustering analysis.

Table 1. miRNA precursors with significantly different expression

miRNA	Located on chromosome	Cell lines	Cancer	Fold-change[a]
miR-205	1q32	SCC17A, SCC17B, SCCD12, SCC10B, SCC5	Head & neck	+36.5
miR-7-3	19p13	SW620	Colorectal	+122
miR-10a	17q21	HCT-8, HCT-15	Colorectal	+28
miR-10b	2q31			
miR-100	11q24	PC3, PPC-1, DU145, TSU-PR1	Prostate (androgen independent)	+4
let-7c	21q21	LNCaP	Prostate (androgen dependent)	+53
let-7c	21q21	H522	Lung	+7
let-7f-1	9q22	T47D, SKBR3, MDA361, MCF7, MDA231, H23, H522, HOP62, A549, H719, HCT8, HCT116, HT29, SW620, HCT15	Breast, lung, colorectal	+7
miR-134	14q32	HL60	promyeolocytic leukemia	−30

[a]Fold-change compared with the mean of the remaining cell lines.

Expression of tissue-specific miRNA in cell lines and tissues

Expression of mature miR-1/miR-133, miR-122 and miR-9/miR-124a are reported to be specific to heart/muscle, liver and brain tissue, respectively (22). Precursors to miR-1, miR-133, miR-122a, miR-9 and miR-124a were expressed to some degree in many of the 32 cell lines studied, although none of these cell lines are from liver, brain, heart or muscle origin (Figure 5 and Supplementary Table 4). The expression of these tissue-specific miRNA precursors varied among the 32 cell lines; some such as miR-9-1, miR-9-2 and miR-133a were expressed at medium to high levels while others such as miR-1, miR-122a and miR-124 were expressed at very low levels (Supplementary Table 4). Northern blots were attempted on the panel of cell lines presented in Figure 6 to both miR-9 and miR-9*. Expression of mature miR-9 or miR-9* was undetectable in these cell lines (data not shown). This demonstrates that high levels of miRNA precursors may not be processed to mature miRNA in some cases.

It is also possible that expression of tissue-specific, mature miRNA is restricted to tissues and not cancer cell lines. To address this, the expression of mature miR-122a, reported to be specific to liver tissue (21), was examined in liver cancer cell lines and in liver tissue from cancer and normal liver. As predicted, the expression of mature miR-122a was negligible in four liver cancer cell lines, but was expressed at very high levels in normal liver tissue (Figure 7A). To determine if the differential expression was due to reduced miR-122a in liver cancer, the mature miR-122a expression was determined in three samples of hepatocellular carcinoma tissue from patients. Mature miR-122a was expressed at high levels in the hepatocellular carcinomas as well (Figure 7A). The expression of two additional 'specific' miRNAs miR-1 and miR-133 [specific to heart and skeletal muscle (21)] were also studied. miR-1 was expressed in the rhabdomyosarcoma cell lines but at much lower levels than in muscle tissue, while expression of miR-133 was restricted to muscle tissue but not the muscle-derived cell lines (Figure 7B). The data presented in Figure 7 demonstrate that at least for these three miRNAs, high levels of tissue-specific, mature miRNA expression is restricted to tissues and not to continuous cell lines derived from the same tissue type.

DISCUSSION

Reported here is an extension of our prior real-time PCR assay (11) to include primers to 222 human miRNA precursors. The miRNA precursor expression was profiled in 32 human cancer cell lines. This is the only study to our knowledge to profile miRNA expression in such a large number of cancer cell lines. We also report a novel method to quantify individual members of nearly identical miRNA isoforms using TaqMan MGB probes. The assay was adapted to a 384-well format, allowing for high-throughput quantification of 196 miRNAs in duplicate per reaction plate.

Since the current number of human miRNAs is relatively small (~222) (the number of human miRNAs has increased to 321 since the initial submission of this manuscript), the PCR assay provides a high-throughput screen for most of the miRNA precursors (in duplicate) using a single reaction plate. Other high-throughput assays, such as cDNA micro arrays, have been used to profile miRNA expression (5–8). The advantage of real-time PCR is that it is more quantitative than cDNA arrays; real-time PCR is able to detect a 2-fold difference in gene expression. The relative sensitivity between cDNA micro arrays and real-time PCR is exemplified by the fact that cDNA micro array expression data is validated by real-time PCR, not the other way around. If the number of human miRNAs remain fairly small, then high-throughput, real-time PCR is a practical means to profile miRNA expression. If the number of human miRNAs increase to ~1000, as recently predicted by Berezikov *et al*. (23), it will then be possible to profile miRNA expression using PCR; however, it may be less practical than micro arrays.

Unsupervised hierarchical clustering of unfiltered data demonstrates that the miRNA expression data produced a near-perfect clustering of the cell lines into the tissue type from which each cell line were ostensibly derived (Figure 5). With the exception of the prostate and breast cancer cell lines, each cell line produced perfect or near-perfect clustering into the respective tissue type (Figure 5). This is remarkable in that the analysis was performed on only 201 genes. Ross *et al*. (24) profiled the expression of ~8000 genes in the 60 cell lines used in the National Cancer Institute screen for anti-cancer drugs (24). This analysis revealed that each of the cell types produced near-perfect clustering into tissues from which each cell line was derived; some cell lines such as leukemia and colorectal clustered into unique branches while the other 10 lines were split between different trees of the dendrogram (24). It is interesting that we obtained similar clustering results as Ross *et al*. (24) by profiling only 201 genes compared with 8000. Explanations include the use of real-time PCR rather than cDNA microarrays and that noncoding miRNAs were profiled rather than protein

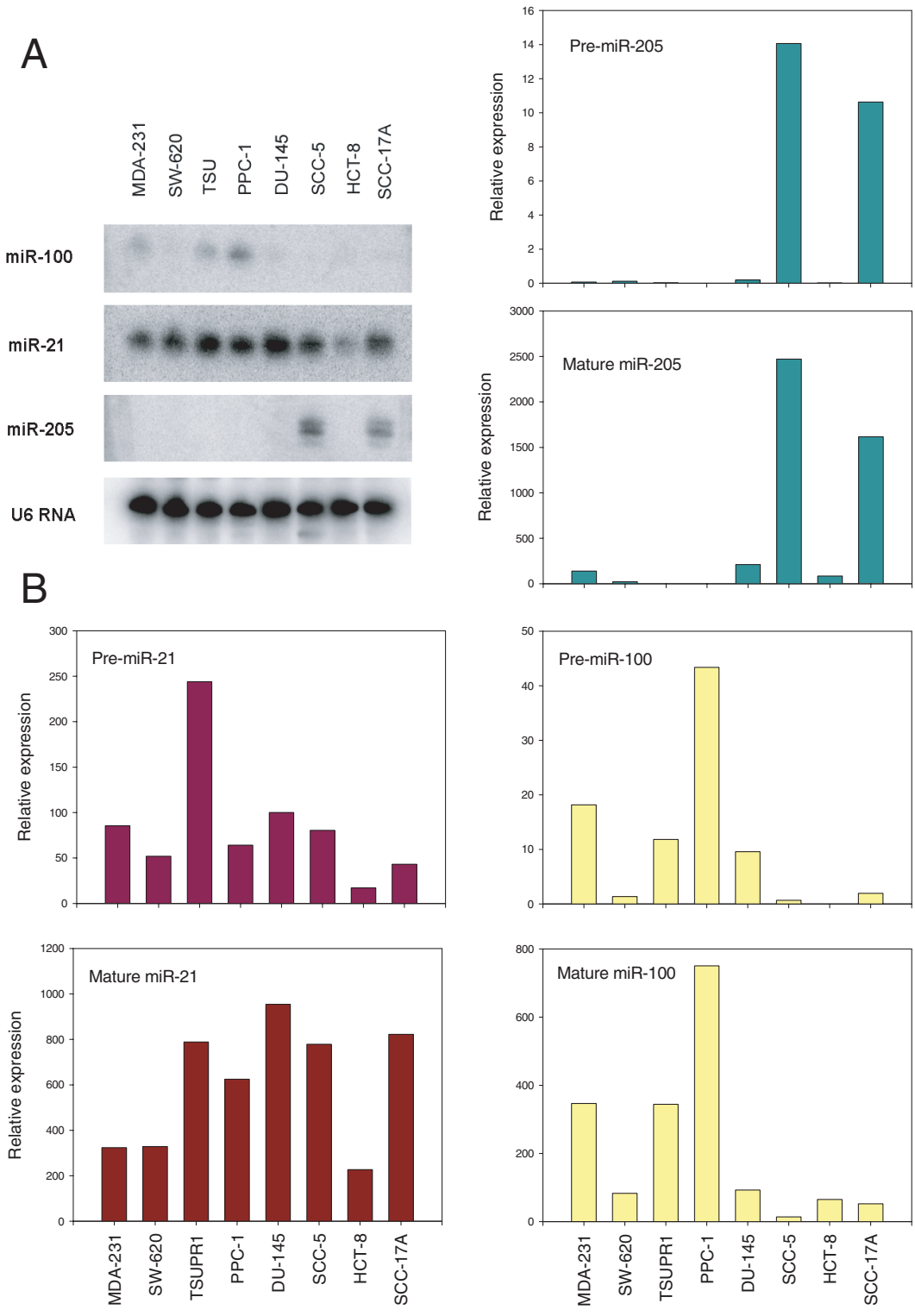


Figure 6. Validation of mature miRNA expression by northern blotting. The expression of three mature miRNAs and U6 RNA was validated in eight cancer cell lines by northern blotting. (**A**) Northern blot of mature miR-100, miR-21, miR-205 and U6 RNA in the eight cancer cell lines listed. The identical blot was stripped and re-probed for each of the RNAs. (**B**) Densitometric analysis of the mature miRNA (northern blot) is compared with the miRNA precursor expression as determined by the real-time PCR assay.

coding mRNAs. Thus, the idea of a tissue-specific miRNA expression signature may have merit, even among cultured cells. In addition to differences in tissue origin, the cell lines were also from three different cell lineages, epithelial (prostate, breast, colorectal, lung and pancreas), hematopoietic and squamous cell (head and neck). Clustering produced complete separation of the squamous cell and hematopoetic cell lines.

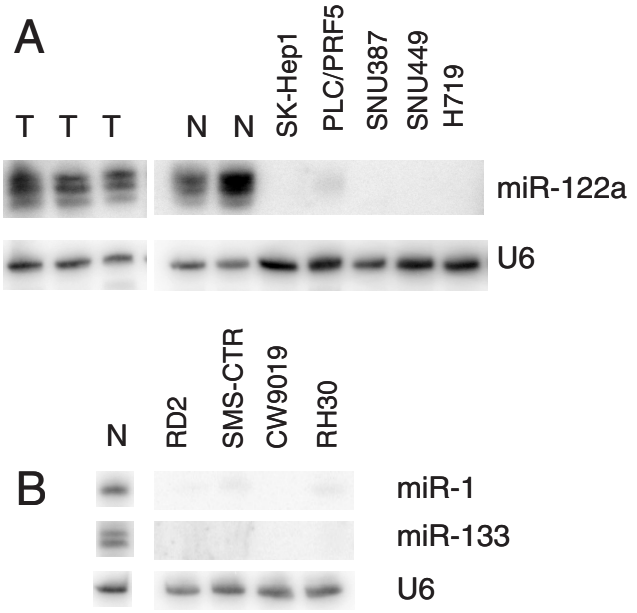


Figure 7. Reduced expression of tissue-specific miRNAs in continuous cell lines compared with tissues. The expression of miR-122a and miR-1-1/miR-133 were reported previously to be specific to liver and muscle tissue, respectively. (**A**) Northern blotting to miR-122a was performed on RNA isolated from specimens of hepatocellular carcinoma from three different patients (T), normal liver tissue (N) and in the liver cancer cell lines SK-Hep1, PLC/PRF5, SNU387 and SNU449, and lung cancer cell line H719. The blots were stripped and re-probed for U6 RNA. (**B**) Total RNA from the rhabdomyosarcoma cell lines RD2, SMS-CTR, CW9019 and RH30 and normal skeletal muscle tissue (N) was probed for miR-1-1, miR-133 and U6 RNA.

Amplification and quantification of the mature miRNA by PCR presents a challenge because the mature miRNA is roughly the size of a standard PCR primer. For this reason, we developed a real-time PCR assay to amplify and quantify the miRNA precursors as a way to predict the levels of the active mature miRNA. Validation of mature miRNA expression was not attempted for each case here; however, validations attempted in this study (Figure 6) as well as prior cases by us (11,25) and others (26) demonstrate that the levels of precursor miRNA often correlate to the mature miRNA. One case for which no correlation exists is the brain-specific miR-9/miR-9*. miR-9 precursors were expressed at very high levels in many of the cell lines (Supplementary Figure 4), however we were unable to detect mature miR-9/miR-9* by northern blotting in eight of these cell lines. It will be interesting to see if the processing of miRNA precursors by specific tissues contributes to high levels of certain miRNAs such as miR-9 in brain.

Another issue that has not been thoroughly addressed in the literature is what constitutes 'miRNA expression'. The difficulty is that northern blotting is currently the gold standard for miRNA detection, yet northern blotting is inherently insensitive. Based upon our extensive analysis of miRNA precursor expression using real-time PCR, we have found that typically only relative precursor expression values >10 are visible by northern blotting (loading 20–30 μg total RNA). Approximately 85% of the miRNA precursors studied here had relative expression values <10, so conceivably, many of these mature miRNAs would be undetectable by northern blotting. But does that mean they are not 'expressed'? It is also possible that many of the miRNAs appearing as green with relative expression values <0.1 (Figure 5) represent background rather than expressed miRNA.

It should be emphasized that the real-time PCR data presented here is for the miRNA precursors and not the active, mature miRNA. The relationship between precursor and mature miRNA expression has not been thoroughly addressed in the literature using sensitive, high-throughput assays. Only until these studies are completed will we have a better understanding of how miRNA processing is regulated in cell lines and in normal and diseased tissues. It is conceivable that the processing of some miRNAs, such as miR-205 and miR-100, are not regulated and that primary transcript levels are processed to mature miRNA in a stoichiometric manner, while others such as miR-9/miR-9* are regulated. Thus, the real-time PCR data presented here should be used as a starting point for those wishing to explore the function of an miRNA in a particular cell line and investigators should begin their study by using northern blotting or perhaps more sensitive assays to ensure that the expression of the mature miRNA is reflected by the precursor expression reported here.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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