Patterns of Known and Novel Small RNAs in Human Cervical Cancer

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Abstract
Recent studies suggest that knowledge of differential expression of microRNAs (miRNA) in cancer may have substantial diagnostic and prognostic value. Here, we use a direct sequencing method to characterize the profiles of miRNAs and other small RNA segments for six human cervical carcinoma cell lines and five normal cervical samples. Of 166 miRNAs expressed in normal cervix and cancer cell lines, we observed significant expression variation of six miRNAs between the two groups. To further show the biological relevance of our findings, we examined the expression level of two significantly varying miRNAs in a panel of 29 matched pairs of human cervical cancer and normal cervical samples. Reduced expression of miR-143 and increased expression of miR-21 were reproducibly displayed in cancer samples, suggesting the potential value of these miRNAs as tumor markers. In addition to the known miRNAs, we found a number of novel miRNAs and an additional set of small RNAs that do not meet miRNA criteria. [Cancer Res 2007;67(13):6031–43]

Introduction
microRNAs (miRNA) are single-stranded RNAs of ~22 nucleotides in length that are generated by the RNase-III type enzyme Dicer from endogenous hairpin-shaped transcripts (1, 2). miRNAs exert at least a part of their biological effects as guides for post-transcriptional gene silencing, producing sequence-specific mRNA cleavage or translational repression that can have dramatic effects on cellular phenotype (3, 4).

To date, there are few miRNAs whose physiologic function has been elucidated in vivo and whose targets are known. Studies in model organisms have revealed that miRNAs are involved in the control of developmental timing, cell proliferation, neuronal cell fate, apoptosis, morphogenesis, fat metabolism, and tumorogenesis, as well as in a variety of patterning processes in plants (1, 5, 6). In mammals, miRNAs have been shown to regulate B-cell differentiation (7), adipocyte differentiation (8), insulin secretion (9), antiviral defense (10), cardiogenesis (11), and tumorigenesis (12, 13).

Accumulating evidence shows that changes in miRNA levels may accompany dysregulated growth and apoptosis in some cancers. Reductions in expression of miR-15a and miR-16, let-7a, or miR-143 and miR-145 have been reported in chronic lymphocytic leukemia (14), lung cancer (15, 16), and colorectal neoplasia (17), respectively. On the other hand, significantly higher levels of miR-155 are present in diffuse large B-cell lymphoma with activated B-cell phenotype (18). Recently, several groups showed that overexpression of the miR-17-92 cluster in mice predisposed to tumor formation and can accelerate progression of tumorigenesis (12, 13, 19).

In addition to indications of potential roles in tumorigenesis, a number of recent studies have pioneered the use of miRNA expression profiles in tumor classification. Strikingly, in one of these studies, a profile comprising 217 previously characterized miRNAs seemed to be more effective in cancer classification, particularly in poorly differentiated tumors, than mRNA microarray profiles containing ~16,000 protein-coding genes (20). In addition, accumulating evidence shows the prognostic potential of miRNA expression profiles in several cancer types (21–23).

Full application of research and clinical tools that derive from miRNA expression profiling will depend on a complete picture of small RNA populations associated with tumors. Given the many biological differences between tumors and normal tissue, one might expect to observe differences between the two samples that would include quantitative variation in known miRNA levels and the presence of novel small RNAs in tumors. Although hybridization-based analyses (e.g., ref. 20) provide perhaps the most rapid method to detect groups of known miRNAs, alternative means are needed for detecting novel small RNAs. Two such methods are (a) cloning of size-fractionated [18–25 nucleotides (nt)] RNAs and (b) computational prediction based on different structural features of miRNAs. The cloning approach has been successfully applied for the identification of >400 miRNAs from human, mouse, zebrafish, Drosophila, and Caenorhabditis elegans (miRBase).5 A large number of these miRNAs formed a data set for designing efficient prediction algorithms for identification of miRNAs in different organisms. Most predictor algorithms depend on evolutionary conservation of miRNA sequences between different species. Such algorithms are limited to detecting conserved miRNAs (24–26).

Very recently, Bentwich et al. applied a different approach that does not depend on sequence conservation, which led them to identify a large number of human miRNAs that are not conserved beyond primates (27). Based on the above studies, it is estimated that the total number of human miRNAs, including nonconserved miRNAs, may be as many as 1,000. However, there are only ~474 human miRNAs listed in the recent release (9.1) of miRBase,6 indicating that the total number of human miRNAs is not saturated. Furthermore, the nonconserved miRNAs are not well characterized and could have somewhat different features, potentially contributing to pattern-recognition challenges in designing algorithms to identify such nonconserved miRNAs.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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5 http://microrna.sanger.ac.uk/index.shtml
6 http://microrna.sanger.ac.uk/
Table 1. Novel candidate microRNAs identified from human cervical cancer cell lines and normal cervical samples

<table>
<thead>
<tr>
<th>Candidate miRNA</th>
<th>Sequence (5'→3')</th>
<th>Number of clones</th>
<th>Size (nt)</th>
<th>Stem-loop structures of putative miRNA precursors ¹</th>
</tr>
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</table>
| miR-374b        | AUAAUUACACCUCUGCUAGUGU | 5               | 22–23    | UG AU C
|                 |                   |                 |          | CCGA G AUAUUACACCUCUGCUAGUGU C
|                 |                   |                 |          | GCUU U UAAUUACCUCUGCUAGUGU A
|                 |                   |                 |          | GU AC C
|                 |                   |                 |          | - CAGUUC - GGG U AGU
|                 |                   |                 |          | ACU AUUGU AGAAGACUC CCC CCGUCAG CG |
|                 |                   |                 |          | UGAA ACCCA UCCUCAG GGG CCGUGGUU GC C
|                 |                   |                 |          | CCC --- A A --- - CGA
|                 |                   |                 |          | G A C UG A U G U G U
|                 |                   |                 |          | CCH GGCUCUGA C AGACCG AGG CACG UACU U"
|                 |                   |                 |          | AAG CCGGAGCU G UCUGUGG CUG AGA UCAG GUU G
|                 |                   |                 |          | G U G C U G C G U
|                 |                   |                 |          | -- A --- -- UU U
|                 |                   |                 |          | A A UAGUA
| miR-934         | UGUCUACUCUGGAGACACUGG | 1               | 22       | C A AAAAA
|                 |                   |                 |          | GCC C A C C CCC CAUCC
|                 |                   |                 |          | GACGGGCGCC GGGGGCGGGGGGGGGGGCCU GU UC C
|                 |                   |                 |          | UGGCCUCUG CCAUGUGCGCUACC CG C
|                 |                   |                 |          | --- C A UG C UCCUC
|                 |                   |                 |          | AA C - - - - - - AA- AG A
|                 |                   |                 |          | AGCUUAGAGGCUCUGGAGGAUCGCAG | 1 22 |
|                 |                   |                 |          | UC GGCAC GGGAC AGUAGAGG GUG UC CAG UC C
|                 |                   |                 |          | AG CCGG U UCCGC UCCAGCUC UCC AC GCC AG C
|                 |                   |                 |          | AG CG GACCAUU G AG A AAGG GAC
|                 |                   |                 |          | A - CCC --- - U C G C CCUCU C UU
|                 |                   |                 |          | AGC CGUC GC CG AGGUCUGGGG GS UUG GC C
|                 |                   |                 |          | UGG GACG GC C CCGAUGCUCCUGCC ACC C CG C
|                 |                   |                 |          | AG UCCACC C U G - - - - UU G
|                 |                   |                 |          | G C C A U - A C A - G C C
|                 |                   |                 |          | GGGAGUGUACCA GU CUAAGAGG ACC GU CA U
|                 |                   |                 |          | CUUCCCGAUGG CA GAUAAUUC C CG U A G U
|                 |                   |                 |          | U A - - A UGC A AC
|                 |                   |                 |          | GCA C C U U U U U G C U C
|                 |                   |                 |          | GCUGGG AGGCAAGGAGGGGCAGGGGC CGGC CG C
|                 |                   |                 |          | GCUC UCUC GACCCUCUG CUCGC G CCC UCG G
|                 |                   |                 |          | AG - U - U - GU AG G
|                 |                   |                 |          | A G C CCCCA - - - - - - - GGGU
|                 |                   |                 |          | GGG GS UGGCUCGG CGGAGGGG CGC CG C CG C
|                 |                   |                 |          | CCAC CG A GC C CGUACGCG UCG CCC GCC CG G
|                 |                   |                 |          | C G G C - - G U G A A C C GAA
|                 |                   |                 |          | C A A AGG A
| miR-940         | AAGGCAGGGGCCCCCGCUCCCCC | 2               | 20–21    | U UACUCA
|                 |                   |                 |          | AUUAAGGAGAGUA CUUUCCGUGUUUUGCGCAUGUGU C
|                 |                   |                 |          | UAAUCUCUCUCAU GAAGAGCAAAACGCCGUCACCGA C
|                 |                   |                 |          | A UCCCCCG
|                 |                   |                 |          | C C - C UCC - - - - - - - C U C UG C
|                 |                   |                 |          | GGGA GUU USAG GGG UGGGGGGACG GC CGUAC C
|                 |                   |                 |          | CCGCU GGAC C ACU CCC ACCUCUGCC CG GUCAGU C G U
|                 |                   |                 |          | A AA A - G - - - - - - - UU C CC GGS
|                 |                   |                 |          | U A U - U AAAUU
| miR-943         | CUGACUGUGCCGCUCCUCAGC | 1               | 21       | U UACUCA
|                 |                   |                 |          | GGUUGGAGGAGUA CUUUCCGUGUUUUGCGCAUGUGU C
|                 |                   |                 |          | UAAUCUCUCUCAU GAAGAGCAAAACGCCGUCACCGA C
|                 |                   |                 |          | A UCCCCCG
|                 |                   |                 |          | C C - C UCC - - - - - - - C U C UG C
|                 |                   |                 |          | GGGA GUU USAG GGG UGGGGGGACG GC CGUAC C
|                 |                   |                 |          | CCGCU GGAC C ACU CCC ACCUCUGCC CG GUCAGU C G U
|                 |                   |                 |          | A AA A - G - - - - - - - UU C CC GGS
|                 |                   |                 |          | U A U - U AAAUU
| miR-944         | AAAAAUGAGUACACUGGAGAG | 1               | 22       | UUACUCA
|                 |                   |                 |          | GGUUGGAGGAGUA CUUUCCGUGUUUUGCGCAUGUGU C
|                 |                   |                 |          | UAAUCUCUCUCAU GAAGAGCAAAACGCCGUCACCGA C
|                 |                   |                 |          | A UCCCCCG
|                 |                   |                 |          | C C - C UCC - - - - - - - C U C UG C
|                 |                   |                 |          | GGGA GUU USAG GGG UGGGGGGACG GC CGUAC C
|                 |                   |                 |          | CCGCU GGAC C ACU CCC ACCUCUGCC CG GUCAGU C G U
|                 |                   |                 |          | A AA A - G - - - - - - - UU C CC GGS
|                 |                   |                 |          | U A U - U AAAUU
| miR-708         | AAGGACUAAACUACUAGCGG | 2               | 22       | UUACUCA
|                 |                   |                 |          | GGUUGGAGGAGUA CUUUCCGUGUUUUGCGCAUGUGU C
|                 |                   |                 |          | UAAUCUCUCUCAU GAAGAGCAAAACGCCGUCACCGA C
|                 |                   |                 |          | A UCCCCCG
|                 |                   |                 |          | C C - C UCC - - - - - - - C U C UG C
|                 |                   |                 |          | GGGA GUU USAG GGG UGGGGGGACG GC CGUAC C
|                 |                   |                 |          | CCGCU GGAC C ACU CCC ACCUCUGCC CG GUCAGU C G U
|                 |                   |                 |          | A AA A - G - - - - - - - UU C CC GGS
|                 |                   |                 |          | U A U - U AAAUU

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(Continued on the following page)
Table 1. Novel candidate microRNAs identified from human cervical cancer cell lines and normal cervical samples (Cont’d)

<table>
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<tr>
<th>Candidate miRNA</th>
<th>Sequence (5’—3’)</th>
<th>Number of clones</th>
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<td>miR-874-3p</td>
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</table>

*Sequences listed represent the observed full-length sequence of each miRNA cloned. The extra bases at the termini of some miRNAs are denoted in bold.

† RNA secondary structure prediction was done using mfold version 3.2. The miRNA sequence is underlined. The actual size of the stem-loop has not been experimentally determined.

In this study, we use direct sequencing to document miRNAs profiles and to identify novel miRNAs in six human cervical cancer cell lines and five normal cervical samples. In addition to a large family of known miRNAs, we show that this approach results in the identification of a number of novel small RNA effectors. Although there are clear similarities between the six cancer cell lines and normal cervix for known miRNAs, there are also clear differences. Our findings suggest a number of specific and consistent alterations in the small RNA profile in cervical cancer.

Materials and Methods

Cell culture. Six human cervical carcinoma cell lines (SW756, C4L, C33A, CaSkI, SiHa, and ME-180) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) at 37°C. CaSki, SiHa, and ME-180 were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) at 37°C. Cancer Res 2007; 67: (13). July 1, 2007 6034 www.aacrjournals.org

To determine if any significant variation in miRNA expression profiles was found between the cancer cell lines and normal cervix, we applied χ² statistics to compare the two groups. P < 0.0001 was considered as significant.

Northern analysis. One microgram of small RNA was fractionated on denaturing 15% polyacrylamide gels, followed by staining with ethidium bromide to determine RNA integrity. Overall RNA levels also served as internal loading controls. Gels were then destained, transferred to Hybond-N+ membranes (Amersham Biosciences) using semi-dry transfer (Hoefer Scientific Instruments) and fixed by UV cross-linking at 1,200 J/m². Subsequently, the blots were washed at 55°C for 15 min each in 2 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS, followed by an incubation in blocking solution.

Statistical analysis. To determine if any significant variation in miRNA expression profile was found between the cancer cell lines and normal cervix, we applied χ² statistics to compare the two groups. P < 0.0001 was considered as significant.

Small RNA isolation and cloning. Small RNA was extracted using mirVana miRNA isolation kit (Ambion). The cloning was done as described by Lau et al. (28), with slight modifications. Purified small RNAs were incubated with 10 μmol/L pre-adenylated 3’-adapter oligomucleotide (Modban, IDT Inc.), 1 x adenylate ligation buffer (ATP-Free), 10% DMSO, and 1 units T4 RNA ligase (New England Biolabs) at 37°C for 1 h. The ligated product was purified on a 12% denaturing polyacrylamide gel, followed by a second ligase reaction with a 5’-adapter oligomucleotide, 5’-ACCGAATTCCTC TTC ACTAA-3’ (upercase, DNA: lowercase, RNA: ChemGenes Corporation) and gel purification. The gel-purified doubly ligated RNA was reverse transcribed using 150 units Superscript II (Invitrogen) and RT primer, 5’-ATTGATGGTGGCTTACAG-3’. The cDNA was amplified by PCR, using the RT primer and a forward primer 5’-CAGCCAACGGGAATTCCTC- CACTAA-3’. A second PCR was done using the RT primer and a second forward primer, 5’-GACGCAACGCGGAATTCCTCATTAA-3’. The PCR product was phenol extracted, ethanol precipitated, and then digested with Ban I (NEB). After further phenol extraction and ethanol precipitation, the digested products were concatenated with T4 DNA ligase (NEB). Concatamers ranging from 600 to 1,000 bp were isolated from a low-melting-point agarose gel, processed with Taq polymerase, and cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colony PCR was done using the M13 forward and reverse primers, and the PCR products were purified using shrimp alkaline phosphatase and exonuclease I (U.S. Biochemical Corporation).

Sequence analysis. Small RNAs obtained by cloning were compared with functionally annotated sequences using BLAST (blastn), 6 BLAT from the University of California at Santa Cruz, 7 miRBase, 9 as well as standard text-matching routines. For each small RNA, the best alignments to a functionally annotated sequence (not more than one error) were used to assign a functional category to the small RNA. The fold-back precursor structure of candidate miRNAs was predicted by mfold (29).

In this study, we use direct sequencing to document miRNA profiles and to identify novel miRNAs in six human cervical cancer cell lines and five normal cervical samples. In addition to a large family of known miRNAs, we show that this approach results in the identification of a number of novel small RNA effectors. Although there are clear similarities between the six cancer cell lines and normal cervix for known miRNAs, there are also clear differences. Our findings suggest a number of specific and consistent alterations in the small RNA profile in cervical cancer.
solution [1× PBS (pH 7.4)/0.05% Tween 20/0.1% SDS/0.5% blocking reagent; Roche] for 1 h and then in streptavidin-alkaline phosphatase conjugate (U.S. Biochemical Corp) for 1 h. After incubation, the blots were washed thrice in each buffer A [1× PBS (pH 7.4)/0.05% Tween 20/0.1% SDS] and buffer B [0.1 mol/L Tris-HCl (pH 9.5), 0.1 mol/L NaCl]. The blots were then incubated with chemiluminescent substrate CDP-Star (GE Healthcare) and exposed to Kodak BioMax XAR film.

Results

Small RNA composition of cDNA libraries. To search for novel candidate miRNAs or other small RNAs and to characterize miRNAs in human cervical cancer and normal cervix, we cloned and sequenced small RNA libraries prepared from RNA in the size range of 18 to 25 nt isolated from six human cervical cancer cell lines and five normal cervixes. A total of 7,303 small RNA clones (5,100 from cervical cancer cell lines and 2,203 from normal cervix) were sequenced (Supplementary Fig. S1 and Table S1). One cell line (C33A) had a higher fraction of rRNAs. This may result from a high fraction of dying cells in this line (site-specific polymorphisms, nonannotated regions in the human genome, PCR/cloning/sequencing errors, or nonhuman biological material present in the culture. Interestingly, none of the small RNA sequences that were identified seemed to correspond to human papillomavirus (HPV) RNA, despite the fact that five of the cell lines are HPV positive.

For normal cervical samples, a total of 706 (32%) clones were annotated as previously identified miRNAs (Supplementary Table S4): 67 previously verified miRNAs, 15 computationally predicted miRNAs but never verified experimentally, and 6 sequences paired with known mature miRNAs that have not been annotated in miRBase. The remaining small RNAs correspond to fragments of rRNA (22%), tRNA (36%), scRNA/snRNA (2%), repeat sequences (2%), mitochondrial (2%), miRNA (1%), and not mapped/unknown (3%; Supplementary Fig. S1B and Table S2). The higher fractions of fragments from degradation of rRNA and tRNA-derived fragments in the clinical samples seem likely to represent general damage or dying cells in the clinical samples; rRNA- and tRNA-derived fragments were not incorporated in the miRNA profile analysis. A total of 30 clones (1%) could be suggested as novel small RNAs, based on the criteria described above. Of these, five could be annotated as novel miRNAs (Table 1), five are miRNA-like molecules with noncanonical hairpin (Table 2), and 20 are without significant hairpin (Table 3).

Identification of novel miRNAs. Seventeen new miRNAs were identified from 26 relevant clones, in which 14 were found in cervical cancer cell lines, and three were found in normal cervical samples (Table 1). Five of these candidates were observed more than once, and two were found in more than one cell line (Table 1 and Supplementary Tables S3 and S4). As expected, the size of these novel miRNAs is in the range of 20 to 24 nt, with a mode of 22 nt. Of the 17 novel miRNAs, 15 are found in other mammalian genomes (but not in invertebrates), whereas two candidates (miR-933 and miR-769-3p) seemed not to be conserved.

Although the 17 novel candidate miRNAs are all unique in sequence, some sequences are similar to previously identified human miRNAs: miR-374b is very similar to miR-374, and miR-708 is similar to miR-28. In addition, the two strands of a novel candidate miRNA, miR-874, were independently identified in different normal cervical samples.

Identification of small RNAs with noncanonical hairpin. Other than the novel candidate miRNAs, we identified nine small

### Table 1. Novel candidate microRNAs identified from human cervical cancer cell lines and normal cervical samples (Cont’d)

<table>
<thead>
<tr>
<th>dG</th>
<th>location</th>
<th>ptr</th>
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</table>
RNAs (from 12 clones) that are perfectly matched to the human genome but do not fully meet the miRNA criteria as described above. We designate these small RNAs (four from cervical cancer cell lines and five from normal cervices) as sRNA-cer (i.e., small RNAs in cervix). Two of these sRNAs were observed more than once (Table 2).

Six sRNAs (sRNA-cer2, sRNA-cer3, sRNA-cer5, sRNA-cer6, sRNA-cer7, and sRNA-cer9) are located in the intergenic regions, whereas three sRNAs are found in the intron or exon of known genes. sRNA-cer1 is located in the exon 9 (NM_003879) or intron 7 (AF009619) of CFLAR (CASP8 and FADD-like apoptosis regulator). sRNA-cer4 overlaps with intron 1 of WDR20 (WD repeat domain 20) and transcribed from the opposite DNA strand. Similarly, sRNA-cer7 is the antisense strand that overlaps with intron 1 of SCARA3 (Scavenger receptor class A, member 3). Notably, most of these sRNAs are not conserved beyond primates.

**Identification of small RNAs without significant hairpin.** We also identified 33 small RNAs from a total of 37 clones that are perfectly matched to the human genome but do not have a significant hairpin structure (Table 3). These sRNAs are 18 to 25 nt in size, with a predominant class in 21 nt. Fourteen of them were found in cervical cancer cell lines, whereas 19 were identified in normal cervical samples. Only four of these sRNAs were observed more than once.

These sRNAs map to different loci in 20 different human chromosomes; only two (sRNA-6 and sRNA-7) are located at the same chromosome region 5q31.2 (56 bp apart). Notably, sRNA-6 and sRNA-7 match the sense strand of a predicted Ensembl candidate small RNAs with noncanonical hairpin identified from human cervical cancer cell lines and normal cervical samples

<table>
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<tr>
<th>Candidate sRNA*</th>
<th>Sequence (5’—3’)†</th>
<th>Sample (number of clones)</th>
<th>Size (nt)</th>
<th>Stem-loop structures of putative miRNA precursors ‡</th>
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* sRNA-cer refers to small RNAs in cervical cells.
† Sequences listed represent the observed full-length sequence of each sRNA cloned.
‡ RNA secondary structure prediction was done using mfold version 3.2. The sRNA sequence is underlined. The actual size of the stem-loop has not been experimentally determined.
Interestingly, this sequence is predicted to have a consensus secondary structure for vault RNA family (Supplementary Fig. S2). Vault RNAs are found as part of the vault ribonucleoprotein complex that has been suggested to play a role in drug resistance (31). The two sRNA sequences identified are paired in the stem-loop structure (Supplementary Fig. S2).

In this class of sRNAs, 19 are located in putative intergenic regions, whereas 14 are in known protein-coding segments. Among the sRNAs that correspond to protein-coding sequences, eight are sense to the intron, and six are antisense to the coding strand of mRNAs [four in the intron, one in the exon, and one in the 3′ untranslated region (3′UTR)]. Notably, one of the intronic sRNA, sRNA-5, is located upstream of miR-33 and in the same intron of SREBF2. The two exonic antisense sRNAs include (a) sRNA-11, which is antisense to exon 16 of BAT2D1 (BAT2 domain containing 1) and (b) sRNA-12, which is antisense to the 3′UTR of MPL (myeloproliferative leukemia virus oncogene).

miRNA expression profiles in human cervical cancer cell lines and normal cervix. To assess the relative abundance level of each miRNA between samples, we compared the relative cloning incidence with respect to the total number of small RNA clones. As shown in Fig. 1, miRNA expression patterns were generally similar among the cell lines and among normal cervixes, although some variations were observed within each group.

We determined the significance of expression variation between normal cervix and cancer cell lines using 2 test (Supplementary Table S5). Of 166 miRNAs expressed in normal cervix and cancer cell lines, six were found to have substantial expression variation between the two groups (Supplementary Table S6). Although let-7b, let-7c, miR-23b, miR-196b, and miR-143 showed significantly reduced abundance in cervical cancer cell lines, miR-21 displayed higher abundance in the cancer group (Fig. 2).

For let-7c, a frequency of 15% to 18% (16.0 ± 1.3) in individual normal cervical samples compares with no detection in three of the cancer cell lines, 0.2% to 0.4% in two lines and 7.2% in one line.

Table 2. Novel small RNAs with noncanonical hairpin identified from human cervical cancer cell lines and normal cervical samples (Cont’d)

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</tbody>
</table>

10 http://www.ensembl.org/
The cloning frequency for miR-196b ranges from 1% to 2% (1.3 ± 0.8) in normal cervical samples, whereas only 0.1% (3 clones out of 3,231) was detected in cancer cell lines. For miR-143, none was found in the cancer group (0/3,231), whereas a total of 7 (out of 706) were detected in the normal group.

**Verification of miRNA expression variations by Northern blot analysis.** To further investigate the miRNA expression variations suggested by cloning, we evaluated the expression level of miR-21 and miR-143 in all normal cervix and cervical cancer cell lines used in the cloning experiments. The Northern blot results revealed that the expression of both miR-21 and miR-143 was significantly different between the normal cervix and cervical cancer cell lines (Fig. 3). In agreement with the cloning data, accumulation of miR-21 was substantially greater in cervical cancer cell lines, whereas miR-143 was substantially more abundant in normal cervix.

### Table 3. Candidate novel small RNAs without significant hairpin identified in human cervical cancer cell lines and normal cervix

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<th>Candidate sRNA*</th>
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<th>Chromosome</th>
<th>Genomic location</th>
<th>Gene/intergenic exon/intron/UTR (S/AS)</th>
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</table>

Total clones

Abbreviations: UTR, untranslated region; S, sense; AS, antisense. *sRNA refers to small RNAs. +Sequences listed represent the observed full-length sequence of each sRNA cloned. The extra bases at the termini of some sRNAs are indicated in bold.
patients) provided by the Columbus (Ohio) Children’s Hospital Gynecologic Oncology Group Tissue Bank. The results are summarized in Supplementary Table S7. For miR-21, expression levels were clearly higher in 21 of the 29 tumor samples compared with their normal counterparts (Fig. 3A). In two cases (G613 and G871), mir-21 expression seemed comparable between the cancer and normal tissues. The remaining six cases were undetermined due to poor detection or uneven RNA yields for the matched samples.

For miR-143, the expression patterns can be divided into several categories (Fig. 3B): (a) absent or barely detectable expression in the tumor samples with substantial expression detected in the matched normal samples (16 of 27 samples tested), (b) lower (but detectable) expression level in the tumor with strong expression in normal counterpart tissue (5 of 27 samples tested), and (c) more abundant expression level in the tumor than that in its normal matched sample (2 of 27 samples tested). The remaining four cases were undetermined (see above).

Discussion

Cervical cancer, the second most common cancer among women worldwide, is frequently caused by specific HPV infections (32). Although participation of HPV proteins (e.g., E6 and E7) in cell transformation is well documented (32), the detailed network of events leading from HPV infection to tumor development has yet to be elucidated. Given that numerous viruses (both DNA and RNA) have been shown to encode small modulatory RNAs (33), and given the changes in endogenous miRNA pattern and tumor-related roles for miRNAs that have been shown (14, 20, 34), we...
thought it appropriate to investigate small RNA profiles in a set of well-characterized cervical cancer cell lines and compared the profiles with normal cervical specimens.

To obtain a definitive and inclusive profile, we used a cloning-based method for profiling RNAs that makes no initial assumptions about the identity of the small RNAs present. Applying this protocol, we found that known miRNAs accounted for a large proportion of small RNAs expressed in each of the cervical cancer cell lines and normal cervix. In addition to these known miRNAs, we identified a number of novel small RNAs with miRNA characteristics, which had not been previously observed in any tissue or cell type. Finally, we identified a significant number of novel small RNAs that do not meet standard miRNA criteria and which may thus be synthesized by distinct mechanisms.

Our analysis of profiles for known miRNAs indicated significant similarities and differences of expression level between the cell lines and normal cervical samples. At least six miRNAs showed substantial expression variations between the two groups, in which the differential expression pattern of two miRNAs were also corroborated by Northern blot analysis. These findings led us to examine the biological relevance of these miRNAs in cervical cancer using a panel of clinical samples. Remarkably, the differential expression pattern of these two miRNAs is consistent in tumors, thus raising the possibility that these miRNAs (or their targets) may provide useful diagnostic tools and may also provide clues to understand the processes that lead to cancer development.

**Increased expression of miR-21 in cervical cancer.** miR-21 was the most abundantly recovered miRNA species in all of the cervical cancer cell lines we examined, showing significantly lower abundance in the normal cervical samples analyzed. Concordantly, the increased expression of miR-21 was also found in a preponderance of tumors. Abundant miR-21 may be a general, albeit not universal, feature of tumor cells. In addition to our analysis, strong miR-21 signals have been reported in several hybridization-based profiles of different tumor types (e.g., glioblastomas, breast cancer) and cancer cell lines (e.g., HCT-116, colorectal carcinoma; HeLa, cervical adenocarcinoma, and glioblastoma cell lines; refs. 34–36). Interestingly, Chan et al. (35) recently reported that suppression of miR-21 in glioblastoma cells can trigger activation of caspases and lead to increased apoptotic cell death. Conversely, Cheng et al. (37) showed that knockdown of miR-21 increased cell growth in HeLa cells. Taken together, these findings suggest that miR-21 may affect different biological processes in different cellular contexts. Interestingly, miR-21 is located in the fragile site FRA17B region, which is one of the HPV16 integration loci at 17q23.2 (38, 39). It is known that HPV integration into the host cell genome can cause genetic alterations (such as deletions, amplifications, or complex rearrangements) and epigenetic alteration, thus intriguing to speculate that the expression of cellular miRNA genes at or near HPV integration sites may contribute to the tumor phenotype.

**Reduced expression of miR-143 in cervical cancer.** The abundance level of miR-143 is strikingly different between normal cervical samples and cervical cancer cell lines, which led us to investigate its association with cervical cancer development. In agreement with the cloning data, the expression of miR-143 is significantly lower in most of the tumors as compared with their normal counterparts. Similarly, mature miR-143 is significantly reduced in different tumor types, e.g., colorectal tumors (17),...
sarcomas, breast, prostate, and lymphoid cancer cell lines (17, 34), suggesting that miR-143 might have suppressor roles in a wide range of tumor cells. It is worthy to note that the only experimentally verified target for miR-143, ERK5 (also known as MAPK7), is known to promote cell growth and proliferation in response to tyrosine kinase signaling (40). Furthermore, abnormal levels of ERK5 expression have been observed in some cancer types (40–42), suggesting its significant role in tumor development. The questions of ERK5 role in cervical cancer cells and its regulation by miR-143 are certainly worthy of further investigation.

No detection of HPV-derived miRNAs. One conceivable result from our analysis would have been the identification of HPV-encoded miRNAs. The cell lines included in this study consisted of five HPV-positive cervical cancer cell lines (SW756, C4I, CaSki, SiHa, and ME-180) and one apparently HPV-negative cell line (C33A). The detection of the HPV genome for these cell lines had been reported (43–45), and was reconfirmed by DNA sequencing (data not shown). We did not identify any viral-encoded miRNAs from the HPV-infected cells by our molecular cloning approach, even after screening ~4,000 clones. Consistent with this observation, no viral-encoded miRNA was predicted from the HPV18 genome by a computational method (46), and no viral-derived small RNAs were detected during latent or productive replication cycle of HPV31 by a cloning approach (47).

Novel miRNAs and other small RNAs. Among the small RNAs cloned from the cervical cancer cell lines and normal cervical samples, we observed a moderate number of previously unconfirmed or unidentified transcripts, which seem by all criteria to be novel miRNAs. The identification of novel miRNAs and other novel sRNAs in these specific tumor cell lines and cell type might indicate that some of these small RNAs are unique to this cell type. Alternatively, these miRNAs might be expressed in cells (or at levels), which escaped detection in previous attempts to catalogue

---

**Figure 2.** Representative miRNAs with significant expression variations between normal cervix and cervical cancer cell lines identified by cloning experiments. The expression was calculated by the fraction of clone number from each pool of library and represented in percentage. Inset, mean fraction of clone number from the miRNA pool for each sample group (N, normal cervix; C, cervical cancer cell lines). *P* values < 0.0001 were considered as significant, determined by *χ*² test. Notably, the expression of miR-143, let-7c, and miR-196b in normal cervix is significantly lower than that in cervical cancer cell lines, whereas the expression of miR-21 is significantly higher in cervical cancer cells.

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11 W.O. Lui and A. Fire, unpublished data.
miRNAs. Whatever the normal pattern of expression for these miRNAs, it is clear that the characterization of miRNA profiles for any tissue or tumor type cannot be complete without an unbiased approach such as miRNA cloning.

Although one might expect some novel miRNAs to be expressed specifically in one tumor type, it is conceivable that others would be expressed in a range of tumor types. Interestingly, we have found several of the novel miRNAs in diverse tumor types. In particular, miR-935, miR-708, and miR-874-3p were also found in human sarcomas; and miR-940 was also found in a renal cell carcinoma cell line.\(^{11}\)

Novel small RNAs that do not meet current miRNA criteria could represent various situations: miRNAs formed by a standard mechanism but failing the arbitrary miRNA criteria due to incompleteness of these criteria, miRNA-like molecules formed by slightly divergent synthetic mechanisms, and other small RNAs such as natural small interfering RNAs (siRNA) that might be formed by completely different mechanisms. Finally, these could represent spurious ssRNA transcripts or common degradation products of longer cellular RNAs. Of these, sRNA-cor3 was also found in a colon cancer cell line, and sRNA-14 was also observed in a renal cell carcinoma cell line,\(^{11}\) indicating that some of these small RNAs are not specific to cervical cancer. Given that tumor genomes use every conceivable mechanism to modulate their own gene expression for the promotion of tumor growth, it would be surprising not to have the siRNA pathway used during tumorigenesis. Further analysis will certainly be required to assess the biological effect of these small molecules. Although the individual small non-miRNAs are rare in cervical cancer cells, their low concentration alone does not rule out their possible biological roles. In particular, an analysis of RNAi effects in *C. elegans* by Pak and Fire (48) show that substantial interference effects can be initiated by a population of siRNAs that are only marginally detectable in cloning experiments.

**Prospects for miRNA discovery and profiling.** Because the immense potential of miRNAs as regulators of gene networks is just beginning to unfold, a detailed molecular analysis of miRNA expression in the process of cancer development is of great interest. As accumulating evidence showing the clinical impacts of miRNA expression profiles (based on a limited number of known miRNAs), a more comprehensive list of miRNAs needs to be identified for cataloging of expression patterns in specific tissue types in physiologic and pathologic conditions. Therefore, the small RNA cloning approach (e.g., Amplified Tag Sequencing; ref. 49) should enable the enumeration and characterization of a more complete set of human miRNAs for subsequent analysis. A high-throughput (e.g., hybridization-based) characterization of known miRNA profiles is thus enabled to identify distinct

---

**Figure 3.** Northern blot analysis of miR-21 expression and miR-143. A, mature miR-21, detected in all cervical cancer cell lines, was barely detected in many of the normal cervical samples. Substantially higher relative expression of miR-21 in tumor was evident in 72% of the cases. B, mature miR-143 was detected in most of the normal cervical samples, but absent or barely detected in all cervical cancer cell lines and in 16/27 (59%) of the cancer samples. In five cases, the expression of miR-143 was relatively lower in cancer than in their matched normal cervical samples, whereas two cases showed relatively more abundance in the tumor samples. Ethidium bromide–stained rRNA bands are shown as loading controls.
signatures in each tumor type. We have certainly seen evidence for such a signature for cervical cancer, and further investigations are certainly warranted to evaluate the application of miRNA profiles as contributory diagnostic/prognostic markers for this tumor type.

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References


Patterns of Known and Novel Small RNAs in Human Cervical Cancer

Weng-Onn Lui, Nader Pourmand, Bruce K. Patterson, et al.


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