Cervical cancer is the result of a multistep process that involves the transformation of the normal cervical epithelium to a preneoplastic cervical intraepithelial neoplasia that is subsequently transformed to invasive cervical cancer (1, 2). Although high-risk human papillomaviruses are associated with cervical cancer (1, 2), human papillomavirus infection alone is not sufficient to induce the malignant transformation. Hence, other unidentified genetic alterations are likely involved. The identification of such genetic alterations would be of considerable importance for the screening and treatment of cervical cancer (3).

MicroRNAs (miRNA) are a recently discovered class of small noncoding RNAs that regulate gene expression (4). Mature 18- to 25-nucleotide-long miRNAs regulate gene expression by catalyzing the cleavage of mRNA (5–10) or repressing mRNA translation (9, 11, 12). The specific roles of miRNAs include the regulation of cell proliferation and metabolism (13, 14), developmental timing (4, 14), cell death (15), hematopoiesis (16), neuron development (17), human tumorigenesis (18), DNA methylation, and chromatin modification (19).

There is increasing evidence that the expression of miRNA genes is deregulated in human cancer (20–26). Altered miRNA expression profiles have been reported in lung cancer (27), breast cancer (28), glioblastoma (29), hepatocellular carcinoma (30), papillary thyroid carcinoma (31), and more recently, colorectal cancer (26). Moreover, some studies have reported that miRNA expression signatures are associated with clinical outcomes of certain diseases (32–34). These data suggest that miRNAs play an important role in a variety of human cancers. However, the molecular basis of miRNA-mediated gene regulation is not fully understood and their role in tumorigenesis remains largely unknown.

Over the past several years, a number of methods for quantifying miRNAs have been described, including Northern blotting, microarrays (35, 36), a modified invader assay (37), a bead-based flow cytometric assay (22), and real-time quantitative PCR (38). Real-time quantitative PCR is more quantitative and sensitive than other high-throughput assays. Here, we present the results of miRNA deregulation in a set of early stage invasive squamous cell carcinomas (ISCC) and normal cervical epithelial tissues, using real-time quantitative PCR array methods.
Materials and Methods

Patients and tissue specimens. Fresh frozen tumor biopsy specimens (n = 10) from patients with primary ISCC (International Federation of Gynecology and Obstetrics stage of IB to IA) were obtained at the time of surgery. A radical hysterectomy with pelvic lymph node dissection was done at the Department of Obstetrics and Gynecology, Samsung Medical Center, between January 2002 and October 2003. The institutional review board of our hospital approved this study (IRB no. 0608095) and informed consent was obtained from the patients. Tumor specimens were immediately snap-frozen at -80°C. Only specimens containing >90% tumor cells, which were examined by a single gynecologic pathologist using H&E staining, were used in the analysis. Table 1 summarizes the patient characteristics.

As a control, normal cervical tissues (n = 10) were obtained from patients undergoing hysterectomy for benign gynecologic disease. Fresh cervical biopsies (5-8 mm³) were obtained before undertaking any surgical procedures. Dispase II (2.4 units/mL; Roche) was used to obtain the normal epithelial tissues alone from the entire cervical tissues including the stroma (39). These biopsies were washed in sterile saline before centrifugation, and the epithelial cell pellets were then used in the analysis.

RNA extraction and reverse transcription. Total RNA was extracted from the ISCC and normal epithelial tissues using an easy-spin total RNA extraction kit (Intron Biotech). The concentration was quantified using the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies). cDNA was synthesized from total RNA using stem-loop reverse transcription primers according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems; ref. 38).

miRNA expression profiling using TaqMan MicroRNA assay. Real-time PCR for miRNA expression profiling was done using an Applied Biosystems 7900HT Sequence Detection system. The expression levels of the 157 human mature miRNAs were measured using the Human Panel Early Access Kit (PN 4365381; Applied Biosystems). Data normalizations were done using miR-16 as the endogenous control according to the manufacturer’s suggestions, miR-16 as a positive control, or cel-lin-4, ath-miR159a, and cel-miR-2 as the negative controls (40). Relative quantification of miRNA expression was calculated by the 

\[\text{Ct}_{\text{target}} - \text{Ct}_{\text{reference}}\]

method (41). The relative expression values were multiplied by 10⁶ in order to simplify the presentation of the data (42).

Real-time quantitative reverse transcription-PCR analysis for DNM2 mRNA expression. DNM2, as an overlapping transcript of miR-199a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous control gene, were used in the same PCR reaction for real-time quantitative reverse transcription-PCR. In order to avoid amplification of the genomic DNA, the primers and probe for amplifying DNM2 and GAPDH were chosen to hybridize at the junction between the two exons as follows: DNM2 (Hs00191900, Applied Biosystems; NM_001005362, exon boundary 13-14, probe 5’-CATCCCAAAT-CAGGGATCCCGCCGG-3’), GAPDH (4310884E, Applied Biosystems). The gene expression ΔCt value of DNM2 from each sample was calculated by normalization with GAPDH and relative quantification values were plotted (43).

Cell lines and transfection of anti–miR-199a. All cell culture reagents were purchased from Invitrogen Life Technologies. The human cervical cancer cell lines, SiHa and ME-180, were obtained from the American Type Culture Collection. SiHa cells were grown at 37°C in 5% CO₂ in MEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). ME-180 cells were maintained in McCoy’s 5A and RPMI 1640.

Cells were transfected with 100 nmol/L of anti–miR-199a (Ambion) or negative control using siPort Neo-FX (Ambion). Three days later, total RNA from the cells was isolated to examine the expression level of miR-199a using the RNA-spin total extraction Kit (Intron Biotech).

Cell viability determined by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assay. Cervical cancer cells (SiHa and ME-180) were plated in a 96-well plate at 4,000 cells/well and then were allowed to grow for 3 days before the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assay. The MTT assay, 1 mg/mL of MTT solution (1 mL of 10 mg/mL MTT in PBS added to 9 mL of serum-free medium) was added to each well. The cells were incubated for 3 h in the dark. The formazan grain was then dissolved in DMSO (Sigma), and absorbance was read at 570 nm using an ELISA plate reader (Bio-Rad). To further assess the effect of anti–miR-199a on cell growth, we treated the transfected cells with 1.5 and 3 μg/mL of cisplatin for 2 days.

Data analysis. SPSS software (version 10.0, SPSS Inc.) was used to perform statistical analysis. The Mann-Whitney U test was used to evaluate the significance between the gene expression of tumor and nonmalignant tissue samples.

Additionally, unsupervised hierarchical clustering was done on the PCR data to investigate the relationships among genes and among samples. Each miRNA raw data Ct were median-centered for all samples before clustering. Hierarchical average-linkage clustering was done by means of the GeneSpring GX software (version 7.3.1, Agilent Technologies), using log-transformed, median-centered gene expression values and the Pearson correlation as similarity metrics.

![Table 1. Clinical background of 10 patients with ISCC of the uterine cervix](https://example.com/table1.png)

<table>
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<tr>
<th>No.</th>
<th>Age</th>
<th>Cell type</th>
<th>International Federation of Gynecology and Obstetrics stage</th>
<th>Tumor size (cm)</th>
<th>Human papillomavirus</th>
<th>LN*</th>
<th>PM †</th>
<th>RM †</th>
<th>Recur</th>
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</table>

* Lymph node metastasis.
† Parametrial invasion.
‡ Resection margin involvement.
miRNA expression in ISCCs and normal cervical epithelial tissues. Among the 157 miRNAs analyzed, there was a significant difference in the expression of 70 miRNAs in comparisons between the ISCCs and normal epithelial tissues ($P < 0.05$), 68 were up-regulated and 2 were down-regulated (Supplementary Table S1). Among these, 10 miRNAs that were the most significantly overexpressed in ISCCs with fold changes of nearly $>100$ and a $P < 0.0001$, were as follows: miR-199-s, miR-9, miR-199a*, miR-199a, miR-199b, miR-145, miR-133a, miR-133b, miR-214, and miR-127. By contrast, only two of the miRNAs, miR-149 (2.974-fold change) and miR-203 (3.704-fold change), showed significant down-regulation.

We used unsupervised hierarchical clustering to classify the samples without using any information on the identity of the
samples (Fig. 1). This procedure resulted in the classification of cancer samples into two major classes based on similarities in miRNA expression. Of interest was the observation of a difference in the LN metastasis between the two clusters \((P = 0.052\) using Pearson \(\chi^2\) test).

miR-199a is an intronic miRNA located in host gene, DNM2 intron 16. We analyzed the overlapping transcripts of the significantly expressed 70 miRNAs (Supplementary Table S1). The analysis was done using the Sanger miRNA registry, which is commonly used to predict human miRNA gene targets. The chromosomal locations of overlapping transcripts were divided into 40 introns, 22 intergenic, 6 3′-untranslated regions, and 2 exons. The gene lists of overlapping transcripts for the top 10 miRNAs are DNM, C1orf61, C20orf166, RP11-771D21.2, and RTL1.

DNM2 mRNA was identified as an overlapping transcript of miR-199-s, miR-199a*, and miR-199a. We found that the nucleotide sequence at DNM2 intron 16 was complementary to the sequences of miR-199a* and miR-199a (Supplementary Fig. S1). Because intronic miRNAs are coordinately expressed with their host gene's mRNA, we evaluated the mRNA level of DNM2 in the same tissues using real-time quantitative PCR. We found that the mRNA level was significantly increased in the ISCC compared with the normal tissues \((P < 0.0001; \text{Fig. 2})\). Therefore, these findings suggest that miR-199a* and miR-199a are intronic miRNAs from the host mRNA, DNM2 intron 16.

miR-127 expression is significantly associated with lymph node metastasis of ISCCs. We also evaluated 10 selected miRNAs in another group of patients (Table 2) to determine whether there was a correlation between any of the expression profiles and clinical features. We selected the 10 miRNAs that were most significantly expressed that had a fold change of nearly >100 and \(P < 0.0001\). There was no significant association between the expression of selected miRNAs and age, the clinical stage, tumor size, and recurrence. However, the expression of miR-127 was increased significantly in patients with lymph node metastasis \((P = 0.0061; \text{Fig. 3})\).

Inhibition of cervical cancer cell growth by anti–miR-199a. In order to evaluate the role of specific miRNAs in cervical carcinogenesis, we selected miR-199a, which is one of the most up-regulated ISCCs (Fig. 4A). The TaqMan real-time PCR revealed that anti–miR-199a significantly reduced the expression of miR-199a in cervical cancer cells, suggesting that anti–miR-199a is efficiently introduced into the cells and acts to knock down miR-199a (Fig. 4B). In addition, we found that this inhibitor reduced cell growth (Fig. 4C). Furthermore, anti–miR-199a–mediated cell growth inhibition was increased in cisplatin-treated cells in a dose-dependent fashion (Fig. 4D and E).

Discussion

In the present study, for the first time, we identified altered miRNA expression in early stage ISCCs and normal epithelial tissues of the cervix. In addition, our findings suggest that miR-127 may be a marker for lymph node metastasis of ISCCs and that miR-199a may be a potential therapeutic target for cervical cancer therapy.

Tissue-specific patterns of miRNA expression have been recently reported; they are thought to reflect embryologic development (22). Several reports showed that specific overexpression or underexpression of miRNAs differ according to the particular tumor types (22–26). Our results showed a highly preferential increase rather than a decrease in the ISCCs compared with normal cervical epithelial tissues. These findings are explained by the fact that miRNA expression is tissue-specific,

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3http://microrna.sanger.ac.uk

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Fig. 2. Real-time quantitative PCR analysis of the mRNA level of DNM2 intron 16, which is the host gene of miR-199a; the mRNA level was significantly higher in ISCCs \((P < 0.0001)\).
as shown by large profiling studies using the tumors of various histotypes (22, 23).

Most human miRNAs are found between protein-coding genes; approximately one-third are located within the introns of annotated mRNAs. These intronic miRNAs are usually in the same orientation as the pre-mRNA, and thus, could be under control of the promoter driving the primary mRNA transcript (44). More than 90 intronic miRNAs have been identified thus far using the bioinformatic approach, but the function of the vast majority of these molecules remains to be determined (45). Intronic miRNAs are usually coordinately expressed with their host gene’s mRNA (44). Our results showed that DNM2 intron 16 is the host gene for miR-199a using bioinformatic analysis, the mRNA of DNM2 was expressed with the intronic miRNA (Fig. 2).

Despite the uncertainty regarding the functional effects of miRNAs, the miRNA expression profile may be used as a prognostic marker for clinical aggressiveness of human cancer. One such example is chronic lymphocytic leukemia. Calin et al. reported a miRNA expression signature composed of 13 mature miRNAs that were associated with prognostic factors and disease progression (34, 46). Recently, a study on pancreatic endocrine and acinar tumors revealed that the overexpression of miR-21 is strongly associated with both a high Ki67 proliferation index and the presence of liver metastasis (47). Our results showed that the expression of miR-127 was significantly higher in the group of ISCCs with lymph node metastasis than in those without metastasis (P = 0.0061; Fig. 3). In addition, there were two major classes of early stage ISCC, identified by hierarchical clustering, which showed differences in lymph node metastasis. However, this study did not include patients with advanced stage and this limits the interpretation of our findings.

It is clear from earlier studies that many of the changes in miRNA expression are not simply a secondary consequence of the transformation process. Rather, loss- or gain-of-function of specific miRNAs seems to be a key event in the genesis of a variety of cancers. Therefore, miRNAs might be potential targets for therapy. The knockdown of overexpressed miRNAs or expression of silenced miRNAs in cancer cells might result in tumor cell death. It was recently reported that a novel class of chemically engineered oligonucleotides, known as “antago-mirs” effectively silenced endogenous miRNAs in vivo (48). In this study, anti–miR-199a reduced cervical cancer cell (SiHa and ME-180) growth and increased cisplatin-induced cytotoxicity (Fig. 4). DNA damage caused by cisplatin might have increased the growth inhibition of anti–miR-199a. In fact, there are some studies demonstrating a positive association

Table 2. Thirty-one patients who were analyzed for the expression of 10 miRNAs which were most significantly expressed in cancers

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<th>International Federation of Gynecology and Obstetrics stage</th>
<th>Tumor size (cm)</th>
<th>Human papillomavirus</th>
<th>LN*</th>
<th>PM †</th>
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<td>-</td>
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* Lymph node metastasis.
† Parametrial invasion.
‡ Resection margin involvement.
between antagomirs and chemosensitivity (49, 50). However, the lack of prediction and identification of an exact target for miR-199a in this study is a major limitation. Future research is needed to resolve these problems.

In summary, we have shown that there is miRNA deregulation in early stage cervical cancer tissues compared with normal cervical epithelial tissues and that miR-127 is associated with lymph node metastasis of the ISCCs. In addition, anti–miR-199a was shown to inhibit cell growth and potentiate the chemotherapeutic response in vitro; this leads us to conclude that miR-199a may be a potential therapeutic target for cervical cancer therapy.

Fig. 3. Real-time quantitative PCR analysis of miR-127 in 31 ISCC samples. The expression of miR-127 was significantly higher in a group of ISCCs with lymph node metastasis (P = 0.0061).

References
Inhibition of cervical squamous cell growth by anti–miR-199a oligonucleotide. (A) Relative expression of miR-199a in cervical squamous cell carcinoma tissues and normal cervical squamous epithelia as detected by TaqMan real-time PCR (*P < 0.0001). (B) Suppression of miR-199a expression by 100 nmol/L of anti–miR-199a in cervical squamous cells (ME-180 and SiHa). (C) Cell growth inhibition by anti–miR-199a. (D and E) Cell growth inhibition in the presence of the anticancer agent cisplatin. Columns, mean of three independent experiments; bars, SE (*, P < 0.05; **, P < 0.01).


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