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# MicroRNA Analysis in Human Papillomavirus (HPV)-Associated Cervical Neoplasia and Cancer

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## Abstract

**Background:** MicroRNAs (miRNAs) are ~22 nt single-stranded, non-coding RNAs that generally negatively regulate their target mRNAs at a posttranscriptional level. Differential expression of miRNAs has been observed in many human cancers.

**Methods:** To study their potential role in the pathogenesis of human papillomavirus (HPV) type 16-associated cervical neoplasia and cancer, we analyzed miRNA expression in cervical tissue from the normal cervix, moderate/severe dysplasia, and invasive squamous cell carcinoma.

**Results:** Using RNA from six cervical cancers, three dysplasias, and four normal samples and the TaqMan® MicroRNA Arrays, we found that 18 miRNAs were overexpressed and 2 underexpressed in cervical cancer compared to normal cervical tissue. We further demonstrated via individual TaqMan® MicroRNA Assays that 8 miRNAs (miRs-16, 21, 106b, 135b, 141, 223, 301b, and 449a) were significantly overexpressed and 2 miRNAs (miRs-218 and 433) were significantly underexpressed in cervical cancer compared to normal cervical tissue. MiR-21, miR-135b, miR-223, and miR-301b were overexpressed in cervical cancer compared to both cervical dysplasia and normal tissue. MiR-218 was similarly underexpressed in cervical cancer compared to dysplasia and normal tissue.

**Conclusions:** Our results suggest that ten miRNAs can delineate cervical cancer from normal cervical tissue, and five miRNAs may have potential as markers for progression from dysplasia to invasive cervical disease.

**Keywords:** MicroRNA; Human papillomavirus; Cervical cancer; Cervical dysplasia; Screening

**Abbreviations:** miRNA: micro Ribonucleic acid; HPV: Human papillomavirus; CaCx: Cervical Cancer; CIN: Cervical Intraepithelial Neoplasia; LEEP: Loop Electrosurgical Excision Procedure; qRT-PCR: quantitative Reverse Transcription Polymerase Chain Reaction

## Introduction

Worldwide, cervical cancer is the most common gynecologic malignancy. The estimated incidence is 493,243 new cases and an associated 273,505 related deaths, making it the third most deadly cancer, behind breast and lung [1]. Cervical cancer is a major health concern in the United States as well. In 2010, there were an estimated 12,200 new cases and an associated 4,210 deaths, accounting for approximately 1% of cancer deaths in women [2]. Developed countries such as the United States have seen a significant decrease in the incidence of invasive cervical cancer during the past 50 years. This is largely due to the widespread use of effective screening techniques. However, the incidence of invasive cervical cancer still remains disproportionately higher in minorities and others with limited access to healthcare.

Due in large part to increased screening for cervical cancer, the incidence of patients diagnosed with cervical dysplasia has increased exponentially over the past 50 years. Currently, it is estimated that between 250,000 and 1,000,000 women in the United States will be diagnosed with cervical intraepithelial neoplasia (CIN) annually [3]. The stepwise progression of mild to moderate to severe dysplasia, and eventually to invasive cervical cancer has been well documented. The management of patients with cervical dysplasia should be focused on the prevention of invasive cervical cancer. There is a huge expenditure required to diagnose, follow and treat patients with cervical dysplasia, in order to prevent the development of invasive cancer.

Human Papillomaviruses (HPVs) are the causative agents in essentially all cervical dysplasia and invasive cervical cancer [4]. There are more than 100 documented HPV types, and approximately 40 (high-risk HPVs) have the propensity to cause malignant changes, in varying degrees, in the anogenital epithelium [5]. HPV infection is extremely common in sexually active individuals, with some reported lifetime incidence rates as high as 80% in women by the age of 50 [6]. While the overall lifetime incidence of HPV infection in women is high, the virus is usually transient and the majority of infections are cleared in less than a year [7]. Nevertheless, some patients are found to have cervical dysplasia that is often persistent and more virulent. The ability to identify which cervical HPV infections will become cancers would markedly reduce the resource utilization currently required to prevent

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cervical cancer. Such a reduction would presumably make cervical cancer prevention more affordable to the third world where it remains a significant burden.

HPVs are small, circular, non-enveloped, double-stranded DNA viruses of approximately 8000 base pairs in size. Upon infection of the basal epithelium, the non-structural early genes E1, E2, E4, E5, E6, and E7 are expressed. Expression of late genes L1 and L2 coincides with a burst of viral replication and transcription; virions are shed at the surface of the epithelium and may infect surrounding tissue [8]. In many cervical cancers, the HPV DNA becomes integrated into the host chromosome, disrupting the E2 regulatory gene and increasing expression of the E6 and E7 oncogenes. E6 and E7 work synergistically to deregulate cell cycle controls through a variety of mechanisms [8]. E6 interacts with many cellular proteins, and it activates telomerase [9]. Importantly, E6 binds the tumor suppressor protein p53, promoting its ubiquitination and proteasomal degradation [10-13]. Binding of p300/CBP inhibits p53 acetylation and further prevents p53-mediated transcription [14,15]. E7 bypasses cell cycle arrest and induces proliferation through interaction and inhibition of pRB family members and constitutive activation of E2F-responsive genes [16-18]. The E7 protein binds to and inactivates the function of pRB and the related tumor suppressor proteins p107 and p130 [19,20]. Expression of E6 and E7 also promotes chromosomal instability, foreign DNA integration, and other mutagenic events [21,22].

MicroRNAs (miRNAs) are small (18-25 nucleotides), non-protein-coding RNAs that regulate gene expression [23]. MiRNAs most often function by binding to the 3′ UTRs of target messenger RNAs (mRNAs), whereby they induce mRNA degradation or translational repression [24]. The functions of miRNAs are still largely unknown, but they appear to be integral in the regulation of cellular gene expression and behavior. Many studies have demonstrated that miRNAs play an essential role in the development of a variety of cancers.

Differentially expressed miRNAs may serve as early biomarkers for the progression of cervical dysplasia. Furthermore, the development of a biomarker panel may be useful in determining which patients with cervical dysplasia are likely to progress to more invasive disease, and it may also be a useful prognostic indicator in patients with invasive cancer. We compared miRNA expression profiles in cervical cancer, dysplasia and normal cervical tissue, and we report the identification of five miRNAs whose deregulation is associated with the progression of HPV-induced disease from mild dysplasia to invasive cancer.

## Materials and Methods

After IRB approval had been obtained, prospective collection of cervical tissue was performed from various grades of neoplasia. Patients with preinvasive and grossly invasive cervical lesions undergoing excision were recruited for adjacent biopsies of the cervical lesion. One biopsy was snap frozen for our analysis and the other was sent to pathology for tissue diagnosis. Patients with microinvasive disease or prior radiation were excluded.

Cervical dysplasia specimens were collected from patients at the time of colposcopy and excision using a similar paradigm of adjacent biopsy techniques. After the LEEP excision was performed, a small sample from the area of suspected dysplasia was removed for miRNA analysis. The LEEP specimen was then sent for pathologic review. In addition to the standard pathologic review, the gynecologic pathologist evaluated the cervical tissue directly adjacent to the tissue that was removed for analysis. We used this analysis to grade the level of dysplasia in our sample. We only analyzed CIN 2 and 3 lesions. Any tissue collected with mild, or no dysplasia, was not processed.

Normal cervical tissue was also collected from controls, age-matched to the cervical cancer patients. This tissue was obtained from the Magee-Womens tissue bank (from a separate IRB) for patients with normal cervical cytology who had undergone a hysterectomy for a benign indication. Normal cervical samples were obtained from the transformation zone, to correspond to that taken from the dysplasia and cancer patients. All cervical tissue was directly evaluated by a gynecologic pathologist.

Once tissue was collected, it was snap frozen and stored at -80°C for later use. Subsequently, a portion of the tissue was removed for DNA isolation and another for RNA isolation. After DNA isolation, tissue samples were confirmed to be either HPV-positive or HPV-negative by PCR analysis using the MY09/MY11 primer set for amplification of the highly conserved HPV L1 structural gene [25]. Amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control, using the forward primer 5′- ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′- TCCACCACCCTGTTGCTGTA-3′. HPV-positive samples were then tested for the presence of HPV type 16 or 18 by PCR analysis of their respective E6 oncogene. The primers used were 5′- ATGCACCAAAAGAGAACTGC -3′ (forward) and 5′ -TTACAGCTGGGTTTCTCTAC -3′ (reverse) for HPV-16 E6 and 5′ -CGGCGACCCTACAAGCTACCTG-3′ (forward) and 5′ -CTGCGTCGTTGGAGTCGTTCC (reverse) for HPV-18 E6. The majority of the tissues were HPV-16 positive, and we proceeded with the analysis of only such samples (dysplasia or cancer). The HPV-negative normal cervical tissues were also confirmed to have completely benign pathology.

Total RNA was isolated from all the cervical tissues using the Ultraspec™ RNA Isolation System (Biotecx, Houston, TX, USA) and analyzed by agarose gel electrophoresis. DNase-I-treated total RNA (1 μg) from the HPV-16 positive tissues was further analyzed by RT-PCR for the expression of the E6 and E7 oncogenes. The HPV-16 E7 primers were 5′-GTAACCTTTTGTTGCAAGTGTGACT-3′ (forward) and 5′ GATTATGGTTTCTGAGAACAGATGG-3′ (reverse). The Advantage® Clontech RT-for-PCR Kit (Clontech, Mountain View, CA, USA) was used according to the manufacturer's instructions, followed by PCR.

We then carried out miRNA expression analysis using the TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers, followed by TaqMan® Human MicroRNA Arrays V2.0 from Applied Biosystems, which are designed for the sensitive amplification and profiling of 667 unique human miRNAs. The arrays also contain primers for three endogenous small RNAs U6, RNU44, and RNU48, which serve as positive controls, as well as primers for an *A. thaliana* miRNA, which serves as a negative control. This approach is based on real-time quantitative PCR and we used 1.5 μg of total RNA per sample for miRNA expression analysis. Amplification of all miRNAs was carried out according to the manufacturer's instructions and the samples were analyzed on the 7900HT Fast Real-Time PCR System (Applied Biosystems).

### TaqMan® MicroRNA array data was deposited in the array express database with accession numbers

The relative quantification of miRNAs was performed using the 2 delta Ct method, where fold change in expression of a gene in an experimental sample is quantified relative to the same gene in a reference sample [26]. We used two-fold as the cut-off ratio for identification of differentially expressed miRNAs. Data analysis was performed first by comparing cervical cancer tissue to normal cervical tissue for all overexpressed and underexpressed miRNAs, using a statistical significance level of p=0.05.

MiRNAs found to be differentially expressed in the cervical cancer or dysplasia samples compared to normal cervical tissue were further analyzed by individual quantitative RT-PCR (qRT-PCR) analysis using the TaqMan® MicroRNA Reverse Transcription Kit and individual TaqMan® MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. The data in these experiments was normalized to the small nucleolar RNU43 levels, and the relative expression levels of the miRNAs were calculated using the 2 delta CT method. These assays were performed in triplicate and independently reproduced a minimum of two times. Statistical significance between groups was calculated via Student's *t*-test.

## Results

Patients were identified for tissue collection from March 2008 until May 2009. We collected and analyzed tissue from 6 patients with cervical cancer, 3 with cervical dysplasia, and 4 with normal cervical tissue. We only analyzed dysplasia samples from patients with pathologically confirmed CIN 2 or CIN 3 lesions. For patients with cervical cancer, we only analyzed samples with squamous cell carcinoma.

Patient characteristics, including pathology and treatment information, are shown in Table 1. Due to the small sample size, we included only those patients with cervical cancer or dysplasia that were found to be HPV-16 positive by PCR analysis (data not shown). Based on RT-PCR, all HPV-16 samples were also found to express the E6 and E7 oncogenes (data not shown).

Based on the TaqMan® MicroRNA Array data from the cervical tissue samples, we found that 20 miRNAs were differentially expressed in cervical cancer compared to normal cervical tissue at $p<0.05$. Of these, eighteen miRNAs were overexpressed and two were underexpressed. The fold change of various miRNAs ranged from 2.8 to 58.2 (Table 2). Unsupervised hierarchical clustering grouped the samples into two categories based on their miRNA expression, delineating cervical cancer from cervical dysplasia and normal cervical tissue. The three dysplasia samples (CIN79, CIN80, and CIN83) exhibited varying levels of expression of the twenty miRNAs, with CIN83 grouping more closely with the normal cervical tissues than CIN79 and CIN80 (Figure 1). The eighteen miRNAs on the top were overexpressed in cervical cancer

| Sample | Specimen Number | Type | Patient Age | Diagnosis | Stage | Treatment |
|---|---|---|---|---|---|---|
| 1 | 32 | Benign | 51 | Uterine Prolapse | n/a | Hysterectomy |
| 2 | 49 | Benign | 39 | H/O Breast Cancer | n/a | Hysterectomy |
| 3 | 167 | Benign | 35 | Menorrhagia | n/a | Hysterectomy |
| 4 | 367 | Benign | 50 | Menorrhagia | n/a | Hysterectomy |
| 5 | 79 | Dysplasia | 56 | CIN 3 | n/a | LEEP |
| 6 | 80 | Dysplasia | 27 | CIN 2 | n/a | LEEP |
| 7 | 83 | Dysplasia | 26 | CIN 2 | n/a | LEEP |
| 8 | 33 | Cancer | 71 | Squamous Cell Carcinoma | 3B | XRT/Chemo |
| 9 | 42 | Cancer | 40 | Squamous Cell Carcinoma | 2B | XRT/Chemo |
| 10 | 47 | Cancer | 52 | Squamous Cell Carcinoma | 3B | XRT/Chemo |
| 11 | 52 | Cancer | 61 | Squamous Cell Carcinoma | 1B1 | Radical Hysterectomy |
| 12 | 57 | Cancer | 38 | Squamous Cell Carcinoma | 1B2 | Radical Hysterectomy |
| 13 | 81 | Cancer | 34 | Squamous Cell Carcinoma | 1B1 | Radical Hysterectomy |

**Table 1:** Patient Characteristics.

| MiRNA | Fold change |
|---|---|
| ***Overexpressed*** | |
| hsa_miR_124 | 58.2 |
| hsa_miR_449a | 35.3 |
| hsa_miR_449b | 30.5 |
| hsa-miR- 301b | 24.3 |
| hsa_miR_517c | 22.4 |
| hsa_miR_545 | 17.4 |
| hsa_miR_223 | 14.2 |
| hsa_miR_135b | 11.1 |
| hsa_miR_21 | 9.6 |
| hsa_miR_512_3p | 9.4 |
| hsa_miR_542_3p | 7.8 |
| hsa_miR_181c | 7.6 |
| hsa_miR_517a | 6.9 |
| hsa_miR_518f | 5.0 |
| hsa_miR_106b | 4.3 |
| hsa_miR_192 | 3.8 |
| hsa_miR_16 | 3.8 |
| hsa_miR_141 | 2.8 |
| ***Underexpressed*** | |
| hsa_miR_433 | -18.6 |
| hsa_miR_218 | -5.6 |

**Table 2:** MiRNAs differentially expressed in cervical cancer tissue compared to normal cervical tissue ($p<0.05$).

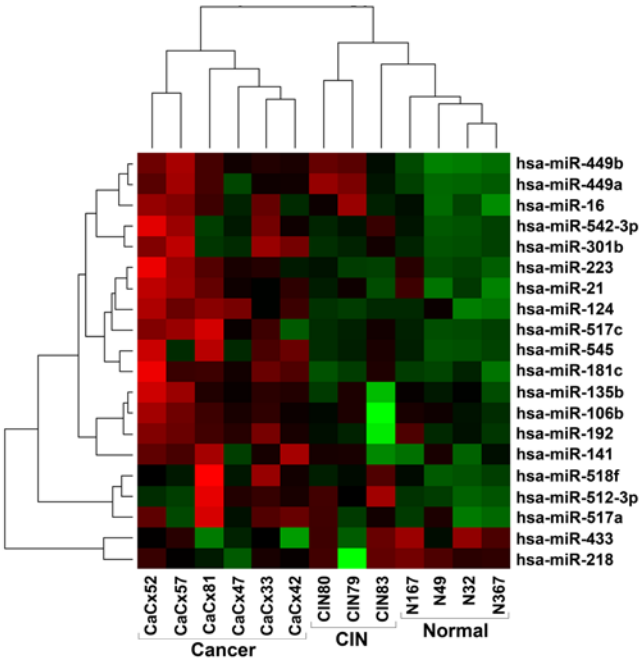


**Figure 1:** Unsupervised hierarchical clustering (p<0.05) of cellular miRNAs with increased (red) or decreased expression (green) in cervical cancer tissue, cervical dysplasia, and normal cervical tissue, based on TaqMan® MicroRNA Array data.

tissues compared to normal cervical tissues. Two miRNAs, shown at the bottom (miR-433 and miR-218), were underexpressed in cervical cancer tissues compared to normal cervical tissues and grouped independently from the overexpressed miRNAs (Figure 1).

To validate the miRNA data obtained from array analysis, we performed individual qRT-PCR analysis of the 20 differentially expressed miRNAs. We identified 10 miRNAs with significant differences between the cancer and dysplasia tissues compared to the normal cervical tissues. The individual qRT-PCR expression plots for these miRNAs, displaying relative expression to U6 snRNA for each sample, are shown in Figure 2. Despite variation within groups, we found that miR-16, miR-21, miR-106b, miR-135b, miR-141, miR-223, miR-301b, and miR-449a were significantly overexpressed in both cervical cancer and dysplasia compared to normal cervical tissue ($p<0.01$). Several miRNAs were overexpressed in cervical cancer tissue compared to both normal and dysplasia tissue. These included miR-21, miR-135b, miR-223, and miR-301b. MiR-21 and miR-135b were significant at $p<0.05$. Interestingly, miR-106b and miR-141 appeared to have greater expression in the dysplasia tissues compared to normal tissues, and intermediate expression in cervical cancer compared to the dysplasia samples ($p<0.01$). MiR-218 and miR-433 were significantly underexpressed in cervical cancer and dysplasia tissue compared to normal cervical tissue (miR-218 at $p<0.01$ and miR-433 at $p<0.05$). Individual qRT-PCR analysis yielded comparable results to the array data for the dysplasia specimens. For many of the miRNAs, such as miR-16, miR-21, miR-135b, miR-223 (overexpressed) miR-218, and miR-433 (underexpressed), CIN83 was similar to the normal cervical tissues, while CIN79 and CIN80 were more similar to the cervical cancer tissues (Figure 2).

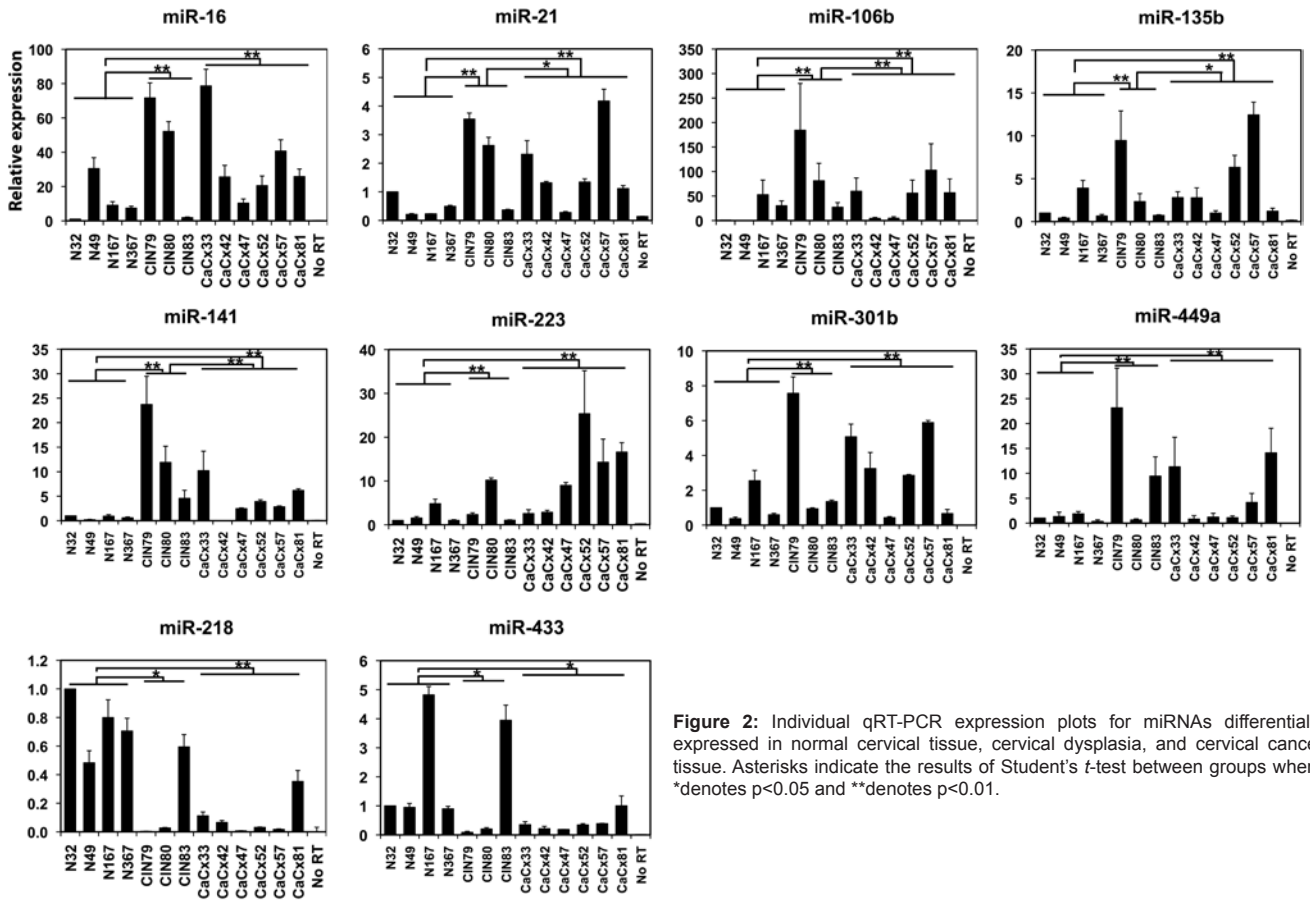


**Figure 2:** Individual qRT-PCR expression plots for miRNAs differentially expressed in normal cervical tissue, cervical dysplasia, and cervical cancer tissue. Asterisks indicate the results of Student's *t*-test between groups where *denotes p<0.05 and **denotes p<0.01.

We identified potential gene targets of the miRNAs we found differentially expressed in our tissue samples. Computationally, miRNAs can bind the 3′ UTRs of hundreds of genes, making target prediction difficult. Therefore, we evaluated gene expression signatures reported by Gius et al., in which they utilized National Cancer Institute ROSP 8K human arrays to identify differentially expressed genes in the normal cervix, cervical dysplasias, and cervical squamous cell carcinomas from both epithelial and underlying stromal tissues [27]. We compared this data with bioinformatically predicted gene targets from MicroCosm Targets, formerly miRBase Targets, which uses an algorithm to find sequences in the 3′ UTR of genes that are complementary to the miRNA seed sequence (the first 2-8 nucleotides at the 5′ end) [28,29]. Thermodynamic stability and conservation of the targets across two or more species are also considered [28,29]. Genes that Gius et al. found decreased in cervical cancer and dysplasia compared to normal cervical tissue were cross-referenced with computational targets for miRNAs that were overexpressed in our study. Likewise, genes that Gius et al. found increased in cervical cancer and dysplasia compared to normal cervical tissue were cross-referenced with computational targets for miRNAs that were underexpressed in our study. Table 3 displays the resulting potential gene targets for the 10 miRNAs that were most significantly over or under expressed in our analysis.

## Discussion

Changes in miRNA expression have been linked to many cancers [30]. They are often found near fragile sites in chromosomes or integration sites of high-risk HPVs [31]. Integration may alter miRNA expression via deletion, amplification, or genomic rearrangement. MiR-21 and miR-106b are located near HPV integration sites [32], which may have implications for their expression in cervical cancer. Depending on the nature of their targets, miRNAs can function as either tumor suppressor genes or oncogenes. For example, overexpression of miRNAs that target oncogenes can lead to increased destruction of these oncogenes and therefore tumor suppression. Conversely, overexpression of miRNAs that target tumor suppressors can result in increased oncogenic activity and tumor formation. MiRNAs have been shown to regulate the oncogenes *Bcl* and *Ras*, as well as the tumor suppressor pRb. MiR-15 and miR-16 were the first miRNAs shown to be associated with cancer; they are underexpressed in chronic lymphocytic leukemia (CLL) [33]. They regulate the *Bcl-2* oncogene, which is overexpressed in many cancers [34]. The *let-7* family of miRNAs regulates the *Ras* oncogenes, which contain activating mutations in about 15-30% of cancers [35]. Down regulation of the *let-7* family of miRNAs results in the up regulation of *Ras*, which is most pronounced in lung cancers [36].

In the current study, we found significant overexpression of 18 miRNAs and underexpression of 2 miRNAs in cervical cancer tissues compared to normal cervical tissues via TaqMan® MicroRNA Arrays (Figure 1). We further demonstrated, via individual qRT-PCR assays, significant overexpression of miR-16, miR-21, miR-106b, miR-135b, miR-141, miR-223, miR-301b, and miR-449a, and underexpression of miR-218 and miR-433 in cervical cancer and dysplasia compared to normal cervical tissue (Figure 2). The above ten miRNAs may be useful in delineating cervical cancer and dysplasia from normal cervical tissue. Importantly, this is one of the few published studies to report differential expression of miRNAs in cervical dysplasia. MiR-21, miR-135b, miR-223, and miR-301b were overexpressed in the cervical cancer tissue compared to both normal and cervical dysplasia tissue. These miRNAs are good candidates for markers of progression from normal to dysplasia to cancer. Loss of miR-218 expression may also be a good marker of progression.

Other studies have reported similar miRNA expression findings to ours for cervical cancer. Using a cloning strategy to identify differentially expressed miRNAs, Lui et al. analyzed the respective cloning frequency of miRNAs in cervical carcinoma cell lines versus normal cervical tissue [37]. They further evaluated miR-21 and miR-143 via Northern blotting in 29 cervical cancer tissues versus adjacent normal cervical tissue. They found significant overexpression of miR-21 and underexpression of miR-143 in cervical cancer compared to normal cervical tissue. Wang and co-workers profiled 455 miRNAs via miRNA microarray in 4 cervical cancer tissues and 4 normal cervical tissues [38]. They found significant overexpression of 18 miRNAs and underexpression of 15 miRNAs cervical cancer tissues compared to normal cervical tissues. In common with our study, they found that miR-16, miR-21, miR-181c, and miR-223 are overexpressed and that miR-218 is underexpressed in cervical cancer compared to normal cervical tissue. Lee et al. analyzed

| MiRNA | Potential Targets |
|---|---|
| ***Overexpressed*** | ***Underexpressed*** |
| miR-16 | CDA, CDC37, NPC1, PPL, PVRL2, TGM1, ACAA1, PPAP2A, RBX1, UCP2, WNT7A |
| miR-21 | ANXA1, CD48, DNAJA2, EIF4G2, EPHX1, IL13RA1, C2, DDAH1, PLA2G4A |
| miR-106b | DPAGT1, NINJ2, TBX19, ATP5F, FUT3, TPT1 |
| miR-135b | ACAA1, DDX58, IL13RA1, SPINK5, AREG, HNRPA1, HNRPAB, NDUFA6 |
| miR-141 | EPHA2, NPC1, BRWD1, DR1, SELE |
| miR-223 | DSG3, EIF4G2, BAG1, RBBP6 |
| miR-301b | PPL, RASAL1, TBX1, TERF2, TGM1, C2, H19, RPS10, SELE |
| miR-449a | CDA, CDC37, NPC1, PPL, PVRL2, TGM1, AREG, ARHGBIB |
| ***Underexpressed*** | ***Overexpressed*** |
| miR-218 | C1ORF41, RTCD1, SHMT2, SSR1, BAI3, CCN1, EBI2, HAS3, IGF2, MMP3, MTIG, PLAUR, PRIM2A, PSMB7, RGS1, RPS29 |
| miR-433 | GPR56, MRPL33, PABPC4, TARBP1, C1S, CD1A, CUL2, ISGF3G, NM1, RPS16, TRIP12 |

**Table 3:** Potential gene targets of miRNAs differentially expressed in cervical dysplasia and cancer.

157 cellular miRNAs via the TaqMan® MicroRNA Human Early Panel Kit (Applied Biosystems) in 10 early stage squamous cell carcinomas and 10 normal cervical samples [39]. They found overexpression of 68 miRNAs and underexpression of two in the cervical carcinomas versus normal cervical tissues. In common with our study, they reported that miR-16, miR-21, miR-135b, and miR-181c are overexpressed in cervical cancer compared to normal cervical tissue. Hu and coworkers analyzed 96 miRNAs in 59 cervical cancer tissues via real-time RT-PCR. They found that miR-9, miR-21, miR-200a and miR-218, and miR-203 were associated with cancer survival [40]. They further evaluated miR-200a and miR-9 in an additional 42 cervical cancer tissues and found them to be predictive of survival. Pereira et al. analyzed 377 known and predicted miRNAs via miRNA microarray in 4 pooled normal cervical samples, 14 cervical dysplasia samples, and 4 squamous cell carcinomas [41]. They reported a great deal of variability in the tissues, but by pooling the normal samples they were able to identify differences in miRNA expression between groups. Interestingly, they found that miR-16 was decreased in the cervical dysplasia samples relative to the normal cervical samples and increased in the cervical cancer samples relative to the cervical dysplasia samples. Of note, the cervical cancer and dysplasia tissues were not characterized by HPV type in the above studies.

In a recent study, Rao et al. investigated 802 known and 122 predicted human miRNAs via CaptialBio mammalian miRNA arrays V 3.0 in thirteen HPV-16 and HPV-18 positive cervical cancers and their adjacent normal tissues [42]. In common with our study, they found that miR-141 is overexpressed and miR-218 is underexpressed in cervical cancer compared to normal cervical tissue. Zhang et al. analyzed miRNA expression via microarray in 5 cervical squamous cell carcinomas that appeared to be HPV-negative and 5 normal cervical tissues [43]. In common with our study, they found miR-21 to be overexpressed in the cervical cancer samples. Yang et al. analyzed the expression of miR-214 via qRT-PCR and found it underexpressed in seven HPV-16 and HPV-18 positive cervical cancers versus matched controls [44]. Li et al. analyzed the expression of pri-miR-34a via semi-quantitative RT-PCR in 32 histopathologically normal cervical samples with high-risk HPV infection, 32 normal samples without HPV infection, 32 CIN lesions with high-risk HPV infection, 12 CIN lesions without HPV infection, and 32 cervical cancers [45]. They found that pri-miR-34a is reduced in cervical cancer and dysplasia compared to normal cervical tissue, in CIN 2 and CIN 3 compared to CIN 1, and also in tissues with high-risk HPV infection compared to those without. Wang et al. also showed via Northern blotting that miR-34a, which is regulated by HPV-16/18 E6 via p53 destabilization, is decreased in three cervical cancers with high-risk HPV compared to age-matched controls [46]. Finally, previous research performed in our laboratory showed that HPV-16 E6 reduces miR-218 expression in cervical carcinoma cell lines, and it is significantly underexpressed in HPV-16 positive cervical dysplasias and cancers [47]. Taken together, the miRNA expression patterns in cervical cancers and dysplasias that are in common between our study and others are overexpression of miR-16, miR-21, miR-135b, and miR-141 and underexpression of miR-218.

Many of the differentially expressed miRNAs in our study, and their potential targets, are differentially expressed in other cancers as well. MiR-16 is underexpressed in CLL, but it is overexpressed in serous ovarian cancer [48] as well as cancer of the head and neck [49]. Calin et al. showed that miR-16 could affect both oncogenes and tumor suppressors [50]. Recently, Kaddar et al. observed that miR-16 can negatively regulate genes that are involved in cell proliferation, such as HMGA1 and caprin-1 in MCF-7 and HeLa cells, but miR-16 induced G1 accumulation occurs only in MCF-7 cells, not in HeLa cells, which contain HPV-18 DNA integrated into the chromosome [51]. The authors speculate that this is most likely due to the status of p53 and Rb, which function normally in MCF-7 cells but are inactivated in HeLa cells. Lack of miR-16 induced G0/G1 accumulation in HeLa cells was also observed by Linsley et al. [52] It is possible that the presence of high-risk HPVs may directly or indirectly affect the expression of miR-16 and its regulatory pathways to promote cell proliferation. WNT7A, an important growth and differentiation factor in the female reproductive tract, is a potential target of miR-16 (Table 3), and it is down regulated in cervical cancer [27]. WNT7A was shown to have an antitumorigenic effect in non-small cell lung cancer [53]. Downstream effectors of WNT7A include Sprouty-4, which inhibits transformed cell growth, migration and invasion, and the epithelial to mesenchymal transition in non-small cell lung cancer [54]. Down regulation of WNT7A may therefore promote these processes and could contribute to cervical tumorigenesis.

MiR-21 is the most highly overexpressed miRNA in numerous cancers. Its overexpression has been observed in the following cancers: ovarian, head and neck, lung, stomach, liver, and pancreas [37,49] [55-58]. MiR-21 down regulates the tumor suppressor PTEN in non-small cell lung cancer [55] and in hepatocellular cancer, in which PTEN regulation leads to overexpression of matrix metalloproteinases MMP2 and MMP9 [59]. These proteins promote cellular migration and invasion. MiR-21 also targets the tumor suppressor genes tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and maspin, and the latter two have been implicated in tumor invasion and metastasis [60,61]. A recent paper demonstrates that miR-21 can regulate PDCD4 in HeLa cells, increasing cellular proliferation [62], which may have implications for the promotion of cervical cancer. MiR-21 may also target annexin 1 (ANXA1) and the eukaryotic initiation factor EIF4G2 (Table 3), which are down regulated in cervical cancer [27]. Reduced expression of ANXA1 is associated with advanced stage breast cancer [63], and down regulation of EIF4G2 correlates with invasive transitional cell carcinoma of the bladder [64]. ANXA1, a calcium and phospholipid binding protein that may be secreted, is involved in various cellular pathways such as inflammation, migration, and proliferation. It is a substrate for EGF receptor tyrosine kinase and inhibits EGF-mediated proliferation [65]. Thus, reduction of ANXA1 by miR-21 would promote cellular proliferation. These data are consistent with miR-21's function as an oncogene in many cancers and in cervical cancer.

MiR-106b is overexpressed in gastric and colorectal cancer, as well as squamous cell carcinoma of the head and neck [56,66,49]. It is part of a cluster of miRNAs, along with miR-93 and miR-25, which is located within an intron of mini chromosome maintenance 7 (MCM7). MCM7, a target of the E2F1 transcription factor, is a marker for cervical cancer and may be induced by both HPV-16 E6 and E7 [67,68]. In gastric cancer, miR-106b targets p21, antagonizing the TGF-beta tumor suppressor pathway. [69]. Additionally, miR-106b may target ninjurin 2 (NINJ2), a cell surface adhesion molecule first identified on neural cells (Table 3) [70], which is also down regulated in cervical cancer [27]. Reduction of ninjurin 2, via overexpression of miR-106b, could contribute to loss of adhesion and increased migration of cervical cancer cells.

MiR-135b is overexpressed in colorectal and prostate cancer [66] [71-73]. A potential target of miR-135b is the serine protease inhibitor SPINK5 (Table 3), which is down regulated in cervical cancer [27]. SPINK5 deficiency causes unregulated epidermal protease activity and degradation of desmoglein 1, which leads to inefficient stratum corneum adhesion and a resultant loss of skin barrier function [74]. Overexpression of miR-135b and loss of SPINK5 could alter

the epithelial architecture of the cervix and promote a tumorigenic phenotype.

MiR-141 is overexpressed in ovarian, prostate, and endometrial endometroid carcinomas[75-77]. Also, Nam et al. reported that overexpression of mir-141 is associated with a poor prognosis in patients with serous ovarian carcinoma [48]. MiR-141 is located on chromosome 12 in a cluster with miR-200c. As part of the miR-200 family of miRNAs, it is regulated by ZEB1 and ZEB2, which in a feedback loop are also regulated by the miR-200 family, mediating the epithelial to mesenchymal transition [78,79]. Overexpression of miR-141 in the cervical epithelium could therefore contribute to cervical tumorigenesis. Other targets include the tumor suppressors BRD3, UBAP1, and PTEN, which are down regulated by miR-141 in nasopharyngeal carcinoma [80].

Overexpression of miR-223 is found in adult T-cell leukemia and adenocarcinomas of the esophagus [81,82]. MiR-223 may target desmoglein 3 (DSG3) (Table 3), which is down regulated in cervical cancer [27]. Desmoglein 3, a desmosomal adhesion molecule, participates in intercellular links via desmosome-intermediate filament complexes and helps to maintain tissue integrity. Reduced expression of desmoglein 3 increases the colony-formation efficiency and proliferative potential of primary keratinocytes [83]. The overexpression of miR-223 in cervical cancer may therefore reduce cellular adhesion and promote proliferation of cancer cells.

MiR-301b is overexpressed in colon cancer tissue [66]. This miRNA may target the RasGAP RASAL1 (Table 3), which is down regulated in cervical cancer [27]. RASAL1 expression is lost in many colorectal cancers, which promotes activation of the *Ras* oncogene [84]. *Ras* activation in HPV-18 E7-expressing organotpic raft tissues increases the invasive potential of the cells via MT1-MMP and MMP9 [85]. Overexpression of miR-301b could potentially increase *Ras* activation and promote cervical cancer via the down regulation of RASAL1.

Conversely, miR-218 is underexpressed in a variety of cancers including breast, ovarian, lung, and gastric cancer [30,31] [86-88]. Polymorphisms in pri-miR-218 are associated with a decreased risk for cervical cancer [89]. MiR-218, which is encoded within Slit genes, can target Robo1 and Robo2, providing a negative feedback loop through Slit-Robo interaction, and thereby regulate vascular patterning [90]. Targeting of Robo1 serves to inhibit metastasis in gastric cancer [91]. MiR-218 inhibits the invasiveness of glioma cells via targeting of IKK-beta [92]. MiR-218 may also target extracellular matrix proteins CCN1 and MMP3 (Table 3), which are up regulated in cervical cancer [27]. CCN1 is overexpressed in several cancers and increases tumorigenicity in mice [93-95]. Underexpression of miR-218 in cervical cancer may promote expression of these genes and contribute to tumorigenesis.

Lastly, miR-433 is underexpressed in gastric cancer [96,97]. Interestingly, miR-433 can potentially target cytoplasmic poly (A) binding protein 4 (PABPC4) (Table 3), which interacts with HPV-16 E6 [98] and is up regulated in cervical cancer [27]. PABPC4 binding of NFX-123 is required for posttranscriptional regulation of hTERT by HPV-16 E6, increasing telomerase activation and cell growth [98]. Underexpression of miR-433 could increase the expression of PABPC4 and augment the growth of E6-expressing cervical cancer cells.

In summary, we identified ten miRNAs whose expression may define a cervical cancer profile. Five of these miRNAs have been identified in other studies. We also identified five miRNAs, miR-21, miR-135b, miR-223, miR-301b, and miR-218, as possible markers of disease progression. The targets of these miRNAs may also be important, and validation of miRNA-mRNA target interaction is the subject of ongoing studies in our laboratory.

Identification of a panel of miRNAs that can be used as early biomarkers in cervical cancer is potentially useful to determine disease behavior and prognosis. They may also provide new targets for anti-cancer therapy. The progression of invasive cervical cancer from mild, asymptomatic HPV infection to advanced stage invasive cervical cancer has been well documented. Although the majority of patients with moderate cervical dysplasia, even if untreated, will not progress to cervical cancer, a significant fraction will. MiRNA expression profiling may enable us to identify those women with more aggressive disease and therefore provide more aggressive treatment for such patients.

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