Differential Expression of MicroRNAs in Uterine Cervical Cancer and Its Implications in Carcinogenesis; An Integrative Approach

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Objectives: Cervical cancer is the second most common cancer in women in developing countries, including India. Recently, microRNAs (miRNAs) are gaining importance in cancer biology because of their involvement in various cellular processes. The present study aimed to profile miRNA expression pattern in cervical cancer, identify their target genes, and understand their role in carcinogenesis.

Methods: Human papillomavirus (HPV) infection statuses in samples were assessed by heminested polymerase chain reaction followed by direct DNA sequencing. Next-generation sequencing and miRNA microarray were used for miRNA profiling in cervical cancer cell lines and tissue samples, respectively. MicroRNA signature was validated by quantitative real-time PCR, and biological significance was elucidated using various in silico analyses.

Results: Cervical cancer tissues samples were mostly infected by HPV type 16 (93%). MicroRNA profiling showed that the pattern of miRNA expression differed with respect to HPV positivity in cervical cancer cell lines. However, target and pathway analyses indicated identical involvement of these significantly deregulated miRNAs in HPV-positive cervical cancer cell lines irrespective of type of HPV infected. Microarray profiling identified a set of miRNAs that are differentially deregulated in cervical cancer tissue samples which were validated using quantitative real-time PCR. In silico analyses revealed that the signature miRNAs are mainly involved in PI3K-Akt and mTOR pathways.

Conclusions: The study identified that high-risk HPV induces similar carcinogenic mechanism irrespective of HPV type. The miRNA signature of cervical cancer and their target genes were also elucidated, thereby providing a better insight into the molecular mechanism underlying cervical cancer development.

Key Words: Cervical cancer, Human papillomavirus, MicroRNA, MiRNA microarray, Small RNA Sequencing
Carcinoma of the uterine cervix is the fourth most common cancer in women, with an estimated 528,000 new cases and 266,000 deaths in 2012. Regular screening methods have been implemented in developed countries to reduce its prevalence, thereby controlling its occurrence. Currently, 80% of new cervical cancer cases occur in developing countries including India, from where one fourth of the world’s cervical cancer cases are reported every year.

Human papillomavirus (HPV), a sexually transmitted virus, is considered as the main etiological agent in the development of cervical cancer. More than 200 different HPV types are identified, which includes the carcinogenesis promoting high-risk HPV types (HR-HPV) such as HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Among HR-HPV types, HPV type 16 is the common oncogenic type followed by HPV type 18. Human papillomavirus types 16 and 18 account for nearly 76.7% of cervical cancers in India. Low-risk HPV groups, including HPV types 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81 are not known to cause cervical cancer. Human papillomavirus types 6 and 11 are the most common low-risk HPV types and are linked to approximately 90% of genital warts. Oncogenic HPV infection is a well-known risk factor for cervical cancer, and its persistence may lead to the progression of cervical intraepithelial neoplasia to cancer. Hence, the identification of oncogenic HPV types is of great importance because of their prognostic significance in patients with abnormal cervical cytology.

MicroRNAs (miRNAs) are small, noncoding RNAs of 18 to 24 nucleotides, discovered in 1993 in the nematode Caenorhabditis elegans. The function of miRNA is to regulate mRNA posttranscriptionally by binding to complementary sequences in the 3’ untranslated region of target messenger RNAs (mRNAs), thereby negatively regulating mRNA translation. Discovery of miRNAs has led to an evolution in various fields of research, and their role in tumorigenesis is becoming more pertinent. The profiling of these small RNAs serves to identify distinct molecular signatures in various cancers, hence aiding in invention of new therapeutic methods, assessing prognostic factors and diagnosis. Recent studies identified that infection with HR-HPVs may induce the deregulation of oncogenic or tumor-suppressive miRNAs, and these observations perhaps give more knowledge on HR-HPV–induced oncogenesis. Identification of a set of differentially expressed miRNAs associated with HPV-induced cervical cancer is significant because these can be useful for developing therapeutic agents. In this study, we focused on the expression profiling of miRNAs in cervical cancer in relation to the HPV infection status.

MATERIALS AND METHODS

Cell Lines

The cervical cancer cell lines CaSki, SiHa, Me180, HeLa, and C33A were kindly provided by Dr Ruby John Anto (Rajiv Gandhi Centre for Biotechnology, Trivandrum, India). The cell lines CaSki and SiHa are HPV type 16 positive, whereas HeLa and Me180 are HPV types 18 and 68 positive, respectively. C33A is negative for HPV infection. The cell lines were cultured in Dulbecco’s modified eagle medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone mixture (Gibco, Life Technologies) at 37 °C in a 5% CO2 incubator.

Clinical Samples

This study has been approved by the Institutional Scientific Review Board and cleared by the Institutional Human Ethics Committee of Regional Cancer Centre, Trivandrum, for collecting the tissue samples from patients (sanction no. 43/2008). The cancer tissue samples were collected from cervical cancer patients (age range, 38–75 years) who reported at the outpatient department of Regional Cancer Centre, and normal cervical tissue samples were collected from patients with normal cervical cytology undergoing hysterectomy procedure for noncancerous problems at the Department of Obstetrics and Gynecology of Sree Avittom Thirunal Hospital, Trivandrum, India. After obtaining written consent from patients, a representative punch biopsy was taken from cervical cancer patients at the time of diagnostic biopsy procedure. Normal cervical mucosa samples were taken from the hysterectomized specimen. A total of 45 cervical cancer tissue samples and 12 normal cervical tissue specimens were included in the study. Clinicopathological details of the subjects included in the study are provided in Table 1, Supplemental Digital Content, http://links.lww.com/IGC/A618. Collected tissue samples were immediately stored in liquid nitrogen until use for experimental workup.

Human Papillomavirus Genotyping

DNA was extracted from cell lines and tissue samples by proteinase K digestion followed by phenol-chloroform isolation method. DNA concentration was estimated using UV spectrophotometer. To check the quality of DNA samples, a 168-base-pair sequence of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was amplified as an internal control. Heminested polymerase chain reaction (PCR) was carried out in 2 consecutive amplification reactions on all
clinical samples for HPV genotyping\(^7\) (Supplemental Digital Content 1, http://links.lww.com/IGC/A614).

**DNA Sequencing**

Human papillomavirus genotyping in the cell lines and tissue samples was done by direct DNA sequencing.\(^7\) Heminested PCR product was purified using GeneElute PCR Clean-up Kit (Sigma), and the sequencing reaction was performed using ABI Big Dye Terminator, version 3.1, cycle sequencing kit (Life Technologies) (Supplemental Digital Content 2, http://links.lww.com/IGC/A615).

**Small RNA Sequencing**

Total RNA was isolated from cervical cancer cell lines using mirVana miRNA isolation kit (Life Technologies) as per manufacturer’s protocol. The quality of isolated RNA was evaluated in Experion analyzer (Bio-Rad Laboratories) and quantitated using UV-vis Spectrophotometer (Biospec-1601, Shimadzu, Japan). Isolated RNA samples were submitted at the next-generation sequencing facility of Centre for Cellular and Molecular Platforms, Bangalore, India, for performing miRNA profiling using Illumina small RNA sequencing technology (Supplemental Digital Content 3, http://links.lww.com/IGC/A616).

**MicroRNA Microarray Profiling**

Total RNA was extracted from 24 tissue samples (18 cervical cancer samples and 6 normal cervical tissues) using Trizol reagent (Takara). The quality and integrity of the total RNA isolated were checked by Experion analyzer (Bio-Rad Laboratories). The samples were outsourced for miRNA microarray analysis to Exiqon, Denmark (Supplemental Digital Content 4, http://links.lww.com/IGC/A619).

**Quantitative Real-Time PCR**

Real-time PCR was performed with miScript SYBR Green PCR Kit (Qiagen, Germany) for validating the expression status of deregulated miRNAs in 30 cervical cancer and 6 normal cervical tissue samples following the manufacturer’s protocols. First-strand cDNA synthesis was carried out with 1 g of total RNA using miScript II RT Kit (Qiagen). Real-time PCR was set up in 7900HT Fast Real-Time PCR system using optical 384-well reaction plate. Gene-specific primer assays (Qiagen) were used for each miRNAs to be validated. Primer assays for U6snRNA, miR-423-3p, miR-191-5p, and miR-103a-3p were used as endogenous controls. All the reactions were performed in triplicate. The expression level of each miRNA was normalized to selected endogenous control. The relative expression was analyzed using 2\(^{-\Delta \Delta Ct}\) method.\(^8\)

**Data Analysis**

**Small RNA Sequencing**

The sequencing data are provided in the standard FASTQ format. The basecall files obtained from the sequencer were converted into FASTQ files and demultiplexed using CASAVA 1.8.2.\(^9\) The illumina adapter sequences were trimmed out from sequence reads using Fastx_toolkit.\(^10\) Also, additional filtering, such as read Q score greater than 30, reads between 15 and 40 nucleotides in length, minimum aligned read length in adapter more than 10, and remove reads without adapter, was also applied in order to obtain high-quality clean reads. For aligning reads to databases such as human genome (GRCh37)\(^11\) and nCPR-seq\(^12\) in combination with miRBase (V20),\(^13\) the study used Bowtie\(^14\) short read aligner. R statistical environment (version 3.3.0) was used to perform data analyses and graphical representation. All sequencing data were normalized with the Bioconductor-DESeq2 package.\(^15\) The Gplots package was used to construct the heat map using unsupervised hierarchical clustering with Spearman correlation coefficients as input and Euclidean distance as the distance metric and complete linkage.\(^16\) Differential expression of miRNAs between various groups was analyzed using MIRNAKEY software.\(^17\) Counting reads mapped to the different known miRNA species in each sample were converted into the normalized RPKM (reads per kilobase per million mapped reads) expression index to allow comparison across experiments. Quantifying differential expression for miRNAs between paired samples, using \(\chi^2\) analysis, thus obtained \(P\) values for differential expression of miRNAs. The calculated \(P\) value was further subjected to Bonferroni correction for multiple hypotheses.

**miRNA Microarray**

Quantified signals were background corrected and normalized using Quantile normalization algorithm. After the background noise correction, miRNAs detected were subjected to threshold filtering. Thus, obtained numbers of present miRNA calls were within the expected range and highly comparable for all samples (data not shown). The miRNAs highly altered in cancer cases have been subjected to unsupervised analysis (Supplemental Digital Content 5, http://links.lww.com/IGC/A620).

**Target Prediction and Pathway Enrichment Analysis**

Target prediction was done by using the algorithms including Target Scan,\(^18\) Diana micro-T,\(^19,20\) and the predicted target genes were mapped into pathways using protein annotation through evolutionary relationship classification system (PANTHER) annotated with gene ontology terms.\(^21\) Relevant pathways were selected according to \(P < 0.05\). Involvement of differentially expressed miRNAs in carcinogenic pathways was assessed using DIANA-miPath online software (Supplemental Digital Content 6, http://links.lww.com/IGC/A621).\(^19\)

**RESULTS**

**Human Papillomavirus Genotyping**

Of the 45 tissue samples analyzed by heminested PCR using consensus primers, 42 were positive for HPV type 16, 2 for HPV type 18, and 1 for HPV type 6 infection. All the normal cervical tissue samples analyzed were negative for HPV infection (Table S1, Supplemental Digital Content, http://links.lww.com/IGC/A618). Because HPV type 16 infection is the most predominant (93%), samples with other than HPV type 16 infection were not included in further studies. Human papillomavirus positivity in cervical cancer cell lines were also reconfirmed using the same protocol. Cell lines CaSki
and SiHa were positive for HPV type 16, HeLa positive for HPV type 18, and Me180 was positive for HPV type 68. Cell line C33a was HPV negative.

**Pattern of miRNA Expression With Respect to HPV Positivity and the Type of HPV Infected in Cervical Cancer Cell Lines**

The miRNA expression pattern was analyzed in cervical cancer cell lines with known HPV infection type, including types 16, 18, and 68, along with an HPV-negative cervical cancer cell line, in order to check whether any differential expression of miRNAs occurred based on the type of HPV infected. First, we tried to identify miRNAs deregulated by HPV infection by comparing miRNA expression pattern in HPV-positive cervical cancer cell lines with HPV-negative cervical cancer cell line. The most significantly altered top 16 miRNAs in relation to HPV positivity in cervical cancer cell lines are shown in Table 1. Then the study also looked on whether any HPV type-specific alterations in miRNA expression occurred in these cell lines with respect to HPV-negative cell line. Initially unsupervised cluster analysis between miRNA expression pattern and HPV infection type was done, and the heat map showed that cell lines with each HPV type can be distinguished based on their miRNA expression pattern (Fig. 1A). The most significantly altered miRNAs with respect to HPV infection type are shown in Table 2. Results revealed that most of the altered miRNAs are common in all types of HPV infection, and only a few showed variations in expression based on the type of HPV infected. Venn diagram was used to compare the miRNA expression pattern in these 3 groups based on HPV types (Fig. 1B). Of 16 HPV-associated miRNAs, 9 were commonly seen in all types, and the remaining ones were differentially expressed. Pathway analysis of these deregulated miRNAs revealed that there is no difference in pathways influenced by different HPV type

**TABLE 1.** Significantly altered top 8 each of up- and down-regulated miRNAs in HPV-positive cervical cancer cell lines compared with HPV-negative cervical cancer cell line

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>Log Fold Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-335-3p</td>
<td>10.3394</td>
</tr>
<tr>
<td>hsa-miR-196b-5p</td>
<td>9.792396</td>
</tr>
<tr>
<td>hsa-miR-31-5p</td>
<td>8.254494</td>
</tr>
<tr>
<td>hsa-miR-34a-5p</td>
<td>8.094333</td>
</tr>
<tr>
<td>hsa-miR-452-5p</td>
<td>6.448743</td>
</tr>
<tr>
<td>hsa-miR-193b-5p</td>
<td>6.285915</td>
</tr>
<tr>
<td>hsa-miR-203</td>
<td>5.9649796</td>
</tr>
<tr>
<td>hsa-miR-1305</td>
<td>−7.00003</td>
</tr>
<tr>
<td>hsa-miR-561-5p</td>
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<td>hsa-miR-181a-3p</td>
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</tr>
<tr>
<td>hsa-miR-218-5p</td>
<td>−5.15906</td>
</tr>
<tr>
<td>hsa-miR-99a-5p</td>
<td>−3.1748838</td>
</tr>
<tr>
<td>hsa-let-7c</td>
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</tr>
<tr>
<td>hsa-miR-9-5p</td>
<td>−1.5244255</td>
</tr>
<tr>
<td>hsa-miR-34c-5p</td>
<td>−1.4135549</td>
</tr>
</tbody>
</table>

Negative value denotes down-regulation.

*Fold change compared with HPV-negative cervical cancer cell line.

and SiHa were positive for HPV type 16, HeLa positive for HPV type 18, and Me180 was positive for HPV type 68. Cell line C33a was HPV negative.

**FIGURE 1.** Cervical cancer cell lines with each HPV type can be distinguished based on their miRNA expression pattern. A, Heat map of cluster analysis of miRNA expression pattern and HPV type in cervical cancer cell lines. Human papillomavirus–negative cervical cancer cell line has a distinct miRNA expression pattern compared with HPV-positive cervical cancer cell lines. B, Venn diagram illustrating the number of miRNAs differentially expressed in the 3 groups (HPV 16-, 18-, and 68-positive cervical cancer cell lines).
infections in cervical cancer cell lines (Table S2, Supplemental Digital Content, http://links.lww.com/IGC/A622). Thus, results indicate that carcinogenic mechanism may be similar in HPV infection irrespective of the HR-HPV types infected.

MicroRNA Expression Profile in Cervical Cancer Tissue

We analyzed the miRNA expression profile in 24 cervical tissue specimens, which includes 18 cervical cancer and 6 normal cervical tissue samples through miRNA microarray. Principal component analysis revealed that the “normal” samples clearly clustered out from the “cancer” samples (Fig. 2A) and thus indicated that miRNA expression profile is significantly deregulated in cervical cancer. The heat map based on the unsupervised hierarchical clustering grouped normal and cancer samples in 2 distinct classes (Fig. 2B). Of the 1038 miRNAs analyzed, a subset of 334 miRNAs differentially expressed with $P < 0.05$ was identified in cancer tissue samples. Volcano plot showed that almost all of the most significantly altered miRNAs are down-regulated in cervical cancer (Fig. 2C). Based on fold change, statistical significance, and array signal intensity, 15 miRNAs, of these 334, have been manually selected for validation purpose. Moderated $t$ test analysis indicated that, of the selected 15 miRNAs, 5 were up-regulated, and 10 were down-regulated in cervical cancer.

Validation of miRNA Expression Pattern Obtained in Microarray

To validate the miRNA data obtained from array analysis, we performed individual quantitative real-time (qRT) PCR for the most differentially expressed 15 miRNAs in an independent set of 30 cervical cancer tissue samples and 6 normal cervical tissue specimens. We performed expression analysis of U6snRNA, miR-423, miR-191, and miR-103a as endogenous controls. MiR-103a-5p exhibited good consistency in expression throughout the samples, and thus, it has been chosen for normalization purposes. Of these 15 miRNAs selected for validation, miR-21-5p, miR-335-3p, miR-196b-5p, and miR-103a were found relatively overexpressed, whereas miR-1184, miR-5587-3p, miR-218-5p, miR-497-5p, miR-377-3p, miR-136-5p, miR-3196, miR-4687-3p, and miR-5572 were down-regulated in cervical cancer. Most miRNAs showed concordance in expression pattern between microarray and qRT PCR except miR-4307 and miR-424-5p. Comparison of log-transformed average expression value of each miRNA in tumor sample attained through real-time PCR was plotted with their respective log fold change of microarray data (Fig. 2D). Thus, these 13 validated miRNAs can be considered as the miRNA signature for cervical cancer.

Target Gene Prediction and Pathway Enrichment of Validated miRNAs

With the 13 validated miRNAs, we performed in silico analysis to determine the biological relevance of these deregulated miRNAs. Biological significance of deregulated miRNAs relies on their effect in targeting protein coding genes. We analyzed the predicted targets of significantly deregulated miRNAs: miR-21-5p, miR-135b-5p, miR-363-3p, miR-429, miR-5572, miR-4687, miR-3196, miR-497-5p, miR-377-3p, miR-1184, miR-5587-3p, miR-136-5p, and miR-218-5p. Putative targets of these differentially expressed miRNAs and related pathways are shown in Table S3, Supplemental Digital Content, http://links.lww.com/IGC/A623.

| Table 2: Significantly altered miRNAs with respect to HPV infection type |
|-----------------------------|-----------------------------|-----------------------------|
| HPV Type 16 Fold Change*    | HPV Type 18 Fold Change*    | HPV Type 68 Fold Change*    |
| hsa-miR-335-3p 10.885       | hsa-miR-335-3p 10.8262      | hsa-miR-205-5p 11.47594     |
| hsa-miR-34a-5p 8.82274      | hsa-miR-34a-5p 8.27682      | hsa-miR-200c-3p 9.926113    |
| hsa-miR-31-5p 7.69271       | hsa-miR-335-3p 7.76708      | hsa-miR-196b-5p 9.863372    |
| hsa-miR-193b-5p 7.65331     | hsa-miR-31-5p 7.62648       | hsa-miR-335-3p 9.286018     |
| hsa-miR-452-5p 7.60289      | hsa-miR-584-5p 7.59648      | hsa-miR-203 8.85427         |
| hsa-miR-365a-5p 6.93631     | hsa-miR-452-5p 7.57826      | hsa-miR-31-5p 6.829689      |
| hsa-let-7f-1-3p 6.75024     | hsa-mir-224-5p 7.3751       | hsa-miR-335-3p 6.455714     |
| hsa-miR-181a-2-3p 6.29441   | hsa-miR-629-5p 6.45757      | hsa-let-7f-1-3p 5.875154    |
| hsa-let-7c -2.0268          | hsa-miR-181a-3p -5.10758    | hsa-miR-218-5p -3.66741     |
| hsa-miR-99a-5p -2.7657      | hsa-miR-34c-5p -4.71152     | hsa-miR-99a-5p -4.81466     |
| hsa-miR-181a-3p -5.6581     | hsa-miR-99a-5p -2.01876     | hsa-miR-99a-5p -5.14938     |
| hsa-miR-218-5p -7.4153      | hsa-miR-218-5p -1.96481     | hsa-miR-561-5p -7.03596     |
| hsa-miR-561-5p -8.877       | hsa-let-7c -1.48598        | hsa-miR-181a-3p -7.12512    |

*Negative value denotes down-regulation.

*Fold change compared with HPV-negative cervical cancer cell line.
We further examined the targets of all up-regulated or down-regulated miRNAs separately to understand the specific signaling pathways involved by these miRNAs using DIANA miRPath. The up-regulated miRNAs influence the important cancer-associated signaling pathways such as PI3K-Akt, Ras, and transforming growth factor β (TGF-β) and also include viral carcinogenesis pathways (Table 3). Interestingly, 64 genes in PI3K-Akt signaling pathway were

![FIGURE 2. MicroRNA expression profile in cervical cancer tissue. A, The principal component analysis plot of cervical tissue samples. The principal component analysis was performed on all samples and on the top 50 miRNAs with the highest SD. The normalized log ratio was used for analysis. Biological difference of the samples was taken as primary component for the variation. Red and blue circles indicate cancer and normal samples respectively. B, Heat map and unsupervised hierarchical clustering. The clustering was performed on all samples and on the top 50 miRNAs with highest SD. The color scale illustrates the relative expression level of a miRNA across all samples: red color represents an expression level below the mean; green color represents expression above the mean. C, Volcano plot depicting significantly deregulated miRNAs. The above volcano plot shows the relation between the logarithm of the P values and the log fold change between cancer and normal. The top selected miRNAs are marked with annotation on the plot. D, A comparison of log fold change ratios obtained from microarray and qRT PCR of 15 candidate miRNAs. The log 2-transformed average expression value of each miRNA in tumor sample attained through real-time PCR (red) was plotted with their respective log fold change of microarray data (blue).](image-url)
targeted by up-regulated miRNAs: miR-21-5p, miR-429, miR-135b-5p, and miR-363-3p (Fig. 3A). These miRNAs mainly target the core candidate genes of PI3K-Akt signaling pathway such as PIK3CA, PTK2, and CREB5, thereby losing the control to mediate cell growth inhibition and promoting tumorigenesis. Although the down-regulated miRNAs can target genes involved in PI3K-Akt, as well as in mTOR pathways, the mTOR signaling pathway can be considered more significant because of its lower \( P \) value (Table 3).

Twenty-four genes in mTOR signaling pathway were targeted by down-regulated miRNAs: miR-1184, miR-377-3p, miR-136-5p, miR-218-5p, miR-4687-3p, miR-497-5p, and miR-5572 (Fig. 3B). Overexpression of these genes enhances the signaling cascade and thereby stimulates the cell proliferation mechanism. It is interesting to note that in the PI3K-Akt pathway the up-regulated and down-regulated miRNAs were found to target distinct genes (Fig. 3A and Fig. 1, Supplemental Digital Content, http://links.lww.com/IGC/A617).

### DISCUSSION

Because more than 200 types of HPV have been identified, the use of type-specific primers for the detection of all clinically relevant HPV in patient samples is impractical and uneconomical.\(^7\) Human papillomavirus genotypes significantly differ in their L1 gene DNA sequences, and these highly conserved HPV L1 gene DNA fragment with hypervariable region for each HPV subtype can be efficiently amplified using heminested PCR using degenerate primers,\(^22\) and genotyping can be performed through DNA sequencing.\(^7\) In the current study, we used degenerate primers such as MY09/MY11 for primary PCR amplification and consensus general primers such as GP6/MY11 for heminested PCR because these can be used to amplify all relevant HPV genotypes and also other variants or mutants.\(^7\) We identified the presence of HR-HPV DNA in all cervical cancer cases supporting the role of HPV as a main etiological agent in the development of cervical cancer. Human papillomavirus type 16 was the commonest genotype noticed in the study (93.3%); besides, it is already known that in India the prevalence of HPV type 16 infection is very high.\(^2\)

Deregulation of miRNA expression has been shown to be associated with many cancers. MicroRNAs are often found near fragile sites in chromosomes or integration sites of HR-HPVs. Integration of HR-HPVs may alter miRNA expression via deletion, amplification, or genomic rearrangement.\(^23\) Expression profiling of miRNAs in cervical cancer cell lines identified a set of HPV-specific miRNAs. Previous studies support the hypothesis that specific miRNA expression signatures in various types of human cancers can be associated with diagnosis, prognosis, and response to chemotherapy. Deregulation of miRNAs is almost similar in cervical cancer with different HPV infection types as the miRNA profiling identified a set of HPV-specific miRNA irrespective of HPV
FIGURE 3. Target gene prediction and pathway enrichment of validated miRNAs. A, Up-regulated miRNAs targeting multiple genes in PI3K-Akt signaling pathway: 4 up-regulated miRNAs are depicted here and their validated targets. Blue boxes represent miRNAs; yellow boxes represent target genes of any one of the miRNA; orange boxes represent target genes of more than 1 miRNA; and green boxes indicate that the genes are not the targets of these miRNA in this context. Figure developed from the output of DIANA miRPath. B, Down-regulated miRNAs targeting multiple genes in mTOR signaling pathway: 7 down-regulated miRNAs are depicted here and their validated targets. Blue boxes represent miRNAs; yellow boxes represent target genes of any one of the miRNA; orange boxes represent target genes of more than 1 miRNA; and green boxes indicate that the genes are not the targets of these miRNA in this context. Figure developed from the output of DIANA miRPath.
infection type. Defining specific role of miRNAs in cancer is crucial, and understanding expressional alterations of these miRNAs is essential to find novel mechanism of activation of known pathways. Pathway analysis of significantly altered miRNAs revealed that most of them are actively involved in carcinogenic pathways. An earlier study on the global mRNA and miRNA profiling in HPV-positive cell lines observed that different HPV types in dissimilar biological conditions may also affect the same pathway, but genes involved may be different. Similarly, another study on the role of miRNA in HPV-associated cancers indicated that a set of miRNAs was shared exclusively by HPV-positive head and neck squamous cell carcinoma and cervical squamous cell carcinoma compared with the number of miRNAs shared exclusively by HPV-negative head and neck squamous cell carcinoma and cervical squamous cell carcinoma. Thus, all these data show that carcinogenic mechanism may be similar in HR-HPV infection irrespective of different HPV types.

The present study validated 15 deregulated miRNAs including 5 up-regulated and 10 down-regulated miRNAs using qRT PCR. Of these 15 miRNAs, 13 of them showed good correlation with microarray data. MiR-21-5p, miR-135b-5p, miR-363-3p, and miR-429 showed a significant up-regulation in cervical cancer cases compared with the normal cervical tissue samples supporting the expanding role of these oncogenic miRNAs in human cervical cancer. Up-regulation of miR-21 has been associated with aggressive progression and poor prognosis in cervical cancer, which suggests that miR-21 might be identified as an independent marker for predicting the clinical outcome of cervical cancer patients. A recent study identified up-regulation of miR-21 in cervical cancer, which in turn down-regulates the expression of PTEN and promotes cell proliferation and cell survival. Little is known about the expression of miR-135b in cervical cancer. Down-regulation of miR-135b markedly inhibited proliferation and arrested cell cycle in cervical cancer cells. APC (adenomatous polyposis coli) gene is a known target gene of miR-135 family, and its up-regulation in colorectal cancer correlated with decreased APC mRNA expression. Mean expression level of miR-363-3p was significantly higher in HPV-positive adenocarcinoma of the uterine cervix. Down-regulation of miR-429 may contribute to colorectal carcinogenesis, as was previously reported, and induces epithelial mesenchymal transition by targeting Onecut2. CRKL gene was identified as a common candidate of miR-429 in breast cancer through combined analysis of expression array results and in silico target search.

Most of the miRNAs analyzed in cervical cancer tissue samples exhibited down-regulation, suggesting their tumor suppressor role. This includes miR-497-5p, miR-377-3p, miR-218-5p, miR-1184, miR-5587-3p, miR-5572, miR-3196, and miR-136-5p. Down-regulation of miR-377, miR-1184, miR-136, miR-4687, and miR-5587 is being reported for the first time in cervical cancer. Stable expression of miR-377 diminished the proliferative, migratory, and the colony-forming capability of melanoma cell lines. Down-regulation of miR-1184 was stated in rectal cancer. However, no other reports are available in cancer cases, suggesting the deregulation of miR-5587 and miR-4687. MiR-136 may play an important role during TGF-β1–induced proliferation arrest by targeting PPP2R2A in keratinocytes. The miR-497 family is also reported to have an important role in HPV pathogenesis. In the present study, miR-497 is the only miRNA shown to be consistently altered in cervical cancer cell lines and cervical cancer tissue. Down-regulation of miR-497 in cisplatin-resistant gastric cancer cell line was associated with the up-regulation of IGF1R, IRS1, and BCL2, suggesting their role as direct targets.

Target gene identification and pathway analysis of up-regulated and down-regulated miRNAs revealed that these miRNAs are regulating genes that are actively involved in carcinogenic pathways. Up-regulated miRNAs such as miR-21-5p, miR-363-3p, miR-135-5p, and miR-429 target the genes involved in PI3K-Akt signaling pathway. Some of the genes such as PIK3CA, COL4A1, PTK2, CREB5, and SGK3 are targeted by more than 1 miRNA. The PI3K/AKT signaling-pathway chiefly controls cell growth, cell division, and apoptosis. Down-regulated miRNAs, on the other hand, targets the genes in mTOR signaling pathway. Previous studies indicate that several miRNAs regulate cervical carcinogenesis by targeting PI3K-Akt and mTOR pathways. For instance, up-regulation of miR-221 can reduce the sensitivity of cervical cancer cells to gefitinib through the PTEN/PI3K/Akt signaling pathway. Likewise, miR-125b and miR-218 suppress cervical tumor growth activity by targeting the PI3K/Akt/mTOR signaling pathway. FOXO1 and p27(Kip1), 2 key effectors of PI3K/Akt signaling, are direct targets of miR-196a. Up-regulation of miR-196a was correlated with advanced tumor stage and poor overall and recurrence-free survival in cervical cancer patients. However, the in silico predictions do not necessarily reflect in vivo conditions, which mandates deeper and more exhaustive biological validation of the target pathways with functional assays.

**CONCLUSIONS**

The present study on cervical cancer cell lines showed that the HR-HPV–associated miRNAs influence similar pathways irrespective of HPV type, thus indicating that carcinogenic mechanism may be similar in all HR-HPV infection. The miRNA signature obtained in cervical cancer tissue also showed some interesting clues on cervical carcinogenesis. Pathways significantly affected by up- and down-regulated miRNAs in cervical cancers are PI3K-Akt and mTOR signaling pathways, respectively. Thus, the present study was able to identify a set of miRNAs associated with HPV infection in cervical cancer, their putative target genes, and related pathways. However, further studies on these deregulated miRNAs and their target genes are essential to validate their biological significance. Also, the present study indicates the therapeutic potential of these altered miRNAs and their target genes in HPV-associated cervical cancers.

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