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ORIGINAL ARTICLE

Novel MicroRNA signatures in HPV-mediated cervical carcinogenesis in Indian women

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Abstract This study aimed to investigate the role of miRNAs in HPV-mediated cervical pre-cancer and cancer cases in Indian population. We analysed the HPV infection and its genotypes in uterine cervical pre-cancer (n=80), cancer (n=200) and normal cervical samples (n=150) by consensus sequence PCR followed by type specific PCRs. Also, microRNA profiling was done in a subset of cervical pre-cancer (n=20), cancer cases (n=50) and normal samples (n=30) by real-time quantitative PCR (qRT-PCR). The prevalence of HPV infection in pre-cancer was found to be 81 % (65/80) and 94 % (188/200) in cancer cases, with most predominant high-risk HPV type-16 (HR-HPV-16) in 83 % of cancer and 91 % of pre-cancer cases, respectively. Whereas in controls, the HPV infection was found to be very low (5 %). The miRNA profiling revealed that in cervical pre-cancer, 100 miRNAs were significantly (p<0.001) differentially expressed with 70 miRNAs upregulated and 30 miRNAs downregulated. In cervical cancer cases, 383 miRNA were found to be differentially expressed (p<0.001), of which 350 miRNAs were upregulated and 33 miRNAs were downregulated. We also observed that 182 miRNAs were differentially expressed (p<0.001) in HPV-16/18-positive (SiHa/HeLa) cell lines compared with HPV-negative (C33A) cell line. In addition, we identified the novel microRNAs such as miR-892b, miR-500, miR-888, miR-505 and miR-711 in cervical precancerous lesions and cervical cancer cases in Indian population. Taken together, the study demonstrates a crucial role of microRNAs in cervical cancer, which may serve as potential early diagnostic markers for cervical carcinogenesis.

Keywords Cervical cancer · HPV · MicroRNAs

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Introduction

MicroRNAs are small non-coding RNA comprising of short sequence of nucleotides (~22 nt) that regulate gene expression [1]. Mature miRNAs often bind to the 3′ UTR region of messenger RNA (mRNA) sequence of target gene leading to mRNA degradation or translational repression depending upon the complementarity between miRNAs and seed sequence of mRNA of that particular gene [2]. Moreover, miRNAs can also bind to 5′UTR region and to the coding region of target gene [3].

To date, more than 1500 human miRNAs have been identified by different microRNA databases using bioinformatics or computational approaches (miRDB, Target Scan, PicTar, miRwalk, etc.). However, majority of them are not validated experimentally and their crosstalks with cancer signalling pathways are yet to be explored. Despite such a small number of microRNAs identified by prediction, databases and/or

experimental studies have analyzed that majority of them code for protein coding genes and are collectively regulated by miRNAs [4, 5]. In addition, a genome-wide analysis indicated that 50 % of the miRNAs are localised in the fragile sites of chromosomes leading to deregulation in gene expression responsible for cancer [4]. Recently, it has also been shown that the oncogenic or tumour suppressor role of miRNAs may lead to their significance in cellular differentiation, cell cycle regulation and apoptosis in carcinogenesis [5, 6]. MicroRNAs can also regulate expression of several of genes at posttranscriptional level.

More recently, the role of miRNAs has been reported in cervical cancer. However, it is also well established that cervical cancer is caused due to persistence infection with HR-HPV [7–10]. Besides viral oncoproteins E6 and E7 of HPV, miRNA deregulation is the contributing factor involved in carcinogenesis and disrupts the function of p53 gene which regulates the posttranscriptional maturation of miRNAs [5]. Recent reports have documented certain miRNAs which are directly or indirectly regulated by HPV and play crucial role in cervical cancer including its diagnosis and prognosis [6, 11].

Cervical cancer is the most common cancer among women in developing countries which contributes 70 % of total cancer burden [12]. In India, cervical cancer is the second most prevalent cancer among women after breast cancer with annual incidence of about 130,000 over 70,000 deaths [7, 9]. Considering the high incidence of cervical cancer in India, the present study was designed to explore the possibility of miRNAs as early diagnostic or prognostic markers in cervical carcinogenesis in Indian population. There are limited data available from India which showed the association of miRNA with cervical cancer. The finding of our present study suggests a crucial role of miRNAs in HPV mediated cervical carcinogenesis. In addition, we tried to establish a dichotomy of miRNA profile between HPV-positive and HPV-negative cell lines which may help in developing novel miRNA-based therapeutic or diagnostic interventions for cervical carcinogenesis.

Materials and methods

Sample collection In the present study, a total of 430 consecutive subjects comprising of 280 cases (80 pre-cancer and 200 invasive carcinoma) and 150 controls were included. The patients were recruited from Lok Nayak Jai Prakash hospital, Safdarjung hospital, New Delhi and Guru Teg Bahadur Hospital, New Delhi with histologically confirmed pre-cancer [high- or low-grade squamous intraepithelial lesion (LSIL/HSIL)] or invasive carcinoma of the uterine cervix. The pre-cancer (N=80) cases comprising of 27 LSIL and 53 HSIL were collected. Classification and grading of the pre-cancer and cancer lesions were done according to Bethesda system and World Health Organization criteria. The tumour stage and histological grade were determined according to the criteria laid down by International Federation of Obstetrics and Gynaecology (FIGO). The study subjects comprised of 21 cases of stage I, 40 cases of stage II, 81 cases of stage III and 58 cases of stage IV and had not undergone any therapy. According to histological grading, tissue biopsies comprising of 75 well-differentiated squamous cell carcinoma (WDSCC), 107 moderately differentiated squamous cell carcinoma (MDSCC) and 18 poorly differentiated squamous cell carcinoma (PDSCC) (Table 1) were studied. The 150 aged and ethnicity-matched control samples (cervical scrapes/tissue biopsy) with normal cervical cytology with no history of cancer were obtained from women who visited the hospital for other gynaecological reasons. Written informed consent was obtained from all the subjects in their native language, and the study was carried out in accordance with the principles of Helsinki declaration. All of the study subjects were personally interviewed for demographic characteristics. The study was approved by the ethics committee of the institute (ICPO-ICMR/IEC/2010/P-003). Once tissue was collected, it was snap frozen and stored at −80 °C for later use. Subsequently, one portion of the tissues sample was removed for DNA isolation and another for RNA isolation.

DNA extraction and HPV genotyping

Genomic DNA was extracted from cervical cancer/pre-cancer tissue biopsies samples as well as control (cervical scrapes/hysterectomy biopsies) samples by using standard proteinase K digestion followed by phenol/chloroform/isopropanol treatment routinely followed in our laboratory [13]. HPV diagnosis was done by PCR amplification using consensus primers MY09 and MY11, and further typing was completed by PCR using type specific primers for HPV 16,18, 6 and 11 [14].

Cell culture miRNA profiling was also done in HPV-positive (SiHa, HeLa) and HPV-negative (C33a) cervical cancer cell line procured from ATCC LGC, Teddington, UK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) and 10 % Foetal Bovine Serum (Sigma, USA), 1 % Penicillin–Streptomycin (Sigma, USA) antibiotic in a humidified incubator at 37 °C temperature with 5 % CO_2.

Table 1 Demographic, clinico-pathological characteristics and HPV status of cancer, pre-cancer and normal controls analysed from North Indian population

Characteristics	Cancer cases *n*=200(%)	Pre-cancer cases *n*=80 (%)	Normal controls *n*=150(%)
Age			
Pre-menopausal	45 (23)	52 (65)	120 (80)
Post-menopausal	155 (78)	28 (35)	30 (20)
P value	*P*<0.001	*P*=0.019	*P*<0.001[a]
Smoking status			
Smokers	12 (6)	6 (8)	2 (1)
Non-smokers	188 (94)	74 (93)	148 (99)
P value	*P*=0.0231	*P*=0.0401	*P*=0.0168[a]
Tobacco chewing status			
Chewers	140 (70)	25 (32)	38 (25)
Non-chewers	60 (30)	55 (69)	112 (75)
P value	*P*<0.001	*P*<0.001	*P*<0.001[a]
Gravida			
<5	106 (53)	58 (73)	93 (62)
>5	94 (47)	22 (28)	57 (38)
P value	*P*=0.1156	*P*=0.146	*P*=0.566[a]
Parity			
<5	120 (60)	59 (74)	89 (59)
>5	80 (40)	21 (26)	61 (41)
P value	*P*=0.98	*P*=0.0424	*P*=0.40[a]
Residence			
Rural	158 (79)	65 (81)	61 (41)
Urban	42 (21)	15 (19)	89 (59)
P-value	*P*<0.001	*P*<0.001	*P*<0.001[a]
Clinical stage			
Stage I and II	61 (30)	ND	ND
Stage III and IV	139 (70)	ND	ND
Pathological grade			
WDSCC	75 (38)	ND	ND
MDSCC	107 (54)	ND	ND
PDSCC	18 (9)	ND	ND
HPV status			
HPV L1	188 (94)	65 (81)	8 (5)
HPV16	165 (88)	59 (91)	4 (50)
HPV18	21 (11)	4 (6)	1 (13)
HPV6	2 (1)	2 (3)	3 (38)
HPV11	–	–	–
HPV16/18 and 6/11 co-infection	–	–	–

[a] Pre cancer + cancer vs controls

miRNA extraction miRNA was isolated from 70 samples consisting of 20 precancerous and 50 cervical cancerous cases of different stages and 30 normal controls using miRNeasy Mini Kit (Qiagen, USA). In addition, miRNA extraction was done in both the HPV-positive and HPV-negative cell lines using the same isolation kit. Analysis of miRNA was done using formaldehyde agarose gel electrophoresis and Bioanalyzer.

cDNA preparation The miRNA isolated was used for cDNA preparation. cDNA was prepared from 1 μg/μl miRNA according to manufacturer's instructions (QuantiMir RT Kit,

System Biosciences). The quantification of cDNA was done by absorbance spectrometer.

qRT-PCR

The prepared cDNA was further used for miRNA expression profiling using QuantiMir RT Kit (System Bioscience) which is designed for amplification of whole genomic miRNA using 1132 miRNA primers. Small endogenous RNAs Human U6 snRNA, RNU43 snoRNA, Hm/Ms/Rt UsnRNA served as positive controls, and qRT-PCR mix without template was used as negative control. The Syber green master mix was used as a detection dye. The PCR conditions were pre incubation at 50 °C for 2 min, 95 °C for 10 min, 2 step amplification at 95 °C for 15 s and 60 °C for 1 min and 72 for 15 s with 50 cycles and the melting conditions were 95 °C for 15 s, 65 °C for 1 min and 97 °C for continuous and cooling at 37 °C for 30 s. Amplification of all miRNAs in samples was carried out according to the manufacture's instruction and was analyzed on the Real-Time Light Cycler 480 (Roche) software. Differentially expressed miRNAs identified by comparing their expression in cervical cancer tissues with that in normal controls using 2 delta Ct method. Each experiment was performed in triplicate and average Ct value was used for the analysis.

Data analysis

The miRNA data was analysed by using the software provided by the System biosciences (Sanger miRBase version 15). In which Ct values of cases and controls were compared to reach normalised miRNA expression level. The relative quantification was determined by taking housekeeping gene as reference genes comprising U6SnRNA, RNU43snoRNA and Hm/Ms/RtU1SnRNA for the Ct values of cases and controls. The Geomean was calculated by average value of above mentioned housekeeping genes for the calculation of ΔCt controls. Furthermore, ΔCt values of cases were calculated by comparing the value of ΔCt of controls. The average value of ΔCt value was calculated as the experiment was done in triplicate and analysed for the standard deviation. The average Ct value was used for the analysis by two paired Student's *t* test for the significant miRNA ($p<0.001$). The difference between the ΔCt of cases and ΔCt of controls gives the ΔΔCt value which was used for the calculation of fold change by $\log_2$ ΔΔCt values.

Statistical analysis

Statistical analysis of data was done by Graph Pad InStat version 3.0 software. The association between various clinico-pathological and demographic parameters with HPV infection in cervical cancer was determined by chi-square test and Fischer's exact test. Whereas the expression profile of miRNAs in cervical cancer tissue biopsies and their fold changes were compared with each stage of cervical cancer, pre-cancer and controls. Additionally, between HPV-positive and HPV-negative cell lines by Student's *t* test, miRNA whose Delta Ct values having $p\leq0.05$ were considered significant.

Results

Demographic and clinico-pathological characteristics

In the present study, demographic characteristics of patients were correlated with clinico-pathological variables of cervical pre-cancer, cancer and normal controls including status of HPV infection (Table 1). The various parameters including age, lifestyle, parity, gravida and tobacco consumption habits either by smoking or chewing and different clinical stages or grades of all the subjects were recorded and used in the study. Tobacco chewing habit was found to be the most confounding factor associated with pre-cancer and cancer ($p<0.001$) 31 % (25/80) and 70 % (140/200) pre-cancer and cancer compared to 25 % (38/150) in control subjects. In addition, rural/urban divide among cases and controls was also found to be associated with increased risk of either cervical pre-cancer or cancer when compared to controls (Table 1).

Prevalence of HPV infection in cervical cancer

The persistence of HR-HPVs infection is the major cause for the development and progression of cervical cancer. Therefore, the initial screening in the studied population was done to detect HPV infection among cervical pre-cancer, cancer cases and controls (Table 1). The data showed that the remarkably high prevalence of HPV infection in both pre-cancer and cancer cases as compared to controls. In pre-cancer lesions, HPV infection was found to be in 81 % (65/80); and in cancer cases, it was 94 % (188/200) where as in controls it was 5 % (8/150). Subsequently, HPV type specific PCRs were performed to determine the subtypes of HPV infection for both high- and low-risk types. The frequency of HR-HPV type 16 in cancer cases was found to be 88 % (165/188) and type 18 was found to be 11 % (21/188) while only 1 % (2/188) was infected with low-risk (LR-HPV) type 6. No co-infection or any other HPV type could be detected in these tumours. However, majority of pre-cancer lesions harboured HR-HPV 16 (91 %, 59/65) and HR-HPV 18 (6 %, 4/65) followed by LR-HPV 6 (3 %, 2/65). In addition, we also tried to establish a correlation of HPV infection with demographic characteristics of the study population. The analysis showed a positive correlation of HPV infection with menopausal status, smoking habits, gravida, parity and residence (rural/urban) of the study

cohort $P \leq 0.05$ with the persistence of HPV infection (Table 2).

Differential expression profile of microRNAs in cervical pre-cancer and cancer cases In the present study, miRNA expression profile was determined in a total of 100 tissue biopsy samples comprising 20 precancerous, 50 cancer and 30 controls. In addition to tissue biopsies, we checked the miRNA expression pattern in established cervical cancer HPV-positive and HPV-negative cell lines.

We profiled 1132 microRNAs that are annotated, to date, by various human microRNA databases by quantitative real-time polymerase chain reactions (qRT-PCR). In cervical pre-cancer lesions, 100 miRNAs were found to be significantly expressed ($p<0.001$). Of which, 30 miRNAs were found to be downregulated and 70 miRNAs were upregulated. Among the deregulated microRNAs in pre-cancer, miR-146a, miR-95, miR-200b, miR-21, miR-3130-5p and miR-576-3p were highly expressed. Whereas miR-34a, miR-1 and miR-23b were highly under expressed (Table 3).

Subsequently, in cervical cancer cases, 383 miRNAs were significantly differentially expressed ($p<0.001$) when compared to normal controls or cervical pre-cancer lesions. Of these, 33 miRNAs were downregulated and 350 miRNAs were upregulated. Interestingly, we observed miR-15a, miR-449b, miR-517a, miR-545, miR-223 and miR-192 were highly upregulated with a fold change more than 30. Similarly, miR-34a, miR-23b, miR-99a, miR-376b, miR-193-5p and miR-423-5p were significantly downregulated (Table 3, Fig. 1).

Furthermore, we analysed our data with respect to disease severity of cervical cancer lesion; the data showed 5 miRNAs, miR-200b, miR-146a, miR-3130-5p, miR-95 and miR-136, were upregulated and miR-29, miR-19a, miR-222, miR-1, miR-29a, miR-222 and miR-19a were highly downregulated more in pre-cancer than early and late stages of cervical cancer. The miRNAs whose expression was found be increased with the advancement of disease include miR-576-3, miR-21, miR-449b, miR-184, miR-17, miR-34a, let-7a and miR-23b were found to be downregulated (Table 4, Fig. 2).

miRNA expression profile in HPV positive & HPV negative cervical cancer cell lines In addition to cervical tissue samples, we investigated the miRNA expression pattern in both HPV-positive and HPV-negative cervical cancer cell lines. Among the cell lines profiled for microRNAs analysis, 182 miRNAs were differentially expressed ($p<0.001$) in HPV-16-positive (SiHa) and HPV-18-positive (HeLa) cervical cell lines when compared with HPV negative (C-33A) cervical cancer cell line. Of these differentially expressed miRNAs, 152 miRNAs were upregulated and 30 miRNAs were downregulated (Table 3). Among the upregulated miRNAs which include miR-21, miR-222, miR-27b, miR-223, miR-19b, miR-141, miR-145 were found to be highly expressed. Also, miR-218, miR-99a, miR-220b, mir-193-5p, miR-1, miR-34a, miR-29a, miR-337-3p and miR-23b were highly downregulated (Table 3).

Differential expression pattern of miRNAs in both cervical cancer cell lines and tissue biopsies We also tried to explore the possibility to find out commonly differentially expressed miRNAs in both cancer cell lines (HPV+ve, HPV−ve) as well as in tissue biopsies. Our data demonstrates that 179 miRNAs commonly expressed in both cell lines and tissues biopsies (Fig. 2). Interestingly, miR-124, miR-21, miR-505, miR-192, miR-15, miR-21, miR-146a and miR-200b expressed highly in tissue biopsies when compared to cell lines. In contrast, miR-125a-3p, miR-27b, miR-19b, miR-145 and miR-222 were expressed more in cell lines. Similarly, miR-99a, miR-34a and miR-193a-5p were highly downregulated in tissue biopsies compared to miR-1, miR-376b, miR-376c, miR-29a, miR-423-5p, miR-892b, miR-218 and miR-337-3p were more under expressed in cell lines (Table 3, Fig. 3). More interestingly, we identified novel

Table 2 Association of HPV infection with demographic variables of cervical pre-cancer, cancer and normal controls

Characteristics	No of patients	HPV infection (%)
Pre-menopausal	217	102 (47)
Post-menopausal	213	159 (75)
P value		*P*<0.0001
Smoking status		
Smokers	20	18 (90)
Non-smokers	410	243 (60)
P value		*P*=0.01
Tobacco chewing status		
Chewers	203	123 (61)
Non-chewers	227	138 (61)
P value		*P*=0.9
Gravida		
<5	257	117 (46)
>5	173	144 (83)
P value		*P*<0.0001
Parity		
<5	268	163 (61)
>5	162	98 (60)
P value		*P*=0.9
Residence		
Rural	284	162 (57)
Urban	146	99 (68)
P value		*P*=0.03

Table 3 Common miRNAs expressed in both cervical tissues and cervical cancer cell lines

S. No	MiRNAs	FC (tissue)		Fold change in cell lines		
		Cancer	Pre-cancer	HPV+		HPV−
				HPV16 (SiHa)	HPV18 (HeLa)	C33a
1	miR-124	22.0	12.0	10.59	9.87	3.98
2	miR-21	35.78	28.93	21.04	16.82	11.32
3	miR-505[a]	16.87	16.89	6.10	5.45	3.1
4	miR-125a-3p	9.63	7.11	12.51	10.21	5.20
5	miR-27b	7.9	10.5	23.25	20.98	11.9
6	miR-141	3.5	4.21	10.10	8.17	2.98
7	miR-19b	12.5	10.5	19.47	16.87	11.78
8	miR-301b	12.2	2.2	2.65	3.31	1.98
9	miR-500[a]	17.23	21.41	5.21	5.11	3.98
10	miR-449b	30.2	21.9	15.18	11.61	9.87
11	miR-15a	35.87	30.13	12.16	10.87	4.13
12	miR-517a	31.76	23.98	5.05	4.22	6.71
13	miR-545	31.1	28.36	11.05	8.76	3.54
14	miR-145	8.98	5.32	20.15	26.98	3.33
15	miR-146a	26.89	34.29	12.98	18.41	7.42
16	miR-711[a]	32.61	30.14	9.31	8.55	4.91
17	miR-888[a]	34.64	29.97	21.41	18.62	10.42
18	miR-223	36.98	30.12	30.85	23.87	19.89
19	miR-95	23.81	29.42	19.32	17.11	8.98
20	miR-135a	21.65	21.65	3.33	2.19	6.87
21	miR-181c	2.15	3.17	2.68	2.98	1.11
22	miR-192	33.1	26.87	9.61	5.98	5.11
23	miR-200b	27.34	36.17	21.34	20.52	10.51
24	miR-3130-5p	28.75	32.65	19.65	18.76	11.11
25	miR-205	32.6	32.6	15.91	13.12	8.98
26	miR-222	3.5	1.5	21.77	18.32	11.45
27	miR-576-5p	36.42	30.13	17.87	15.41	8.61
28	miR-99a	−10.21	−15.92	−4.78	−9.10	−1.98
29	miR-34a	−38.98	−29.73	−15.98	−14.23	−9.8
30	miR-193a-5p	−21.31	−20.87	−12.74	−11.98	−3.2
31	miR-1	−32.96	−36.87	−21.22	−7.86	−2.87
32	miR-376b	−12.87	−11.2	−2.74	−5.81	−1.2
33	miR-23b	−27.63	−20.91	−22.12	−19.51	−3.2
34	miR-29a	−26.78	−30.91	−17.86	−12.23	−4.21
35	miR-376c	−27.81	−21.11	−12.9	−16.2	−1.2
36	miR-423-5p	−25.87	−21.98	−12.91	−8.1	−2.3
37	miR-218	−7.1	−5.3	−12.87	−9.2	−1.09
38	miR-337-3p	−2.7	−1.9	−17.52	−12.82	−4.5
39	miR-892b[a]	−27.61	−21.98	−11.91	−9.32	−3.2

[a] Novel miRNAs reported in cervical pre-cancer and cancer cases and cell lines

microRNA signatures in cervical cancer tissue biopsies which include miR-500, miR-888, miR-505, miR-711 (upregulated) and miR-892 (downregulated) (Table 3, Fig. 1).

Discussion

In India, cervical cancer is a leading cause of cancer-related deaths among women contributing approximately one fifth of

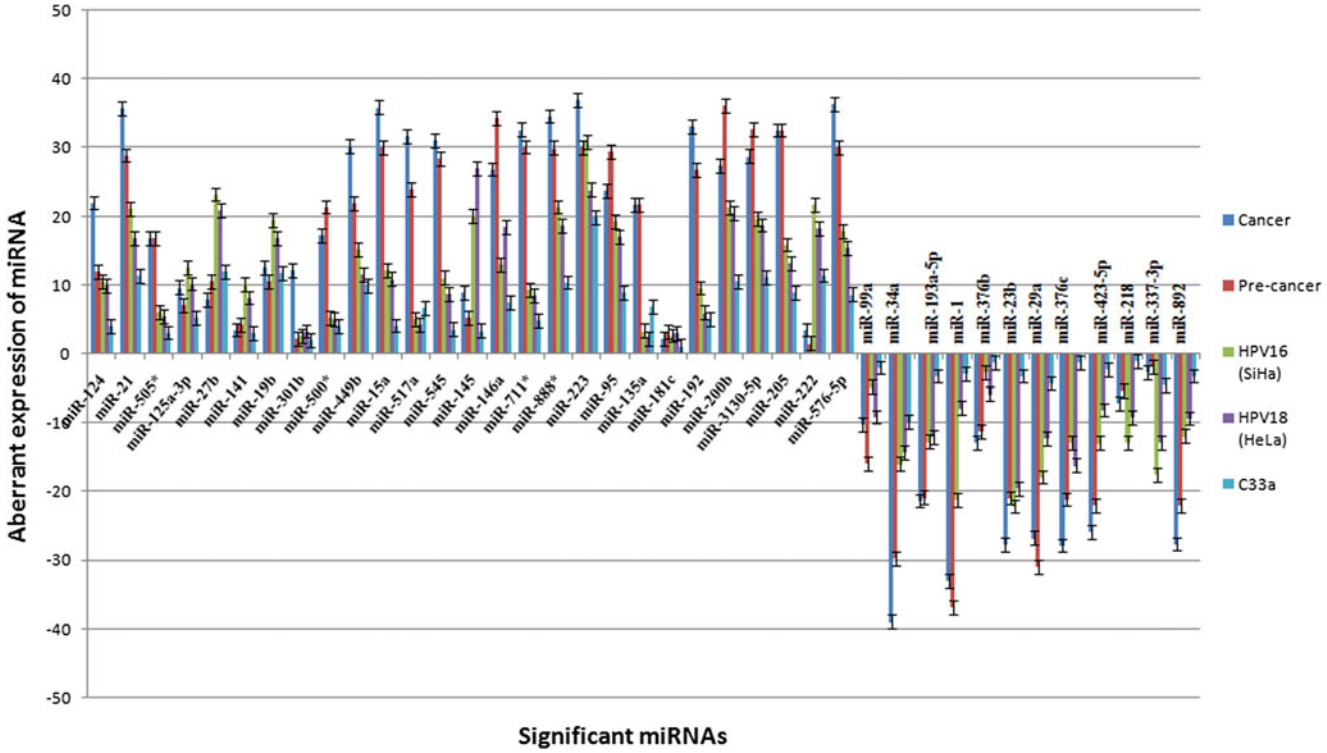

Fig. 1 Aberrantly expressed microRNAs in pre-cancer, cancer and controls by quantitative real-time PCR (qRT-PCR) performed in triplicate (as mentioned in 'Materials and Methods' section). The relative quantification of expressed miRNAs was done by average of 2 delta Ct method and fold change was calculated by $2^{-\Delta\Delta Ct}$ values

the total cancer burden globally [12]. It is well established that Human Papilloma Virus is the major etiological factor for the genesis of cervical cancer [7, 9]. In addition, there is utmost need to identify biomarkers which could essentially detect the disease at early stages. Therefore, our study tried to find the inducible role of host microRNAs which have been shown to have crucial role in carcinogenesis including cervical cancer. The aberrant expression pattern of miRNA expression has been found in various type of malignancies breast cancer [15], lymphoma [16], colorectal [17], glioma [18] and prostate cancer [19] including cervical cancer [20, 21]. The association of HPV with regulation of miRNAs (e.g. miR-34a) is well

Table 4 miRNA expression pattern in the progression of cervical cancer ($p<0.001$)

miRNA	Pre-cancer	Stage I + stage II	Stage III + stage IV	Expression
let7a	−12.43	−22.65	−30.12	Downregulation
miR-146a	34.29	31.98	21.80	Upregulation
miR-95	29.42	26.90	20.70	Upregulation
miR-200b	36.17	30.69	24.00	Upregulation
miR-21	28.93	33.81	35.77	Upregulation
miR-34a	−29.73	−37.99	−39.98	Downregulation
miR-1	−36.87	−34.98	−30.94	Downregulated
miR-3130 -5p	32.65	31.58	25.92	Upregulation
miR-576-3p	30.13	34.79	37.98	Upregulation
miR-29a	−30.91	−28.99	−24.49	Downregulation
miR-449b	21.90	28.15	32.25	Upregulation
miR-23b	−20.91	−25.43	−29.83	Downregulation
miR-136	22.12	19.87	11.23	Upregulation
miR-19a	−12.91	−11.21	−9.87	Downregulation
miR-222	−19.87	−12.18	−11.23	Downregulation
miR-184	13.45	14.82	22.16	Upregulation
miR-17	3.90	8.95	15.21	Upregulation

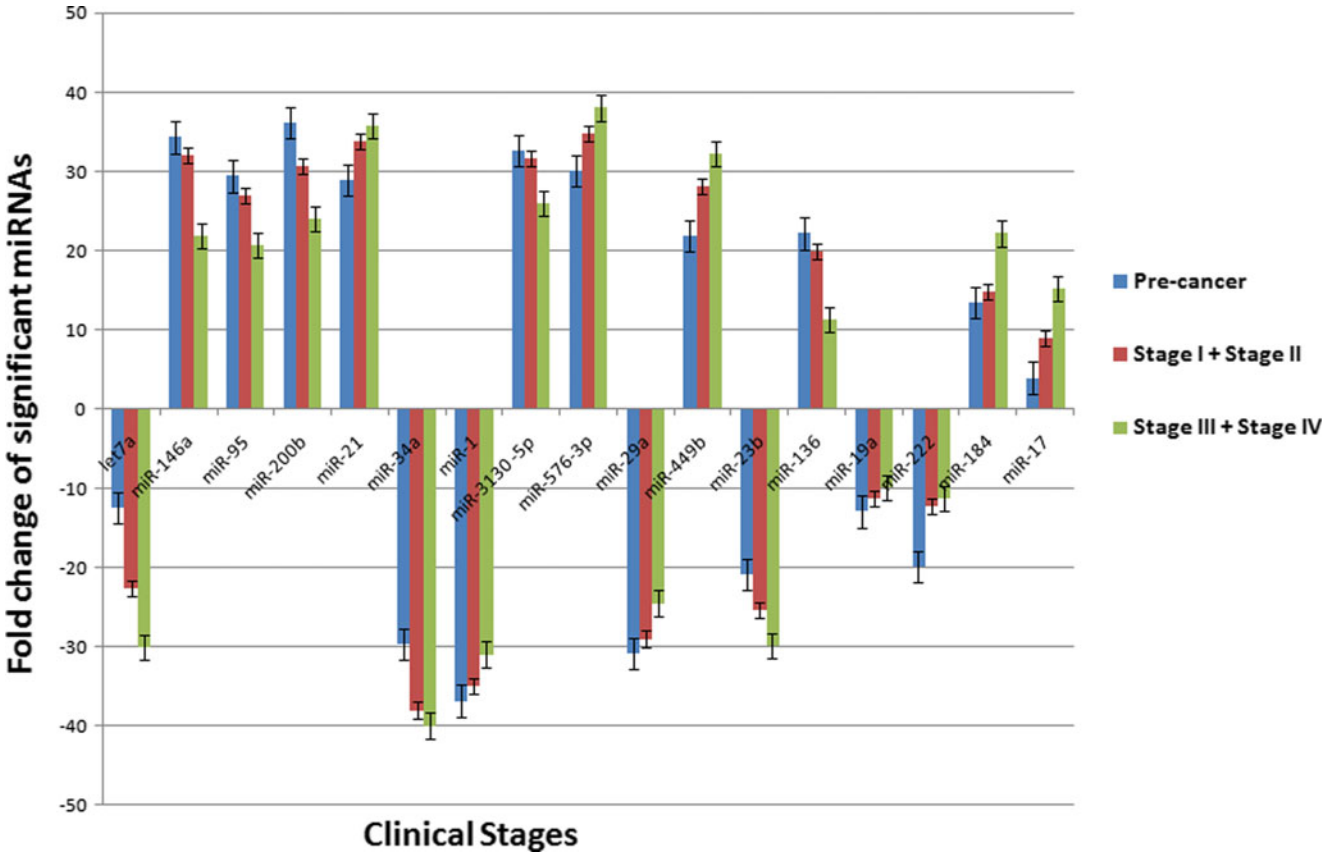

Fig. 2 Distribution of microRNAs having specific pattern with the clinical stage of cervical cancer showing mean ± SE with significant p value ($p<0.001$) of fold change in different stages in the progression of cervical cancer

established and has shown to be induced by HR-HPV E6 in the p53-dependent pathway and could be an early-onset event in the development of cervical cancer [22]. Also, studies have indicated that HPV infection is a pre-requisite for transformation of host cells through complex regulatory network via early oncogenic proteins of HR-HPVS [23].

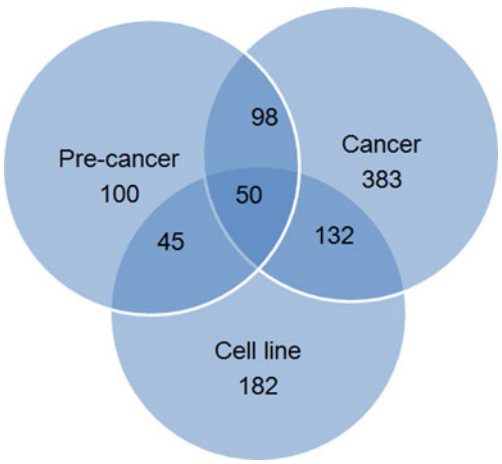

Fig. 3 Venn diagram showing commonly significant expressed miRNAs in tissue biopsies (pre-cancer vs controls, cancer vs controls and pre-cancer vs cancer respectively) and in HPV-positive (SiHa, HeLa) and HPV-negative (C33a) cervical cancer cell lines

Therefore, our study also aimed to determine the differential expression profile of miRNAs among HPV-infected cervical pre-cancerous and cancer cases. In the present study, we have screened a large number of miRNAs (N=1132) which are annotated in miRNA human database, comprising of reported miRNAs in cervical cancer and many which are not being reported in cervical carcinogenesis till date. The profiling of genome-wide miRNAs explains the role of HPV infection in the regulation of expression of miRNAs. We observed a distinct pattern and large number of significantly deregulated miRNAs (N=383) in cancer cases and in pre-cancerous lesions (N=100). Among the deregulated microRNAs, miR-146a, miR-95, miR-200b, miR-3130-5p had a fold change greater than 20 in both cervical pre-cancer and cancer cases. Interestingly, miR-200b showed higher expression pattern exclusively in pre-cancer lesions. On the contrary, miR-200b has previously been shown to be downregulated in nasopharyngeal [24] and hepatocellular carcinoma [25] but upregulated in ovarian [26] and bladder cancer [27] which is consistent with current study. In addition, miR-200b has been demonstrated to be inhibitor of pTEN gene which has been shown to have potential role in the progression of carcinogenesis [28]. Though the levels of miR-200b were higher in cancer, it can be speculated that the increased levels of miR- 200b in

cervical pre cancer may be due to initiation of metaplastic activity and increased cell proliferation to prepare pre-cancer cells to progress into cancer stages by chemo-sensitivity [29] along with HBV viral infection (Liu et al.). The study also demonstrates upregulation of miR-21 both in cervical pre-cancer and cancer. miR-21 has been studied extensively and more recently has been found to have carcinogenic potential in HPV-meditated cervical carcinogenesis [20]. It promotes cellular transformation required for tumour formation and invasion by post-transcriptionally down regulated PDCD4 expression [30] and pTEN [31]. The present study also suggests a crucial role of well studied miR-146a, which is found to be upregulated in cervical cancer cases. In contrast, miR-146a has shown to be downregulated in prostate cancer [32] and hepatocellular carcinoma [33]. However, difference in pathophysiology of cancers and also role of hormone driven cancers and HPV or HBV viral infections could likely contribute to difference in expression pattern [34]. Moreover, we also demonstrate a higher level of yet another oncomir miR-205, which is associated with function of severity of cervical lesion by promoting cellular proliferation and migration [35] but has shown to be downregulated in hepatocellular carcinoma by elevating the expression pattern of CD24 [36]. Similar to our results, miR-205 has found to be upregulated in oral squamous carcinoma [37] and lung carcinoma by suppressing the expression of pTEN gene [38]. In the present study, we also demonstrate the downregulation of miR-34a and miR-1 in cervical pre-cancer and cancer lesions. HPV type 16 has been shown to alter the expression of miR-34a in cervical cancer [21] by interacting with oncogenic HPV E6 protein via p53-dependent pathway [22]. Also, miR-34a has been shown to reduce cell viability and invasion by directly targeting E2F3, which in turn enhances the expression of survivin protein [39]. The global analysis of exosomal miRNAs has revealed that viral oncogenes of HPV (E6&7) provides E6/E7-dependent growth of HPV-positive cells which in turn deregulates cellular proliferation, apoptosis [40]. The ectopic expression of miR-34 has been shown to induce cell cycle progression by downregulating gene promoters necessary for cell cycle regulation and coordinates the activation multiple transcriptional targets in combination with other effectors to inhibit inappropriate cell proliferation [41]. In the present study, expression level of miR-34a and miR-23b was found to be highly under-expressed and may be due to its interaction with HPV oncoprotein E6 that may suppress p53 activity as evidenced by the higher fold change of −38.98 and −27.63, respectively. In addition, we observed downregulation of miR-29a, with preferential decrease from cancer to pre-cancer lesions. Zhao and colleagues had demonstrated the role of miR-29a in gastric cancer and oral squamous cell carcinoma by participating in apoptosis [42, 43]. Bioinformatic approaches have also explained the putative HPV-induced miRNA-mRNA regulatory network of miR-29a by targeting transcription factor YY1 and CDK6 which promotes malignant transformation induced by HPV and restrains cellular proliferation and enhance apoptosis [44]

Similarly, miR-218 has shown to be downregulated by HPV16 E6 via targeting LAMB3 and appears to be a crucial modulator of cancer cell processes through inhibition of signalling [45, 46]. Similar to their observations, we could also demonstrate a reduced expression of miR-218. Interestingly, more reduced expression was observed in HPV-positive cervical cancer cell lines when compared to cervical pre-cancer and cancer tissue biopsies.

We also observed some of the miRNAs which were highly upregulated in tissue biopsies of cervical lesions but did not showed any change with respect to the severity of cancer lesion either clinically or histologically.

Since infection with high risk has been shown to have carcinogenic potential by activation of its two oncoproteins E6 and E7, we also analysed the expression pattern of microRNAs in HPV-positive and HPV-negative cell lines. The analysis found some of the common microRNAs which include miR-449b, miR-15a, miR-517a, miR-545, miR-223, miR-192, miR-150 and miR-181c to be upregulated, and miR-99a, miR-193b, miR-376c and miR-23b were found to be downregulated (data not shown) but showed difference in expression pattern or fold change. Interestingly, the present study reports five miRNAs (miR-892b, miR-500, miR-888, miR-505 and miR-711) which are not yet been reported in cervical cancer. A recent report also suggests that miR-892b is found to upregulated in per-urethral vagina wall in post-menopausal women [47]. These observations clearly suggest the difference in cellular characteristic that could play an important role in transformation of normal epithelial cell to carcinogenic one, as in the case of HPV infection where squamo-columnar iunction (transformation zone) provides favourable niche for viral propagation and could define the downregulation of miR-892b including its upregulation in retinoblastoma pathogenesis analysed by in silico approach [48]. Recent reports depicted that miR-500 has been proven as a biomarker in hepatocellular carcinoma [49] and has shown to be elevated and upstream of the nuclear factor-κB gene in gastric cancer [50]. miR-888 has been found to be over-expressed in endometrial cancer by targeting its tumour suppressor progesterone receptor [51]. However, in breast cancer cell line MCF-7, the elevated level of miR-888 has been found to potentially target E-cadherin gene [52]. Similarly, earlier studies have demonstrated upregulation of miR-505 in colorectal cancer [53] and breast cancer [54]. Our data corroborate their findings and demonstrate upregulation of miR-505 in both cervical pre-cancer and cancer cases. Conversely, circulating levels of miR-505 have found to be downregulated in biliary cirrhosis [55]. Increased level of miR-711 was also found in this study. We also observed yet another similar result as reported in T cell lymphoma [56]. The other miR-205 found to be

upregulated in both cervical pre-cancer and cancer cases. This microRNA has shown to be frequently deregulated in many cancers including cervical cancer and is a predictive biomarker with dual edged sword as a tumour suppressor or an oncogene depending on cellular niche [57].

Apart from molecular signatures associated with cervical carcinogenesis, there seems to be other confounding cofactors which can potentially increase the risk of development of cervical cancer progression and may help in providing favourable environment for persistent high risk infection to develop invasive cancer state [7]. Among these factors, the present study demonstrates a significant association of cervical pre-cancer and cancer cases with tobacco consumption (Table 1). Our earlier studies and other studies have demonstrated an association of demographic characteristics with cervical cancer and HPV infection [58–61].

Thus, to conclude, the present study suggests a potential role of microRNAs in cervical cancer and might be valuable biomarkers for cervical cancer detection and progression with their translational potential into clinical applications.

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Compliance with ethical standards

Conflicts of interest None

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