RESEARCH COMMUNICATION

Prognostic Significance of HPV Physical Status and Integration Sites in Cervical Cancer

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Abstract

Background: Human papilloma virus (HPV) infection is the major cause of cervical cancer and integration of HPV DNA into the host cell genome is believed to be essential for malignant transformation. MiRNAs are a class of 19-24 nt non-coding RNAs that regulate gene expression primarily through post transcriptional repression or m-RNA degradation in a sequence specific manner. The aim of this study was to determine the frequency of HPV16 and 18 integrated and episomal forms and to evaluate its prognostic significance in invasive cervical carcinoma cases and to detect by in-silico approach MiRNAs near HPV integration sites (within <3Mb). Methods: HR-HPV 16 and 18 typing was performed by Nested Multiplex PCR (NMPCR) and HPV 16 and 18 physical status (integrated and episomal forms) was determined by Amplification of Papillomavirus Oncogene Transcripts (APOT) assay. Nested PCR products of the APOT assay were resolved on a 2% agarose gel and the PCR products of interest were excised and sequenced. In silico analysis was done to identify the Fragile sites and MiRNAs' near integration sites of the HPV. Results: Episomal forms were more common with the HPV16 type and integrated forms with the HPV18 type (p=0.011). Patients with tumors having the episomal forms had a better disease free survival than those with integrated forms of HPV16 type, but this did not reach statistical significance. We detected 53 miRNAs near integration sites, of which 39 have been reported to be associated with cancers. The incidence of miRNAs near HPV integration sites was 78.3%, being more common with HPV16. Conclusion: This is the first study from India to provide the physical status of HPV16 and HPV 18 in cervical cancers, to assess their prognostic importance and to identify FRA and MiRNAs' near HPV integration sites.

Key Words: Cervical cancer - HPV physical status - HPV integration - MiRNA - prognosis

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Introduction

Cervical cancer is the most common cancer among Indian women and in women in most developing countries (Parkin et al., 2005). The crude incidence rate (CIR) in Chennai is 19.8/100,000 as per MMTR (Madras Metropolitan Tumor Registry). Human papillomavirus (HPV) is the major risk factor responsible for the development of more than 99% of cervical cancers. HPV16 and HPV18 are the most common subtypes associated with cervical cancer, with HPV16 accounting for approximately 60 - 70% of all cervical cancers (McPhillips et al., 2004) and HPV18 in another 10–20% (Braun et al., 2004). In India over 99% of cervical cancer cases harbor high risk (HR)-HPV infection (Franceschi et al., 2003), the HPV16 type being the most common.

Human papillomavirus can exist in cervical epithelial cells as episomal, integrated or mixed forms (episomal and integrated). Viral integration into the host-cell genome occurs downstream of the early genes E6 and E7, often in the E1 or E2 region; this disruption results in the loss of negative-feedback control of oncogene expression by the viral regulatory E2 protein (Woodman et al., 2007). In low-grade cervical lesions, the majority of HPV genomes persist in episomal state, whereas in high-grade lesions and invasive carcinomas, up to 88% of cervical cancers have integrated HPV DNA (Ziegert et al., 2003). In HPV16 associated neoplasia, viral DNA may persist in mixed episomal and integrated forms. In contrast, HPV18 genomes are reported to be exclusively integrated in the host genome (Shera et al., 2001).

Chromosomal fragile sites (CFS) (FRAs) are loci which show gaps or breaks on metaphase spreads of cells that have been grown in the presence of inhibitors of DNA replication (Wilke et al., 1996). Cytogenetic mapping of multiple integration sites suggest that HPV integration occurred preferentially in bands containing CFSs in invasive cancers (Thorland et al., 2000). MicroRNAs (miRNA) are small non-coding RNA of 19–24 nucleotides in length that were discovered >12years ago by Ambros and colleagues (Garzon et al., 2006). miRNA regulates gene expression by translational repression, mRNA

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cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation. Recent studies showed that some miRNAs regulate cell proliferation and apoptosis processes and can act as oncogenes or tumor suppressors. Up to one-third of human mRNAs are predicted to be miRNA targets (Zhang et al., 2006). More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs may play a more important role in the pathogenesis of a limited range of human cancers (Zhang et al., 2007). A significant number of miRNAs are near the Human Papilloma Virus (HPV) Integration Sites (Calin et al., 2004).

The aim of our present study was to determine the prognostic value of HPV physical status in patients with cervical carcinoma and to perform in-silico identification of FRAs and known miRNAs near the HPV integration sites.

Materials and Methods

Study Population

A total of 121 cervical carcinoma biopsies were collected from patients attending Cancer Institute (WIA), Adyar, Chennai, after obtaining an informed consent for the study. The study was cleared by the Institutional Ethical Committee. Biopsy specimens collected were bisected; one portion was submitted for standard histopathologic diagnosis, while the other portion was stored in RNA later at -70°C for subsequent molecular analysis. The stage of the disease was classified according to International Federation of Gynecology and Obstetrics (FIGO). Of the 121 patients, 118 patients were treated with radiation therapy, two patients had radiation therapy followed by surgery and one patient received chemotherapy concurrently with radiotherapy.

RNA and DNA Isolation

RNA from biopsy samples were isolated using RNeasy mini kit (Qiagen, Germany) according to manufacturer's protocol. DNA was precipitated from the first flow through, using 3M sodium acetate (pH 4.5) and two volumes absolute alcohol, centrifuged at 12000rpm for 10min, washed twice with 70% alcohol, air dried and re-suspended in distilled water (Klaes et al., 1999).

Typing for HPV16 and HPV18

HPV16 and 18 typing was done using Nested Multiplex Polymerase Chain Reaction (NMPCR) technique (Sotlar et al., 2004). SiHa DNA for HPV16 and HeLa DNA for HPV18 (positive controls) and C33A DNA (negative control) were included.

cDNA Synthesis

One µg of total RNA was reverse transcribed using an oligo (dT)17 primer coupled to a linker sequence (dT)17p3 with 200U of MMLV (Superscript II) for one hour at 42˚C in a 20µl final reaction volume. ABL PCR was then routinely performed to check for RNA integrity and cDNA quality.

Amplification of Papillomavirus Oncogene Transcripts (APOT) assay

The APOT assay, used to detect integrated and episomal forms, is based on the structural differences among the 3' ends of viral oncogene transcripts (Klaes et al., 1999) and was done as per the published literature. Positive (SiHa for HPV16 and HeLa for HPV18) and negative (C33A) controls were included with each run.

Sequence Analysis

Fifty µl of the Nested PCR products were resolved on a 2% agarose gel containing ethidium bromide. The PCR products of interest were excised from agarose gel and extracted using Qiagen gel extraction kit. The amplimers were sequenced directly using a Big-Dye Terminator Cycle Sequencing Reaction kit v3.0 (Applied Biosystems, Foster City, USA). The Sequencing products were loaded and run on a ABI 310 DNA sequencer. Sequence data was analyzed using BLASTN program.

Follow –up

Clinical review for follow-up cases was performed in the Cancer Institute (WIA), at 3 monthly intervals for the first three years, 6 monthly reviews for the next 2 years and then yearly or earlier if there were any symptoms. In case of symptoms or suspicious pathologic findings, further diagnostic procedures were initiated, including interventional biopsies for definitive diagnosis.

MiRNA identification

There are now 706 human miRNAs annotated in the miRNA registry (http://www.sanger.ac.uk/Software/Rfam/mirna/). For precise location of miRNAs, all the known miRNAs were used as queries to BLAST search against the Homo sapiens genome (BUILD 36.1), at www.ncbi.nlm. nih.gov_genome_guide_human. A distance of less than 3Mb was considered to define close vicinity for HPV integration sites and miRNAs (Calin et al., 2004). Common fragile sites (n=89) and Rare fragile sites (n=28) are listed in the Genome Database (GDB).

Statistical Analysis

The relation between the HPV physical forms and the clinico-pathologic parameters were evaluated using Chi-square test or Fisher's exact test. Survival probability was estimated by using Kaplan-Meier method. Logrank test was employed to test for statistical significance of survival curves. Cox proportion hazard model was used to elicit the prognostic factors for disease free (DFS) and overall (OS) survival in the univariate analysis. Survival time was measured in months from the date of diagnosis to the date of last follow-up or death or recurrence or loss to follow up whichever was earlier. Each patient's clinical status was determined to the last available date. Statistical analyses were done using SPSS, version 13.0.

Poisson regression model was clustered on the samples to obtain the incidence rate ratios of miRNAs at HPV integration sites. MiRNAs were the "events" and the region of HPV16 and 18 integration sites were the "length" measured taken as less than 3Mb. STATA v10 was used for completing the computations.

Table 1. Frequency of HPV Subtypes and HPV Physical Status

HPV typing Status	Total	N	%
	121		
HPV16 positive		88	72.7
HPV18 positive		25	20.7
HPV16 and 18 Negative		8	6.6
HPV physical status data available	87		
HPV16		67	70.5
HPV18		20	21.0
HPV16	67		
Integrated		38	56.7
Episomal		28	41.8
Integrated and Episomal		1	1.5
HPV18	20		
Integrated		16	80.0
Episomal		2	10.0
Integrated and Episomal		2	10.0
No: taken for survival studies	75		
HPV16		58	77.3
HPV18		17	22.7
HPV16	58		
Integrated		32	55.2
Episomal		25	43.1
Integrated and Episomal		1	1.7
HPV18	17		
Integrated		14	82.3
Episomal		1	5.9
Integrated and Episomal		2	11.8

Results

The details of the HPV types and their physical status are given in Table 1. Among the 87 patients in whom the physical status of HPV was known, 75 patients (58-HPV16 and 17- HPV18 positive) had completed the prescribed potentially curative treatment and had the follow up data and were included for prognostic associations with the HPV type and their physical status. Patient's age ranged from 22-65 years with a mean age of 45.0 ± 9.7 (mean ± SD) years for HPV 16 type and mean age of 42.7±8.9 (mean± SD) years with a age range of 30-60years for HPV 18 type. The prevalence of adenocarcinoma and adenosquamous cell carcinomas was statistically significant (p=0.001) in HPV 18 type compared to HPV 16 type. Episomal forms were significantly higher in HPV 16 (43.3%) than in HPV 18 (10.0%) (p-value =0.011).

Association of HPV 16 and 18 physical statuses with clinicopathological parameters is shown in table 2. Mean age of patients with HPV 16 episomal forms (48.5±9.2) was significantly higher than HPV 16 integrated forms (42.5±9.4) (p=0.02). In older age group (more than 50 years old), HPV 16 episomal forms were higher than integrated forms with a statistical significance (p=0.041). However, neither HPV 16 and 18 genotypes nor HPV physical status were statistically significantly associated with other clinicopathological parameters assessed in this study.

Adenocarcinoma and adenosquamous cell carcinomas showed a 2 fold higher risk of failure than squamous cell carcinomas (p=0.167). Stage was also important with locally advanced stages doing poorly compared to early

Table 2. HPV Physical Status and Clinico-pathological Parameters

Parameter	HPV 16			HPV18		
	I(n=38)	E(n=28)	I+E(n=1)	I(n=16)	E(n=2)	I+E(n=2)
Age						
<=50	27(71.1)	12(42.9)	1(100)	11(68.8)	1(50.0)	2(100)
>50	11(28.9)	16(57.1)	-	5(31.3)	1(50.0)	-
p-value		0.041			0.535	
Stage						
Early	8(21.1)	8(28.6)	-	6(37.5)	-	1(50.0)
Adv	30(78.9)	20(71.4)	1(100)	10(62.5)	2(100)	1(50.0)
p-value		0.664			0.517	
Histopathology						
SCC[1]	25(65.8)	23(82.1)	1(100)	10(62.5)	1(50.0)	1(50.0)
SCC[2]	8(21.1)	5(17.9)	-	-	-	-
AC	5(13.2)	-	-	6(37.5)	1(50.0)	-
Clear	-	-	-	-	-	1(50.0)
p-value			0.31		0.041	
Tumor Grade						
Inter	3 (7.9)	4(14.3)	-	2(12.5)	1(50.0)	-
High	35(92.1)	24(85.7)	1(100)	14(87.5)	1(50.0)	2(100)
p-value		0.663			0.308	

I = Integrated; E = Episomal; I+E = Mixed; Adv, locally Advanced; 1and LCNK; 2and LCK; 3/ASC; Clear, clear cell, Inter, intermediate

stage. HPV18 type had a 40% higher risk of failure compared to HPV16 type (p=0.444). Episomal forms compared to integrated forms of HPV16 had 27% lower risk of failure but this did not achieve statistical significance (p=0.49). Univariate analysis of clinico-pathological parameters showed no significant association with the prognosis of disease.

In a follow up period of 48 months, Kaplan Meier and univariate analysis revealed no statistical significance for Overall (OS) or Disease free survival (DFS) when compared to the HPV type or their physical status.

In a total of 57 HPV integration sites (HPV16=39; HPV18=18), we found miRNA or fragile sites in/near 37 HPV integration sites (HPV16=25; HPV18= 12). Identified miRNAs in HPV16 and 18 at gene and intergene regions and exonic and intronic regions are summarized in the Table 3. The list of identified miRNAs and fragile

Table 3. MiRNA and Fragile Sites at HPV Integration Sites

Total HPV Integration Sites (57)			
HPV16 (39) (68.4%)		HPV18 (18) (31.6%)	
MiRNA + Fragile sites	No MiRNA + fragile sites	MiRNA + Fragile sites	No MiRNA + Fragile sites
25(64.1%)	14(35.6%)	12 (66.7%)	6(33.3%)

MiRNAS and Fragile sites AT Genic and Intergene Regions					
HPV16 (25) (64.1%)					
Gene (15) (60%)			Intergene (10) (40%)		
MiRNA	MiRNA+FRA	FRA	MiRNA	MiRNA+FRA	FRA
11 (73.3)	2(13.3)	2(13.3)	3 (30)	3(30)	4(40)
Exon (5) (38.5) Intron (8) (61.5)					
HPV18 (12) (66.7%)					
Gene (6) (50%)			Intergene (6) (50%)		
MiRNA	MiRNA+FR	FRA	MiRNA	FRA	
3(50)	2(33.3)	1(16.6)	5 (83.3)	1(16.6)	
Exon (1) (20) Intron (4) (80)					

Table 4. Fragile and miRNAs at Gene Regions of HPV Integrations

HPV type	HPV Integration sites	Related Protein	Fragile site		Closest miRNAs	Distance Mirnas (MB)
16	1q21.2	GOLPH3L	FRA1F	1q21	-	-
16	15q26.3	LRRC28	-	-	mir 7-2	2.7
16	17q23.1	TUBD1	FRA17B	17q23.1	mir 301a	0.7
					mir 454	0.72
					mir 301a	0.7
					mir 142	1.5
					mir 21	0.01
18	7p22.1	FOX K1	FRA7B	7p22	mir 589	0.76
16	17q12	C-CR7	-	-	mir 144	0.51
					mir 338	0.93
					mir 451	0.51
					mir 10a	2.8
					mir 193a	2.1
16	11p13	MPPED2	FRA 11E	11p13	-	-
16	1q43	NID1	-	-	mir 1537	0.18
18	10q24.33	SH3PXD2A		--	mir 608	2.6
					mir 936	0.43
					mir 609	0.6
					mir 146b	1.1
					mir 1307	0.02
18	10q24.33	SH3PXD2A		--	mir 609	0.63
					mir 146b	1.1
					mir 608	2.6
					mir 936	0.46
					mir 1307	0.19
16	20q13.13	SLC9A8	-	-	mir 645	0.75
					mir 1259	0.55
16	15q23	THSD4	-	-	mir 629	1.4
					mir 630	1.1
16	17q11.2	PHF12	-	-	mir 144	0.07
					mir 451	0.07
					mir-193a	2.6
18	12q22	TMCC3	-	-	mir 331	0.69
					mir 492	0.21
16	3q28	TP73	-	-	mir 28	1
					mir 944	0.1
16	3q28	FAM79B	-	-	mir 28	0.62
					mir 944	0.51
16	5q32	CSK1A1	-	-	mir 103-1	2.7
					mir 143	0.09
					mir 145	0.09
					mir 218-2	2.9
					mir 378	2
					mir 582	0.47
					mir 584	0.46
16	17q21	BRCA 1	-	-	mir-10a	0.33
					mir-152	0.2
					mir-1203	0.08
16	18q21.31	ATP8B1	FRA 18B	18q21.31	mir 1-2	2.2
					mir 133a-1	2.2
18	7q36.2	RHEB	FRA7I	7q36	mir 671	2.2
18	8q24.3	GPAA1	FRA8D	8q24.3	-	-
16	6P12.2	PKHD1	--		mir 2060.4	8
					mir 133b	0.48

Table 5. Fragile and miRNAs at Intergene Regions of HPV Integrations

HPV type	HPV Integration sites	Fragile site		Closest miRNAs	Distance Mirnas (MB)
16	14q32.2	-	-	mir 127 cluster	1-0.009
16	5p14.1	FRA5E	5p14	-	-
18	3q28	-	-	mir 28	1.20
			-	mir 944	0.08
18	3q28		-	mir 28	0.70
			-	mir 944	0.43
16	22q13.1-13.2	FRA22A	R/Fol	22q13 mir 658	2.50
			-	mir 659	2.50
			-	mir 1281	0.71
18	10q25.2	FRA10E	10q25.2	-	-
		FRA 10B	R/BrdU	10q25.2	-
16	22q13.1-13.2	FRA22A	R/Fol	22q13 mir 658	2.50
			-	mir 659	2.50
			-	mir 1281	0.71
18	1q41	-	-	mir 215	1.50
			-	mir-194-1	1.50
			-	mir-664	1.60
16	2q22.3	FRA2K	R/Fol	2q22.3	- -
16	1q32.1	-	-	mir-135b	0.25
18	1q32.2-41	-	-	mir-205	0.01
16	12q22	-	-	mir 492	2.70
16	10q21.3	FRA 10C	C/BrdU	10q21 mir-1296	0.08
18	8q24.21	-	-	mir-1205	0.80
			-	mir-1206	0.85
			-	mir-1207	0.89
			-	mir-1208	0.99
16	1p36.1	FRA1A	C/Aph	1p36.1	- -
16	22q13.1-3.2	FRA 22A	R/Fol	22q13 mir 658	2.50
			-	mir 659	2.50
			-	mir 1281	0.71

sites at HPV16 and 18 integration sites is given in Tables 4 and 5.

We detected 16 fragile sites in/near 57 HPV integration sites. The fragile sites were detected in 11/39 (28.2%) in HPV16 and 5/18 (27.8%) in HPV18 positive samples, of which 11 were common fragile sites (7 in HPV16 & 4 in HPV18) and 3 were rare fragile sites (2-HPV16 & 1-HPV18).

In the 53 miRNAs identified, one miR-127 cluster was found. In addition, 8 miRNAs (miR-194-1, miR-671, miR-1203, miR-1205, miR-1206, miR-1207, miR1208 and miR-1537) were reported in human miRregistry but no expression studies or target genes were reported till date. Literature search provided evidence for expression studies on the remaining 44 miRNAs. Twenty five identified miRNAs (miR1-2,miR 7-2,mir 10a,miR-21, miR-28,mir-103-1,miR-133a-1, miR-133b,mir 135b, miR-142, miR-143, miR-144, miR-145, miR-146b,miR-152, miR-205, miR-206, miR-215, miR-218-2,miR 301a, miR-331, miR-338, miR-451, miR-936, miR-944) were reported to be expressed in cervical tumor cells. Expression of 14 identified miRNAs (miR-193a, miR-378,miR-454, miR-492, miR-582, miR-584, miR-589, miR-608, miR-609, miR-629, miR-630, miR-645, miR-658, miR-659) were found in other tumor types and 5 identified miRNAs were associated with embryonic stem cells. In 11 miRNAs, identified target genes have been reported (Viktor et al., 2008; Park et al., 2008; Schaefer et al., 2009; Cheng et al., 2005).

The relative incidence rate of miRNAs near HPV16 integration is 1.4 times higher than in HPV18, commonly in the genic regions than in the intergene regions (p=0.015)

and again more common in the intronic regions (1.6 times higher) compared to the exonic regions (p value 0.05). In HPV18, miRNAs' were 2.5 times more likely in the intronic regions than in exonic regions (p=<0.001).

Discussion

The HR-HPV types, especially 16 and 18 are major etiological agents for cervical cancer. We found HPV16 (72.7%) and 18 (20.7%) positivity, to be in agreement with the previous published results from India (Franceschi et al., 2003). While HPV18 type has been reported to be mostly integrated (upto 80%) (Woodman et al., 2007), reports on the episomal status of HPV16 have been varied, ranging from 20 – 40% (Das et al., 1992; Arias-Pulido et al., 2006). In our study we found the frequency of HPV16 episomal forms to be 41.8% in cervical cancers and integrated forms in 56.7%, suggesting that integration might not be an important event in progression of early dysplasia to invasive carcinoma, in HPV16. Sathish et al (2004) had reported the frequency of HPV16 episomal forms to be 68% in both CIN and invasive cases of cervical cancer.

Univariate association showed that the HPV18 type was more prevalent in adenocarcinoma and adenosquamous cell carcinoma which is in agreement with other studies (Tong et al., 2007). Significant association was shown between HPV subtypes and HPV physical status (p=0.011), with a higher frequency of episomal forms in HPV16 type (Shera et al., 2001). We found that the episomal forms of HPV16 type were higher in patients above 50 years of age (p=0.041). The HR-HPV16 and 18 types were found to be prognostic factors for survival (Lai et al., 2007) but was not seen in our study, which is in agreement with Tong et al (2007).

Fragile sites are genomic regions prone to chromosomal breaks that facilitate foreign DNA integration. Some studies have identified the relationship between the fragile sites and the HPV integration site (Wentzensen et al., 2004). Frequency of HPV16 and 18, integration at fragile sites has been reported to be around 28% (Thorland et al., 2000).

We have identified by in-silico approach, 53 miRNAs within a range of less than 3Mb distance from the HPV integration sites. Of the 53 miRNAs one is a miR-127 cluster, on chr.14q32, a HPV16 integration site. It is the largest miRNA cluster identified to date comprising of 52 members and this region of chr.14q32.1 is a frequent target of translocations and inversions in T cell leukemias (Huppi et al., 2007).

HPV16 integration found at Chr. 17q23.1, is also the location for common fragile site of FRA17B C/Aph. The miR-21, miR-142, miR-301a and miR454 were present at this site and our results are similar to the earlier reports (Calin et al., 2004; Luiet al., 2007). miR-21 is overexpressed in a wide variety of human cancers including breast, colon, lung, ovarian, and glioblastoma multiforme. Its overexpression has been experimentally shown to lead to caspase activation and increased apoptotic cell death (Mirnezami et al., 2009). PTEN, caspase, BCL-2, tropomyosin and Programmed Cell Death 4 (PDCD4) are gene targets for miR-21 (Viktor et al., 2008; Park et al., 2008).

In the 53 miRNAs', the largest group of 7 miRNAs (miR-103-1, miR-143, miR-145, miR-218-2, miR-378, miR-582, miR-584) were identified at HPV16 integration site 5q32. miR-143 and mir-145 are down regulated in cervical cancer and have been found in several other cancers, including colorectal cancer and B-cell lymphoma (Reshmi et al., 2008). DR4 and DR5 are predicted targets (Viktor et al., 2008) and RAS E2F3, BCL-2, MCL-1 are target genes for mir-145 (Park et al., 2008). Mir-218-2 is down regulated in cervical cells and in colon, stomach, prostate, and pancreas cancers, but not in lung and breast carcinomas (Volinia et al., 2006) and inhibits cell growth (Cheng et al., 2005). MiRNA 103-1 is up-regulated in all cancers expect breast and is induced in hypoxic environment (Kulshreshtha et al., 2007).

The HPV integration at 3q28 has been seen with both HPV16 and 18. In HPV18, integration at 3q28 is at an intergene regions and in HPV16 it is at the genic region involving TP73 and TPRG1, which are members of p53 family of transcription factors. The miRNAs miR-28 and miR-944 are found at 3q28 HPV integration site, both the miRNAs have been reported to be expressed in cervical cells (Lui et al., 2007). The HPV18 integration at 1q41 had miRNAs', mir-215, mir194-1 and mir-664, of which miR-215 and mir-194-1 have been reported at HPV16 integration site (Calin et al., 2004).

This study represents data on the frequency of HPV16 and 18 integrated and episomal forms and the association of clinico-pathological parameters with HPV genotypes and HPV physical status. HR-HPV types 16 and 18 and their physical status were not found to be significant prognostic factors. However, given the size of the study there is a need to examine a larger sample, before any conclusions can be drawn. To our knowledge this is the first insilico work of miRNAs location, based on experimental data of HPV integration sites from India. Among the 39 miRNAs' identified, 25 of them have been reported to play a role in cervical cancer. Additional expression studies of miRNA genes at or near HPV integration sites could help understand their function in gene regulation, which in turn can help develop biomarkers and newer targets for therapy.

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